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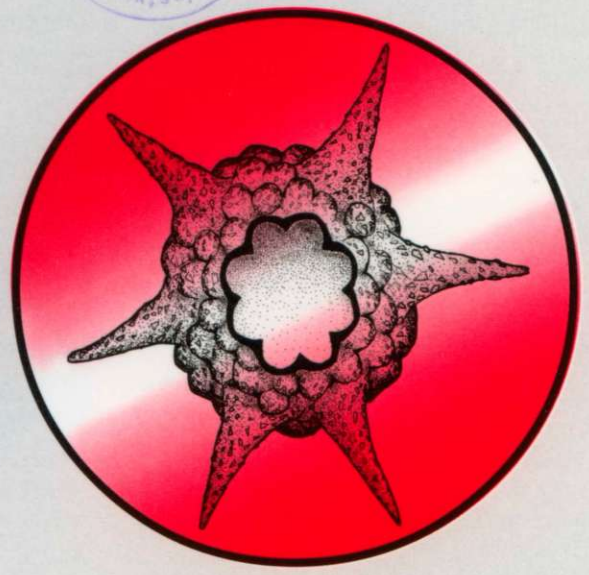

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REVIEW ARTICLE

Ciliate Mating Types and Their Specific Protein Pheromones

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Summary. The determination of a number of pheromone structures from species of *Euplotes* provided direct evidence that these cell type-specific signals are represented by families of homologous proteins, consistently with their genetic control through series of single-locus multiple alleles. Due to their structural homology, unequivocally manifested by the organization of similar three-dimensional topologies, pheromones can thus compete with one another to effectively bind to their cells of origin in autocrine fashion, or to other conspecific cells in paracrine fashion. The cell response to these different pheromone interactions will accordingly vary, reproductive (mitotic proliferation) in the former case, mating (sexual) in the latter.

Key words: chemical signalling, ciliates, growth factors, protein families, protein structures, reproduction, sex.

INTRODUCTION

In the wake of the milestone report by Sonneborn (1937) ("Sex, sex inheritance and sex determination in *Paramecium aurelia*") that conjugation of *P. aurelia* involves the intra-specific differentiation of two genetically distinct mating types, a functional equivalence of mating types of ciliates with sexes of any other (micro)organism has for long been widely accepted. Each mating type would be represented by "a strain of conspecific individuals not able to undergo [mating] fusion with each other, but only with members of a complementary mating type" (e. g., Hausmann *et al.* 2003), and the mating type-distinctive signal molecules would behave like "sex substances" (e. g., Kuhlmann and Heckmann 1989) able "to induce chemical interaction and mutual stimulation between cells complementary for mating union and fertilization" (e. g., Miyake 1981, 1996). These concepts are also the foundation of a model, known as the "gamone-receptor hypothesis" (Miyake 1981, 1996), predictive of the mechanism of action of the first two of these signal molecules, denoted as "Gamone-1" (G-1) and "Gamone-2" (G-2), that have been isolated from *Blepharisma japonicum*. G-1 has been characterized as a glycoprotein with a sequence that has now been determined to be of 272 amino acids plus six sugars (Sugiura and Harumoto 2001); G-2, instead, is a 3-(2'-formylamino-5'-hydroxybenzoyl)lactate, i. e., a possible tryptophan derivative (Jaenicke 1984, Miyake 1996). In spite of the fact that G-1 and G-2 presume quite different genetic determinants - in addition to being chemically unrelated, G-1 is species-specific while G-2 is shared in common by a variety of

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species of *Blepharisma* (Miyake and Bleyman 1976, Miyake 1996) these molecules have been designated to distinguish cells representing two "complementary" mating types, i. e., Mt-I and Mt-II, and presumed to behave as symmetric inducers of sexual cell pairing: G-1 by binding and activating its cognate receptor on Mt-II cells, and G-2 by binding and activating its cognate receptor on Mt-I cells.

Objective reasons, however, exist, as has been pointed out (Nanney 1980, Luporini and Miceli 1986, Dini and Nyberg 1993), in contradiction with the equivalence between ciliate mating types and sexes; hence, in contradiction to the related "gamone-receptor" interpretation of the activity of the *B. japonicum* G-1 and G-2 signals in the terms only of sexual factors that are synthesized by cells of one cell type to target another cell type. Essentially, these reasons are that: (i) rather than being binary and dimorphic like sex, ciliate mating type systems are usually highly multiple (open?) and polymorphic, like the self-incompatibility systems of flowering plants and some mating type systems of fungi (Casselton 2002, Charlesworth 2002); (ii) effective mating takes place in many ciliates between cells of the *same* mating type (homotypic mating, or selfing), just as it takes place between cells of *different* mating types (heterotypic mating); (iii) every mating cell, independently of whether it represents one or the other mating type, generates one migratory and one stationary gamete nuclei, and it is this behavioral divergence between gamete nuclei that has to be credited with the properties of a sexual, "male"/"female" differentiation.

To these reasons we can now add the knowledge, that is the object of this review, of various aspects of the biology and structure of the mating type signals that have been studied, under the denomination of "pheromones", in three distinct species of *Euplotes*, i. e., *E. raikovi*, *E. octocarinatus*, and *E. nobilii*, the first two of which are species widely distributed in temperate waters (marine and lacustrine, respectively) and the third one is restricted to the marine costal waters of Antarctica. These *Euplotes* species, more properly than *B. japonicum*, can collectively be regarded as instructive models to search into the evolutionary and functional meanings of the ciliate mating type mechanism. While in *B. japonicum* we are substantially ignorant of the genetic basis of the mating types and of the associated glycoprotein and tryptophan-derivative signals, the genetic basis of *Euplotes* mating types has been determined to be represented by series of alleles that segregate at a single, highly polymorphic Mendelian *mat* (mating-type) locus (e. g., Dini and Luporini 1985, Luporini *et al.* 1986, Heckmann and Kuhlmann 1986, Dini and Nyberg 1993). And consistently with this genetic control, *Euplotes* pheromones all appear to be represented by proteins that are homologous members of species-specific families (Raffioni *et al.* 1992); hence, within each family, characterized by structures that are globally similar, yet distinct from one another due to local structural specificities (Luginbühl *et al.* 1994, Liu *et al.* 2001, Zahn *et al.* 2001). These relationships of structural homology that unequivocally exist between mating type-specific pheromones should be kept as a firm and central notion in any attempt to rationalize the function and mechanism of action of ciliate pheromones and, more in general, the evolutionary meaning of the ciliate mating type systems.

Euplotes pheromone synthesis and secretion

Euplotes cells synthesize and secrete their pheromones constitutively throughout the entire life cycle, regardless of whether they are, or are not in a developmental or physiological stage suitable for undertaking a mating process (Luporini *et al.* 1992, 1996). Pheromone gene transcription and pheromone secretion are already shown by cells that are at the very beginning of their clonal life cycle (Luporini *et al.* 1992), when they are usually regarded as "sexually immature" or "adolescent" because of their incapacity to mate (Kuhlmann and Heckmann 1989), as well as by cells that are growing (reproducing) asexually (mitotically) in the presence of food (Vallesi *et al.* 1995), hence in a physiologically inadequate stage to engage in sex.

The rates of pheromone production, as can be assessed by quantitative analyses of protein purified from the cell supernatant, vary significantly among species as well as, within a given species, among mating types (Luporini *et al.* 1986). Pheromones of *E. octocarinatus* (designated Phr-1, Phr-2, and so forth) are produced in minimal amounts, that vary from 0.36-0.38 µg/l (Phr-3 and Phr-4) to 0.5 µg/l (Phr-1) (Weischer *et al.* 1985, Schulze-Dieckhoff *et al.* 1987), with respect to those of *E. nobilii* (designated E*n*-1, E*n*-2, and so forth), and *E. raikovi* (designated E*r*-1, E*r*-2, and so forth) in which the production is in the range from 50 µg/l (E*n*-2) to 150 µg/l (E*n*-1) (Alimenti *et al.* 2002, 2003a), and from 20 µg/l (E*r*-20) to 330 µg/l (E*r*-1) (Raffioni *et al.* 1987, 1992), respectively.

In *E. raikovi*, quantitative variations in pheromone production between cells of different mating types appear to be closely correlated with, and are presumably

caused by, different degrees of amplification of the pheromone (mating-type) genes in the cell macronucleus. This correlation has been established more precisely for the pheromones E*r*-1, E*r*-10, E*r*-2, and E*r*-20, through the utilization of cultures grown at a density of about 10^4 cells/ml for 4-5 days in the presence of food and then left to enter early starvation for an additional 2 days. Pheromone E*r*-1, that is produced in an amount that is about 2-fold those of E*r*-2 and E*r*-10 (330 µg/l *versus* 140 and 180 µg/l, respectively), and about 10-fold that of E*r*-20 (20 µg/l), is specified by a gene whose macronuclear copy number has been counted to be about 2-fold the copy numbers of the E*r*-2 and E*r*-10 genes ($2.5\text{-}2.9 \times 10^4$ *versus* $1.6\text{-}1.8 \times 10^4$ and $0.9\text{-}1.2 \times 10^4$, respectively) (La Terza *et al.* 1995), and about 10-fold the copy number of the E*r*-20 gene ($2\text{-}3 \times 10^3$) (unreported data). These gene-copy numbers hold independently of whether cells carry a homozygous or heterozygous allelic combination at their *mat* locus, and appear to be established at the beginning of the life cycle, when cells develop their new macronucleus from the products of the synkaryon divisions (La Terza *et al.* 1995).

In a functional context, these inter-mating type quantitative variations in pheromone production are usually regarded to be of secondary relevance. Most attention is in fact focused on the diversification in the pheromone structures that can more directly be associated with the cell capacity, or incapacity, to effectively interact to form mating pairs. Nevertheless, they greatly condition which type of mating pair - heterotypic or homotypic, i. e., between cells of *different* mating types, or of the *same* mating type - will prevalently, or exclusively, be formed in a mating mixture, both pair types being *equally* formed and "fertile" in *Euplotes* as in many other ciliates (Heckmann and Kuhlmann 1986) (only *Blepharisma* homotypic pairs are known to be "sterile" and to remain united for days without switching on any meiotic process). In mating type combinations involving cells characterized by similar rates of pheromone production, heterotypic pairing is preponderant. Instead, the combinations that involve cells with dissimilar rates of pheromone production usually generate a prevalence of homotypic pairs, and the proportions of these pairs relative to each one of the two mating types of the combination closely reflect the inter-mating type differences in pheromone production. In general, it holds that: the lower the amount of pheromone that cells secrete, the higher the proportion of homotypic pairs that they form, and, *vice versa*, the higher the amount of pheromone secreted, the lower the proportion of homotypic pairs formed.

These observations have a close counterpart in the measures of pheromone activity, that traditionally are only referred to the paracrine (sexual) pheromone activity and, hence, based on mating inducing assays carried out (according to a procedure originally devised for *Blepharisma* gamones) by monitoring the minimal concentration required for a given pheromone to induce the formation of at least one mating pair between cells secreting another pheromone (Miyake 1981). However, assays carried out with cells suspended in the (virtual) absence, or in the presence of their secreted pheromone, give rise to values of pheromone activity that may change by even three orders of magnitude, i. e., from a range between 10^{-13} and 10^{-11} M in the former case to a range between 10^{-9} and 10^{-8} M in the latter, that is also the range in which fall the values of pheromone binding affinities assessed on the basis of *in vitro* analysis (Ortenzi and Luporini 1995).

Physicochemical features, cytoplasmic precursors, and primary structures of *Euplotes* pheromones

Twenty unique amino acid sequences of *Euplotes* pheromones have so far been structurally determined, either by direct chemical analysis of purified protein preparations, or molecular cloning of the relevant coding genes: nine of 37-51 residues from *E. raikovi* (Raffioni *et al.* 1992, Luporini *et al.* 1995, Vallesi *et al.* 1996, Di Giuseppe *et al.* 2002), nine of 85-109 residues from *E. octocarinatus* (Brünen-Nieveler *et al.* 1991, 1998; Meyer *et al.* 1991, 1992; Möllenbeck and Heckmann 1999), and two of 52-60 residues from (a single strain of) *E. nobilii* (Alimenti *et al.* 2002, 2003a). While pheromones of *E. octocarinatus* belong to strains showing intra-specific mutual mating compatibility (i. e., cells of a strain can mate with, and their pheromones can induce mating between, cells of any other strain), *E. raikovi* pheromones belong to two distinct groups of strains that are mutually mating incompatible, and denoted "PR" and "GA" (with reference to their collection sites, i. e., Porto Recanati and Gaeta on the Eastern and Western coasts of Italy, respectively). In practice, pheromones (E*r*-1, E*r*-2, E*r*-7, E*r*-10, and E*r*-11) of the PR strains can induce mating between PR cells, not between GA cells (E*r*-11 representing a partial exception); similarly pheromones (E*r*-20, E*r*-21, E*r*-22, and E*r*-23) of the GA strains can induce mating between GA cells, but not between PR cells (cells secreting E*r*-11, again, repre-

senting a partial exception). Because of this behavioral (mating) strain diversification, *E. raikovi* pheromones are also regarded as classifiable into distinct protein subfamilies, particularly apt to reveal the structural specificities that condition their varied spectra of activity (Vallesi *et al.* 1996).

As summarized in Table 1 *Euplotes* pheromones are all rich in cysteines (typically, six in *E. raikovi*, eight in *E. nobilii*, and ten in *E. octocarinatus*), and possess acidic isoelectric points (between 3.25 and 4.08). Only *E. nobilii* pheromones denote appreciable differences in polar and hydrophobic amino acid composition in relation with their cold-adaptation (Alimenti *et al.* 2003b), that usually requires a reduction of the protein hydrophobicity associated with increased solvent accessibility and molecular flexibility (e. g., Marshall 1997). They are in fact significantly more polar and hydrophilic than *E. octocarinatus* and *E. raikovi* pheromones, and show a mean value of the aliphatic index (indicative of the degree of thermostability) that is markedly lower than in the two other species (i. e., 24.1 *versus* 50.3 and 65.4, respectively).

As occurs in many secreted protein hormones, also *Euplotes* pheromones (as well as the glycoprotein G-1 of *B. japonicum*) are synthesized as inactive protein precursors (pre-pro-pheromones), that denote a typical signal-peptide/pro-segment/mature-protein construction from which the active pheromones have to be liberated by proteolytic cleavages of the signal-peptide and pro-segment (Miceli *et al.* 1989, 1991; Meyer *et al.* 1991; Sugiura and Harumoto 2001). A salient feature of these pheromone precursors (not yet known in *E. nobilii*) is a remarkable degree of intra- and inter-specific sequence identity that distinguishes both the signal-peptide (to a major extent) and the pro-segment (to a minor extent) from the secreted pheromone forms that are themselves rather variable. This strict structural conservation of the signal-peptide and pro-segment has presumably functional reasons, correlated with the production of membrane-bound pheromone isoforms that in *E. raikovi* have been identified as the cell effective pheromone binding sites and receptors (Ortenzi *et al.* 2000). These isoforms are co-synthesized together with the secreted forms through a process of differential splicing of the primary transcripts of the pheromone genes and incorporate the entire pheromone precursor at their carboxyl region, while extending their amino terminal region by the addition of a new sequence (Miceli *et al.* 1992, Di Giuseppe *et al.* 2002). Due to this incorporation, the signal-peptide and pro-segment become the isoform (pheromone-receptor) trans-membrane and anchoring domains; it thus appears necessary to impose them unique conformational changes to avoid enzymatic processing and removal.

Upon looking closer at the multiple sequence alignment of the pheromone precursors, shown in Figs 1 and 2, it appears that the signal peptide sequences contain no amino acid substitution at all in *E. octocarinatus* and only a dozen substitutions in *E. raikovi*, furthermore mostly concentrated in pheromone Er-23 that represents a somewhat deviant member of the *E. raikovi* pheromone family (as discussed below). At the inter-species level, they present closely comparable lengths (19 residues in *E. raikovi versus* 16 in *E. octocarinatus*) and more than 50% positions that are fully conserved. Functionally, all the canonical signal-peptide features are therein recognizable, including a crowding of positively charged and hydrophobic residues and a processing site for signal peptidases represented by the dipeptide Ala-Phe.

The degree of the pro-segment sequence identity in *E. octocarinatus* is such that there is only one substitution throughout the first 29 positions (the structurally "eccentric" pheromone Phr4 excluded); while in *E. raikovi* (Er-23 excluded) it varies from 50 to 94%. At inter-specific level, the variations appear limited to: (i) the double length that the pro-segment acquires in *E. octocarinatus* in respect to *E. raikovi* (basically 36 *versus* 18 residues); (ii) the processing site for the release of mature pheromone forms, that changes from the *E. raikovi* dipeptide Arg-Asp (with three substitutions of Arg with Gln, Gly, or Lys, and one of Asp with Gly) to the *E. octocarinatus* dipeptide Lys-Asp/Tyr/Gly. Nevertheless, in both species there is a repetition (from two times in *E. raikovi* to six in *E. octocarinatus*) of the motif Arg/Lys-Xxx-Xxx-Ser (with occasional changes of Arg/Lys with Gln or Met and of Ser with Glu or Thr), that may be of common functional importance for post-translational modifications.

In the secreted pheromones, the degree of sequence identity drops drastically, even to reach intra-specific values as low as 25-30% that are usually seen as a twilight zone to infer true relationships of sequence homology. It seems likely that at the origin of the pheromone diversification between *E. raikovi* and *E. octocarinatus* there is an event of gene duplication. This is strongly suggested by the fact that, as is also the case of the pro-segment, the pheromone sequences in *E. octocarinatus* are practically twice as large as in *E. raikovi*.

Table 1. Basic physicochemical properties of *Euplotes* pheromones.

Pheromones	Total residues (n)	Cysteine residues (n)	Isoelectric point pI	Charged residues (%)	Polar residues (%)	Hydrophobic residues (%)	Glycine residues (%)	Aliphatic index[1]
Er-1	40	6	3.71	20.0	45.0	30.0	5.0	53.75
Er-2	40	6	3.83	12.5	42.5	35.0	10.0	44.00
Er-7	40	6	3.83	12.5	45.0	35.0	7.5	46.50
Er-10	38	6	4.08	18.4	42.1	31.6	7.9	51.32
Er-11	39	6	3.45	12.8	41.0	43.6	2.6	87.69
Er-20	37	6	3.33	13.5	45.9	37.8	2.7	84.32
Er-21	37	6	3.37	10.8	40.5	45.9	2.7	108.11
Er-22	37	6	3.77	16.2	40.5	40.5	2.7	89.73
Er-23	51	10	3.33	9.8	50.9	21.6	17.6	23.14
En-1	52	8	3.25	11.5	59.6	23.0	5.8	18.85
En-2	60	8	3.50	15.0	50.0	25.0	10.0	29.33
Phr1	99	10	3.58	20.2	40.4	34.3	5.1	70.00
Phr1*	98	10	3.56	15.3	44.9	33.7	6.1	64.80
Phr2	101	10	3.52	19.8	42.6	29.7	7.9	43.47
Phr2*	102	10	3.41	17.6	43.1	31.4	7.8	47.84
Phr3	99	10	3.88	21.2	47.5	26.3	5.1	48.28
Phr3*	99	10	3.96	19.2	52.5	22.2	6.1	43.33
Phr4	85	8	3.64	15.3	43.5	34.1	7.1	38.00
Phr5 [1]	108	10	3.92	13.9	47.2	28.7	10.1	48.70
Phr5 [2]	109	10	3.49	14.7	44.9	28.4	11.9	48.26

Er, En, and Phr pheromones are from *E. raikovi*, *E. nobilii*, *E. octocarinatus*, respectively. [1]Calculated according to Ikai (1980), as relative volume occupied in globular proteins by aliphatic side chains.

Conservation is substantially limited to the amino-terminus usually occupied, in *E. raikovi* in particular, by an Asp residue (that likely serves as recognition for the proteolytic pro-pheromone processing), and to the cysteine positions. The retention of these positions is obviously maximal at the intra-specific level (with the partial exception of *E. octocarinatus*), and less stringent at the inter-specific one. In the case of *E. nobilii* and *E. raikovi*, that are species with close phylogenetic kinship (Di Giuseppe and Dini, personal communication), the six Cys residues (designated I to VI) distinctive of *E. raikovi* pheromones (Er-23 with ten cysteines excepted) find their counterparts in six of the eight Cys residues of *E. nobilii* pheromones, thus implying an inter-species substantial retention of the disulfide bond pattern Cys-I/Cys-IV, Cys-II/Cys-VI, and Cys-III/Cys-V established in *E. raikovi* (Stewart *et al.* 1992). Between the pheromone families of *E. raikovi* and *E. octocarinatus*, that are species branching phylogenetically rather distant from each other (Bernhard *et al.* 2001, Petroni *et al.* 2002, Song *et al.* 2004), the adoption of some architectural motifs in common seems to be much more dubious. There is in fact only a convincing match of the disulfide bonded Cys-I, Cys-III, Cys-IV and Cys-V of *E. raikovi* pheromones with four of the six fully conserved Cys residues in *E. octocarinatus* pheromones.

Three-dimensional structures of *E. raikovi* pheromones

The molecular architecture was determined for six *E. raikovi* pheromones (of the nine characterized at the level of their primary sequences), i. e., Er-1, Er-2, Er-10, and Er-11 of the PR subfamily, plus Er-22 and Er-23 of the GA subfamily. These determinations were carried out by NMR spectroscopy (Brown *et al.* 1993; Mronga *et al.* 1994; Ottiger *et al.* 1994; Luginbühl *et al.* 1994, 1996; Liu *et al.* 2001; Zahn *et al.* 2001) and, in the case of Er-1, also by X-ray crystallography (Weiss *et al.* 1995). As illustrated in Fig. 3, these pheromones (except Er-23) show constructions that closely mimic one another, *regardless* of the extent of divergence that may

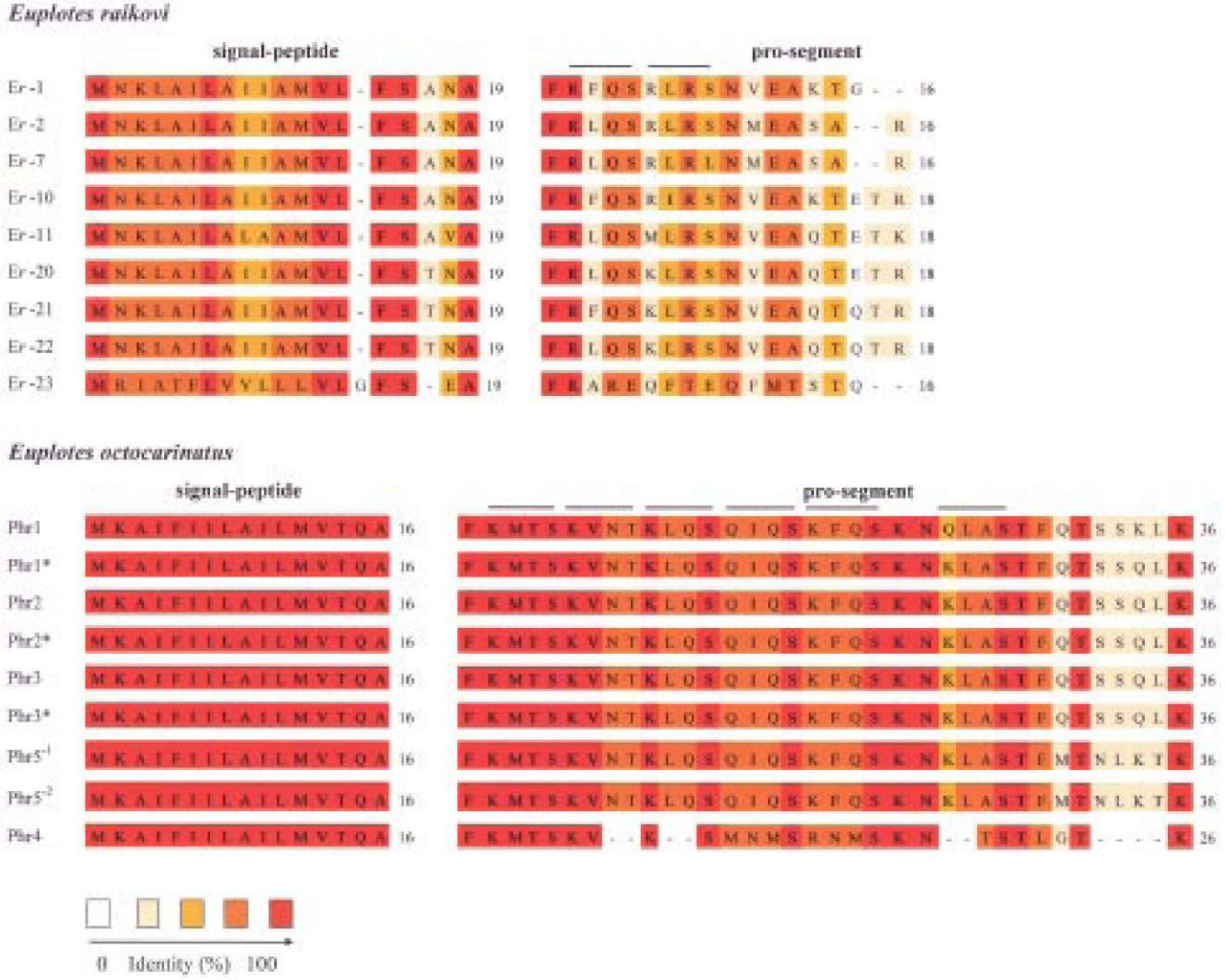
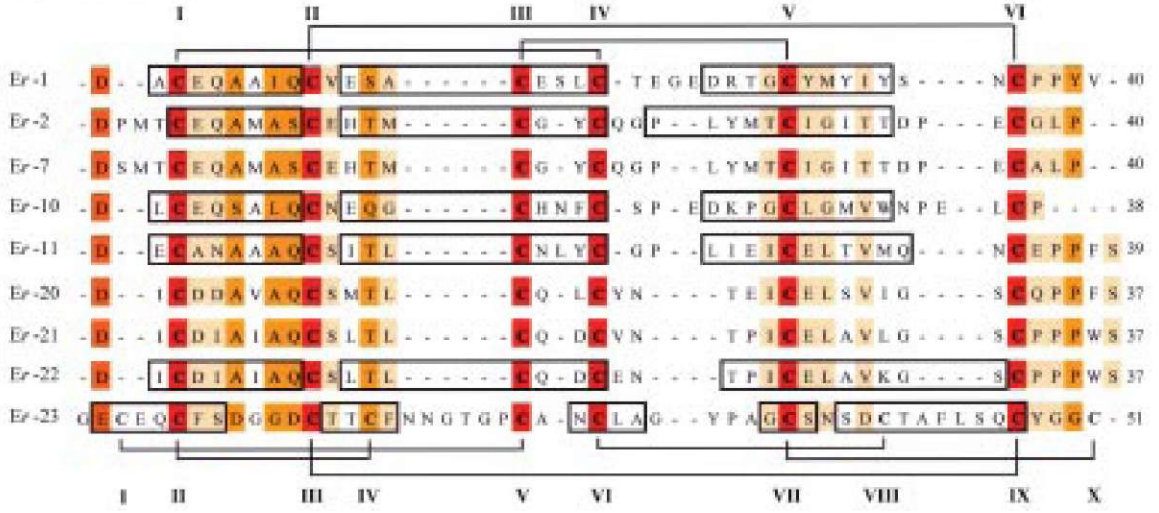


Fig. 1. Multiple sequence alignment of the *E. raikovi* and *E. octocarinatus* pheromone precursors. The alignment is based on the Clustal X program (Jeanmougin *et al.* 1998) and maximized by gap insertions. The numbers of residues of each sequence are reported on the right, and a motif repetition is indicates by lines. Sequence identities are colour-coded using a gradient from white (< 40% identity) to red (100% identity).

Fig. 2. Multiple sequence alignment of *E. raikovi*, *E. nobilii*, and *E. octocarinatus* secreted pheromones. Criteria for arranging sequence alignments and identities are as in Fig. 1. In the *E. raikovi* pheromones, the sequence segments organized in helical structures are enclosed in boxes, while the half-cystine residues are indicated by progressive roman numerals and connected by lines according to their disulfide pairings.

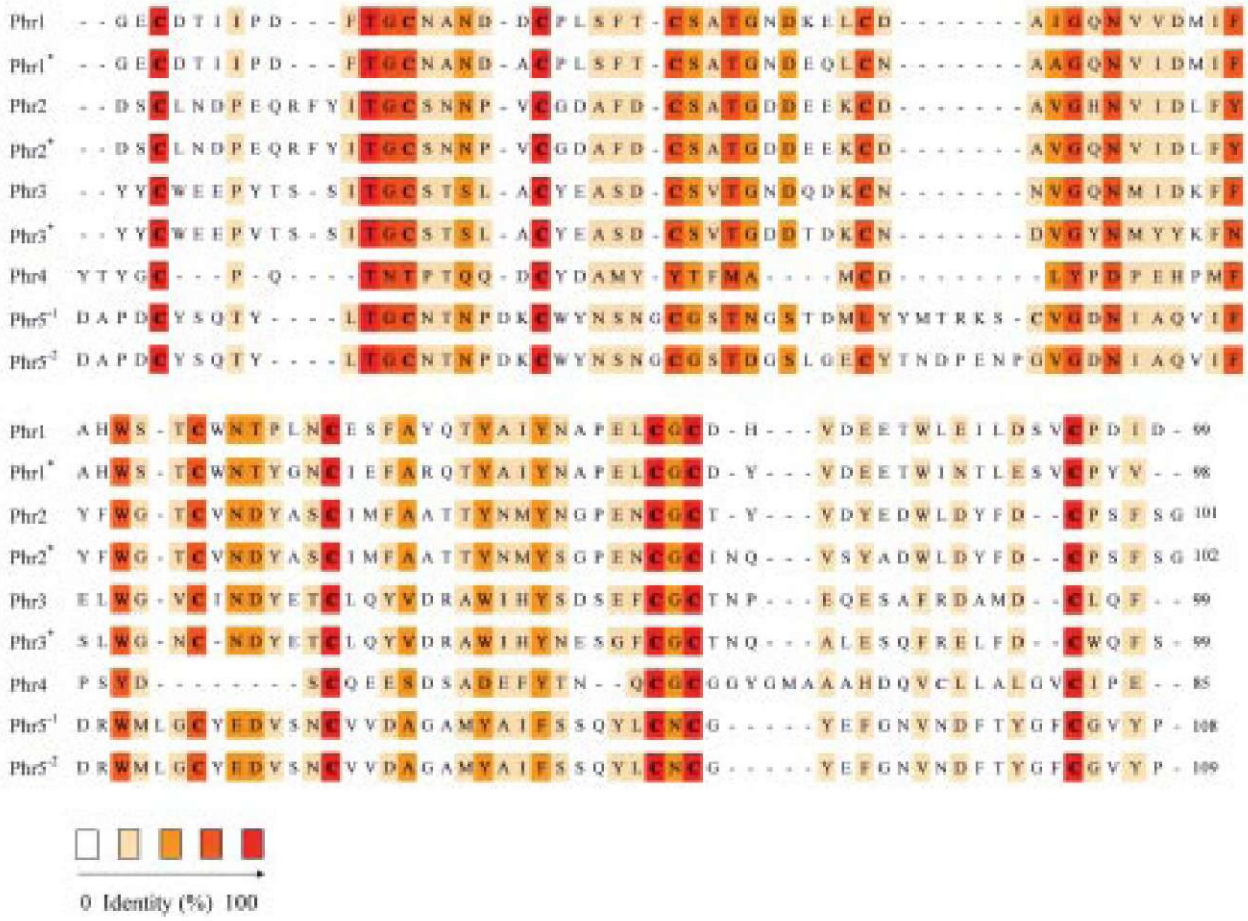
separate their primary sequences. These constructions all conform to a unique model shaped like a pyramid, in which the base is triangular and the vertical edges are provided by three helices, designated 1 to 3 starting from the molecule amino-terminus; they are united by two extended loops of 2-4 residues and show appreciably different lengths and an up-down-up topological orientation. Helices 1 and 3 are uniformly in regular α-organization, while helix 2 tends to be less regular and to appear as a short stretch of distorted 3_{10}-helix turns. Compactness and stability of each pheromone construction are ensured by the three strictly conserved disulfide bridges, two of which (Cys-I/Cys-IV, and Cys-III/Cys-V) connect helix 2 (facilitated in this connection by its

Euplotes raikovi



Euplotes nobilii

Euplotes octocarinatus



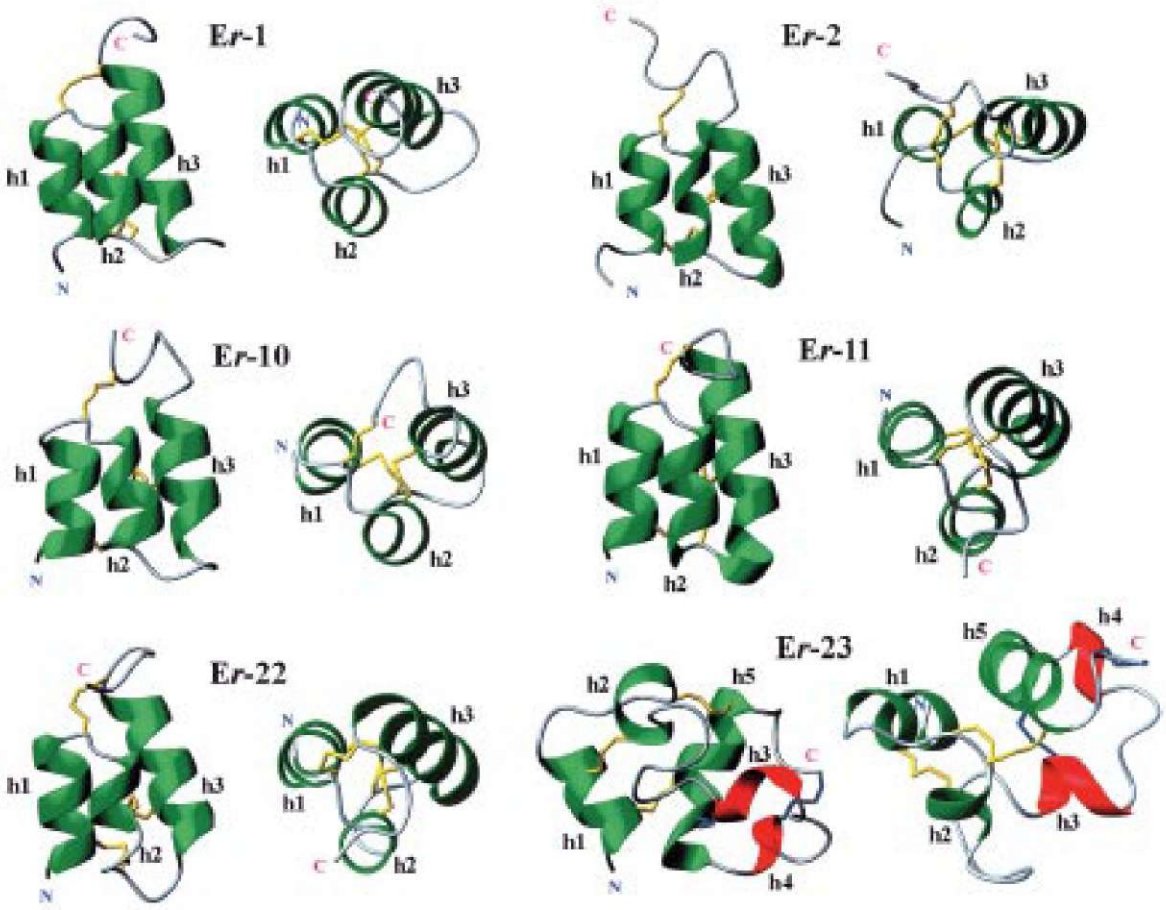


Fig. 3. Comparison of the three-dimensional structures of *E. raikovi* pheromones. Each pheromone is represented in frontal and top views prepared with the program MOLMOL (Koradi *et al.* 1996). The helices (h) are numbered progressively from the molecule amino-terminus (N) to the carboxy-terminus (C). The three helices conserved in all the molecules are coloured green and their three interconnecting disulfide bridges are shown in yellow. The two helices and the two disulfide bridges that are unique to Er-23 are coloured red and blue, respectively. The atom coordinates of each pheromone, deposited at the Protein Data Bank (PDB), are to following: Er-1, 1ERC; Er-2, 1ERD; Er-10, 1ERP; Er-11, 1ERY; Er-22, 1HD6; Er-23, 1HA8.

distorted structure that ensures a proper orientation for the disulfide bonds of its Cys III and Cys IV residues) to helices 1 and 3, while the third one (Cys-II/Cys-VI) ties the carboxy-terminal tail to the top of helix 1. The close spatial proximity of the two inter-helix disulfide bonds is such to cause a rather exceptional situation, i. e., in each pheromone the formation of quantitatively minor conformations represented by disulfide isomers generated by all the three possible disulfide pairings between any two of the four Cys residues I, III, IV, and V (Brown *et al.* 1993, Luginbühl *et al.* 1994).

On this common conformational backbone, every pheromone then locally imposes its personal hallmarks to acquire its own architectural specificity (as discussed in great detail by Luginbühl *et al.* 1994, for pheromones Er-1, Er-2, and Er-10). More direct contributions to the construction of these hallmarks, that are likely of great significance also for the pheromone function, appear to be provided by variations in the organization of: (i) helix 2, (ii) the loop connecting helices 2 and 3, and (iii) the carboxy-terminal tail immediately following the last (VI) Cys residue positioned at the end of helix 3. In any case it is this tail (nevertheless absent in Er-23, whose sequence ends with a disulfide bonded Cys residue) that more markedly than the other two structures assumes aspects varied enough - in relation to, (i) its length (spanning from one to five residues), (ii) spatial arrangement, and (iii) number of Pro residues (from one to three,

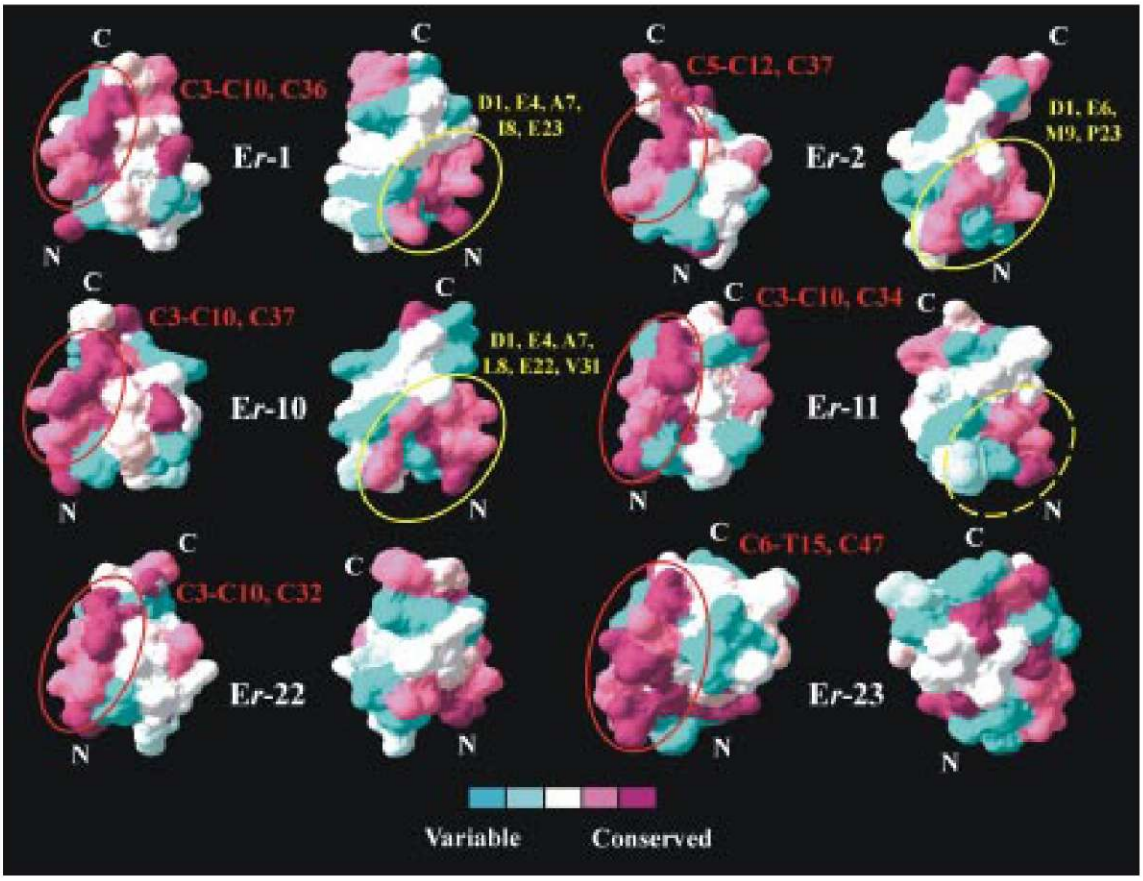


Fig. 4. Comparison of the surface conservation patters of *E. raikovi* pheromones, obtained by using the Rate4Site method (Pupko *et al.* 2002; Glaser *et al.* 2003). The molecular surfaces, prepared with the Swiss-PdbViewer program (Guex and Peitsch 1997), are color-coded as follows: dark violet, maximal conservation; white, average conservation; dark turquoise, maximal variability. The approximate spatial locations of the molecule amino- and carboxy-terminus are indicated by N and C, respectively. Each pheromone is presented in two orientations: in the left one, helices 1 and 2 are in the viewer front, while in the right one there is helix 3. However, in Er-1, Er-2, Er-10, Er-11, and Er-22, the axis of helix 1 in nearly vertical, while in Er-23 this axis is rotated approximately 90° counter-clockwise in the left orientation, and approximately 90° clockwise in the right orientation. The surface patch conserved in all pheromones is delimited by red ovals, while the surface patch conserved in pheromones of the PR subfamily (Er-1, Er-2, Er-10, and Er-11) and not conserved in pheromones of the GA subfamily (Er-22 and Er-23) is delimited by yellow ovals (in Er-11, dashed line indicates a less extended conserved patch). The amino acid residues yielding the conserved surfaces are reported jointly with the numbers indicating their positions in the primary structures.

and causing the appearance of conformational isomers containing *cis*- and *trans*-peptide bonds) - to be at the same time distinctive of every single pheromone and discriminative between the PR and GA pheromones. In Er-1, Er-2 and Er-10, that are more typical members of the PR subfamily, the tail terminal residue appears similarly settled over the top of helix 3; instead, in Er-22, representative of the GA subfamily, it appears dislocated over the top of helix 2. Intriguingly, an Er-22-like dislocation is shown also by Er-11 that, although a member of the PR subfamily, still manifests a weak capacity to induce mating between cells of the GA strains.

The Er-23 case

Notwithstanding that Er-23 is, (i) synthesized by a structurally complete gene regularly amplified to thousands of macronuclear copies, (ii) secreted in amounts (50-60 µg/l) comparable with the other pheromones from the same wild-type strain that co-releases Er-22 (hence, a strain heterozygous at its *mat* locus for the two relevant coding genes), and (iii) participates effectively in cell signaling between cells of the GA strains, it is set quite apart from the standard structure of the other *E. raikovi* pheromones because of its sequence elonga-

tion from 37-40 to 51 residues and its increase in the cysteine number from six to 10 (Di Giuseppe *et al.* 2002). Clearly (see Fig. 3), also the molecular architecture of Er-23 markedly deviates from the basic fold of the *E. raikovi* pheromone family, as it has to accommodate the addition of the new stretch of 11 residues (Zahn *et al.* 2001). This accommodation essentially occurs at level of the central domain of the Er-23 architecture, and determines a new fold based on the presence of five helices (rather varied in extension and organization) that become fastened together by five disulfide bridges provided by the following Cys-residue combinations: I-V, II-IV, III-IX, VI-VIII, and VII-X.

Although the Er-23 three-dimensional structure is unique, also this structure appears to be reconcilable with the three-helix bundle topology distinctive of the *E. raikovi* pheromone family (Zahn *et al.* 2001). Its helices 1, 2, and 5 are in fact arranged in an up-down-up fashion just like the three helices distinctive of the family model (see Fig. 3), and their connections are ensured by three disulfide bonds (i. e., Cys-I/Cys-V, Cys-II/Cys-IV, and Cys-III/Cys-IX) that are spatially equivalent with those of the other family members.

Even more convincing support for this structural reconciliation of Er-23 with all the other *E. raikovi* pheromones is provided by a comparative analysis of the functionally important regions exposed on the surface of the whole set of the determined pheromone structures. This analysis is based on algorithmic tools, of which the original one is referred to as "Conservation Surface-mapping" (ConSurf) (Armon *et al.* 2001) and an implemented version of it as "Rate4Site" (Pupko *et al.* 2002, Glaser *et al.* 2003), that fit extremely well with the case of the *E. raikovi* pheromone family. They in fact map the rate of evolution of the molecular surface of homologous proteins (better, if this homology is founded on recognized family links) with known three-dimensional structures, and their ultimate target is the identification of surface hot spots and patches that are likely to be in effective contact with other protein domains, nucleic acids, or ligands. Clearly, the rationale underneath these tools is that surface residues of key importance for the protein binding activity should be conserved throughout evolution, just like buried residues that are crucial for the maintenance of the protein fold.

As shown in Fig. 4, the most pronounced conserved surface domain, generated by a cluster of 8-10 residues, appears in common between Er-23 and all the other pheromones. In Er-23, these residues are provided by the second half of helix 1, helix 2, the helix-1/helix-2 connecting loop, and include Cys-IX; in all the other pheromones, they are provided by helix 1 and include Cys-VI. It appears, in addition, that Er-23, like its subfamily member Er-22, lacks another principal surface domain that, instead, is conserved in the members of the PR subfamily (significantly, to a major extent in Er-1, Er-2, and Er-10, and to a minor extent in Er-11), and is formed by a cluster of residues positioned in the cleft delimited between the internal faces of helices 1 and 2.

Conclusions

The experimental utilization of *Euplotes* has determined decisive progress in our knowledge of the structure and biology of ciliate pheromones, for long limited only to the "gamones" of *Blepharisma*, i. e., a ciliate to be regarded as rather "eccentric" for a number of aspects (first of all, functional and genetic) of its mating types (granted that intra-specific mating type systems did actually evolve in *Blepharisma* and its relative heterotrichs, all of which appear to systematically pursue intra-specific "selfing" in nature). This progress widens our view on the evolutionary significance of ciliate mating type systems, and permits us to better appreciate that the conceptual and functional complexity of these systems is far from the one that is binary and complementary of sex but, rather, appears close to the multiple and polymorphic one of self/non-self recognition systems evolved in multi-cellular life forms.

The characterization of the three-dimensional conformations of a significant number of pheromones from *E. raikovi*, in which mating types have a clearly established genetic basis, has, in particular, made it possible to appreciate visually the major implication that arises from Mendelian analyses of the ciliate mating type systems, i. e., that ciliate pheromones are cell type-specific markers represented by intra-specific families of *structurally homologous* proteins. This structural pheromone homology strongly argues against a model of pheromone activity based on the interaction, uniquely finalized to a cell mating (sexual) purpose, between a pheromone of one cell type and a cognate receptor of it carried by another cell type (Miyake 1981, 1996). Rather, it suggests that pheromones are to be regarded as signal molecules capable of binding, in competition with one another, to each others' cognate receptors; hence, capable of eliciting a variety of context-dependent cell responses, as is the case of cytokine and growth factor networks in more complex multi-cellular organisms.

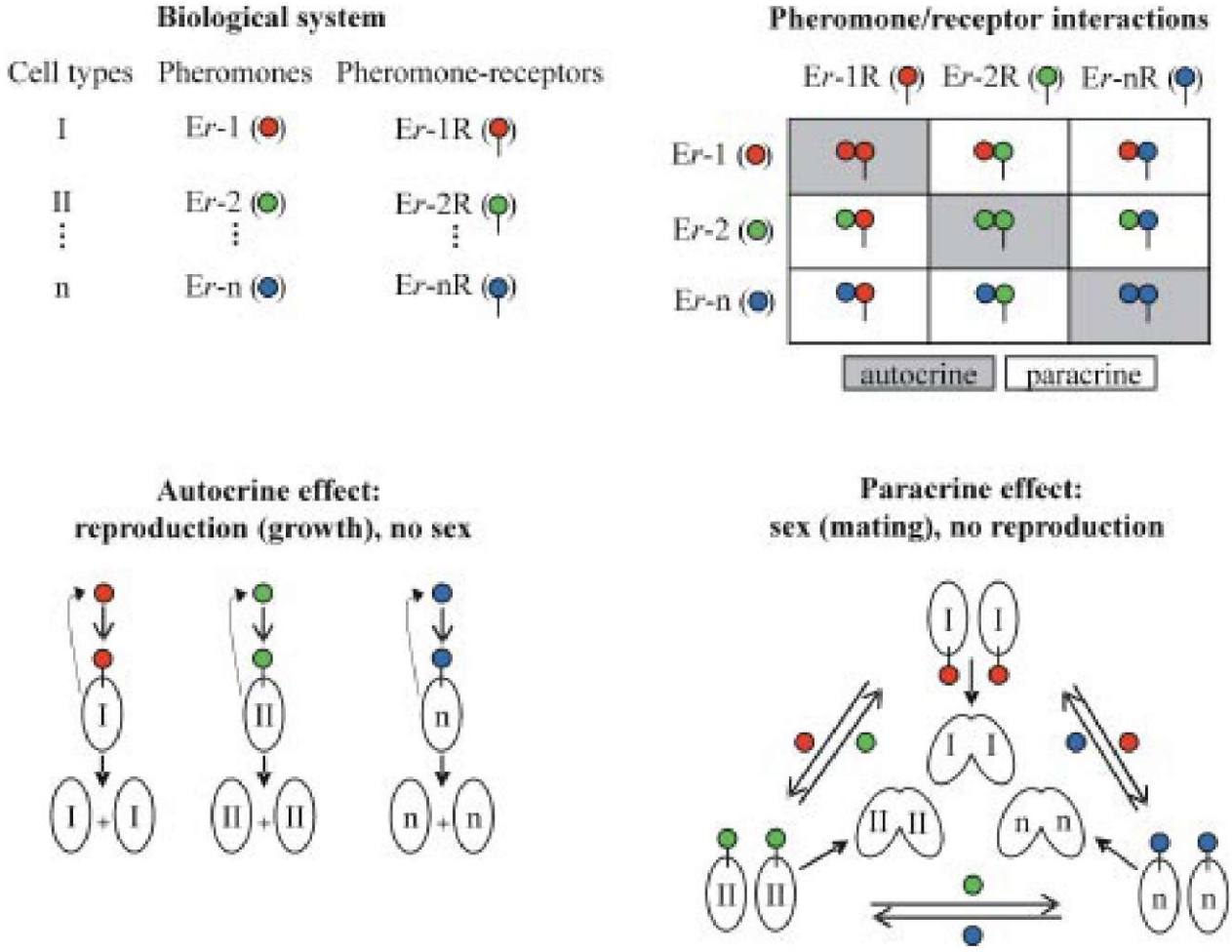


Fig. 5. Summarizing picture of the pheromone biology of *E. raikovi*. The multiple cell (mating) types of the biological system (I, II, … n) are each one represented distinct from the others by its secreted pheromone *and* cognate membrane-bound receptor, whose extracellular domain is structurally equivalent (same color) with the pheromone (both molecules arising from the same gene via alternative splicing). The other panels visualize any possible autocrine (homotypic) and paracrine (heterotypic) pheromome/pheromone-receptor interactions and the relevant effects of these interactions.

In *E. raikovi*, in addition to acting as mating signals (paracrine activity); pheromones have been shown able to promote the vegetative (mitotic) proliferation of the same cells from which they are secreted (Vallesi *et al.* 1995). The pre-requisite to carry out this autocrine mitogenic activity, that we can reasonably regard as being evolutionarily and biologically primary in respect to the paracrine one associated with sex, is that cells secreting a pheromone must be able to synthesize also the receptor for this pheromone. The meeting of *E. raikovi* with this requirement represents, at the same time, an insightful instance of parsimony and elegance, and a unique key to explain how new functional mating types (i. e., cells distinguished by a new pheromone and its cognate receptor) can arise (and also how "old" mating types can disappear) in those mating-type systems which are usually classified, in particular in *Euplotes* and other hypotrichs, as "open" because of the virtually unlimited number of different mating types that they comprise (Ammermann 1982, Valbonesi *et al.* 1992, Dini and Nyberg 1993). By alternatively splicing its primary transcripts, each pheromone gene generates *both* a soluble pheromone (whose release into environment requires proteolytic processing of the cytoplasmic precursor) and its cognate receptor represented by a longer pheromone isoform that utilizes the un-cleaved signal peptide of the cytoplasmic precursor to remain anchored to the cell surface (Miceli *et al.* 1992, Ortenzi *et al.* 2000). The extra-cellular domain of this receptor, in each cell type structurally equivalent to the secreted phero-

mone, functions as ligand (pheromone) binding site (Weiss *et al.* 1995, Ortenzi *et al.* 2000); the structurally unique intra-cellular domain contains sequence motifs that are an object of research interest for enzymatic and transduction activities.

Thus, in conclusion, as diagrammatically summarized in Fig. 5, *Euplotes* (and reasonably other ciliates as well) proves to have very proficiently invested in its pheromone/mating-type systems to control both the basic (quantitative and qualitative) aspects of life, i. e., reproduction and sex. To reproduce (i. e., multiply mitotically), or to mate (i. e., engage in a sexual process with inevitable detriment to reproduction) is an alternative that cells can apparently decide in relation to which type of pheromone-receptor association, i. e., autocrine *versus* paracrine, turns out (in the time and space) to be more effective on their surface. Insightful information on how this association occurs at the molecular level essentially relies on the determination of the Er-1 pheromone crystallographic structure (Weiss *et al.* 1995), and functional analyses of Er-1 binding to recombinants of the membrane-bound Er-1 isoform (receptor) (Ortenzi *et al.* 2000). The challenge for the future is to strengthen this information and, more demanding, shed light on the regulatory molecules and events underlying these varied, i. e., reproductive and sexual, cell responses.

Acknowledgments. This review is dedicated to Prof. Kurt Wüthrich (ETH, Zurich), Nobel Prize for Chemistry in 2002. His and his collaborators' contribution to the determination of pheromone three-dimensional structures was fundamental to our progress in understanding the biology of these molecules and, more in general, the evolutionary significance of the ciliate mating types. Research on pheromones of *E. raikovi* and *E. nobilii* was financially supported by grants from the Ministero dell'Istruzione, dell'Università e della Ricerca, and the Programma Nazionale di Ricerche in Antartide.

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ACTA
PROTOZOOLOGICA

Morphology, Biometry and Distribution of *Difflugia biwae* Kawamura, 1918 (Protozoa: Rhizopoda)

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Summary. The freshwater testate amoeba *Difflugia biwae* Kawamura, 1918, isolated from Mulan Lake, Hubei Province, China is investigated using light microscopy and scanning electron microscopy. The morphology, biometry and distribution of this little known species are supplied. After careful comparison with three other similar species, including *D. delicatula* Gauthier-Lièvre *et* Thomas, *D. elegans* Penard and *D. oblonga caudata* Štěpánek, we believe that the characteristics of smooth fusiform shell, conspicuous great collar flare (always larger than body-width) around the aperture, constriction behind the aperture and a somewhat curved aboral horn set *D. biwae* apart from all other *Difflugia* species. This species shows a great diversity in total length, collar height and aboral horn length which have high variability (CV between 11.76 and 24.52). However, body width, collar diameter, neck width, body length and aperture diameter are fairly constant with low variability (CV between 5.34 and 8.79) which shows a remarkable uniformity of *D. biwae*. Also, the size frequency distributions of both body width and body length yield bell-shaped (normally distributed) curves and indicate that *D. biwae* is a size-monomorphic species, characterized by a main-size class and a small size range. *D. biwae* is probably endemic to East Asia (China and Japan) because it has such a large size (165-306 μm) that it would have been easily found in Europe and North America, if it were there. Consequently, *D. biwae* must have a restricted geographical distribution, disproving the old hypothesis that microscopic organisms are cosmopolitan.

Key words: biometry, *Difflugia biwae*, distribution, morphology, Testacea.

INTRODUCTION

Difflugia biwae Kawamura, 1918 was first found in Lake Biwa, the largest freshwater lake in Japan (Kawamura 1918). Subsequently, "*D. biwae* is endemic to Lake Biwa, Japan" has been a fundamental paradigm for nearly a century (Toshihiko 1979, Nishino and Watanabe 2000, Tsugeki *et al.* 2003). However, *D. biwae* has not been seen since 1987 in the waterbodies of Lake Biwa (Ichise *et al.* 1996). More recently, Tsugeki *et al.* (2003) showed that the remains of *D. biwae* occurred abundantly before 1960, but decreased dramatically thereafter and were not found at all after 1980 in the sediments of Lake Biwa. Thus the fact that *D. biwae* seems to be extinct in Lake Biwa was affirmed in both the waterbodies and the sediments.

A very short description of *Difflugia biwae*, together with a simple line drawing, was originally supplied in Japanese by Kawamura (1918, 1927). Many years later, both the description and the line drawing of *D. biwae* have been presented in more than three monographs in

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Japanese (Abe *et al.* 1957, Toshihiko 1979, Okada *et al.* 1981). However, the later data gave us no more information than those reported by Kawamura in the original literature. Unfortunately, little emphasis has been given to detailed study of its morphometrical characterization in all previous investigations. Further, the comparisons of this species with similar species were never perfectly defined. Interestingly, we have observed abundant material of living specimens of *D. biwae* with a high population density in Mulan Lake, Hubei Province, China during our investigation on the testate amoebae of the Yangtse River Valley. The purposes of this work are (1) to characterize morphologically and biometrically *D. biwae* using abundant material, and (2) to discuss the geographical distribution of *D. biwae*.

MATERIALS AND METHODS

Difflugia biwae Kawamura was collected from Mulan Lake, Hubei Province, China on 24-25 July, 2003, and on 28-29 July, 2004. The information of the lake was given by Yang *et al.* (2004). Specimens for light microscopy (Zeiss Axioplan 2 imaging System, Germany) and scanning electron microscopy (X-650 HITACHI, Japan) observations were prepared following the procedure of Yang *et al.* (2004). To visualize the number and position of the nucleus, we incubated the samples with 1.0 μg/ml 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in PBS for 10 min and viewed them under UV fluorescence.

Eight morphometric characters were portrayed in Figs 1 and 2. All measurements were made at middle magnification (320x) using an ocular micrometer. Statistical analysis was performed using the computer program STATISTICA, version 6.0.

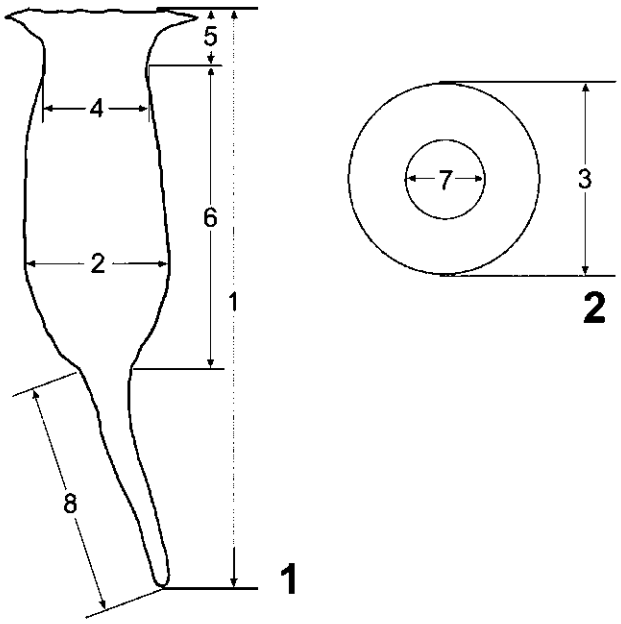
RESULTS AND DISCUSSION

Morphology

The shell is brown, fusiform, 2.1-3.7 collar diameters in body length (Figs 3-5, 15). Surrounding the circular oral aperture is a conspicuous low-funnel-shaped (95°-125°) collar with a ragged margin (Figs 3-6, 15); the body is circular in cross section, narrowest below the collar and gradually swelling to broadest in the position of the posterior 0.20-0.25 of the body length, then narrowing abruptly towards the aboral horn, its greatest diameter 0.60-0.98 collar diameter (Figs 3-5, 15); the aboral horn is stout, tubular and somewhat curved, variable in length, 0.16-0.50 of the total length, and gradually tapering to a blunt tip (Figs 3-5, 7, 8, 15). One to seven colourless long finger-like pseudopodia from the protoplasmic body may protrude through the aperture (Figs 8, 10). A gas vacuole was often observed in living specimens (Fig. 8). Such specimens can right themselves either by extrusion of the long pseudopodia attaching to the substrate (Fig. 10), or by a combination of the pseudopodia and gas vacuole formation. The cytoplasm commonly occupies the greater part of the shell cavity and usually attaches to the internal walls by one or more threads of ectoplasm (Fig. 11); sometimes the cytoplasm can extend into the aboral horn (Fig. 12). Reproduction was sometimes encountered, but the evidence (two shells joined at their oral apertures, Fig. 13) could not be used to determine if it was sexual or asexual because the high optical density of the shells obscured details of the nuclei in these specimens. Although the shell is only semitransparent or opaque, the fluorescent light micrograph stained with DAPI clearly shows that single spherical nucleus is generally located in the posterior of the protoplasmic body and a few tiny algae are sometimes attached to the surface of the shell (Fig. 14). The nucleus is ovular with many nucleoli and has a diameter range of 25-30 μm (Fig. 9).

Study under light microscopy reveals the shell wall is thin, uniform in thickness, composed of fine sand granules with flattish pieces of quartz and muddy particles to produce a smooth surface (Figs 3-5). It has been illustrated by scanning electron microscopy that shells are covered with variously shaped particles, apparently of exogenous origin as determined from the irregularity of the shapes of included particles (Figs 16-18). However, the details of the surface of the shell are visibly different along the anterior body, the posterior body and the aboral horn. The reason is because the particles constituting the anterior portion of the shell covering are small to medium, angular and polymorphic to give a smooth appearance (Fig. 16); the posterior region is composed of small angular particles and medium flattish pieces of quartz to give a smoother appearance (Fig. 17); the aboral horn is made of small flattish pieces of quartz to give the smoothest appearance (Fig. 18). It appears that *Difflugia biwae* is able to select and arrange the building material according to size and shape to construct a species-specific shell. This is in good agreement with that reported by Meisterfeld (2000) in the genus *Difflugia*. No cement structures are recognizable in the scanning electron microscope.

In this study, we found that the typical shape of the shell of *D. biwae* in lateral view is fusiform in outline with a straight neck, a conspicuous great collar flare and a long aboral horn (Fig. 3). Furthermore, the morphological characters of the typical specimens from Mulan



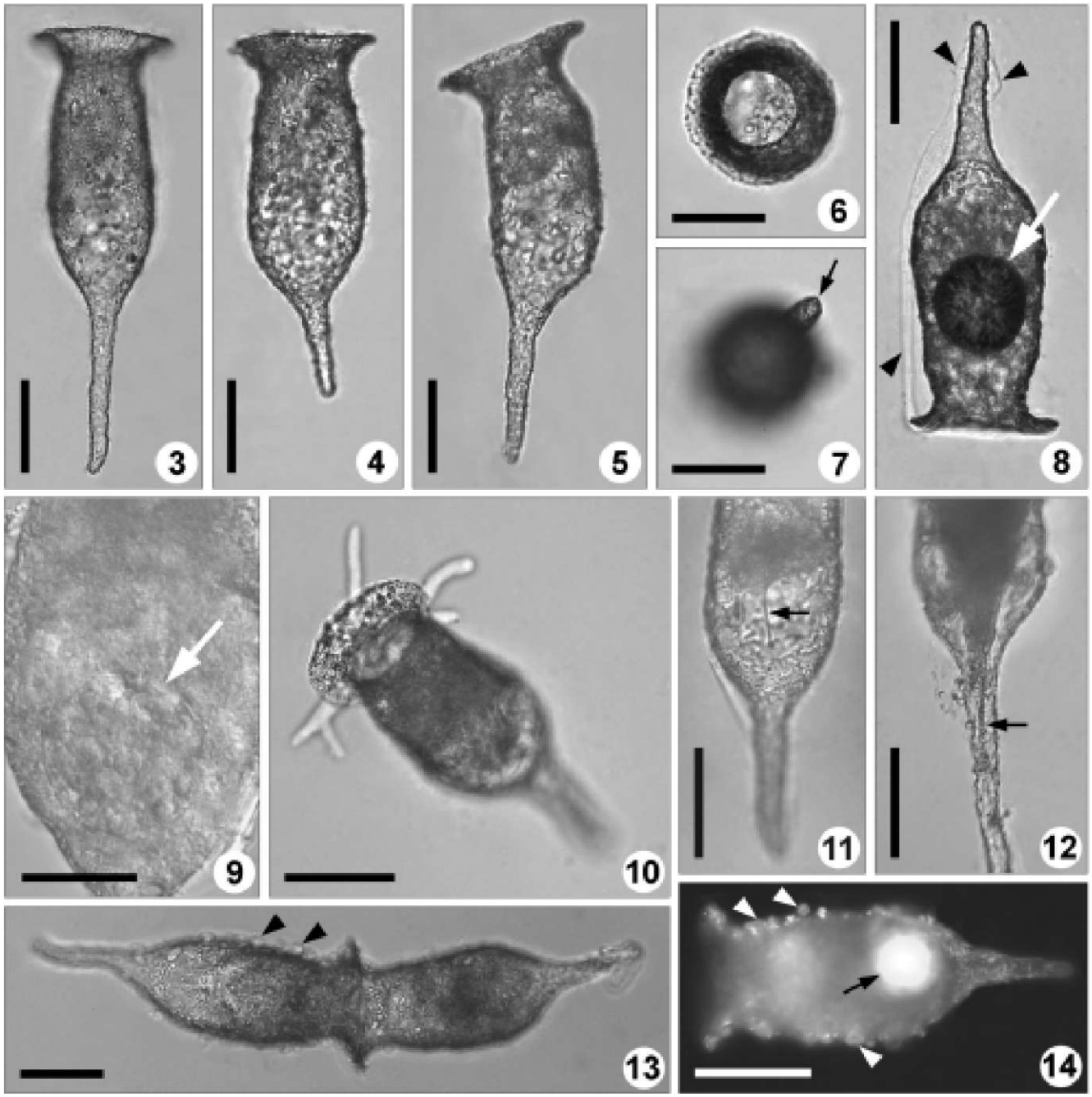
Figs 1, 2. Shell outline and position of measured axes used in this study. 1 - total length; 2 - body width; 3 - collar diameter; 4 - neck width; 5 - collar height; 6 - body length; 7 - aperture diameter; 8 - aboral horn length.

Lake agree well with those reported from Lake Biwa (Kawamura 1918, 1927). However, none of the previous descriptions touched on the atypical shapes of the shell of *D. biwae* (Kawamura 1918, 1927; Abe *et al.* 1957; Toshihiko 1979; Okada *et al.* 1981). Our examination suggests that among the atypical shapes found are those with a short aboral horn (Fig. 4) and those with an oblique neck (Fig. 5). Nevertheless, the population from Mulan Lake bears a strong resemblance to the original in terms of the following characteristics: its fusiform smooth shell furnished with a conspicuous great collar flare (always larger than body-width) around the aperture and a usually curved aboral horn, its long thin pseudopodia, its single spherical nucleus located in the posterior position and its lacustrine habitat (Kawamura 1918, 1927). Unfortunately, Kawamura (1918, 1927) was not very precise in the original illustration of the pseudopodia of living specimens in *D. biwae*, and because of this its typical locomotive form on the substrate was overlooked. The resting form of the specimens as shown in Fig. 8 is similar to that already illustrated by Kawamura (1918, 1927). One or two pseudopodia always extend from the aperture to the back of the shell. In fully extended condition, the pseudopodia may exceed the point of the aboral horn along the shell (Fig. 8). During locomotion two to seven long, sometimes branched, finger-like pseudopodia are seen to be projected out from the aperture (Fig. 10).

Biometry

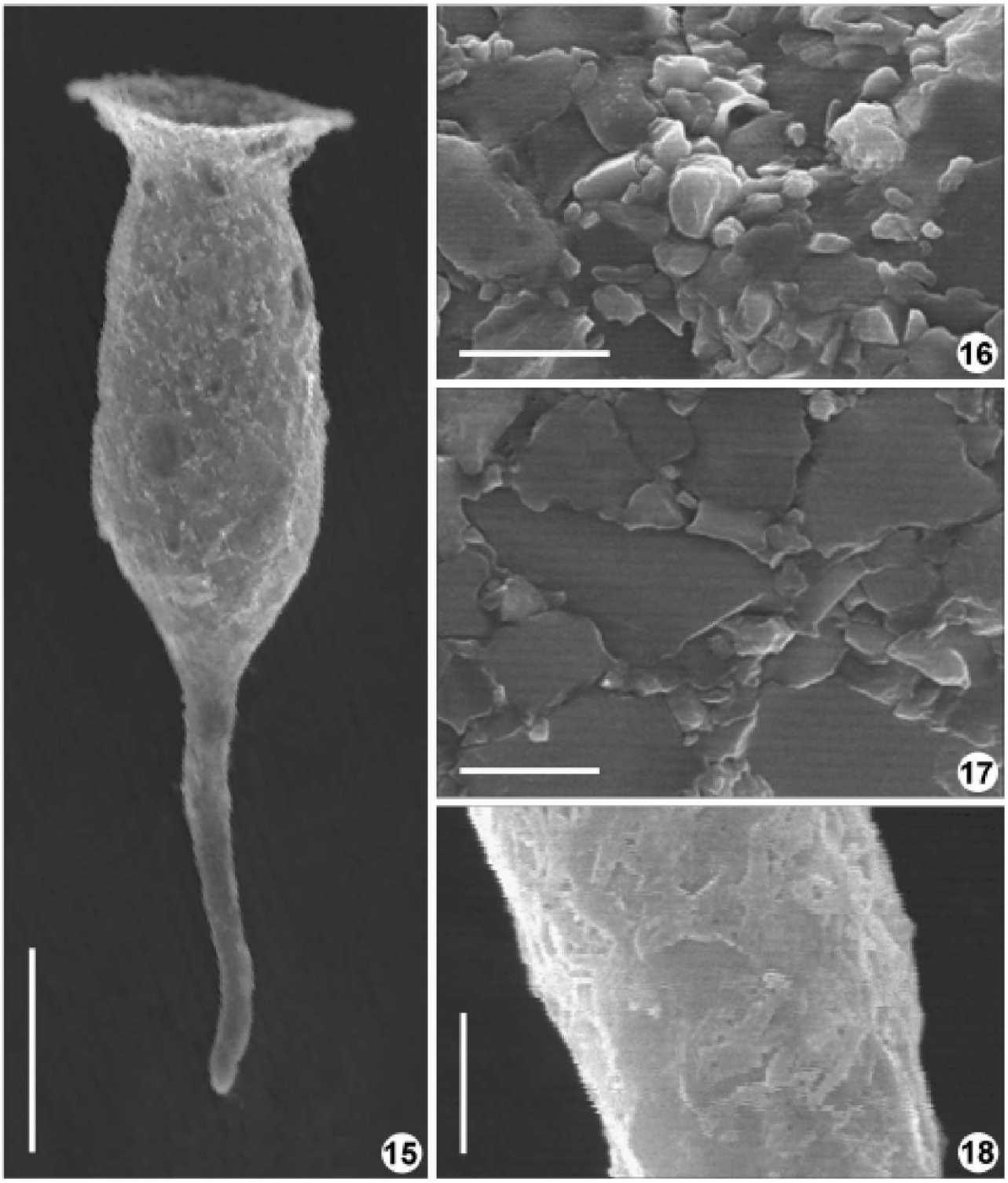
Table 1 shows the detailed morphometric characterization of *Difflugia biwae* according to our studies. The species shows a great diversity in total length, collar height and aboral horn length which have high variability (CV between 11.76 and 24.52). However, body width, collar diameter, neck width, body length, and aperture diameter are fairly constant and have low variability (CV between 5.34 and 8.79) (Table 1). The coefficients of variation of body width and body length are the lowest, and show a remarkable uniformity of *D. biwae* in body size. Also, the size frequency distributions yield bell-shaped (normally distributed) curves and indicate that *D. biwae* is a size-monomorphic species, characterized by a main-size class and a small size range (Figs 19, 20). Table 2 illustrates that more than half (57%) of all the relationships between morphometric characteristics in *D. biwae* are positively correlated at $p < 0.05$, especially TL is strongly positively correlated with HL at $p < 0.001$ ($r = 0.9383$; $n = 100$), while NW is highly positively correlated with AD at $p < 0.001$ ($r = 0.9432$; $n = 100$).

Biometric analysis is the most important part of phenotypic analysis of testate amoebae, as organisms with few morphological characters (Bobrov and Mazei 2004). However, we learned that the size of *D. biwae* is 300-400 μm in total length (including aboral horn) from previous studies (Kawamura 1918, 1927; Abe *et al.* 1957; Okada *et al.* 1981). Thus, total length in the population from Lake Biwa is greater than that in the population from Mulan Lake (300-400 μm *vs.* 165-306 μm). This discrepancy may mainly be due to differences in the variation of the investigated specimens. In the current study, 100 specimens including one kind of typical shape of shell of *D. biwae* and two kinds of atypical shapes (specimens with a short aboral horn and those with an oblique neck) were randomly selected for the morphometric investigation, whereas only a few specimens with typical shape of shell were probably involved in Kawamura's study (Kawamura 1918, 1927). Furthermore, the variation of the aboral horn length was not mentioned at all in any previous studies (Kawamura 1918, 1927; Abe *et al.* 1957; Toshihiko 1979; Okada *et al.* 1981). However, our results clearly showed that the aboral horn length (HL) has the highest value of the coefficient of variation (24.52%), and it is strongly



Figs 3-14. LM photographs of *Difflugia biwae*. **3-5** - lateral views of three different specimens with a long aboral horn (3), a short aboral horn (4) and an oblique neck (5); **6** - apertural view showing the circular aperture is surrounded by a conspicuous great collar flare; **7** - bottom view showing the aboral horn (arrow); **8** - lateral view showing the pseudopodia (arrowheads) and the gas-vacuole (arrow); **9** - view showing the ovular nucleus (arrow); **10** - lateral-oblique view showing the pseudopodia; **11** - living specimen showing the cytoplasm attached to the internal walls of the shell (arrow); **12** - living specimen showing the cytoplasm extended into the aboral horn; **13** - view showing reproduction (shells joined at their oral apertures) and some algae attached to the surface of the shell (arrowheads); **14** - lateral view showing the single nucleus (arrow) and the algae (arrowheads) (Fluorescence microscopy with DAPI stain). Scale bars 20 μm (9); 50 μm (3-8, 10-14).

positive with total length (TL) (Tables 1, 2). Accordingly, the total length of *D. biwae* is largely dominated by the length of the aboral horn. The intrinsic reason for the variation of the aboral horn length in *D. biwae* is still not well understood. One possibility is that it was due to the direct and indirect effect of the local environmental



Figs 15-18. SEM photographs of *Difflugia biwae*. **15** - lateral view; **16** - detail of the surface of the anterior portion of the shell covering, note the close interlocking of small to medium angular and polymorphic particles to give a smooth surface; **17** - detail of the surface of the posterior portion of the shell covering, note the close interlocking of small angular particles and medium flattish pieces of quartz to give the smoother surface; **18** - detail of the surface of the aboral horn, note the close interlocking of small flattish quartz to give the smoothest surface. Scale bars 5 μm (16-18); 50 μm (15).

Table 1. Morphometric characteristics of *Difflugia biwae* from Mulan Lake, China.

Characters[1]	$\bar{x}$	M	SD	SE	CV	Min	Max	n
Total length (1)	227.6	227	26.76	2.68	11.76	165	306	100
Body width (2)	60.5	60	3.23	0.32	5.34	53	69	100
Collar diameter (3)	78.2	78	6.76	0.68	8.64	63	101	100
Neck width (4)	45.1	45	3.04	0.30	6.74	37	56	100
Collar height (5)	22.9	23	5.08	0.51	22.18	11	36	100
Body length (6)	121.3	122	7.90	0.79	6.51	100	142	100
Aperture diameter (7)	35.4	35	3.11	0.31	8.79	27	45	100
Aboral horn length (8)	85.0	83	20.84	2.08	24.52	29	153	100

[1]Numbers 1-8 in parenthesis designate features as shown in Figs 1 and 2. Data based on random selection of specimens. Measurements in μm. CV - coefficient of variation in %, M - median, Max - maximum, Min - minimum, n - number of individuals investigated, SD - standard deviation, SE - standard error of the mean, $\bar{x}$ - arithmetic mean.

Table 2. Correlation coefficients between morphometric characteristics in *Difflugia biwae* from Mulan Lake, China. TL - total length; BW - body width; CD - collar diameter; NW - neck width; CH - collar height; BL - body length; AD - aperture diameter; HL - aboral horn length (see Figs 1 and 2).

Characters	TL (1)	BW (2)	CD (3)	NW (4)	CH (5)	BL (6)	AD (7)	HL (8)
TL (1)	——							
BW (2)	0.1935	——						
CD (3)	0.4607***	0.2618**	——					
NW (4)	0.1326	0.2763**	0.2700**	——				
CH (5)	0.3824***	0.1659	0.5128***	0.0487	——			
BL (6)	0.5994***	0.1155	0.1907	0.2278*	-0.0205	——		
AD (7)	0.1264	0.2665**	0.3222**	0.9432***	0.1356	0.2109*	——	
HL (8)	0.9383***	0.1604	0.3712***	0.0917	0.2266*	0.4052***	0.0594	——

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

factors (e.g. water chemistry and trophic status) of the habitat where the population was formed. Another possibility is that the aboral horn length likely represents a normal variation in growth form of this species because all other morphometric characteristics of the shell, with the exception of collar height and total length, and the basic shape of the shell are consistent with the existence of just a single phenotype.

Comparison with similar species

In terms of the shell shape and the possession of an aboral horn, *Difflugia biwae* resembles *D. delicatula* Gauthier-Lièvre *et* Thomas, 1958 (Gauthier-Lièvre and Thomas 1958), *D. elegans* Penard, 1890 (Ogden 1979) and *D. oblonga caudata* Štěpánek, 1952 (Štěpánek 1952). However, *D. biwae* differs from *D. elegans* and *D. oblonga caudata* in having smooth appearance of the shell (*vs.* rough in *D. elegans* and *D. oblonga caudata*) and in having a longer aboral horn (more than 25% of the body length *vs.* less than 20% in *D. elegans* and *D. oblonga caudata*) (Table 3). Although the shell shape and structure of *D. biwae* are most similar to those of *D. delicatula* in having a smooth and fusiform shell with a long aboral horn, it can be clearly distinguished by having a greater total length in excess of 165 μm (*vs.* 75-100 μm in *D. delicatula*) and by having the wide flat collar which is always larger than its body-width in diameter (*vs.* always less than body-width in diameter in *D. delicatula*) (Table 3). So *D. biwae* is considered to be a distinct species in having smooth fusiform shell with a wide flat collar, constricted neck, and long aboral horn.

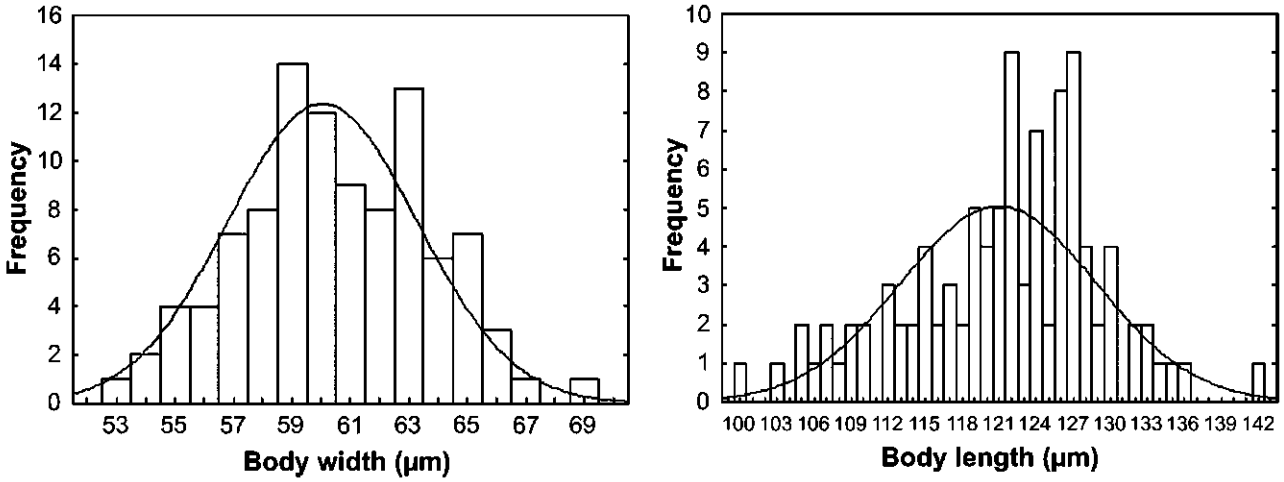


Fig. 19. Histogram showing the size frequency of body width (character 2 as shown in Fig. 1) in *Difflugia biwae*.
Fig. 20. Histogram showing the size frequency of body length (character 6 as shown in Fig. 1) in *Difflugia biwae*.

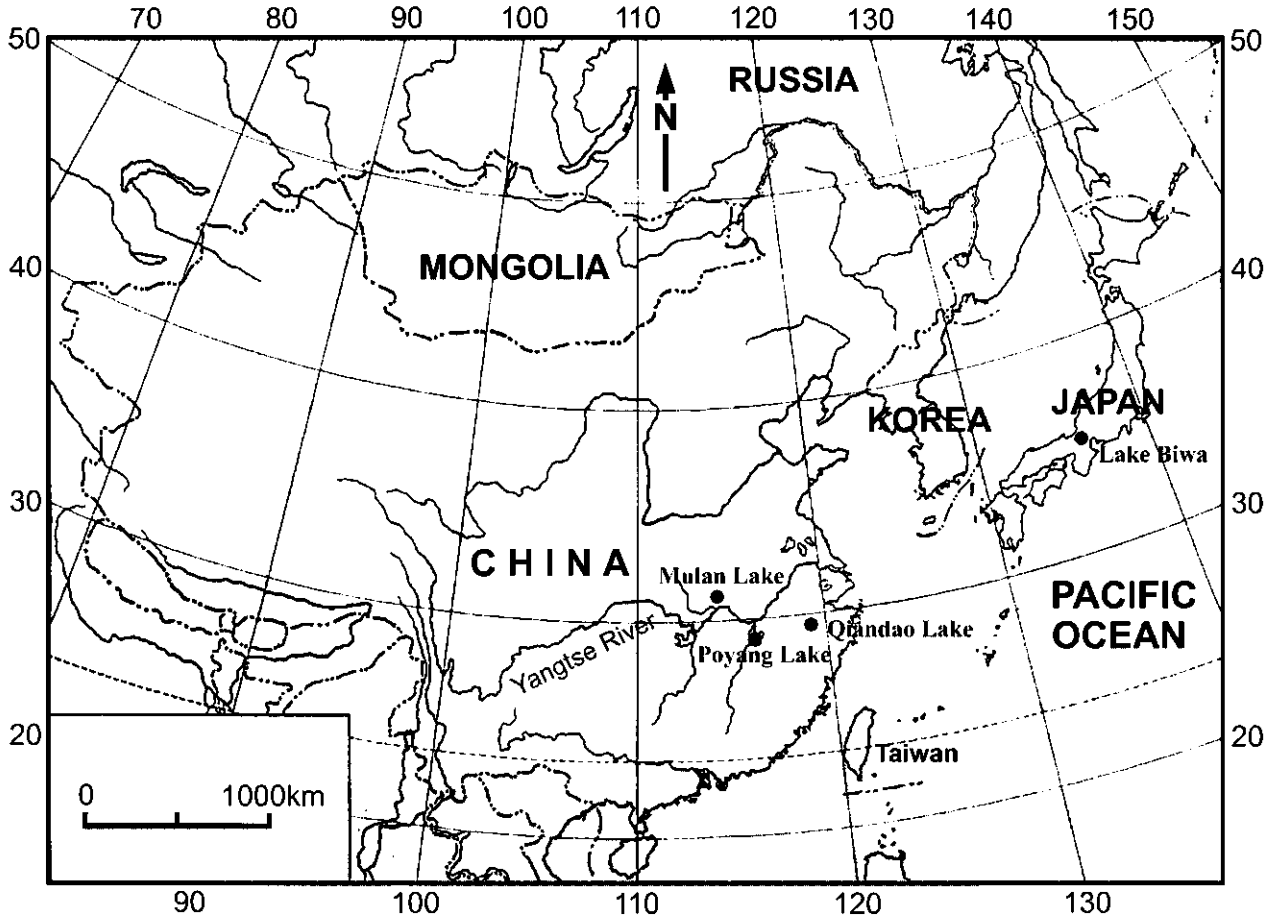


Fig. 21. Distribution of *Difflugia biwae* indicated by filled circles in the East Asia.

Table 3. Comparison of *Difflugia biwae* with three closely-related species. All measurements in μm. (? - data not available)

Characters	*Difflugia biwae*	*D. delicatula*	*D. elegans*	*D. oblonga caudata*
Total length	165-306	75-100	113-158	287
Body width	53-69	28-35	69-99	105
Collar diameter	63-101	?	?	90
Neck width	37-56	?	?	56
Collar height	11-36	8-9	?	?
Body length	100-142	?	?	?
Aperture diameter	27-45	15	35-55	70
Aboral horn length	29-153	20-22	?	42
Shell outline	smooth; elongate fusiform with a conspicuous collar flare and a long aboral horn	smooth; elongate fusiform with a small collar flare and a long aboral horn	rough; elongate oviform with a small collar flare and a short aboral horn	rough; elongate oviform with a conspicuous collar flare and a long aboral horn
Shell composition	small to medium polymorphic particles and flattish quartz	small to medium polymorphic particles and flattish quartz	small to large pieces of angular quartz and often diatoms	medium sherds and sand grains
Collar diameter > Body width	yes	no	no	no
Neck	long and constricted	short and constricted	short and constricted	long and constricted
Sample location	China	Africa	England	Slovakia
Data resource	present study	Gauthier-Lièvre and Thomas 1958	Ogden 1979	Štěpánek 1952

Distribution

Many testate amoebae species have cosmopolitan distribution, at least at the morphological level, but others have restricted distribution (Foissner 1987, 1999; Beyens and Meisterfeld 2001). At the beginning of the twentieth century, the aquatic testacean species *Difflugia biwae* Kawamura was first found in Lake Biwa, Southern Honshu, Japan (Kawamura 1918, Tsugeki *et al.* 2003), but it was not found everywhere other than in three Chinese lakes: Qiandao Lake, Zhejiang Province (Li and Yu 2001), Poyang Lake, Jiangxi Province (Wang *et al.* 2003) and Mulan Lake, Hubei Province (present work) up to now (Fig. 21). Furthermore, it is so large (165-306 μm) that it would have been easily found in Europe and North America, if it were there. Distribution of the *D. biwae* is limited, as far as it is known at present, to only the deep lakes in East Asia (Japan and China). Consequently, *D. biwae* must have a restricted geographical distribution, disproving the old hypothesis that microscopic organisms are cosmopolitan (Finlay 2002). According to Finlay (2002), the abundance of individuals in microbial species is so great that dispersal is rarely restricted by geographical barriers, but geographical

barriers to dispersal apply increasingly to organisms above 1-10 mm in size, thus excluding nearly all protists. However, Wilkinson (2001) argued that the ubiquity-biogeography transition gradient starts at 100-150 μm, rather than 1 mm, thus including some larger testate amoebae. It is safe to affirm that our results agree with the opinion of Wilkinson because *D. biwae* always exceeds 150 μm in size.

Regarding its typical habitat, *D. biwae* is considered to be among the typical inhabitants of the deep lake (Kawamura 1927). Recently, Tsugeki *et al.* (2003) showed that *D. biwae* has been extinct in Lake Biwa due to eutrophication caused by nutrient input. So far, only China has the living organism of this special and interesting species.

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Free-living Amoebae Serve as a Host for the *Chlamydia*-like Bacterium *Simkania negevensis*

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Summary. Members of the novel family Parachlamydiaceae are commonly observed in free-living amoebae (FLA) as host cells. Therefore, we examined the potential of 14 different species of free-living amoebae to serve as hosts of the *Chlamydia*-like bacterium, *Simkania negevensis*, previously isolated as a contaminant from a cell culture in Israel (Kahane *et al.* 1993, 1995). The inoculum of the obligate intracellular agent was prepared from Buffalo Green Monkey (BGM) cells. The infection of *Acanthamoeba* strain HLA and of *Naegleria clarki* (N-DMLGo) revealed typical morphological stages of a *Chlamydia*-like life cycle, including the presence of elementary and reticulate bodies, as could be shown by electron microscopy. Subsequent infection studies with an *Acanthamoeba*-adapted *Simkania* isolate showed that also *Balamuthia mandrillaris* and one of two *Hartmannella* strains supported the growth of *Simkania. Balamuthia* can be considered as an experimental host for mass production of elementary bodies. This is based on the finding that the host amoebae expelled great numbers of bacteria leading to a long-term survival of the infected trophozoites. The observation that *Simkania negevensis* can survive and replicate within at least four of tested FLA species suggests that various free-living amoebae may serve as survival and multiplication vehicles supporting the spread of these pathogens in aquatic environments. The concept that *Simkania* may fall into the group of environmentally preadapted pathogens is discussed as well.

Key words: *Acanthamoeba, Balamuthia, Chlamydia,* endoparasite, *Hartmannella,* host range, *Neochlamydia, Simkania negevensis,* ultrastructure, *Waddlia.*

INTRODUCTION

Chlamydiae are well known as important obligate intracellular pathogens. They are the causative agents of a variety of diseases in humans and animals, e.g. infections of the eye, as well as of the respiratory and genital tracts. The life cycle of chlamydiae is characterized by the development of reticulate bodies (RBs) that divide intracellularly by binary fission and the more resistant elementary bodies (EBs) that are specialized for transmission to another cell of the same host and to a new host (Moulder 1984).

Recent isolation of several novel *Chlamydia*-related bacteria from contaminated cell culture (Kahane *et al.* 1993, 1995), from an aborted bovine foetus (Dilbeck *et al.* 1990, Rurangirwa *et al.* 1999, Henning *et al.* 2002)

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and from free-living amoebae (Michel *et al.* 1994, Amann *et al.* 1997), has led to the reclassification of the order Chlamydiales and to the establishment of the families Simkaniaceae, Waddliaceae, and Parachlamydiaceae respectively (Everett *et al.* 1999, Poppert *et al.* 2002). Since the members of the Parachlamydiaceae, such as *Parachlamydia* (Amann *et al.* 1997), *Neochlamydia* (Horn *et al.* 2000), Halls Coccus (Birtles *et al.* 1997) and various hitherto unnamed strains isolated in the U.S. (Fritsche *et al.* 1993, 2000) multiply within free-living amoebae (FLA), the question arose as to whether other members of the Chlamydiales could also grow within FLA* This issue has been addressed in various host range studies i.e. for *Chlamydophila pneumoniae* or *Waddlia chondrophila* as the type species of *Waddliaceae*. While *C. pneumoniae* could be observed to survive and replicate within *Acanthamoeba polyphaga* for about 14 days (Essig *et al.* 1997), the *Waddlia* isolate was shown to be able to multiply permanently within various amoebal hosts (Michel *et al.* 2001b, 2004). Similarly, *A. polyphaga* could be successfully infected with *Simkania negevensis*, an organism which was originally isolated as a contaminant from a cell culture (Kahane *et al.* 1993, 1995; Lieberman *et al.* 1997) and formerly designated "Z"-organism. The infection resulted in a stable host-parasite association (Kahane *et al.* 2001). In the present study we aimed to confirm these results by evaluating a broader range of FLA as potential hosts as we had previously described for *Waddlia* (Michel *et al.* 2001b, 2004). The question of whether FLA - that are widely spread in nearly all known freshwater habitats - may serve as natural hosts and vehicles is of considerable interest since *S. negevensis* was shown to be the cause of bronchiolitis in infants (Kahane *et al.* 1998) and was also associated with community-acquired pneumonia (cap) in adults (Lieberman *et al.* 1997). It is widely spread in the Negev region of Israel, and seropositivity for *Simkania* has meanwhile been reported from Canada, Great Britain, and the Americas (cit. in Kahane *et al.* 2001). The findings presented in this article may help to identify the environmental reservoir and may therefore contribute to our knowledge on the epidemiology of this organism.

MATERIALS AND METHODS

Origin and maintenance of *Simkania negevensis*. Buffalo Green Monkey (BGM) cells were used for cultivation and maintenance of *Simkania* strain ATCC VR-1471, strain Z, Lot # 1171209. Cells were weekly passaged in serum-free medium (SF-3, CytoGen, 35764 Sinn / Germany) without antibiotics and with cycloheximide, were transferred in intervals of 6 weeks.

Protozoan strains and culture. The protozoa used in this study are listed in Tables 1 and 2. *Naegleria gruberi* (CCAP 1518/1c) and *N. lovaniensis* (Aq/9/45D) were kindly provided by Johan De Jonckheere/ Brussels, the *Balamuthia mandrillaris* strain CDC: V039 was provided on Vero cells by courtesy of Klaus Janitschke/ Berlin. In order to replace the Vero cells as host cells, a cell-free medium that had been developed by Michel and Janitschke (1996) [a modification of SCGYE-medium (De Jonckheere 1977)] was used. The *Acanthamoeba* strain HLA was originally isolated from the cornea of a keratitis patient and has been kindly provided by Horst Aspöck, Vienna. The *Dictyostelium* strain Sö-P2 was isolated from soil by M. Steinert, Würzburg, who made it available to us. The *Naegleria* strain N-DMLGo isolated from a garden pond in Bad Hönningen has recently been sequenced and identified as *Naegleria clarki* by Julia Walochnik/Vienna (Walochnik *et.al.*, in preparation). The protozoan cultures were maintained on non-nutrient agar (NNA) seeded with *Enterobacter cloacae* according to Page (1988). *N. gruberi*, *N. lovaniensis*, and *Hartmannella vermiformis* (OS 101) were grown axenically on SCGYE-medium according to De Jonckheere (1977).

Growth of bacteria within protozoa. The cell suspension of a 7-day-old infected BGM culture was passed through a membrane filter with a pore size of 1.2 µm. It was rich in elementary and reticular bodies representing a superabundance since these tiny stages are not countable, and since they do not produce colony forming units (cfu) on nutrient agar Alternatively, the bacterial inoculum was prepared from heavily infected acanthamoebae, strain HLA, grown axenically, by freeze-thawing and subsequent passage through a 1.2 µm filter. Bacterial elementary and reticulate bodies from 100 µl filtrate were added to 25 ml culture flasks containing trophozoites in SCGYE-medium forming a monolayer in a 24h log-phase culture. The same inoculum was added to agar plates with trophic forms preincubated for 24 h as well. After 48 h at 28°C, the infected amoebal cultures were inspected daily for a time period of 10 days. The only exceptions were the two *Dictyostelium* strains incubated at room temperature. Morphological alterations and symptoms of infection as seen by light microscopy were compared with uninfected host cells. In order to obtain protozoa-adapted *Simkaniae*, heavily infected *Acanthamoeba* trophozoites were submitted to freeze-thawing. The released EB's and RB's were filterpurified from amoebal debris and utilized for further cocultivation assays.

Electron microscopy. Infected amoebal host cells were harvested 5 days post infection. The cell suspension was centrifuged for 10 min at 600 g. The resulting pellet was fixed in 3% glutaraldehyde (1 h),

* The results had been presented at the 20th Annual Meeting of the German Protozoological Society at Bonn-Röttgen (see Michel *et al.* 2001a).

transferred to 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide and embedded in Spurr resin. Thin sections were stained with 1% lead citrate and examined using a Zeiss EM 10 electron microscope.

RESULTS

Cocultivation of *S. negevensis* and protozoa. In order to evaluate representatives of the three important genera *Acanthamoeba, Naegleria* and *Hartmannella* as potential hosts for *Simkania negevensis*, we analysed the capability of the bacteria to multiply intracellularly in various strains (Table 1) by light microscopy, phase contrast microscopy and transmission electron microscopy. Bacterial inocula were prepared from infected BGM cells, and the results of the coincubations on both NN-agar plates and in axenic SCGYE-medium are summarized in Table 1. After 5 days of cocultivation, one of four *Naegleria* strains tested (*N. clarki*, N-DMLGo) and one of two *Acanthamoeba* strains (HLA) showed characteristic signs of infection, such as rounding up of cells, inhibition of cell migration and the presence of intracellular coccoid bacteria. Infected trophozoites of *Acanthamoeba* were not able to form cysts whereas the infected naegleriae still formed cysts which did not contain endocytobionts. These features resemble previous observations of *Neochlamydia- or Waddlia*-infected host cells (Horn *et al.* 2000, Michel *et. al.* 2004). Subsequent subcultures showed that the association of *Simkania* and its respective experimental host was stable during the time of observation. Since the degree of infection was highest with *Acanthamoeba* strain HLA, this host-parasite combination was maintained indefinitely for months and was chosen as an adaptation model of these bacteria to a protozoan host.

Subsequently, *Simkania* was isolated and filter-purified from axenically grown acanthamoebae which were jammed with intracellular bacteria after three serial transfers. Various other strains of FLA were cocultivated

Table 1. Cocultivation results of *Simkania negevensis* with various promising candidates of free-living amoebae. After three transfers on *Acanthamoeba*-strain HLA the protozoa-adapted *Simkania* strain "Z-P" was cocultivated with amoeba species shown in Table 2. Cysts - cysts were formed but did not harbour endocytobionts.

Amoeba species	Strain	Source	Intracellular growth
Naegleria gruberi	CCAP 1518/1e	Unknown	-
Naegleria lovaniensis	Aq/9/1/45D	Aquarium/Belgium	-
Naegleria sp.	N- Beck	Aquarium/Germany	-
Naegleria clarki	N-DMLG$_o$	Garden pond/Germany	+++ cysts: -
Acanthamoeba castellanii	C$_3$ ATCC 50739	Potable water reservoir	-
Acanthamoeba castellanii	HLA	Keratitis case/ Vienna	+++ → Z-P
Hartmannella vermiformis	A$_1$Hsp$_o$	Host of *Neochlamydia*	-

Table 2. Cocultivation assays of the protozoa-adapted strain "Z-P" of *Simkania* together with various further strains of free-living amoebae and two isolates of the slime mould *Dictyostelium discoideum*.

Amoeba species	Strain	Source	Intracellular growth
Naegleria lovaniensis	Aq/9/1/45D	Aquarium/Belgium	-
Willaertia magna	NI$_1$CL$_1$	Pond/India	-
Acanthamoeba castellanii	C$_3$ ATCC 50739	Potable water reservoir	-
Acanthamoeba lenticulata	45	Human nasal mucosa	-
Hartmannella vermiformis	Os 101	Physiotherap. bath	+++
Hartmannella vermiformis	C3/8	Surface water	-
Balamuthia mandrillaris	CDC: V039	*Papio sphinx*. Brain	+++
Dictyostelium discoideum	Berg$_{25}$	Human nasal mucosa	-
Dictyostelium discoideum	Sö-P$_2$	Surface water	-

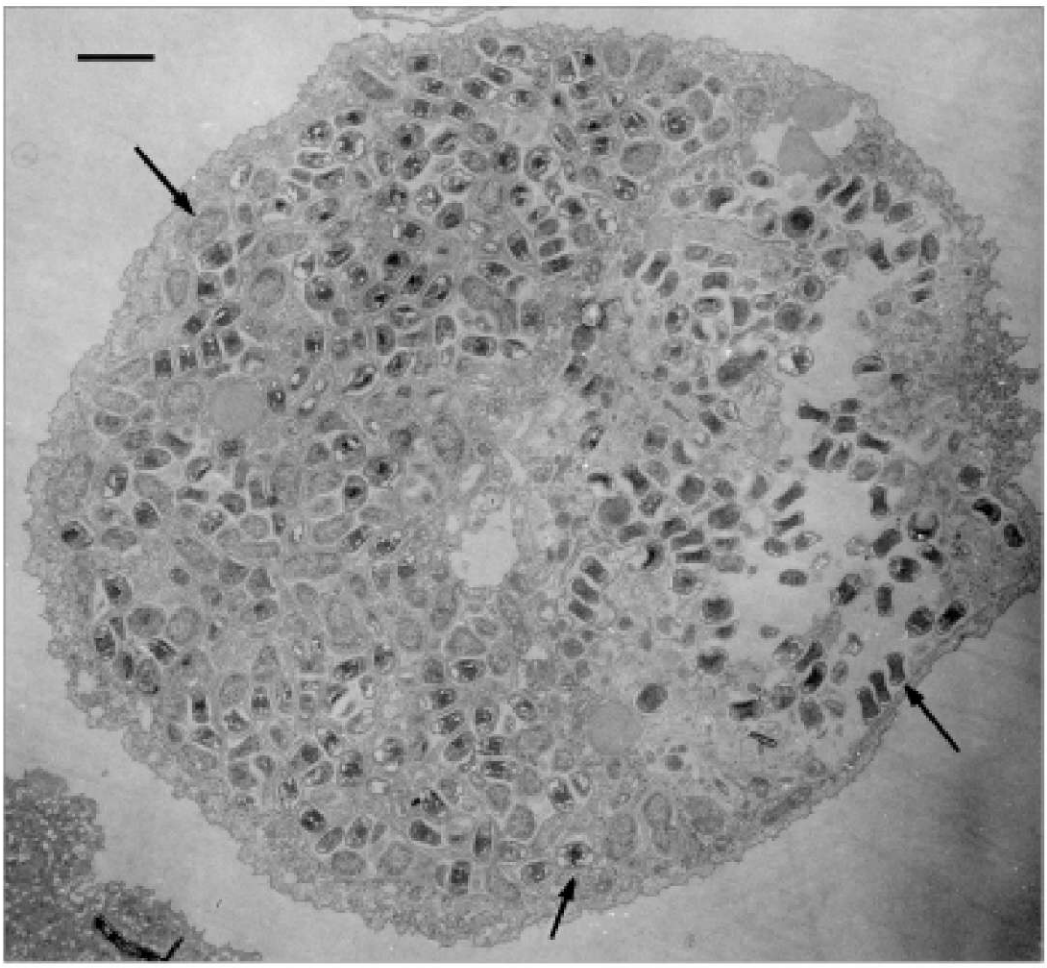

Fig. 1. Overview of an infected *Acanthamoeba*, strain HLA, jammed with numerous organisms of *Simkania negevensis* (arrows) as a result of multiplication of the endoparasite in a period of six days. Scale bar 1.0 μm.

with the bacteria-rich suspension (Table 2). As a result of the testing of 8 strains belonging to 6 different genera only *Hartmannella vermiformis*, strain Os101 and *Balamuthia mandrillaris* grown in SCGYE-medium proved positive within a period of 3-4 days. In addition to the confirmation of a cytoplasmatic infection with Simkaniae as seen by phase contrast microscopy, both strains of these unrelated amoebae lost their ability to form cysts, which proves that they were actually infected. These associations of amoebal hosts and endocytobionts turned out to be stable infections over many passages. Since the trophozoites of *Balamuthia* began shedding elementary bodies 3 to 5 days post infection, they survived because they could not be overgrown by the endoparasites. The number of expelled EBs in the medium increased to such an extent that this host-parasite combination was suited perfectly for mass production of EBs as the infectious stages of *Simkania*. In contrast, strains of *Willaertia magna*, a second *Hartmannella* strain designated C3/8, and two strains of the cellular slime mould *Dictyostelium* were resistant to infection. The same was true for *Naegleria lovaniensis* and *Acanthamoeba* strain C3, infection of which had been attempted by cocultivation with the protozoa-adapted *Simkania* cells.

Electron microscopy. Acanthamoebae of strain HLA were fixed on the 6th day after infection with a suspension of *Simkania* stages from BGM-cell culture. Electron microscopy revealed heavily infected trophozoites which were jammed with organisms belonging to different stages of the *Chlamydia*-like developmental cycle (Fig. 1). This reflects the high replication rate of these

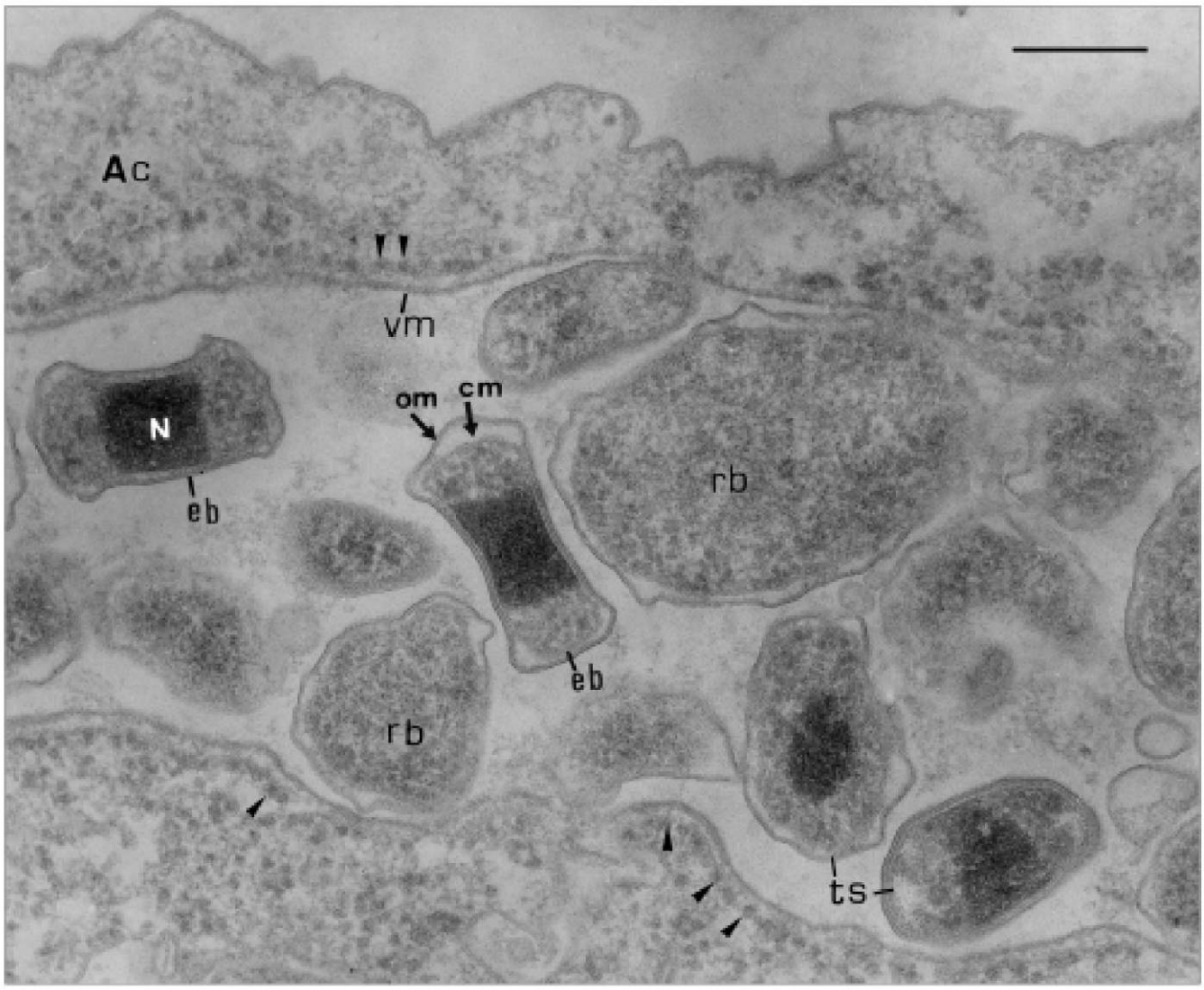

Fig. 2. Detail of an *Acanthamoeba* trophozoite (Ac) harbouring different developmental stages of *Simkania* replicating within a membrane-bound vacuole (vm). The elementary bodies (eb) contain electron-dense nuclear material (N) and ribosomes. They are surrounded by the outer membrane (om) at some places separated from the inner cytoplasmic membrane (cm). They are considered as Gram-negative. The significantly larger reticulate stages (rb) have less dense fibrillar nuclear material and more ribosomes. They are also surrounded by an obviously more flexible trilaminar envelope. Transitory stages (ts) show intermediate traits between EBs and RBs. Remarkable are distinct ribosomes within the host's cytoplasm which appear arranged like a string of pearls beneath the vacuolar membrane (arrowheads). Scale bar 0.25 μm.

intracellularly growing bacteria. Even at this low magnification, at least two kinds of developmental stages of the endocytobiont could be distinguished as exemplified in Fig. 2, which shows even three stages. They are located within an elongated, membrane-bound vacuole of the host amoeba. Replication is performed by the reticulate bodies, which finally transform *via* a transitory stage (ts) to the elementary bodies representing the infectious stage. The RBs containing numerous ribosomes appear pleomorphic with a flexible outer membrane. The characteristic shape of the mature EBs is quadrangular or dumbbell-shaped. As far as we know, this unique outline is different from the EBs of all other *Chlamydia*-like bacteria. Eb's contain electron-dense nuclear material (N) and ribosomes. They are enveloped by the outer membrane, which may be separated from the inner cytoplasmic membrane at some places. These stages appear Gram-negative. The transitory stages being still pleomorphic like the RBs already contain an increasing amount of electron-dense nuclear matter. In

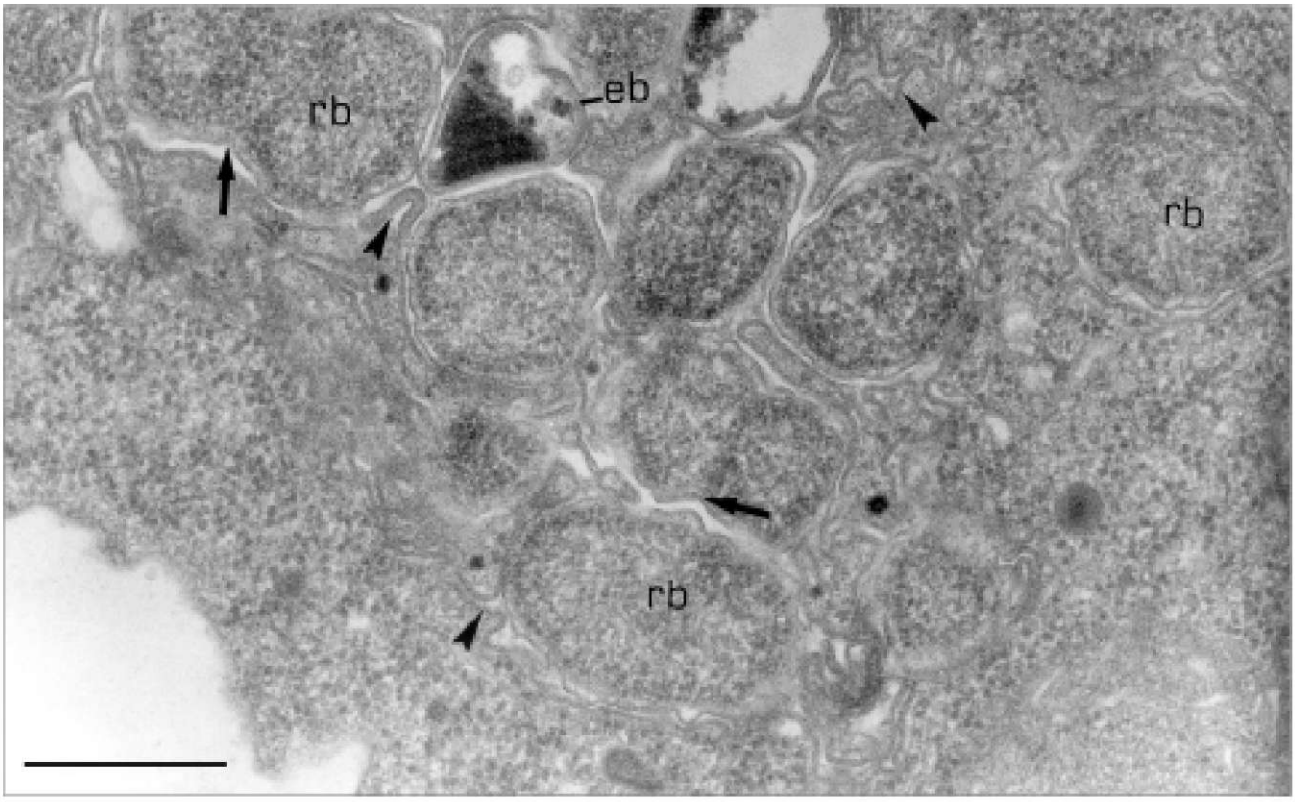

Fig. 3. *Naegleria*, strain DMLG, harbouring mainly reticulate bodies (rb) of *Simkania* replicating by binary fission (arrows). A single elementary body (eb) can also be distinguished. The endoparasites are closely surrounded by a membrane of the host amoeba. They have connections with wrinkled laminar structures found in the vicinity of the enclosed endocytobionts (arrowheads). Scale bar 0.5 μm.

the host amoeba a regular row of ribosomes was observed beneath the vacuolar membrane, appearing like a string of pearls. This unusual phenomenon could be seen in nearly all micrographs inspected so far (not shown).

Since in addition to this *Acanthamoeba* strain amoebae of the genera *Hartmannella, Naegleria*, and *Balamuthia mandrillaris* could be infected successfully with *Simkania*, at least one micrograph of a section of an infected *Naegleria* we have presented here. The only susceptible strain N-DMLG harboured numerous *Simkania* stages, mainly RBs and few EBs (Fig. 3). Since the host membrane surrounded the endoparasites closely, the bacteria appeared to be located within the cytoplasm as known from other endoparasites, such as *Legionella*-like amoebal pathogens (Llap's), for instance. RBs with signs of binary fission could be observed as indicated by a distinct division furrow. In the vicinity of the enclosed parasites an irregular system of laminar structures with unknown function could be observed as well. In contrast to infected acanthamoebae, the pearl-string-like assembly of ribosomes seen beneath the vacuolar membrane was not found within infected naegleriae.

DISCUSSION

Since most members of the family Parachlamydiaceae had been commonly observed in free-living amoebae before their taxonomic identification (Michel *et al.* 1994, Amann *et al.* 1997, Horn *et al.* 2000, Fritsche *et al.* 2000) they were placed into the newly established family Parachlamydiaceae which was reserved for this sister group of the Chlamydiaceae (Everett *et al.* 1999). The ability to grow intracellularly within protozoa could have preadapted these bacteria as pathogens of higher eukaryotes (Corsaro *et al.* 2002, Görtz and Michel 2003) in the same manner as assumed previously for *Legionella pneumophila* (Harb *et al.* 2000). This startling idea was the rationale for attempts to associate other members of the Chlamydiales primarily with acanthamoebae (Essig

et al. 1997, Kahane *et al.* 2001), but in the case of *Waddlia chondrophila* also with other species of FLA (Michel *et al.* 2001 a, b, 2004). Accordingly we started cocultivation assays of *S. negevensis* and various FLA of diverse taxonomic positions. As a result, not only could the findings of Kahane *et al.* (2001) be partially confirmed with acanthamoebae as experimental hosts, but also some different amoebal species (Tables 1, 2) could be successfully infected with long-term persistence and exponential growth of the endoparasitic simkaniae. On the other hand, Kahane's observation of *Acanthamoeba* cysts harbouring stages of *Simkania* could not be observed in the case of the susceptible strain HLA since it had lost its cyst-forming capacity as a result of infection. Similar observations have been made in the case of acanthamoebae naturally infected with *Parachlamydia* (Michel *et al.*1994). The fact that only one of four *Naegleria* strains was permissive to infection may be explained by the history of this host strain N-DMLGo - recently identified as *Naegleria clarki* (Walochnik *et. al.*, in preparation) which had originally harboured simultaneously two unidentified Gram-negative endocytobionts (Michel *et al.* 1999). Perhaps this isolate is a *Naegleria* strain susceptible to various endocytobionts. This original host strain was cured from its twofold infection step by step by a combined application of elevated temperatures and antibiotics and by utilisation of the flagellate transformation test as well (Michel *et al.*, in preparation). As a result of these measures they resumed the ability to form cysts proving that they got rid of their cytoplasmatic fraction of endoparasites (Peb), since infected trophic forms were unable to form cysts. The loss of the intranuclear population of parasites (Pn) could be observed by phase contrast microscopy without problems and was confirmed by electron microscopy. Interestingly, the parasite-free amoebae again lost their capability to form cysts if they were infected with simkaniae. The observation that only one of four *Naegleria* species and one of two *Acanthamoeba* strains were susceptible to infection underlines the need to expose more than one strain of a genus to these infectious agents. In the case of *Hartmannella vermiformis* it was even shown that only one strain of the same species was susceptible (OS101) whereas a second one was totally resistant to infection. Similar differences were obtained during recent investigations on the susceptibility of various FLA to *Waddlia chondrophila* (Michel *et al.* 2004). Only one of six *Acanthamoeba* strains tested proved positive and one of two *Dictyostelium dicoideum* strains could be infected permanently, consequently within the same species of slime mould. *Simkania* and *Waddlia* were both able to invade several unrelated species of FLA and to replicate in them, thus establishing a persistent host-parasite association. This is in contrast to *Chlamydophila pneumoniae* for instance, which survives no longer than a fortnight within its experimental host (Essig 1997). Consequently, it is justified to assume that the experimental hosts of *Simkania* and *Waddlia* identified in the present investigation or earlier (Michel *et al.* 2004), respectively, may play this role also in nature. This hypothesis would provide an explanation for the possible function of the susceptible host as a transportation vehicle and reservoir of these recently detected relatives of the Chlamydiaceae. Another important aspect is the assumption that the adaptation to intracellular survival and replication may have occurred in the course of evolution long before these bacteria were able to infect warm-blooded animals and humans (Harb *et al.* 2000, Corsaro *et al.* 2002). Comparing the host ranges of both bacteria, some significant differences can be emphasized. Four strains of FLA were susceptible to infection with *Simkania* - whereas *Waddlia* was found to be able to infect eight different strains. Both were infectious for at least one strain of *Acanthamoeba, Naegleria* and *Hartmannella*. The most remarkable differences were (a) the high susceptibility of *Balamuthia* for *Simkania* but not for *Waddlia* and (b) the ability of *Waddlia* to infect one of two strains of *Dictyostelium* whereas both tested strains of this cellular slime mould proved to be resistant to infection with *Simkania*. With *Balamuthia mandrillaris* a suitable host has been identified for laboratory mass production of *Simkania* elementary bodies as an alternative model for maintaining these endocytobionts in long-term cultures, providing high yields of bacteria for various scientific purposes. Thus *Balamuthia* was shown to serve as an experimental host of an obligate intracellular bacterium. Similar experience had been made earlier, when this pathogenic amoeba could be successfully infected with strain "Knic", a Gram-negative coccoid bacterium isolated from *Naegleria* sp. and resembling *Ehrlichia* species (Michel *et al.* 2000). Only recently, *Balamuthia* was also shown to be permissive to infection with *Legionella pneumophila* after in vitro cocultivation with this pathogenic agent of Legionellosis (Shadrach *et al.* 2004) well known to survive and replicate within acanthamoebae, amongst others (Rowbotham 1980). Although *Balamuthia* proved to be very susceptible to infection with *Simkania* in vitro, it does presumably not play an important epidemiological

role for the dispersal of *Simkania* and other intracellular bacteria since it has been isolated only once from the environment (Schuster *et al.* 2003) in contrast to frequently found FLA, such as acanthamoebae, hartmannellae or naegleriae. Each positive in vitro result obtained from cocultivation assays of FLA with certain pathogens is of great theoretical and practical value as a laboratory model. But their putative occurrence under natural conditions remains speculative unless infected host protozoa will be actually isolated from the environment. This was, for example, true for *Legionella pneumophila*, which first had been found to be able to replicate within *Acanthamoeba polyphaga* as a result of experimental cocultivation (Rowbotham1980).

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Redescription of *Cochliopodium vestitum* (Archer, 1871), a Freshwater Spine-bearing *Cochliopodium*

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Summary. A freshwater lobose rhizopod *Cochliopodium vestitum* (Archer, 1871) (Himatismenida) was re-isolated and studied. New diagnosis for this species, based on the light- and electron-microscopic data is provided. The most peculiar feature of this species is fine spines visible with light microscopy, which originate from the top of some of the scales comprising the tectum, as revealed with electron-microscopy. Comparison of *C. vestitum* with the other spine-bearing cochliopodiums (*C. echinatum* and *C. longispinum*) suggests synonymy of these species, and *C. vestitum* remains the only valid spine-bearing *Cochliopodium*.

Key words: amoebae, *Cochliopodium*, Himatismenida, scales, systematics, ultrastructure.

INTRODUCTION

At the border of 19-20th centuries, four species of *Cochliopodium*, carrying long thin radial spines on the cell surface were described. These were *C. vestitum* (Archer, 1871) Archer, 1877, initially *Amphizonella vestita*; *C. pilosum* Hertwig et Lesser, 1874; *C. echinatum* Korotneff, 1879; and *C. longispinum* West, 1901. Later Archer (1877) proved the species name *C. pilosum* to be a junior synonym of *C. vestitum*. Other species were relatively well recognized and mentioned as distinct species (Leidy 1879, Penard 1902, Awerintzew 1906). However, Cash and Hopkinson (1909), for example, considered *C. echinatum* and *C. longispinum* to be synonyms. Descriptions of these four species do not contain a complete set of light-microscopic features and none of them has been re-isolated and studied using electron microscopy. Thus, the taxonomic status of these species remains ambiguous. The nature of spines visible with light microscopy (LM) on the surface of these amoebae remains unclear. This paper reports on the study of two spine-bearing strains of *Cochliopodium*, identified as *C. vestitum*. Rede-

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scription of this species and its comparison with the other spine-bearing *Cochliopodium* spp. are provided.

MATERIALS AND METHODS

Two strains of *Cochliopodium vestitum* were isolated from the upper layer of the bottom sediments in Leshchevoe Lake (Valamo Archipelago, Ladoga Lake, North-Western Russia) in September 1998 and from the upper layer of bottom sediments and washings of *Sphagnum* sp. at the bank of an unnamed lake at Srednii Island (Chupa Inlet, Kandalaksha Bay, The White Sea) in September 1999-2001. Samples were inoculated in the 90 mm Petri dishes with Prescott and James medium (Prescott and James 1955) and one wheat grain per dish. Amoebae found in the samples survived several transfers into the fresh Prescott and James medium, while attempts of cloning were not successful in all cases. For transmission electron microscopy (TEM) amoebae were fixed at room temperature while adhering to the surface of the previously polymerized Epon 812 resin with 0.5% phosphate-buffered OsO_4 for 10 min, followed by 2.5% glutaraldehyde for 20-30 min and 1% OsO_4 for 1 h. After dehydration, the specimens were embedded in Epon 812 resin. Sections were stained with uranyl acetate and Reynolds' lead citrate. Whole mounts of the scales were made by treatment of the cells with 0.5% Triton X-100, followed by several washes with distilled water and placement on formvar-coated grids.

RESULTS

Cochliopodium vestitum (Archer, 1871) emend.

Diagnosis: Length in locomotion 39-70 µm (mean 59 µm), breadth 48-74 µm (mean 65 µm), length : breadth ratio 0.6-1.08 (mean 0.9); in locomotion rounded to oval, with numerous subpseudopodia at the anterior margin of hyaloplasm. Very high dome-shaped granuloplasmic mass. One nucleus of vesicular type, 13-15 µm in diameter (mean 14 µm), nucleolus 6-8 µm in diameter (mean 7 µm). Scales consist of a quadrangular base plate, 4 vertical stalks cross-connected at two levels and a wide funnel-shaped top part formed by 4 concentric rings and 24 radial spokes originating from the vertical stalks. Some of the scales with a sharp spine (10-14 µm in length) originating from the center of a top part. Side of the base plate 0.6 µm, diameter of the top part 0.8-0.9 µm, height of the scale 0.71 µm.

Observed habitats: Freshwater pools in Counties Westmeath and Tipperary, Great Britain (Archer 1871); freshwater Absecom pond (New Jersey) and China Lake (Wyoming), North America (Leidy 1879); lakes in Novgorod and Tver' regions, central Russia (Awerintzew 1906); bottom sediments of a freshwater Leshchevoe Lake at the Valamo Archipelago, Ladoga Lake, bottom sediments and patches of *Sphagnum* sp. at the bank of a freshwater lake at Srednii Island (Chupa Inlet, Kandalaksha Bay, the White Sea), North-Western Russia.

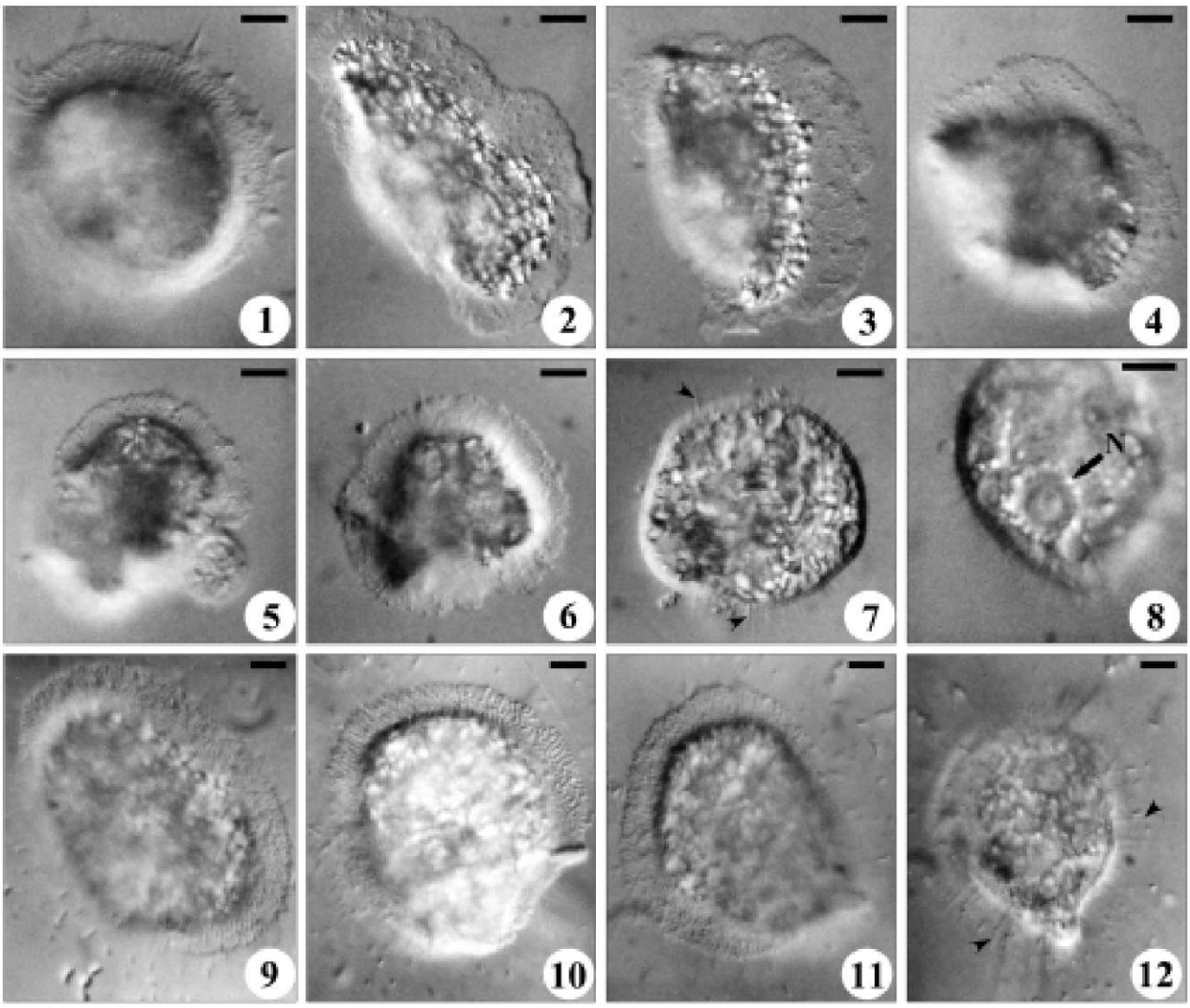
Type material: Since neither Archer nor later researchers made slides of this species, I establish the neotype. Type slides are deposited with the collection of preparations of the Biological Research Institute of the Saint-Petersburg State University, accession numbers 953 (neotype) and 954 (paraneotype).

Differential diagnosis: None of the *Cochliopodium* spp. known to date possesses scales with the spines easily visible with LM.

Description: Both Ladoga and the White Sea strains were identical in LM features. Amoebae were covered by the tectum, with scales distinctly seen as the large granules on the dorsal surface of hyaloplasm (Figs 1-4, 9-11). Anterior and lateral margins of the hyaloplasm often extended beyond the border of scales. Numerous thin hair-like spines were seen radiating from the surface (Figs 7, 12). In adhering amoebae these spines were seen only when the granuloplasm was in focus (Fig. 12).

The locomotive form was rounded, oval or rarely drop-shaped (Figs 1-4, 9). Central granuloplasmic mass surrounded with the flattened hyaloplasmic veil was normally shifted back to the posterior end of the body, "hiding" narrow posterior margin of the hyaloplasm when amoebae were observed from the top. Hyaloplasm had a uniform width in anterior and lateral parts, narrowing posteriorly. Anterior and lateral margins of hyaloplasm were very dynamic and formed flattened or conical subpseudopodia 8-10 µm in length (Fig. 1). While the amoeba was advancing, subpseudopodia moved to, and finally retracted at the posterior end of the cell. Often subpseudopodia split or fused together from the tips to the bases. Posterior part of the hyaloplasm was very narrow, with the straight or slightly curved edge, sometimes with few trailing filaments. When the cell changed the direction of locomotion, part of the posterior edge formed broad adhesive expansion. In slower movement amoebae were rounded, with almost smooth margin of hyaloplasm (Figs 5, 10).

Non-directly moving amoebae were more rounded than locomotive, sometimes irregularly triangular (Figs 6, 11). The granuloplasm occupied a central position, and the hyaloplasm was wider than in the locomotive cells. Stationary amoebae were hemispherical or bell-shaped, rounded in above view, with the hyaloplasmic veil completely retracted (Fig. 7). In culture dishes amoebae

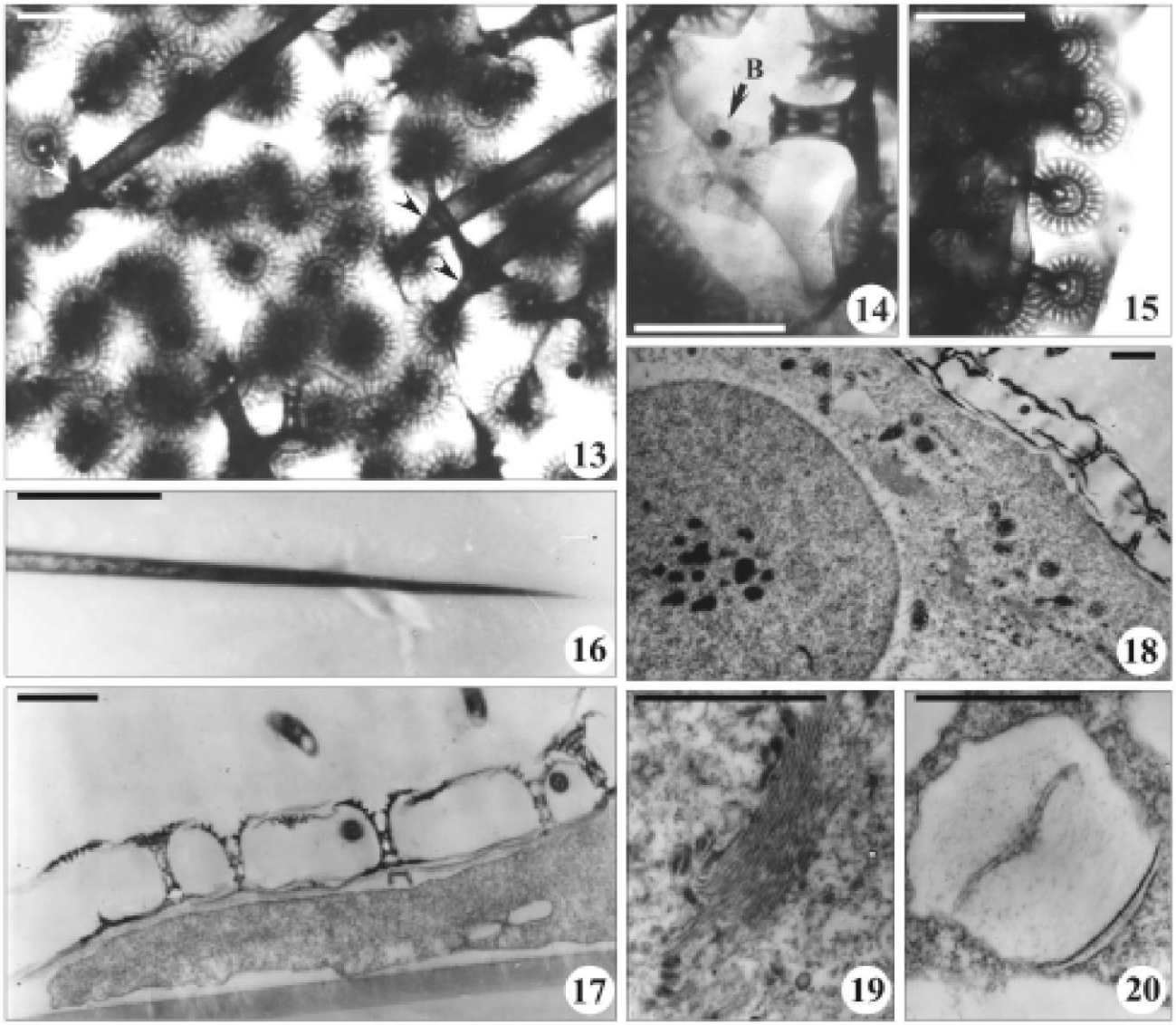


Figs 1-12. *Cochliopodium vestitum*, LM micrographs (**1-8**, White Sea strain; **9-12**, Valamo strain). **1-4, 9** - locomotive forms; **5, 10** - amoebae during slower movement; **6, 11** - non-directly moving amoebae; **7** - stationary amoeba; **8** - nucleus (N) in a living cell; **12** - granuloplasm of the adhering amoeba showing spines on the surface of tectum. Spines in Figs 7 and 12 arrowheaded. Scale bars 10 μm.

floated spontaneously. Floating form was spherical, with several long slender hyaline pseudopodia, formed in a compact bundle from the small scale-free area on the cell surface (Fig. 21). Pseudopodia slowly bent, but were never seen to branch or anastomose.

Nucleus (Fig. 8) was spherical, with the layer of refractile perinuclear granules and a large central nucleolus, which in living and stained amoebae often looked pale and non-homogeneous. The granuloplasmic crystals had a shape of the hexagonal plates about 5 μm in size (Figs 2, 3, 7). Numerous transparent vacuoles of various sizes were seen close to the ventral surface of the cell. Amoebae fed on bacteria and small eukaryotes (flagellates, unicellular algae, and amoebae). Encystment was never observed in cultures.

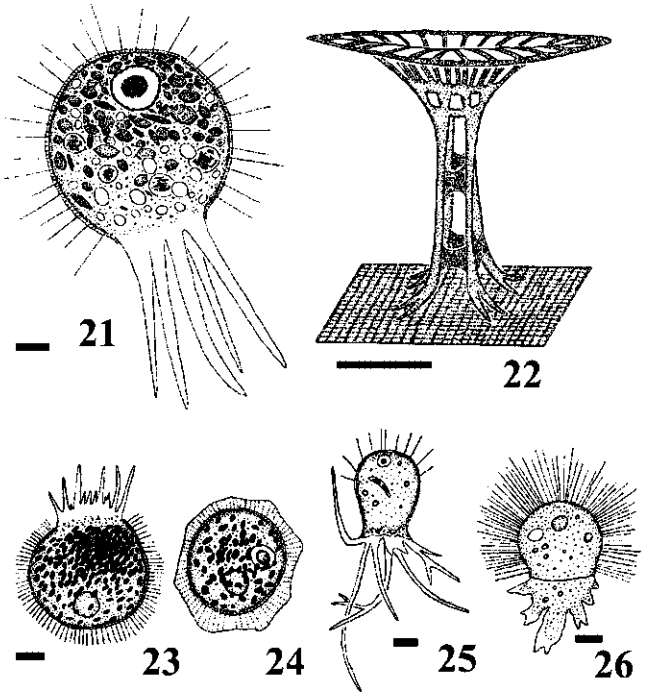
The base plates of the scales (Fig. 22) were grid-shaped (Fig. 14) with the mesh size about 30-40 nm. The stalks of the central column were almost straight, slightly curving outwards at the point of attachment of the top part (Figs 14, 17). Every stalk attached to the base plate with a bird-foot structure having two digits. Near the base and in the middle neighboring stalks were joined together with wide connectives. At the top, stalks continued into the top part of a scale (Figs 13, 15, 17), which started from the first concentric ring. The stalks were attached to this ring, and above it each stalk gave 2



Figs 13-20. *Cochliopodium vestitum*, White Sea strain, TEM micrographs. **13-15** - whole mounts of the scales (B - base plate of a scale in Fig. **14**); **16** - tip of the spine in a whole mount preparation; **17** - tectum in the section through the hyaloplasmic veil of an adhering amoeba; **18** - overview of the nucleus and cytoplasm; **19** - dictyosome; **20** - scale under formation in a Golgi-derived vesicle. Spine-bearing scales in Fig. 13 are marked with arrowheads. Scale bars 1 μm.

branches, altogether forming a basket of 8 radial spokes. These spokes were attached to the second concentric ring, above which every spoke branched into 3, altogether forming a wide funnel of 24 radial spokes cross-connected with two more concentric rings, the outermost one and the one in the middle. In the whole mounts some of the scales were seen to have such a typical structure, but possessed a long sharp-ended electron dense spine, originating from the center of a top funnel (Figs 13, 16). Diameter of the base of a spine was 0.3 μm.

Scales on the surface of the plasma membrane densely overlapped between each other with their bases and top parts. The space between the plasma membrane and the scale layer (*ca* 100 nm) was filled with the fuzzy material, which was never seen on the ventral surface of the cell (Fig. 17). Nucleus in the sections had a central nucleolus, consisting of numerous irregularly rounded electron-dense granules of various sizes (Fig. 18). A well-developed Golgi complex was seen near the nuclear envelope, lying dorsally to the nucleus (Fig. 18). It consisted of several dictyosomes, each composed of



Figs 21-24. Drawings of *Cochliopodium vestitum*. **21** - floating form, Valamo and White Sea strains; **22** - scale, White Sea strains; **23** - floating form after Archer (1871); **24** - adhering form after Archer (1871). Scale bars 10 μm (21, 23, 24); 0.25 μm (22).
Figs 25, 26. Drawings of: **25** - *Cochliopodium echinatum* after Korotneff (1879); **26** - *C. longispinum* after West (1901). Scale bars 10 μm (25); 20 μm (26).

several tens of flattened cisternae (Fig. 19). Dictyosomes were surrounded with the numerous vesicles containing various materials, and sometimes scale under formation (Fig. 20). In contrast to some other species of *Cochliopodium* (e. g. Yamaoka *et al.* 1984, Kudryavtsev 2004, Kudryavtsev *et al.* 2004), the "Golgi attachment", an electron-dense dictyosome-associated structure resembling a MTOC was never seen in the sections. Mitochondria had electron-dense matrix and tubular cristae.

DISCUSSION

Identification

This *Cochliopodium* is comparable only with those species, which were reported to carry LM-visible spines on the surface of tectum, since it is impossible, that anyone observing this species could have overlooked this feature. Among such species the present strains mostly resemble *C. vestitum* (Archer, 1871) Archer, 1877. These strains are similar to this species in the size and the shape of adhering and floating amoebae (Figs 23, 24). Archer (1871) stated that in his species "hair-like processes" (spines) were up to 20 μm in length and had "very variable degree of development" (*op. cit.*, p. 115). Therefore Bark (1973) suggested that Archer studied more than one species. However, in the studied strains the spines were also poorly seen in locomotion, and their observed length depended on the angle of view. The nature of the surface spines in Archer's amoebae seems to be identical to that of my strains, since Archer (1871) observed the spines detached from the surface of amoebae "with slightly capitate lower extremity" (*op. cit.*, p. 123), the same was observed in my strains. "Irregularly scattered, generally elliptic, or rounded ... grayish, or somewhat purple colored, sharply and darkly bounded, and clear and shiny bodies" (Archer 1871, p. 112) of *C. vestitum* may correspond to the crystals of my strains. Chlorophyll granules in the cytoplasm of *C. vestitum* considered as a very characteristic feature of this species, and never observed in my strains, might have originated from the ingested food. Archer (1877) rejected this idea, describing at the same time feeding of his amoebae on unicellular green algae and reporting the observation a strain which did not contain chlorophyll (Archer 1871). Later descriptions of *C. vestitum* mostly agree with that made by Archer (Leidy 1879, West 1901, Penard 1902, Cash and Hopkinson 1909), except the length of the spines, which is normally indicated as being 3.7-5 μm (West 1901, Cash and Hopkinson 1909) and diameter of the cell in Penard's (1902) strain indicated as 35 μm. However, comparison of the length of spines in the figures with the cell diameter in descriptions suggests that they were longer than indicated in the text, at least as long as 10 μm. My strains fit these later descriptions as well; therefore, it is possible to identify them as *C. vestitum*.

Synonymy of the spine-bearing *Cochliopodium* spp.

As mentioned earlier, *C. pilosum* Hertwig et Lesser, 1874 is certainly the synonym of *C. vestitum*. Hertwig and Lesser (1874) only cite Archer's (1871) description and provide a new name for a species, which they transfer to a new genus. This mistake has already been corrected (Archer 1877, Leidy 1879), and *C. pilosum* is not a valid species since 1877.

Cochliopodium echinatum has initially been described without any data on the cell dimensions and locomotive morphology (Korotneff 1879). After that it

was observed by Penard (1902), who provided the diameter of the floating cell. Measurements of the figures made by Korotneff (Fig. 25) and Penard show that the spines of *C. echinatum* were approximately 10-15 μm in length, which is identical to *C. vestitum*. My strains of *C. vestitum* are highly similar to *C. echinatum*, at least in those features which were described by the researchers mentioned (*op. cit.*). West (1901) described another spine-bearing species, *C. longispinum* (Fig. 26), for which Cash and Hopkinson (1909) suggested a synonymy to *C. echinatum*. Both West, and Cash and Hopkinson state that *C. longispinum* differs from *C. vestitum* in the more delicate "shell", wider "shell mouth", broader pseudopodia, lack of chlorophyll granules in the cytoplasm and longer spines (23-29 μm). However, the thickness of tectum observed with bright field LM may depend on the conditions of observation. "Width of the shell mouth" is outstandingly variable in *Cochliopodium* amoebae, given the nature of their "shell" (tectum). Breadth of the pseudopodia is also variable, depending on the state of an amoeba, and the presence of chlorophyll granules in the cytoplasm depends on the diet of the cell. Thus, the only stable difference between *C. vestitum* and *C. longispinum* is the length of the spines, but this seems to be not enough for reliable identification of the species. Therefore, I propose a synonymy of *C. vestitum*, *C. echinatum* and *C. longispinum*, and *Cochliopodium vestitum* (Archer, 1871) remains the only valid species by priority.

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Taxonomic Studies on Three Marine Pleurostomatid Ciliates: *Kentrophyllum verrucosum* (Stokes, 1893) Petz, Song *et* Wilbert, 1995, *Epiphyllum soliforme* (Fauré-Frémiet, 1908) gen. n., comb. n. and *Amphileptus sikorai* sp. n., with the Establishment of a New Genus *Epiphyllum* (Ciliophora: Pleurostomatida)

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Summary. The morphology of living cells and infraciliature of three marine pleurostomatid ciliates, *Kentrophyllum verrucosum* (Stokes, 1893) Petz, Song *et* Wilbert, 1995, *Epiphyllum soliforme* (Fauré-Frémiet, 1908) gen. n., comb. n. and *Amphileptus sikorai* sp. n., collected from mariculture ponds near Qingdao (Tsingtao), China, have been investigated. Based on the present work and on previous data, an improved diagnosis for the genus *Kentrophyllum* Petz, Song *et* Wilbert, 1995 is suggested: leaf-shaped Amphileptidae without conspicuous neck-like region and having numerous somatic kineties on both cell sides; spines along ventral and dorsal margins; somatic ciliature on the right side forming sutures; one peripheral kinety consisting of dikinetids and forming a complete circle surrounding cell margin on right side. The new genus *Epiphyllum* is diagnosed by the following combination of characters, which separates it from other pleurostomatids: (1) sutures present on both right and left sides; (2) peripheral kinety present; (3) numerous somatic kineties on both sides of cell; (4) cell without typical neck-like portion, hence generally oval or leaf-like in body outline, although it also exhibits high levels of metaboly; (5) presence of peribuccal papillae along left side of oral slit; (6) spines absent. Redefined diagnoses for *K. verrucosum* and *E. soliforme* are also supplied. As a new species, *Amphileptus sikorai* can be distinguished by: medium-sized marine *Amphileptus* about 90-200 × 20-60 μm *in vivo*, leaf-shaped body with a conspicuous hyaline fringe, no neck-like region; 14-17 left and 13-18 right somatic kineties; three perioral kineties bending to dorsal side at anterior end; two macronuclear nodules; two or three contractile vacuoles positioned dorsally in mid-body; bar-shaped extrusomes densely arranged in hyaline fringe along cell margin. Based on the data available, a new key to some closely related genera of pleurostomatids is suggested.

Key words: *Amphileptus sikorai* sp. n., *Epiphyllum* gen. n., *Kentrophyllum*, marine ciliates, morphology and infraciliature.

INTRODUCTION

Laterally compressed pleurostomatous ciliates with a slit-like cytostome are free-living and commonly found in a variety of habitats all over the world (Kahl 1931, Dragesco 1960, Borror 1963, Agamaliev 1967, Hartwig 1973, Fryd-Versavel *et al.* 1975, Wilbert and Kahan 1981, Foissner 1987, Li 1990, Song 1994, Martin-Cereceda *et al.* 1995).

Although numerous species in this group have been described, early studies were based almost exclusively on live observations (Kahl 1931; Dragesco 1954, 1965; Vuxanovici 1959, 1961; Canella 1960), which has led to great difficulties in species identification and taxa separation as noted by several researchers (Wilbert and Kahan 1981, Foissner 1984). Only in the last three decades have silverstain methods been employed to reveal the infraciliature of pleurostomatids (Fryd-Versavel

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et al. 1975; Foissner 1977/78, 1984; Song and Wilbert 1989; Song 1993; Song and Gao 1994; Foissner and Leipe 1995; Foissner *et al.* 1995; Petz *et al.* 1995; Song *et al.* 2004). Nevertheless, many forms remain unknown in terms of their infraciliature and hence need reinvestigation using modern methods.

Compared with the freshwater pleurostomatids, investigations on marine forms using modern methods are particularly lacking (Song 1991, Petz *et al.* 1995, Song *et al.* 2004, Wilbert and Song 2004,). Recently, during investigations carried out on mariculture ciliate fauna in north China, one new and two poorly known pleurostomatids were isolated from marine culturing ponds near Qingdao. Detailed morphological observations of the three taxa were carried and the results of these investigations are presented here.

MATERIALS AND METHODS

Samples were collected (on 4th January, 2002; 5th August, 2002; and 10th April, 2003) from three mariculture ponds near Qingdao (Tsingtao, 36°08'N; 120°43'E), China.

Living cells were isolated and observed using bright field and differential interference contrast microscopy. The infraciliature was revealed by the protargol impregnation method according to Wilbert (1975). Living individuals were examined at 100× to 1000× magnification; measurements were carried out with an ocular micrometer; drawings of stained specimens were performed at 1250× with the aid of a camera lucida. Terminology is mainly according to Corliss (1979) and Foissner and Leipe (1995). The following terms need to be explained in order to avoid any confusion:

Metaboly: Movements of the cell that involve rapid contortions and/or contractions.

Peribuccal papillae: Wart-like protuberances on the cell surface, densely spaced along the left edge of oral slit in some leaf-shaped pleurostomatids.

Peripheral kinety: Homologous with perioral kinety, as a single and continues row around the cell margin on the right side. It is usually composed of closely arranged dikinetids.

Spine(s): This term describe here the cytoplasm structure found in some pleurostomatids, which is unmovable and spine-like, usually sparsely distributed along the ventral and dorsal margin except the cytostome regions. This structure should be detected *in vivo*.

Suture: The centrally positioned slit-like, glabrous area formed by the shorted somatic ciliary rows, typically on the right side (referred to by some authors as the "spica").

RESULTS AND DISCUSSION

Order: Pleurostomatida Schewiakoff, 1896
Family: Amphileptidae Bütschli, 1889
Genus: *Kentrophyllum* Petz, Song *et* Wilbert, 1995

Based on the Antarctic organism *Kentrophyllum antarcticum*, Petz *et al.* (1995) established the genus *Kentrophyllum* and gave a brief definition as follows: "medium-sized to large Amphileptidae, very flat. Having spines along ventral and dorsal margins. Right perioral kinety surrounding almost entire body". According to the present studies, we supply here an improved diagnosis.

Improved diagnosis: Leaf-shaped Amphileptidae without conspicuous neck-like region and with numerous somatic kineties on both cell sides; spines along ventral and dorsal margins; somatic ciliature on the right side forming sutures; one peripheral kinety consisting of dikinetids and forming a complete circle surrounding cell margin on right side.

Remarks: We have here introduced two additional diagnostic features for this genus, *viz*: the possession of sutures on the right side and the leaf-shaped body shape (i.e. without typical neck-like region) into the definition. We believe these features to be the most reliable criteria for recognizing this genus, notwithstanding the fact that body shape is seldom used in generic diagnoses. It also seems reasonable to include in the diagnosis the numerous somatic kineties on both cell sides, a feature that is present in all *Kentrophyllum* spp. so far described. The term peripheral kinety (instead of perioral kinety) is suggested because this truly specialized structure is unique and clearly different from the typical perioral kinety present in some other pleurostomatids.

Hence, this genus is circumscribed by: (1) the very flat, leaf-shaped body; (2) the peripheral kinety; (3) sutures on the right side; (4) spines along cell margin; (5) both sides densely ciliated. In addition, this group is possibly exclusively marine although further studies are required in order to confirm this.

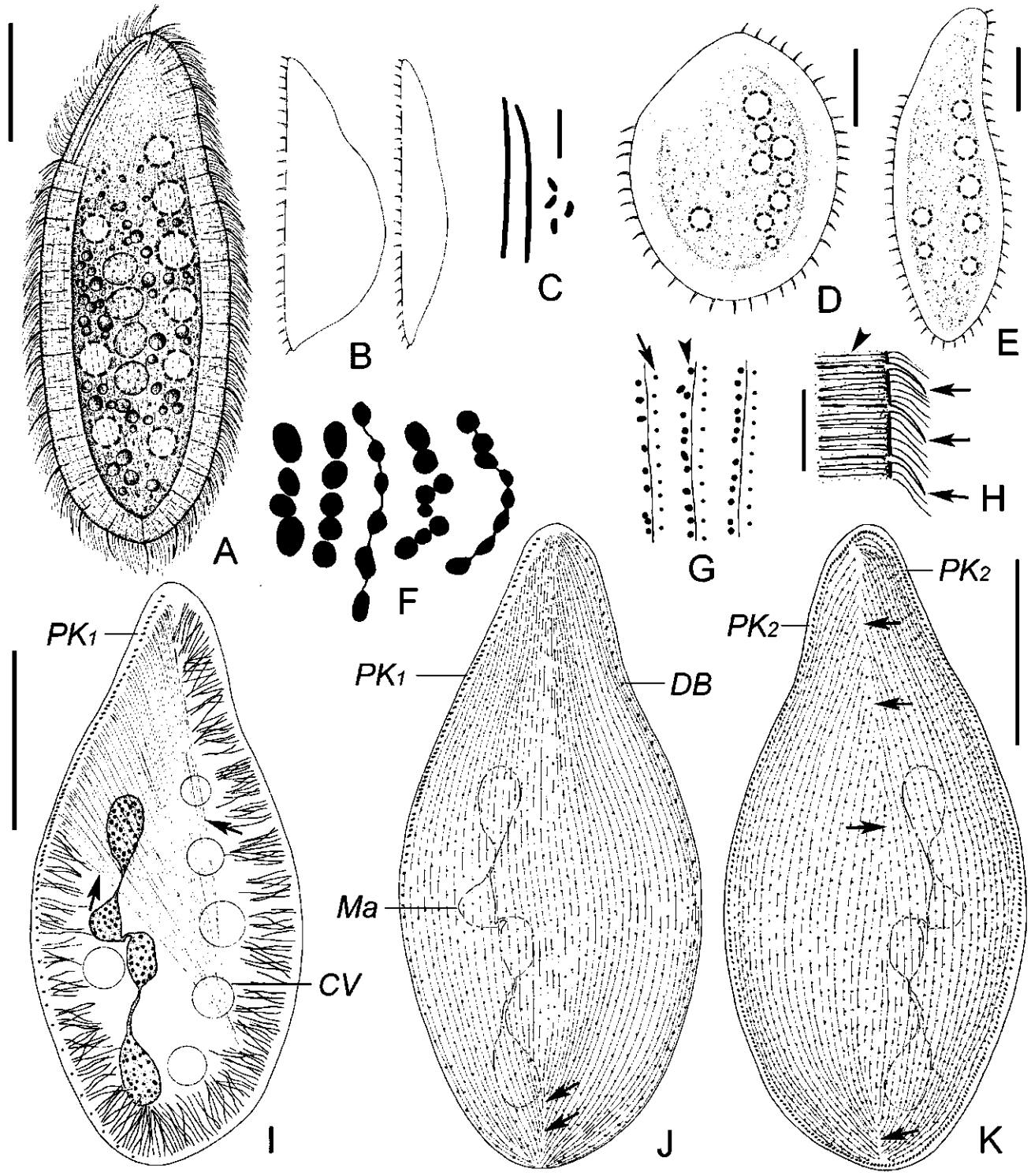
Kentrophyllum verrucosum (Stokes, 1893) Petz, Song *et* Wilbert, 1995 (Figs 1-3, Table 1)

Basionym: *Loxophyllum* (*Litosolenus*) *verrucosum* (Stokes, 1893) Kahl, 1931

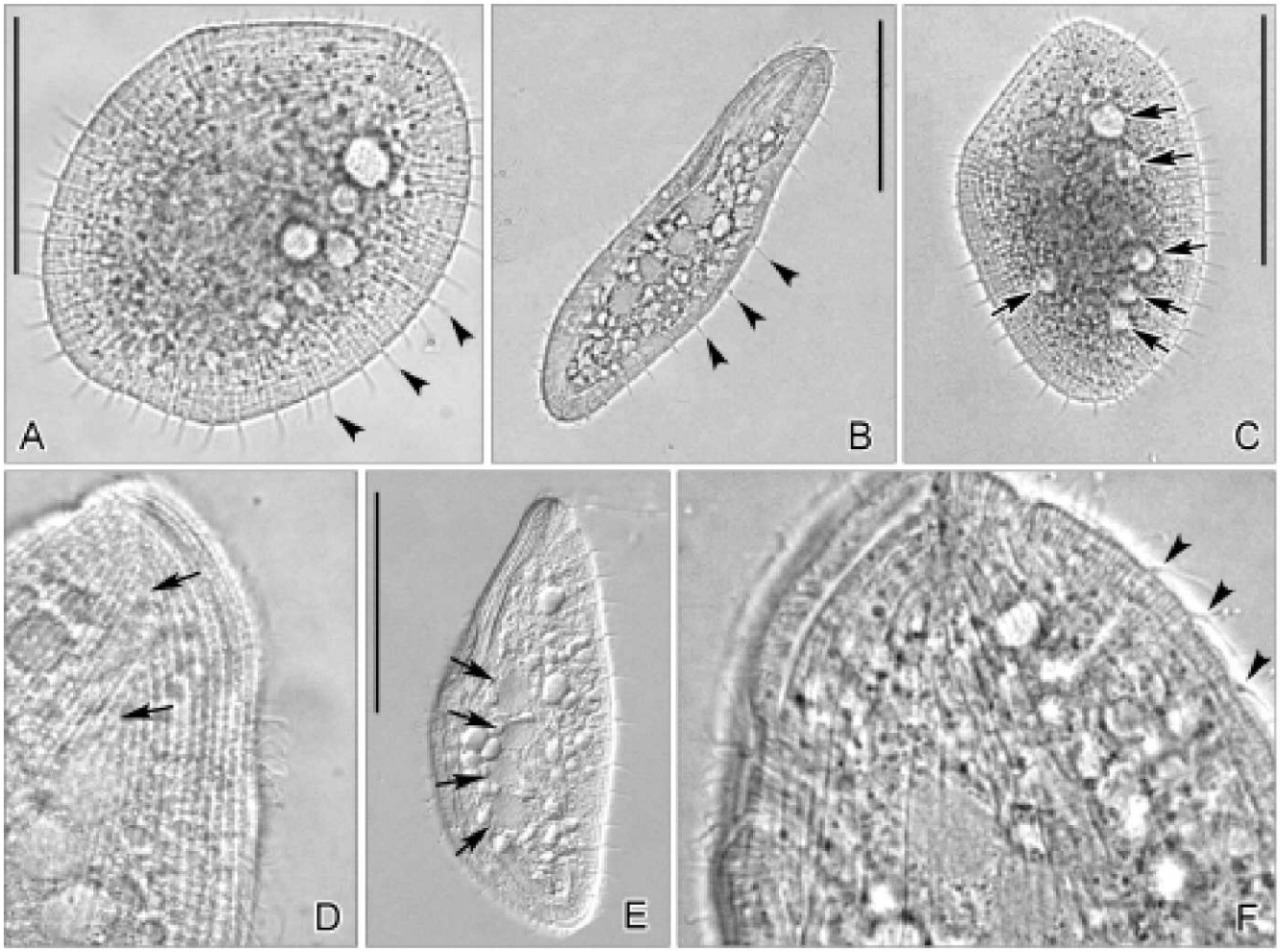
Syn. *Loxophyllum multinucleatum* Ozaki *et* Yagiu, 1943

This species has never been described using silverstain methods, hence the ciliature is hitherto unknown. Based on the Qingdao population as well as the previous reports, we here give a new definition and description:

New diagnosis: Leaf-shaped marine *Kentrophyllum*, *in vivo* about 90-250 × 60-160 μm. Cell exhibits high



Figs 1A-K. Morphology and infraciliature of *Kentrophyllum verrucosum*, from life (A-E, H) and after protargol impregnation (F, G, I-K). **A** - left view of a typical individual; **B** - lateral views; **C** - two types of extrusomes; **D** - showing typical body shape of stationary individual, note the contractile vacuoles; **E** - extended body shape when moving; **F** - macronuclear nodules; **G** - showing the short, spindle-shaped extrusomes (arrowhead) distributed along ciliary rows (arrow); **H** - to show the long, bar-shaped extrusomes (arrowhead) and the spines (arrows); **I** - left side view, showing the nematodesmata (arrows), and the extrusomes surrounding the body margin except the oral area; note the nuclear apparatus and the variation in size of the contractile vacuoles; **J, K** - infraciliature of left and right sides respectively of the same individual, arrows indicate the sutures; note the peripheral kinety (i.e. PK_2), which forms a loop that surrounds the body on right side. CV - contractile vacuole, DB - dorsal brush kinety, Ma - macronucleus, PK_1 - perioral kinety 1, PK_2 - perioral kinety 2 (i.e. peripheral kinety). Scale bars 5 μm (C); 40 μm (D, E, I, K); 50 μm (A).



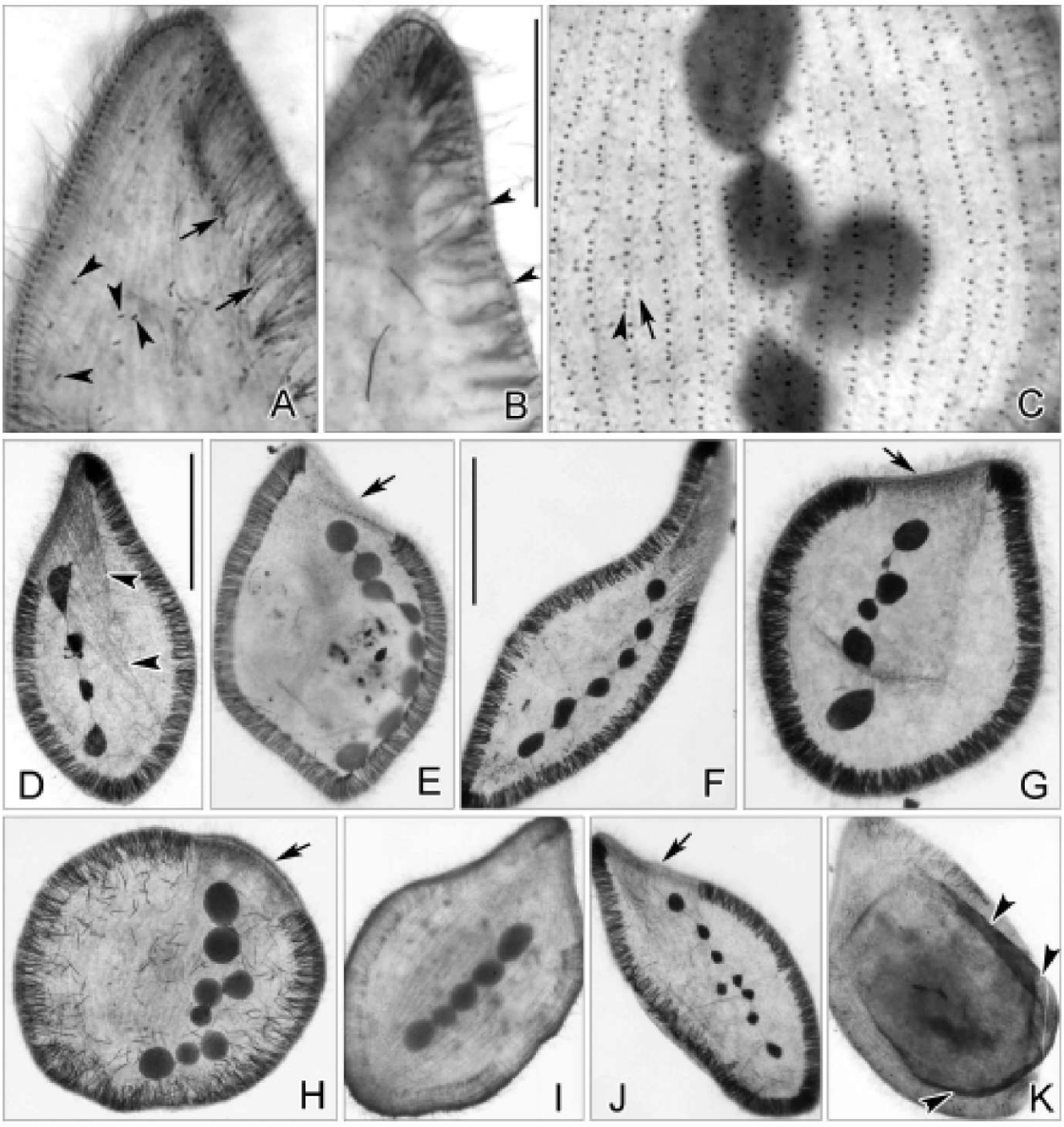
Figs 2A-F. Photomicrographs of *Kentrophyllum verrucosum* from life. **A, B** - views of left side, showing typical body shapes when stationary (A) and moving (B), arrowheads indicate spines which surround the entire body except oral area; note the extrusomes concentrated in the hyaline fringe surrounding the cell; **C** - left side view, arrows marks contractile vacuoles; **D** - the anterior part of the right side, note the conspicuous suture (arrows); **E** - view of left side (interference contrast), arrows indicate macronuclear nodules; **F** - the anterior end of left side, arrowheads mark the spines. Scale bars 70 μm (A); 90 μm (B); 100 μm (C, E).

levels of metaboly. 30-48 right, and 35-53 left somatic kineties with sutures on the right and the posterior left sides. About 6-10 contractile vacuoles along ventral and dorsal margins. With 2-9 (usually 4) macronuclear nodules. Two types of extrusomes. Dorsal brush kinety extending entire length of body.

Deposition of slides: Since no silver impregnated specimen are known to exist, one voucher slide of protargol impregnated specimens is deposited in the Natural History Museum, London, UK with registration No. 2004:3:22:1. A second slide (No. Lin-02-08-5-1) is deposited in the Laboratory of Protozoology, Ocean University of China.

Ecological features: Found in a shrimp-farming pound (5 August 2002), water temperature at sampling was about 27°C, salinity *ca* 22‰, pH *ca* 8.1.

Redescription: Cell highly metabolic with body shape strongly variable: from slender to completely oval, generally no typical form (Figs 1A, D, E; 2A-C; 3D-K). When gliding on the bottom of the Petri dish, often broadly leaf-shaped (Fig. 1A) to more or less sigmoid (Fig. 1E), with anterior end slightly narrowed. Size highly variable, about 90-250 × 60-160 μm *in vivo*, usually around 150-180 × 70-90 μm; the body size of a cell 130 × 100 μm can extend to 240 × 60 μm (Figs 1A, D, E; 3D-J). Laterally compressed about 3-1.5:1, right side



Figs 3A-K. Photomicrographs of *Kentrophyllum verrucosum* after protargol impregnation. **A** - anterior part of left side, note the densely spaced peripheral kinety (i.e. PK_2), arrows indicate the long extrusomes, arrowheads mark short extrusomes scattered in the cytoplasm; **B** - anterior end of left side, arrowheads mark the dorsal brush; **C** - middle part of right side, to show the distribution of ciliary rows (arrow shows example) and short spindle-shaped extrusomes row (arrowhead shows example); **D-J** - different body shapes, note the distribution of long extrusomes and the macronuclear nodules, arrows mark the cytostome, and arrowheads (D) indicate nematodesmata; **K** - view of left side, arrowheads demonstrate the conspicuous vault. Scale bars 30 μm (B) 50 μm (D) 80 μm (F).

Table 1. Morphological characterization of *Kentrophyllum verrucosum* (1st line), *Epiphyllum soliforme* (2nd line) and *Amphileptus sikorai* (3rd line). Data based on protargol impregnated specimens. All measurements in μm. Abbreviations: CV - coefficient of variation, M - median, Max - maximum, Mean - arithmetic mean, Min - minimum, n - number, SD - standard deviation.

Characters	Min	Max	Mean	M	SD	CV	n
Body length	88	240	169.8	168	42.19	24.8	25
	58	184	111.4	120	40.33	36.2	25
	88	184	128.4	128	23.46	18.3	25
Body width	50	160	89.8	96	30.18	33.6	25
	42	92	58.6	56	12.50	21.3	25
	24	56	41.8	40	7.10	17.0	25
Length of oral slit	32	100	62.4	64	16.49	26.4	25
	20	66	41.9	40	14.96	35.7	25
	20	28	25.2	26	2.38	9.5	25
Length of nematodesmata	88	160	125.9	120	21.92	17.4	14
	40	82	58.7	56	11.46	19.5	11
	45	88	70.6	80	17.32	24.6	9
Length of the long extrusomes	10	13	10.9	10	1.13	10.4	25
	6	8	7.12	6	0.93	13.0	25
	9	12	10.1	10	0.54	5.4	25
Number of right somatic kineties	30	48	39.1	40	4.88	12.5	21
	32	46	38.7	38	3.12	8.1	25
	13	18	14.6	14	1.38	9.5	23
Number of left somatic kineties	35	53	43.1	44	6.01	14.0	16
	34	56	45.5	45	6.59	14.5	22
	14	17	15.4	15	0.76	5.0	19
Number of macronuclei	2	9	4.8	4	1.63	33.6	25
	4	6	4.2	4	0.44	10.3	25
	2	2	2	2	0	0	25
Length of macronucleus	10	32	15.7	16	4.85	31.0	25
	10	24	14.6	12	4.44	30.5	25
	10	22	16.3	16	2.46	15.1	25
Width of macronucleus	7	14	10.1	10	2.13	21.0	25
	8	19	12.4	10	3.48	28.1	25
	8	17	12.9	12	2.28	17.7	25

(i.e. side in contact with substratum) flat, left slightly to distinctly vaulted, mostly due to the presence of food material (Figs 1B, 3K), and with many inconspicuous longitudinal shallow grooves. Fringe flat, thin and hyaline, about 20 μm in width, with long, uniformly distributed extrusomes (Figs 1A, D, E; 2A-C). Two kinds of extrusomes: type 1, *ca* 2 μm long, spindle-shaped, most distributed regularly along ciliary rows, some scattered in cytoplasm (Fig. 1C; arrowheads in Figs 1G; 3A, C). Type 2, *in vivo* 18-20 μm long, bar-shaped, densely arranged, mostly concentrated in the hyaline fringe and surrounding entire cell excluding buccal area, some scattered in cytoplasm (Fig. 1C, arrowhead in Fig. 1H, arrows in Fig. 3A). Those extrusomes that lie at the bases of the spines often appear slightly stronger than others when observed *in vivo*, although no clear difference could be detected after staining (Figs 1A, H; 2A, C; 3A, H).

Pellicle thin. Cytoplasm gray to dark in central region of cell, depending on food supply, often containing many large (3-7 μm across), shining globules (Fig. 1A).

When observed *in vivo* numerous (30-50) immobile spines visible; spines needle-shaped, 8-12 μm in length, evenly spaced on ventral and dorsal margins, but absent in area of cytostome; difficult to detect after protargol impregnation (arrows in Fig. 1H; arrowheads in Figs 2A, B, F). Cytostome extending about 33% of cell length as indicated by nematodesmata, which originate from kinetosomes of perioral rows and are recognizable as delicate fibrils after protargol impregnation terminating in posterior 1/5 of body (Figs 1I, 3D). Peribuccal papillae not observed.

About 6-8 contractile vacuoles (CV), most (5 or 6) along the dorsal margin, 1-3 along the ventral margin (n = 10). Position of CVs seemingly not fixed: in oval forms CVs often packed together (Figs 1D, 2A) or well

away from the margin region (Fig. 2C), while in slender specimens CVs clearly in rows (Figs 1A, E); diameter of CVs up to18 μm when fully expanded (Figs 2A-C).

Two to nine (mostly 4) macronuclear nodules (Ma) near central region of cell; nodules spherical to ellipsoid, 10-30 × 7-14 μm in size after fixation and interconnected by funiculus (Figs 1A, F; 2B, E; 3D-J). Nucleoli round, distributed throughout nodules (Fig. 1I). Micronucleus not observed.

Movement by slowly gliding on substrate, rarely swimming in water.

Somatic ciliature as shown in Figs 1J, K; 3A-C. 30-48 (mean 40) right kineties densely ciliated, cilia about 10-12 μm long; intermediate kineties noticeably shortened anteriorly, forming a distinct anterior suture that extends >50% body length, while the posterior suture is inconspicuous (Figs 1K, 2D). Left side also densely ciliated, about 35-53 (mean 44) somatic kineties with cilia about 6-8 μm long; nearly half the kineties anteriorly shortened and terminating at perioral kinety 1 (PK_1). In central region, some kineties shortened posteriorly, giving the impression of a suture in subcaudal area (arrows in Fig. 1J). Dorsal brush kinety (DB) extending over entire length of body, composed of regularly spaced dikinetids (Fig. 1J, arrowheads in Fig. 3B). Two perioral kineties (PK_{1-2}): PK_1, at left of oral slit, formed of densely spaced dikinetids in anterior half and loosely spaced monokinetids in posterior half (Fig. 1J); right perioral kinety, or peripheral kinety (PK_2), forming a closed loop, composed of densely spaced kinetosome pairs and surrounds entire body (Fig. 1K).

Comparison: *Kentrophyllum verrucosum* (Stokes, 1893) was known for a long time as *Loxophyllum verrucosum* (Stokes, 1893) Kahl, 1931 until Petz *et al.* (1995) transferred it to the current genus. Although it has been reported or mentioned on at least two other occasions (Dragesco 1965, Czapik and Jordan 1976), the infraciliature has never previously been described. To summarize the previous data, this species has the following combination of features: (1) medium-sized body with 3-9 macronuclear nodules and several contractile vacuoles along dorsal margin; (2) with spines and bar-shaped extrusomes surrounding nearly entire body; (3) the right side is densely ciliated, with about 35-40 ciliary rows; (4) marine habitat (Table 2).

Our population corresponds to previous descriptions of *Kentrophyllum verrucosum* in most respects, i.e. the body shape and size, presence of spines, several macronuclear nodules, several to many contractile vacuoles near dorsal margin, the number of somatic kineties, and its marine habitat (Stokes 1893, Dragesco 1965, Czapik and Jordan 1976). A conspicuous difference in our population is the presence of (usually) 1-3 contractile vacuoles located near ventral margin, which are not mentioned in the previous reports. However, it should be noted that in many marine ciliates including pleurostomatids, the number and position of the contractile vacuoles is difficult to discern. In some taxa no contractile vacuoles are visible *in vivo* although excretory pores are revealed by silver impregnation. Alternatively, contractile vacuoles may be readily detectable in freshly isolated specimens but not after culture whereas in other cases the reverse is true (present authors, unpublished; Wilbert and Song 2004). Finally, it is not uncommon for contractile vacuoles to be present but inactive, or to pulse with very long time intervals (>10 minutes) and thus to be overlooked. In conclusion, we believe that early descriptions of the number and positions of contractile vacuoles in marine pleurostomatids should be treated with caution.

In addition, the peribuccal papillae, which are present in the population described by Dragesco (1965), were not present in our form nor were they mentioned in the original description (Stokes 1893). Furthermore Kahl (1931) only observed some eyelash-brush-like cilia regularly arranged along the oral slit in the German population. We therefore believe that the presence or absence of peribuccal papillae is probably a population-dependent character.

Among the known nominal *Kentrophyllum* spp, *K. verrucosum* is most similar to *K. setigerum* (Quennerstedt, 1867) Petz, Song *et* Wilbert 1995, which has been repeatedly investigated but never using modern methods (Sauerbrey 1928, Kahl 1931, Wang and Nie 1932, Dragesco 1960, Petran 1963) (Table 2). Apart from the variable number of macronuclear nodules (2-9 *vs.* 4 in *K. setigerum*), *K. verrucosum* also differs from *K. setigerum* in having more somatic kineties on the right side (30-48 *vs. ca* 22).

Kentrophyllum pseudosetigerum (Dragesco, 1954) Petz, 1995 is also similar to *K. verrucosum*. The former can be distinguished, however, by having more macronuclear nodules (*ca* 12 *vs.* 2-9 in *K. verrucosum*) and fewer somatic kineties (16-17 *vs.* 30-48 in *K. verrucosum*) (Dragesco 1954, 1960) (Table 3).

Considering the other 3 congeners, *Kentrophyllum verrucosum* differs from *K. raikovi* (Dragesco, 1965) Petz, 1995 in having a much smaller body size (90-250

vs. 360-500 μm) and the regular distribution of contractile vacuoles (*vs.* many CVs dispersed throughout the body in the latter), while both *K. fibrillatum* (Dragesco, 1954) Petz, 1995 and *K. antarcticum* Petz, 1995 invariably have only 2 macronuclear nodules (*vs.* usually 4 in *K. verrucosum*) (Table 3).

In 1943, Ozaki and Yagiu reported a new species from the Japan Sea under the name *Loxophyllum multinucleatum*. This name, however, was preoccupied by a marine species found in Germany, *Loxophyllum multinucleatum* Kahl, 1928, which lacks spines and is possibly a valid species of *Loxophyllum* although its infraciliature remains undescribed (Kahl 1928). Considering the general appearance of *L. multinucleatum* Ozaki *et* Yagiu, 1943 *in vivo*, i.e. the body shape and size, the number of macronuclei (5-14), the number and position of contractile vacuoles, the presence of spines surrounding the cell margin, and the habitat, we believe that this organism is conspecific with *K. verrucosum*.

Genus: *Epiphyllum* gen. n.

Diagnosis: Amphileptidae with flat body, generally leaf-shaped but highly variable and without neck-like portion; numerous somatic kineties on both cell sides; having peripheral kinety on right side; sutures present on both left and right sides; spines absent.

Type species: *Epiphyllum soliforme* (Fauré-Fremiet, 1908) comb. n.

Etymology: Composite from the Greek words *Epi* (upon, on the surface of) and *phyllum* (leaf). Neuter gender.

Remarks: Generally, the new genus *Epiphyllum* most closely resembles *Kentrophyllum* Petz, Song *et* Wilbert, 1995. It differs from the latter, however, in the absence of spines, which are characteristically around the body margin in *Kentrophyllum* (Petz *et al.* 1995).

The most conspicuous characteristics of *Epiphyllum*, which separate it from other related pleurostomatid genera (e.g. *Amphileptus*, *Opithodon*, *Loxophyllum*, *Litonotus*, *Acineria*), are the presence of sutures on both left and right sides and the possession of a *Kentrophyllum*-like ciliature pattern and morphology, i.e. numerous ciliary rows on the left side, presence of peripheral kinety, flat body lacking a neck-like region, absence of dorsal warts etc. (Foissner 1984, Augustin *et al.* 1987, Foissner and Foissner 1988, Song and Wilbert 1989).

***Epiphyllum soliforme* (Fauré-Frémiet, 1908) comb. n. (Figs 4-6, Table 1)**

Basionym: *Loxophyllum soliforme* Fauré-Fremiet, 1908

New diagnosis: Generally leaf-shaped marine *Epiphyllum* 50-200 × 40-100 μm *in vivo* with 2-5 contractile vacuoles along dorsal margin in mid-region. 32-46 right and 34-56 left somatic kineties. 4-6 macronuclear nodules. Two kinds of extrusomes (short, spindle-shaped and long-bar shaped). Dorsal brush kinety terminating at anterior 1/3 of cell length. Peribuccal papillae present.

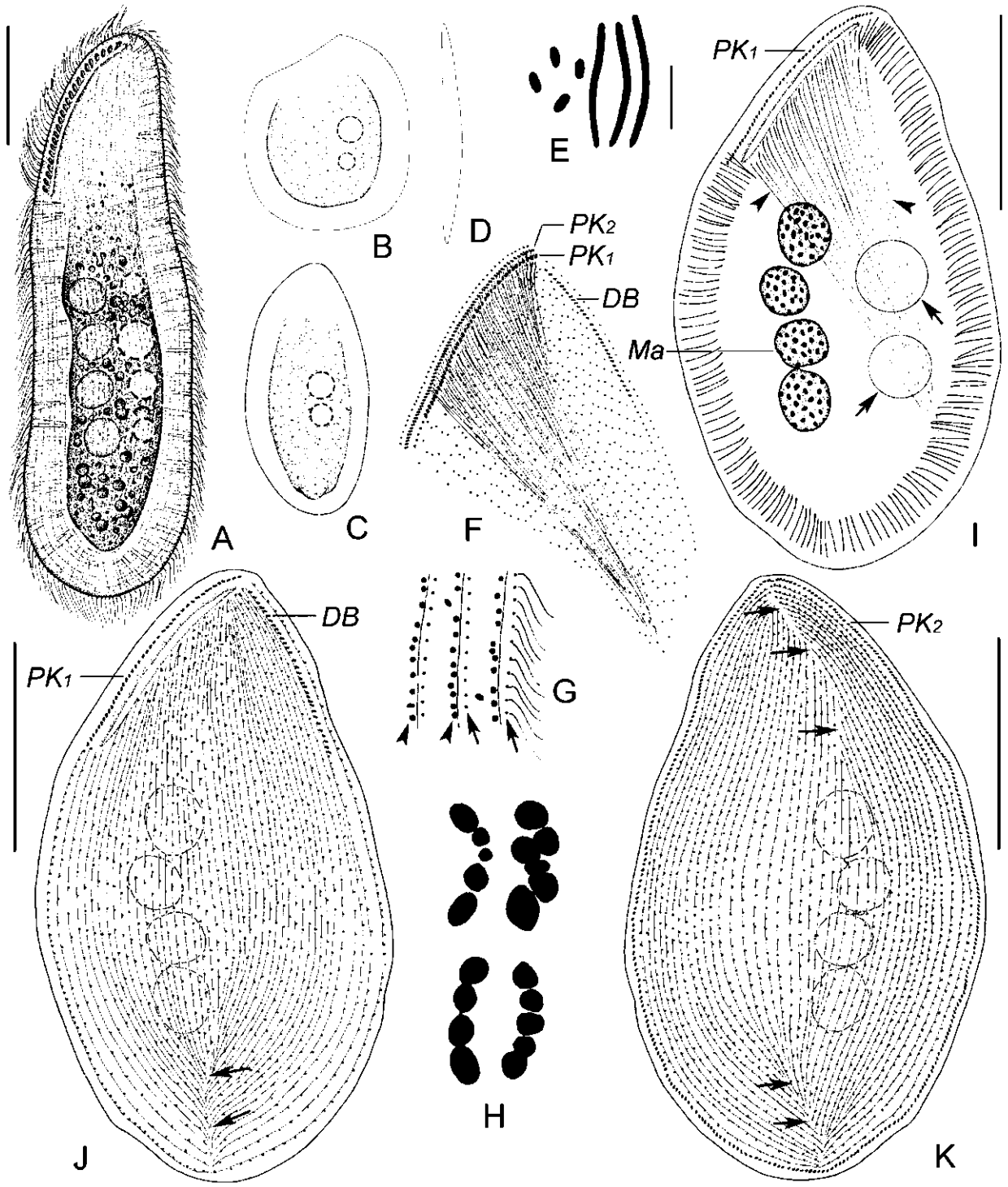
Ecological features: Isolated from a water-filter-system for fish-farming ponds (4 January 2002), water temperature about 25°C, salinity *ca* 31‰, pH *ca* 7.8.

Slide deposition: One voucher slide with protargol impregnated specimens is deposited in the Natural History Museum, London, UK with registration number 2004:3:22:2. A second slide is deposited in the Laboratory of Protozoology, Ocean University of China (slide number: Lin-02-01-04).

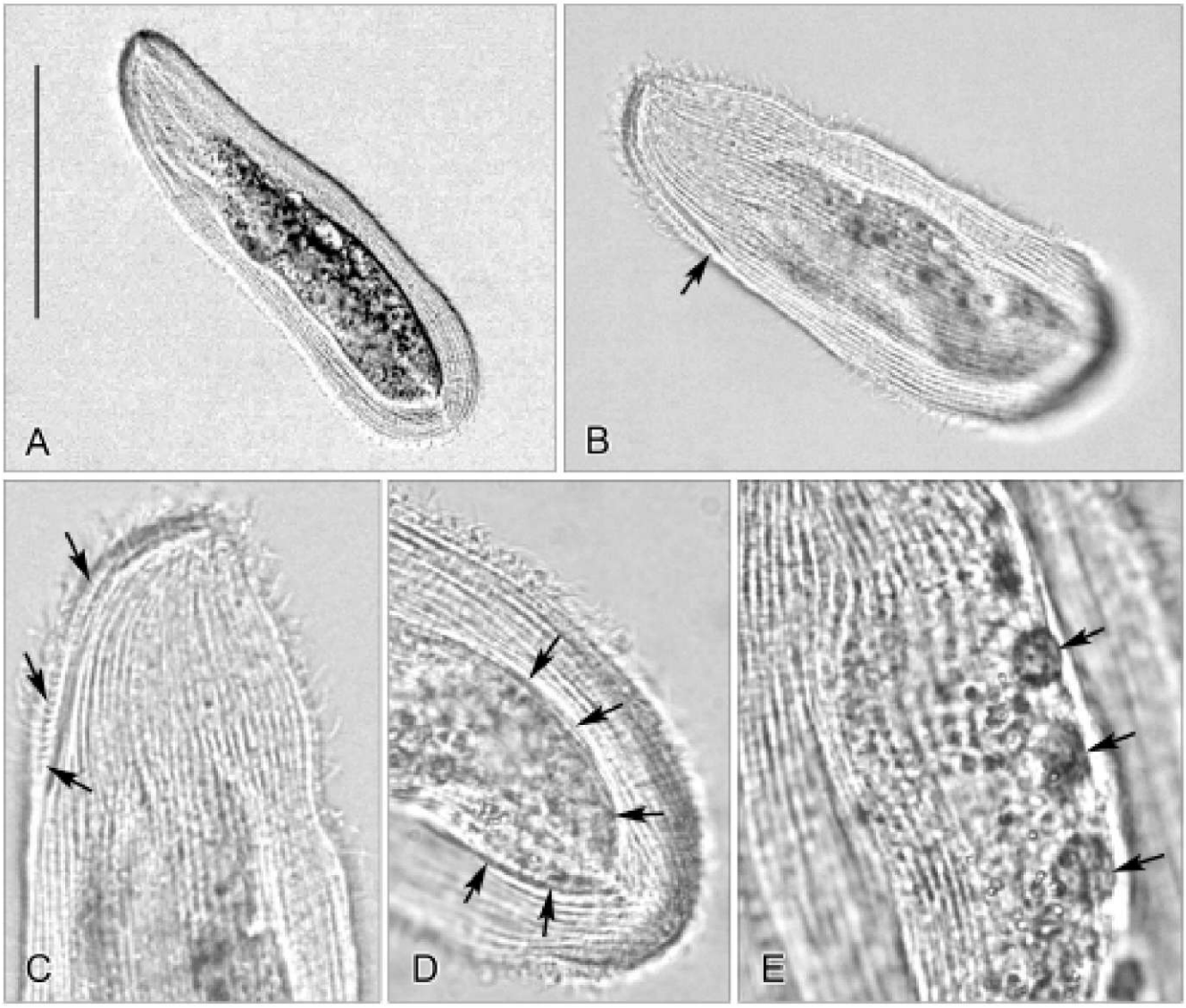
Redescription: Cell exhibits metaboly, with highly variable shape: when gliding on substrate, body broad to slim leaf-shaped, anterior portion often slightly narrowed, but clearly no neck-like region (Figs 4A, C; 5A, B); when stationary, body oval-shaped or nearly round (Figs 4B, 6H-L). Size variable, 50-200 × 40-100 μm *in vivo*, usually about 120-150 × 50-60 μm. Usually highly compressed laterally about 4-6:1; right side flat, left slightly vaulted with many inconspicuous longitudinal shallow grooves (Figs 4D; 5B, E). Fringe thin and hyaline, about 20 μm in width, and surrounding entire cell (Figs 4A-C, 5A-B). With two types of extrusomes (Fig. 4E): type 1 short spindle-shaped, about 2 μm long, mostly distributed regularly along ciliary lines, some scattered in cytoplasm (arrowheads in Figs 4G, 6F); type 2 bar-shaped, 15-17 μm long *in vivo*, mostly packed in hyaline fringe and uniformly distributed, some scattered in cytoplasm (Figs 4A, I; arrows in Fig. 6F).

Pellicle thin. Cytoplasm slightly yellow-coloured when observed at low magnification, but no coloured granules observed under high magnification; often containing many shining globules (3-5 μm in diameter) in central opaque region (Fig. 4A). No spines around cell margin.

Cytostome extending to 33% of cell length as indicated by nematodesmata, which are about 50-75% of



Figs 4A-K. Morphology and infraciliature of *Epiphyllum soliforme* gen. n., comb. n. from life (A-D) and after protargol impregnation (E-K). **A** - left view of a motile individual, note the peribuccal papillae along the left side of oral slit; **B, C** - the body shape of stationary (B) and motile (C) individuals; **D** - left-lateral view; **E** - two kinds of extrusomes; **F** - showing the perioral kineties, nematodesmata, and dorsal brush kinety; **G** - showing the short, spindle-shaped extrusomes (arrowheads) distributed along the ciliary rows (arrows); **H** - macronuclear nodules; **I** - left side view showing the nematodesmata (arrowheads), the contractile vacuoles (arrows), and the nuclear apparatus, note the long extrusomes surrounding the margin except cytostome area; **J, K** - the infraciliature of left and right sides respectively, arrows mark the three typical sutures; note the peripheral kinety on right side (i.e. PK_2) surrounding the whole body thus forming a loop. DB - dorsal brush kinety, Ma - macronucleus, PK_1 - perioral kinety 1, PK_2 - perioral kinety 2 (i.e. peripheral kinety). Scale bars 5 μm (E); 40 μm (A, I-K).



Figs 5A-E. Photomicrographs of *Epiphyllum soliforme* gen. n., comb. n.from life. **A, B** - views of left side, showing the typical body shapes when moving, arrow marks the posterior end of the oral slit; **C** - anterior region of left side, arrows demonstrate the peribuccal papillae along the left side of oral slit; **D** - posterior part of left side, arrows mark the bulge; **E** - central part of left side, arrows show the contractile vacuoles. Scale bars 80 μm (A, B).

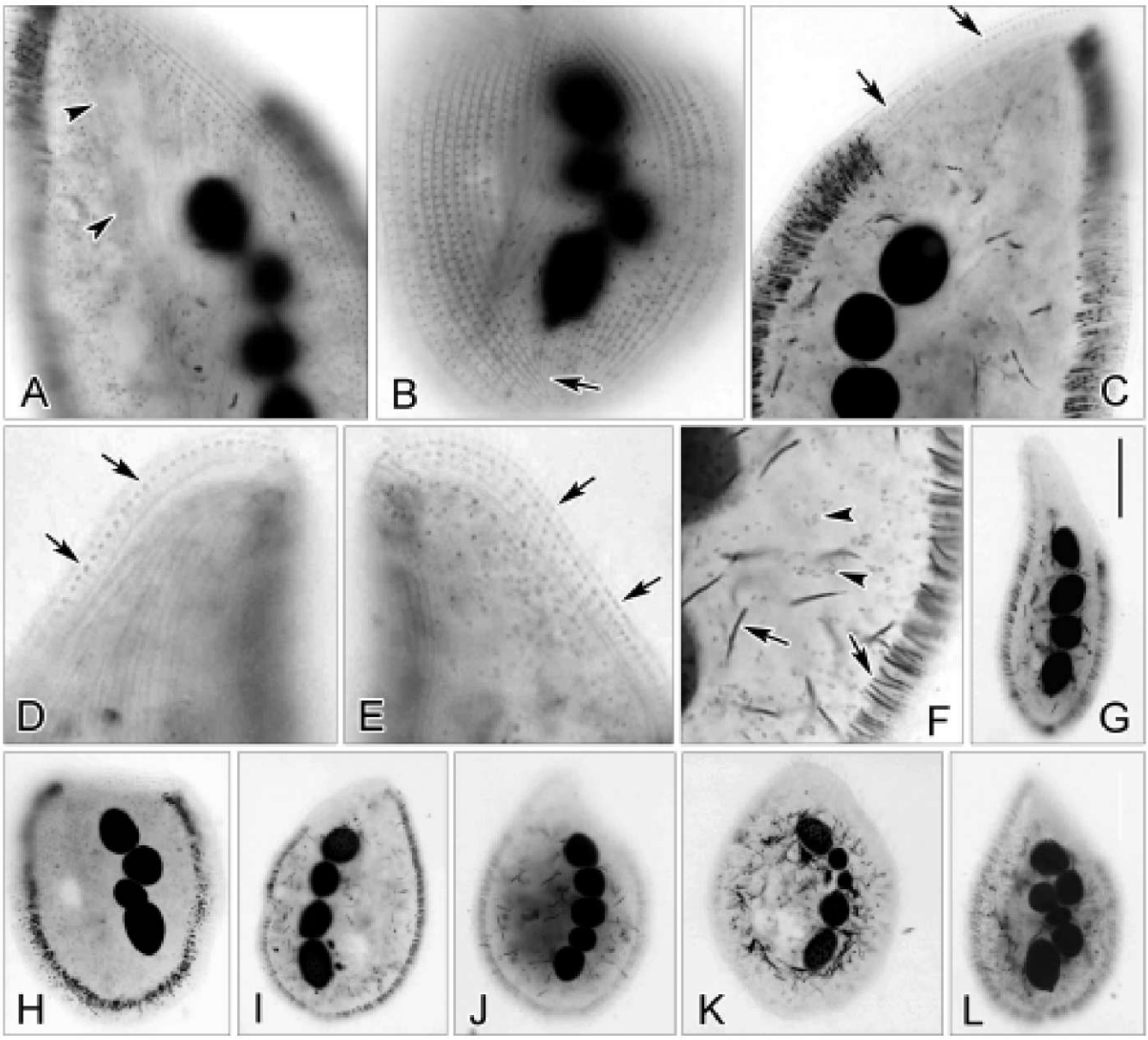
body length (Figs 4I, F). About 20-40 densely spaced peribuccal papillae along left side of oral slit, 2-3 μm in length, and difficult to detect after protargol impregnation (Fig. 4A, arrows in Fig. 5C).

About 2-5 contractile vacuoles (CV) located together near central region of cell and slightly displaced towards dorsal margin of cell (Figs 4A, 5B). Size of CVs highly variable, 8-15 μm in diameter (n = 10); normally 2-4 vacuoles, often only two of which keep pulsing in stationary cells (Figs 4A-C, 5E).

Nuclear apparatus near ventral margin and comprises 4-6 (usually 4) macronuclear nodules (Ma), slightly ellipsoid, about 10-24 × 8-19 μm in size after fixation (Figs 4A, I, H; 6G-L). Nucleoli round (Fig. 4I). Micronucleus not observed.

Movement by slowly gliding on substrate, rarely swimming in water and often stationary.

Somatic kineties as shown in Figs 4F, J, K; 6A-E. About 32-46 (mean 38) right kineties densely arranged, cilia about 4-6 μm long; intermediate kineties shortened



Figs 6A-L. Photomicrographs of *Epiphyllum soliforme* gen. n., comb. n. after protargol impregnation. **A, B** - infraciliature of right side, arrowheads indicate the nematodesmata, arrow demonstrates the posterior suture; **C** - anterior section of left side, arrows mark perioral kinety 1; **D** - anterior end of left side, arrows point to perioral kinety 1; **E** - anterior part of right side, arrows indicate the peripheral kinety (i.e. PK_2); **F** - the distribution of two kinds of extrusomes: short spindle-shaped (arrowheads) and long bar-shaped (arrows); **G-L** - different body shapes, note the distribution of long extrusomes and the macronuclear nodules. Scale bars 30 μm (L); 40 μm (G).

at both ends, forming a distinct anterior suture, that is >33% of body length, and a conspicuous subcaudal posterior suture (arrows in Figs 4K, 6B). Left side also densely ciliated, about 34-56 (mean 45) left kineties, which terminate anteriorly at perioral kinety 1 and also form a typical posterior suture in subcaudal area (arrows in Fig. 4J). Dorsal brush kinety (DB) extending to 33% of body length, comprising densely spaced dikinetids (Fig. 4J). Two perioral kineties (PK_{1-2}): PK_1 along left side of oral slit, with regularly spaced dikinetids in anterior 1/3 of body (*viz.* oral area) and continues posteriorly as monokinetids; PK_2, or peripheral kinety, composed of closely spaced dikinetids and surrounds entire right side of body (Figs 4I-K, 6C-E).

Table 2. Morphometrical comparison of different populations of *Kentrophyllum verrucosum* and *K. setigerum*. 1 - species, 2 - body length (in μm), 3 - number of macronuclear nodules, 4 - number of contractile vacuoles, 5 - distribution of extrusomes, 6 - distribution of spines, 7 - peribuccal papillae, 8 - number of right somatic kineties, 9 - number of left somatic kineties, 10 - source.

1	2	3	4	5	6	7	8	9	10
K. verrucosum	*ca* 200	4	many*	clustered**	sparse	absent	-	-	Stokes (1893)
K. verrucosum	200-250	3-6	several	clustered	sparse	absent	-	-	Kahl (1931)
K. verrucosum	130-300	4-9	many	uniform	sparse	present	36-40	*ca* 30	Dragesco (1965)
K. verrucosum	-	6-8	1 or 2	uniform	dense	present	35-40	-	Czapik and Jordan (1976)
K. verrucosum	90-250	2-9	6-10	uniform	dense	absent	30-48	35-53	Original
K. setigerum	-	4	3-4	-	sparse	-	-	-	Quennerstedt (1867)
K. setigerum	150-600	1	*ca* 5	clustered	sparse	present	-	-	Sauerbrey (1928)
K. setigerum	100-350	4	7	uniform	dense	present	-	-	Kahl (1931)
K. setigerum	105	4	several	uniform	dense	absent	-	-	Wang *et* Nie (1932)
K. setigerum	80-330	4	several	uniform	dense	present	*ca* 22	-	Dragesco (1960)
K. setigerum	90-220	4	many	uniform	dense	present	-	-	Petran (1963)
K. setigerum	85-240	4	*ca* 4	uniform	(absent)	absent	-	-	Jones (1974)

* Many: >10; ** Clustered: gathered in groups.

Comparison with similar species: This species was originally found in France in a brackish marsh near the Bay of Biscay by Fauré-Fremiet (1908) who gave a detailed description based on living observations and named it *Loxophyllum soliforme*. It has subsequently been reported only once (Kahl 1931) but without a detailed description. According to these two reports, this species has the following features: (1) leaf-shaped body, about 90 × 30 μm in size; (2) with wide hyaline fringe containing extrusomes; (3) no spines around body; (4) with small refringent nodules ("peribuccal papillae") along the edge of oral slit; (5) four macronuclear nodules and one large contractile vacuole in mid-body near dorsal margin; (6) marine habitat. Thus the infraciliature was hitherto unknown.

The Qingdao population corresponds very well with these features. Some individuals in our samples were slightly larger (50-200 × 40-100 *vs.* 90 × 30 μm) but this is almost certainly a population-dependent variation. The only significant difference between the two populations is the number of contractile vacuoles: our form has 2-5 CVs in a single dorsally located group near the mid-body (*vs.* one CV mentioned in previous reports). As discussed above (see description for *Kentrophyllum verrucosum*), there might be various reasons for this apparent difference. Therefore, the Qingdao population should be identified as *Loxophyllum soliforme* which is assigned to the new genus *Epiphyllum* as *E. soliforme* (Fauré-Fremiet, 1908) gen. n., comb n.

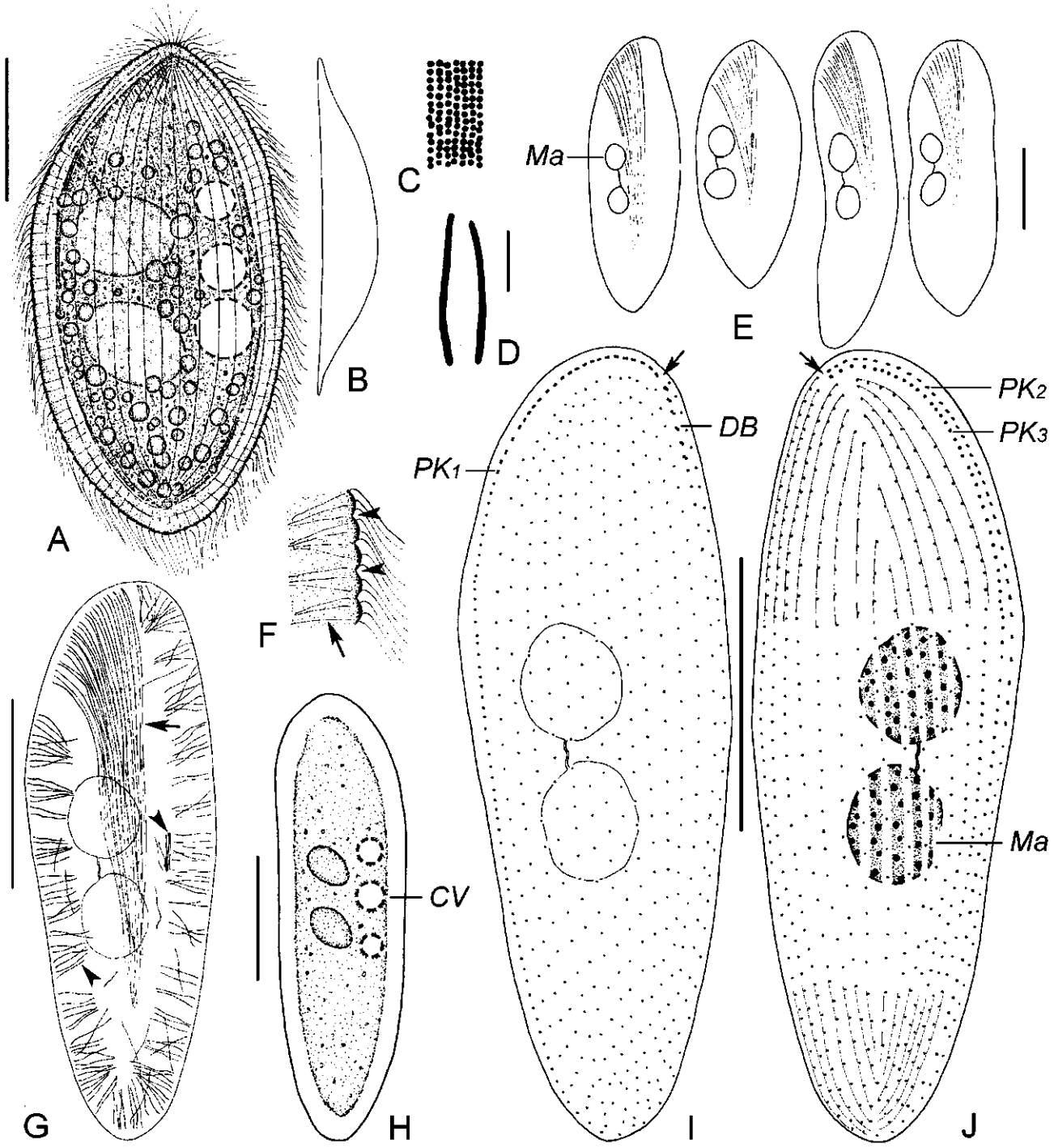
Considering its general morphological characteristics, *Loxophyllum hohuensis* Wang *et* Nie, 1933 found in China, is similar to *Epiphyllum soliforme*. However, it can be separated from the latter by its larger body size (200-280 *vs.* 50-200 μm in *E. soliforme*) and the fresh water habitat (*vs.* marine) (Table 4).

Other morphologically similar forms to which *E. soliforme* should be compared include: *Loxophyllum variabilis* Dragesco, 1954, *L. kahli* Dragesco, 1960, *L. elongatum* Tucolesco, 1962, *L. compressum* Dragesco, 1965, *L. asetosum* Burkovsky, 1970, and *L. schewiakoffi* Burkovsky, 1970. Although the infraciliature remains unknown for all these taxon, *Epiphyllum soliforme* can be easily distinguished from them by the combination of body size, the number of macronuclear nodules, the ciliary pattern and the number and arrangement of the contractile vacuoles (Table 4).

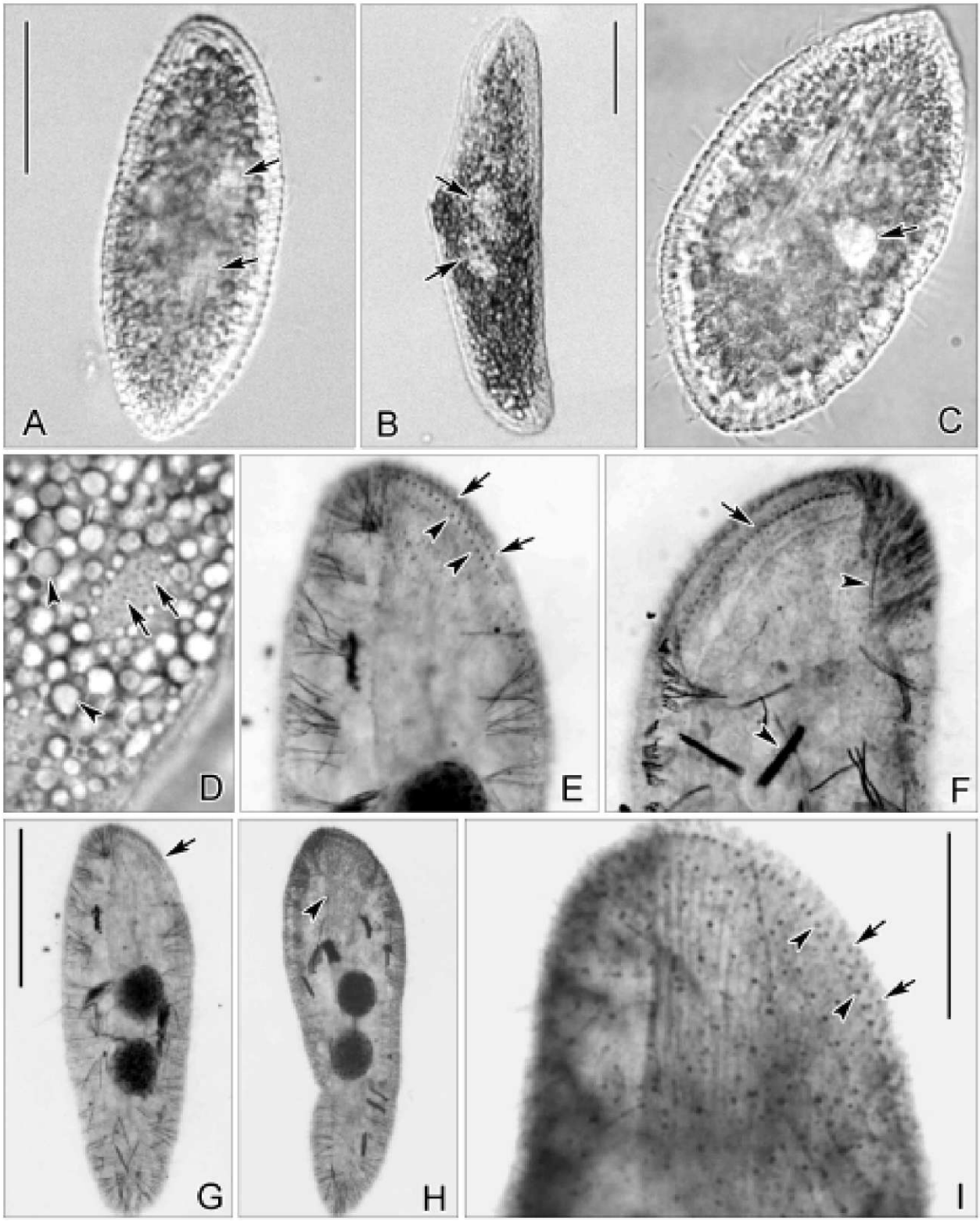
Genus: *Amphileptus* Ehrenberg, 1830

Amphileptus sikorai sp. n. (Figs 7, 8; Table 1)

Diagnosis: Medium-sized marine *Amphileptus* about 90-200 × 20-60 μm *in vivo*, leaf-shaped body with a conspicuous hyaline fringe, no neck-like region; 14-17 left and 13-18 right somatic kineties; three perioral kineties bending to dorsal side at anterior end; two macronuclear nodules; two to three contractile vacuoles positioned dorsally in mid-body; extrusomes bar-shaped, densely arranged in hyaline fringe along cell margin.



Figs 7A-J. Morphology and infraciliature of *Amphileptus sikorai* sp. n. from life (A-C, F, H) and after protargol impregnation (D, E, G, I, J). **A** - left view of a motile individual; **B** - lateral view; **C** - cortical granules; **D** - extrusome; **E** - different body shapes; **F** - to demonstrate the bar-shaped extrusomes (arrow) and the slightly notched pellicle (arrowheads); **G** - left side view, showing the nematodesmata (arrow) and the extrusomes (arrowhead) surrounding entire body except oral area; **H** - left side view, note the hyaline fringe and the location of the contractile vacuoles and macronuclear nodules; **I, J** - infraciliature of left and right sides respectively, arrows indicate the anterior end of the perioral kineties. CV - contractile vacuole, DB - dorsal brush kinety, Ma - macronucleus, PK_1 - perioral kinety 1, PK_2 - perioral kinety 2, PK_3 - perioral kinety 3. Scale bars 5 μm (D); 30 μm (E); 40 μm (A, G, H-J).



Figs 8A-I. Photomicrographs of *Amphileptus sikorai* sp. n. from life (A-D) and after protargol impregnation (E-I). **A, B -** right (A) and left (B) side views of different shaped individuals, arrows indicate large macronuclear nodules; **C -** left side view, arrow marks single, large contractile vacuole, note the notched margin; **D -** to show the cytoplasmic globules (arrowheads) and cortical granules (arrows); **E -** anterior portion of right side, arrows indicate perioral kinety 2, arrowheads demonstrate perioral kinety 3; **F -** anterior end of left side, to show the extrusomes (arrowheads) and perioral kinety 1 (arrow); **G, H -** right side views, arrow points to cytostome and arrowhead to nematodesmata. **I -** infraciliature of anterior end of right side, showing perioral kinety 2 (arrows) and 3 (arrowheads). Scale bars 10 μm (I); 40 μm (A, B, G).

Table 3. Morphometrical comparison of *Kentrophyllum verrucosum* and other similar marine species. 1 - species, 2 - body length (in μm), 3 - number of macronuclear nodules, 4 - number of contractile vacuoles, 5 - distribution of extrusomes, 6 - distribution of spines, 7 - peribuccal papillae, 8 - number of right somatic kineties, 9 - number of left somatic kineties, 10 - source.

1	2	3	4	5	6	7	8	9	10
K. fibrillatum	90	2	1	uniform	dense	-	-	-	Dragesco (1954)
K. fibrillatum	up to 90	2	1	uniform	dense	present	*ca* 24	20-22	Dragesco (1960)
K. pseudosetigerum	170	*ca* 12	1	uniform	dense	present	16-17	-	Dragesco (1954)
K. pseudosetigerum	180	*ca* 12	1	uniform	dense	present	-	-	Dragesco (1960)
K. raikovi	360-500	11-14	many*	clustered**	dense	present	*ca* 40	-	Dragesco (1965)
K. antarcticum	200-310	2	3-5	uniform	sparse	absent	29-40	24-27	Petz *et al.* (1995)
K. multinucleatum	100-140	5-14	5-6	uniform	dense	absent	-	-	Ozaki and Yagiu (1943)
K. verrucosum	90-250	2-9	6-10	uniform	dense	absent	30-48	35-53	Original

* many: no specific number was given but > 20 were illustrated; ** clustered: gathered in groups.

Table 4. Morphometrical comparison of *Epiphyllum soliforme* and some similar *Loxophyllum* spp. 1 - species, 2 - body length (in μm), 3 - number of macronuclear nodules, 4 - number of contractile vacuoles, 5 - distribution of extrusomes., 6 - peribuccal papillae, 7 - number of right somatic kineties, 8 - number of left somatic kineties, 9 - habitat, 10 - source.

1	2	3	4	5	6	7	8	9	10
E. soliforme	50-200	4-6	2-5	uniform	present	32-46	34-56	marine	Original
L. hohuensis	200-280	4	4-5	uniform	absent	-	-	fresh water	Wang and Nie (1933)
L. variabilis	100-260	5-7	1	uniform	present	16-17	-	marine	Dragesco (1954, 1960)
L. kahli	120	11-12	2	uniform	present	-	22-24	marine	Dragesco (1960)
L. elongatum	170-180	1?	10-12	uniform	absent	-	-	marine	Tucolesco (1962)
L. compressum	280	6-7	4-5	uniform	absent	14-16	*ca* 10	marine	Dragesco (1965)
L. asetosum	80-160	2-6	6-12	-	-	-	-	marine	Burkovsky (1970a)
L. schewiakoffi	80-100	1	-	uniform	-	25-30	-	marine	Burkovsky (1970b)

Ecological features: Collected from an abalone-farming pond (10 April 2003), water temperature about 15°C, salinity *ca* 30‰, pH *ca* 8.0.

Type slides: One permanent slide of protargol impregnated specimens is deposited as a holotype in the Natural History Museum, London, UK with registration No. 2004:3:22:3. One paratype slide (No. Lin-03-04-10) is deposited in the Laboratory of Protozoology, OUC, China.

Dedication: We dedicate this new species to our distinguished colleague, Dr Jerzy Sikora, Nencki Institute of Experimental Biology, Warszawa, in recognition of his academic and editorial contributions to protozoology.

Morphology and infraciliature: Cells often slender to broad leaf-shaped with both ends bluntly pointed, but without the neck-like region that is commonly seen in most *Amphileptus* spp. (Figs 7A, E; 8A-C). Size highly variable, about 90-200 × 20-60 μm *in vivo*, mostly around 120-150 μm in length. Cells also highly flattened (about 3-5:1), right side flat, left vaulted and with conspicuous longitudinal shallow grooves (Figs 7A, B). Transparent margin relatively narrow (about 10 μm wide), opaque central region dominant (Figs 7A, H; 8A-C).

Pellicle thin and slightly notched (Figs 7F, 8C). Extrusomes about 12-15 μm long, bar-shaped, more or less in groups, concentrated in hyaline fringe and surrounding entire cell except oral region (Figs 7A, D, F, G; arrowheads in Fig. 8F). Cortical granules tiny and colourless (< 0.8 μm across), packed in extremely dense regular pattern on left surface (Figs 7C, 8D).

Cytostome about 15-25% of cell length, as indicated by nematodesmata which are long and highly developed,

and often extend to 30-80% of body length (Figs 7E, 8E-I). No peribuccal papillae observed.

Cytoplasm often packed with numerous differently sized greasily shining globules (1-6 μm across) (arrowheads in Fig. 8D), which render the main part of body completely opaque, even at high magnification (Figs 8A-C).

Contractile vacuoles 2-3 in number, always in a single group along the dorsal margin near mid-body (Figs 7A, H; 8C). Constantly with two macronuclear nodules that are oval-shaped, about 15-25 μm (*ca* 10-22 μm after fixation) in length and inter-connected by funiculus; *in vivo*, often appear as two large hyaline areas in mid-body region, slightly displaced towards ventral margin (Figs 7A, J; 8B). Nucleoli small, numerous and evenly distributed (Figs 7J, 8G). Micronuclei not observed.

Movement by gliding on substrate and often swimming by slowly rotating around longitudinal axis. Generally insensitive to mechanical stimuli.

All somatic kineties relatively densely spaced and cilia loosely arranged (Figs 7I, J). Usually 13-18 right kineties (mean 14), intermediate kineties conspicuously shortened anteriorly, forming a distinct anterior suture, *ca* 33% of body length; posterior suture inconspicuous (Fig. 7J). About 14-17 somatic kineties on left side, all of which terminate anteriorly at perioral kinety 1 (PK_1) (Fig. 7I). Dorsal brush kinety (DB) composed of a few close-set basal body pairs and continue posteriorly as a row of monokinetids (Fig. 7I).

Three densely ciliated perioral kineties (PK_1, PK_2, PK_3) as shown in Figs 7I, J. PK_{1-3} curving around anterior end and bending slightly to dorsal side (arrows in Figs 7I, J). PK_1 comprises dikinetids in oral region and continues posteriorly as monokinetids (7I, arrow in Fig. 8F). PK_2 composed of dikinetids, extending to about 1/2 of cell length and continues posteriorly as a row of monokinetids (arrows in Figs 8E, I). Basal bodies (monokinetids) in PK_3 often strongly and argentophilic (arrowheads in Figs 8E, I).

Comparison and discussion: With respect to its body shape and size, the number of macronuclei, and the position and number of contractile vacuoles, *Amphileptus sikorai* most closely resembles *Loxophyllum trinucleatum* Mansfeld, 1923, *Amphileptus salmica* (Burkovsky, 1970) Foissner, 1984 and *Opisthodon niemeccense* Stein, 1859. The former two can easily be separated from *A. sikorai* by their smaller body length (60-90 *vs.* 90-200 μm in *A. sikorai*), number of macronuclei (3 *vs.* 2 in *A. sikorai*) and number of contractile vacuoles (8-10 *vs.* 2-3 in *A. sikorai*) (Mansfeld 1923, Kahl 1931, Burkovsky 1970a, b). *Opisthodon niemeccense* is similar to *A. sikorai*, especially in terms of its morphology *in vivo*, e.g. the body shape and size, both with two macronuclei. However, *A. sikorai* can be distinguished by having more contractile vacuoles (2-3 *vs.* 1 in *O. niemeccense*), no pit-like depression at the anterior end (*vs.* present in *O. niemeccense*), and three perioral kineties (*vs.* two in *O. niemeccense*) (Penard 1922, Kahl 1931, Foissner 1984).

Key to the identification of selected pleurostomatids: Based on the data available, a key to some closely related pleurostomatid genera is suggested:

1. Somatic ciliature with suture(s).....2
Somatic ciliature without suture(s).....6
2. Only right somatic ciliature with suture(s).....3
Both right and left somatic ciliature with sutures.....5
3. Anterior end not rolled up or immersed into a pit-like depression.....*Amphileptus*
Anterior end rolled up or immersed into a pit-like depression.....4
4. Anterior end rolled up and curved to the left.....*Amphileptiscus*
Anterior end immersed into a pit-like depression.....*Opithodon*
5. With spines surrounding whole body.....*Kentrophyllum*
Without spines.....*Epiphyllum*
6. Dorsal warts with grouped extrusomes present.....*Loxophyllum*
Dorsal warts absent.....7
7. Anterior end not rolled up.....*Litonotus*
Anterior end rolled up and curved to the left.....*Acineria*

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ACTA
PROTOZOOLOGICA

Two New Marine Pleurostomatid Ciliates from China, *Loxophyllum jini* and *L. qiuianum* (Ciliophora: Pleurostomatida)

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Summary. The morphology of two marine pleurostomatid ciliates, *Loxophyllum jini* sp. n. and *L. qiuianum* sp. n., collected from coastal waters near Qingdao (Tsingtao), China, were investigated using observations *in vivo* and following protargol impregnation. *Loxophyllum jini* is identified by its large body size (about 300-600 μm long *in vivo*), multi-macronuclear nodules (8-17), several (3-7) contractile vacuoles along the dorsal margin of cell, and the somatic kineties on left (9-14) and right (13-20) side. As a new species, *L. qiuianum* distinguished from its congeners by a combination of characters including the marine habitat and the distribution of extrusomes, the terminal position of contractile vacuole and the numbers of somatic kineties on both the right and left sides (19-30 and 8-13).

Keywords: *Loxophyllum jini* sp. n., *L. qiuianum* sp. n., marine ciliates, morphology and infraciliature.

INTRODUCTION

Bilaterally compressed pleurostomatid ciliates are commonly reported from a variety of geographical habitats all over the world (Kahl 1931, Dragesco 1960, Borror 1963, Agamaliev 1967, Hartwig 1973, Fryd-Versavel *et al.* 1975, Foissner 1987, Wilbert and Kahan 1981, Li 1990, Song 1994, Martin-Cereceda *et al.* 1995, Lin and Song 2004, Lin *et al.* 2005).

Although many taxa in this group have been described, studies carried out before the last quarter of the 20th century were based almost exclusively on live observations (Kahl 1931; Vuxanovici 1959, 1961; Dragesco 1954, 1965; Canella 1960), which has led to great difficulties in species identification and separation within the *Litonotus-Amphileptus-Loxophyllum* complex (Wilbert and Kahan 1981, Foissner 1984). Following the application of modern methods such as silver staining in the study of pleurostomatids, several new genera have been established in recent decades including: *Pseudoamphileptus* Foissner, 1983, *Kentrophyllum* Petz, Song *et* Wilbert, 1995, *Siroloxophyllum* Foissner *et* Leipe, 1995, *Amphileptiscus* Song *et* Bradbury, 1997, *Apoamphileptus* Lin *et* Song, 2004 and *Epiphyllum* Lin, Song *et* Warren, 2005.

The most conspicuous features of the species-rich genus *Loxophyllum* include: (i) the ciliary pattern on the right side, i.e. right somatic kineties, anteriorly shortened

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along the perioral kineties (*vs.* shortened in the median area, thus forming suture in *Amphileptus*, *Kentrophyllum*, *Amphileptiscus*, etc.); (ii) extrusomes distributed along both ventral and dorsal margins, and those on the dorsal margin usually clustered to form warts (*vs.* extrusomes absent on the dorsal margin in *Litonotus* and *Acineria* and never clustered to form warts) (Kahl 1931, Song 1993, Foissner and Leipe 1995, Foissner *et al.* 1995, Petz *et al.* 1995, Lynn and Small 2002).

During a recent survey of the marine ciliate fauna in northern China, two new members of *Loxophyllum* were isolated from coastal waters of the Yellow Sea near Qingdao, China. The results of these investigations are presented here.

MATERIALS AND METHODS

Samples were collected (14th October, 2002, and 4th June, 2004) from two different locations in coastal waters of the Yellow Sea near Qingdao (Tsingtao), China. Specimens were isolated from glass slides that had been immersed in the water for up to 10 days, after which time they were transferred to Petri dishes with sea water from the sampling site and transported to the laboratory. The slides were maintained at room temperature for two days as raw cultures together with small ciliates and flagellates in the original water which acted as a food source.

Living cells were isolated and observed using bright field and differential interference contrast microscopy. The infraciliature was revealed by the protargol impregnation method according to Wilbert (1975). Living individuals were examined at 100× to 1000× magnification; measurements were carried out with an ocular micrometer; drawings of stained specimens were performed at 1250× with the aid of a camera lucida. Terminology is mainly according to Corliss (1979) and Foissner (1984). The following term is here defined because of its importance in separating the new taxa from their marine congeners. **Warts (Wa)**: present in most *Loxophyllum* species as papillary protrusions along the dorsal margin of cell, each filled with a cluster of extrusomes.

RESULTS

Order: Pleurostomatida Schewiakoff, 1896
Family: Amphileptidae Bütschli, 1889
Genus: *Loxophyllum* Dujardin, 1841

Loxophyllum jini sp. n. (Figs 1-3, Table 1)

Diagnosis: Large marine *Loxophyllum* about 300-600 μm long *in vivo* with conspicuously slender body; approximately 11 macronuclear nodules and 3 micronuclei; about 9-14 left and 13-20 right somatic kineties; several (*ca* 3-7) contractile vacuoles along the dorsal margin of cell; extrusomes bar-shaped, uniformly distributed along the hyaline edges of both ventral and dorsal margins; without warts.

Type locality and ecological features: Collected (4 June 2004) from coastal waters of the Yellow Sea near Qingdao (Tsingtao), China, 36°08'N; 120°43'E. Water temperature *ca* 17°C, salinity 29‰, pH *ca* 7.9.

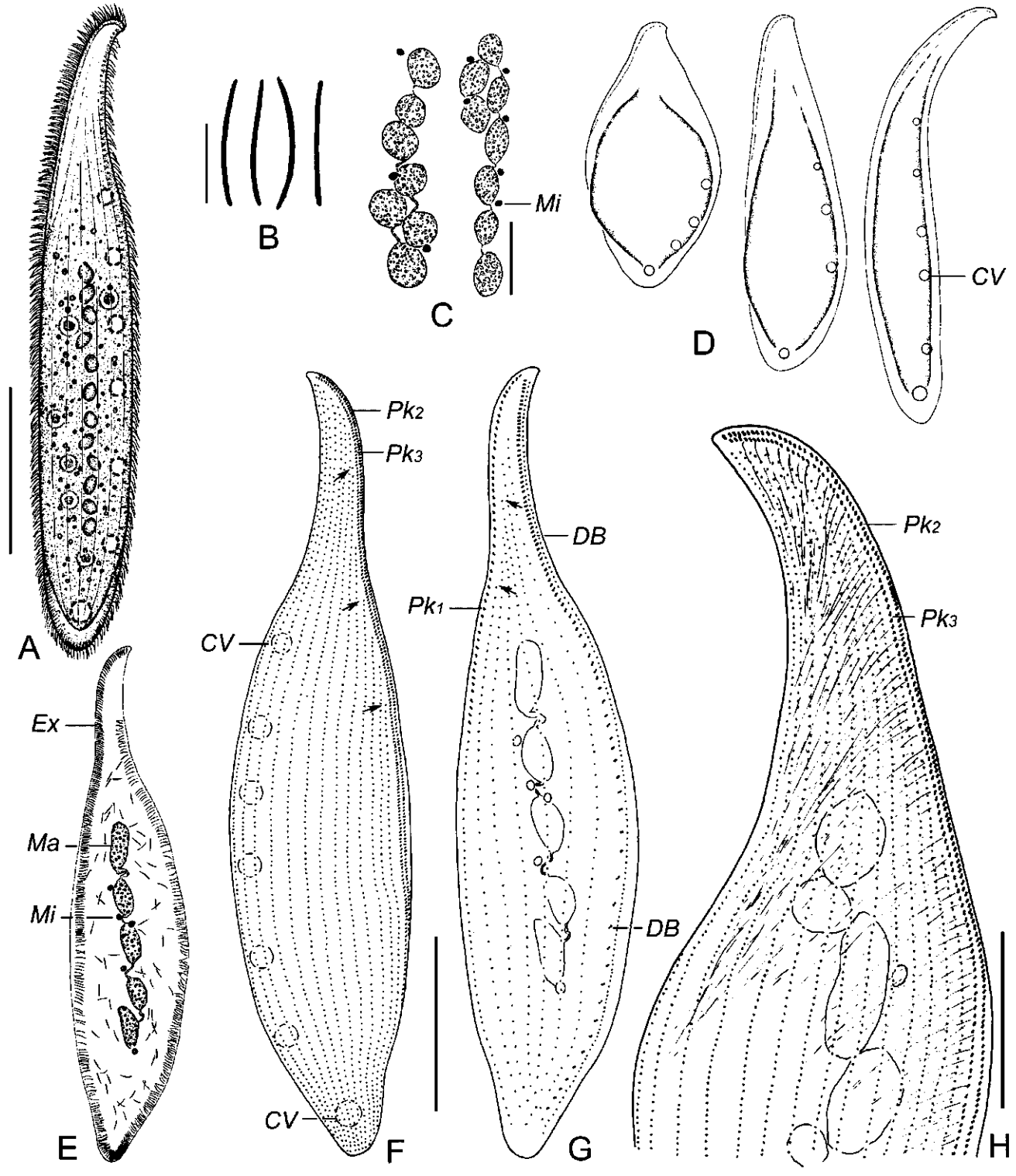
Type slides: One permanent slide of protargol impregnated specimens is deposited as a holotype in the Natural History Museum, London, UK with registration No. 2005:2:2:1. One paratype slide (No. Lin-04-6-4) is deposited in the Laboratory of Protozoology, OUC, China.

Dedication: We dedicate this new species to our distinguished colleague, Prof. Lipei Jin, Sun Yat-Sen University, Guangzhou, China, in recognition of his academic contributions to ciliatology.

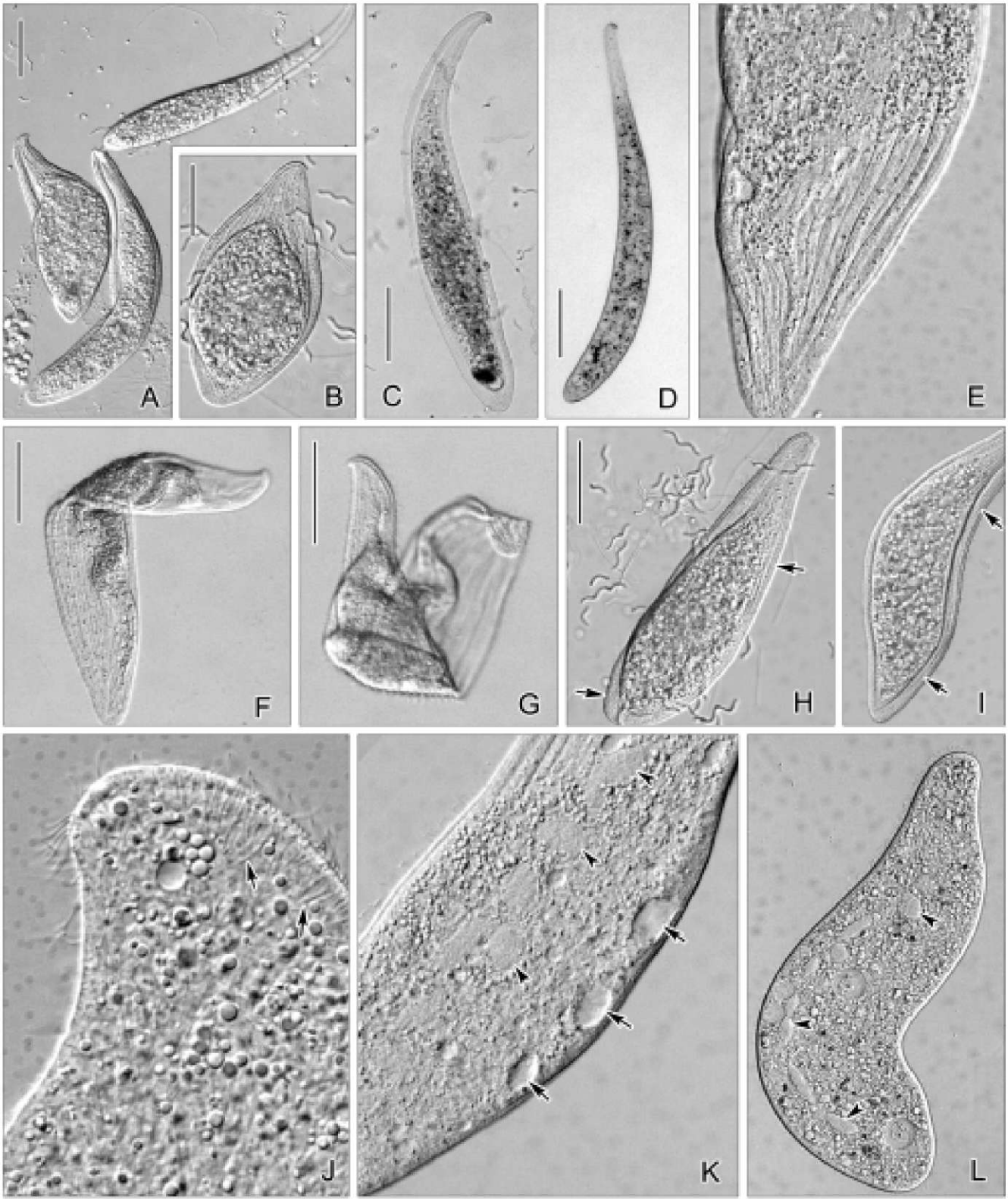
Morphology and infraciliature: Size highly variable, 300-600 × 50-150 μm *in vivo*, usually 400-500 μm in length (for morphometrics see Table 1). Cell very flexible, variable in shape from extremely slender rod-shaped when extended to broadly leaf-shaped when contracted (Figs 1A, D; 2A-D, F-I). Leaf-shaped body often with well-defined thin and hyaline fringe (Figs 2A-C, H, I). Laterally compressed about 3-1.5:1; right side flat; left slightly to distinctly vaulted, mostly due to the presence of ingested food material (Figs 2A-C). Pellicle with many inconspicuous longitudinal shallow grooves (Fig. 2E). Right side densely ciliated, cilia *ca* 6 μm long; cilia sparsely distributed on left side, difficult to detect in life.

Cytoplasm slightly grayish, often with numerous tiny (usually 3-4 μm across) greasily shining globules, which render the main part of the body opaque (Figs 2A-J). Extrusomes bar-shaped, slender, straight to slightly curved, 8-10 μm long *in vivo* (Fig. 1B), evenly distributed along entire ventral and dorsal margins with the exception of the anteriormost region of the dorsal margin, some scattered in cytoplasm (Figs 1B, E; 2J). Food vacuoles few in number, 10-15 μm across. Several (3-7) contractile vacuoles (CV) about 5-10 μm in diameter, pulsating infrequently, positioned along the posterior 3/4 of the dorsal margin; the largest CV usually terminally positioned (Figs 1A, D, F; 2K).

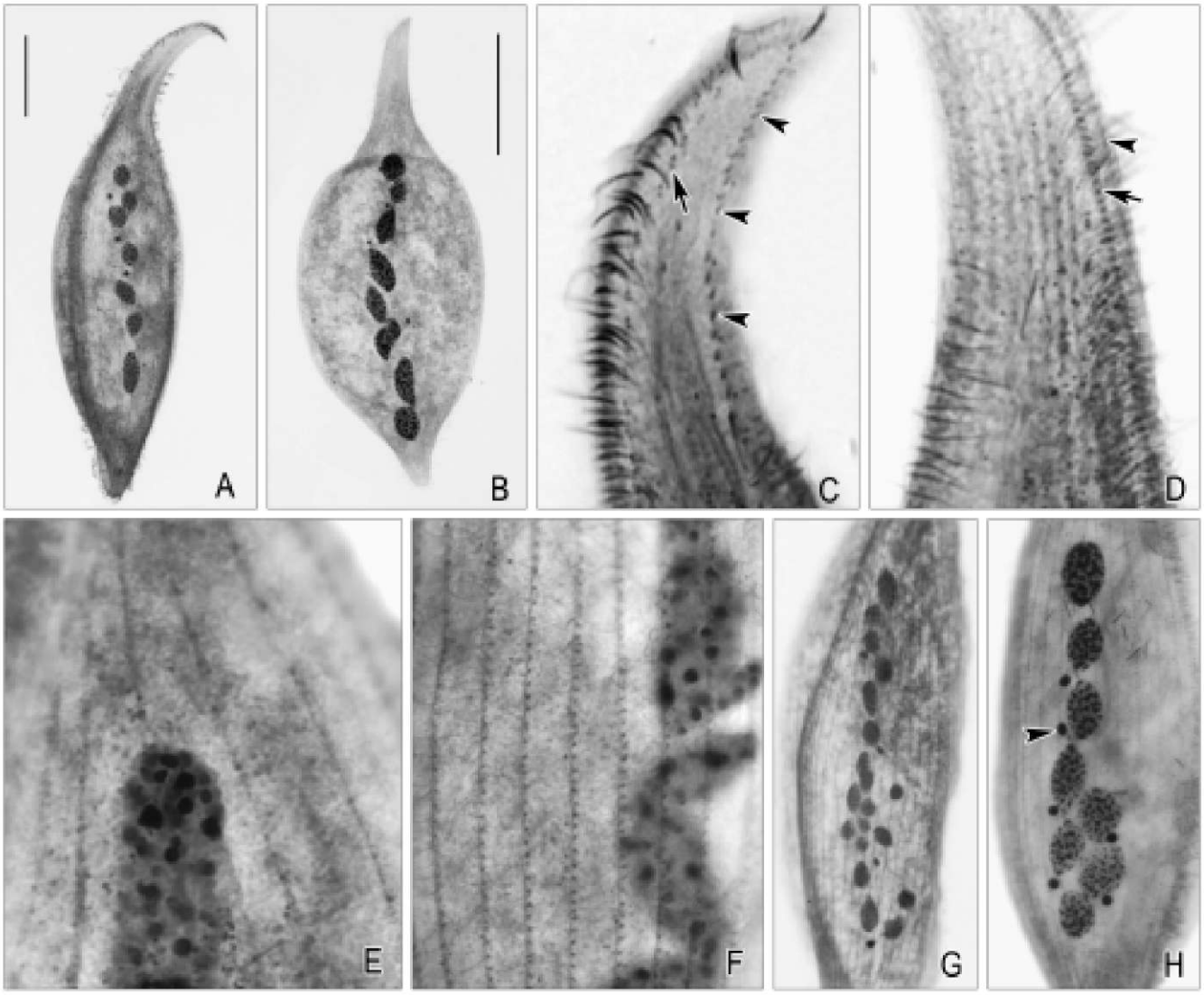
Many (8-17) macronuclear nodules, elongate to ellipsoid, 10-40 × 8-20 μm in size, interconnected by funiculus, located in central region of cell, usually detectable *in vivo* under differential interference contrast microscopy, and with 2-6 oval micronuclei (*ca* 5 μm long) between them (Figs 1C, E; 3A, B, G, H).



Figs 1 A-H. Morphology and infraciliature of *Loxophyllum jini* sp. n., from life (A, B, D) and after protargol impregnation (C, E-H). **A** - left view of a typical individual; **B** - extrusomes; **C** - nuclear apparatus; **D** - shape variations of the same individual, note the contractile vacuoles and the hyaline edge; **E** - nuclear apparatus and distribution of extrusomes; **F, G** - infraciliature of right (F) and left (G) sides of the same specimen, arrows mark the somatic kineties terminating along the perioral kineties; **H** - right view showing the detailed structure around the cytostome, note the developed nematodesmata. Abbreviations: CV - contractile vacuole, DB - dorsal brush kinety, Ex - extrusome, Ma - macronucleus, Mi - micronucleus, PK_1 - perioral kinety 1, PK_2 - perioral kinety 2, PK_3 - perioral kinety 3. Scale bars 5 μm (B); 40 μm (C); 50 μm (H); 100 μm (A, F).



Figs 2 A-L. Photomicrographs of *Loxophyllum jini* sp. n. from life. **A, B -** views of normal body shape when extended and contracted; **C, D** - two typical body shapes when the cell is fully extended; **E** - left view of the posterior region of the cell showing the shallow grooves; **F, G** - showing typical cell contortions; **H, I** - noting the grayish central body and the hyaline edge (arrows); **J** - right view of the anterior region of the cell, showing the fine and densely spaced extrusomes (arrows) ; **K, L** - differential interference contrast microscopy, showing the nuclear apparatus (arrowheads), arrows mark the contractile vacuoles. Scale bars 100 μm.



Figs 3 A-H. Photomicrographs showing the infraciliature of *Loxophyllum jini* sp. n. after protargol impregnation. **A, B**- specimens showing the typical nuclear apparatus; **C** - anterior left side, arrows mark the dorsal brush, arrowheads indicate the perioral kinety 1, note the distribution of the extrusomes along the dorsal margin; **D** - view of right side, showing perioral kineties 2 (arrow) and 3 (arrowhead); **E, F** - somatic kineties on the left (E) and right (F) sides; **G, H** - nuclear apparatus, arrowhead depicts one of the micronuclei. Scale bars 100 μm.

Generally insensitive to disturbance. Movement by slowly gliding on substrate, rarely swimming.

Somatic kineties as shown in Figs 1F-H, 3A-H. Perioral kineties 2 and 3 ($PK_{2,3}$) to right of oral slit, both consisting of closely spaced kinetosome pairs, i.e. dikinetids (Figs 1F, 3D), and both terminating near posterior end of cell (Fig. 1H). Perioral kinety 1 (PK_1) to left of oral slit, with widely spaced basal body pairs in anterior half and continues posteriorly as a row of monokinetids (Figs 1G, 3C). Right side with 13-20 kineties (mean 18, including $PK_{2,3}$), somatic kineties densely arranged and terminate anteriorly along PK_3 (Fig. 1F, arrows). Left side more sparsely ciliated, with 9-14 kineties (including PK_1), somatic kineties terminating anteriorly along PK_1 (Fig. 1G, arrows). Dorsal brush kinety (DB) composed of basal body pairs and extends to posterior end of cell (Figs 1G, 3C). Nematodesmata well developed, all originating from the kinetosomes of the right perioral kineties and extending along the cytopharynx into the cytoplasm (Fig. 1H).

Comparison: It is generally agreed that the shape and distribution of the extrusomes, the number and position of the contractile vacuoles, the pattern of the ciliature and the nuclear apparatus have great diagnostic

Table 1. Morphological characterization of *Loxophyllum jini* sp. n. (1st line) and *L. qiuianum* sp. n. (2nd line). Data based on protargol impregnated specimens. All measurements in μm. Abbreviations: M - median, Max - maximum, Mean - arithmetic mean, Min - minimum, n - number of specimens, SD - standard deviation.

Characters	Min	Max	Mean	M	SD	n
Body length	344	480	408.5	400	46.32	16
	168	320	252.0	248	39.72	25
Body width	64	160	82.8	80	23.49	16
	80	144	112.9	120	17.91	25
Number of right somatic kineties*	13	20	17.5	18	1.55	16
	19	30	23.2	23	2.93	25
Number of left somatic kineties**	9	14	11.9	11	1.71	13
	8	13	10.2	10	1.52	25
Number of macronuclear nodules	8	17	11.2	11	3.17	16
	1	3	2.1	2	0.40	25
Length of macronuclear nodules	11	43	26.3	22	9.39	16
	28	72	44.6	44	9.33	25
Width of macronuclear nodules	8	18	14	16	2.94	16
	18	32	25.2	24	4.10	25
Number of micronuclei	2	6	3.6	3	1.03	16
	1	1	1	1	0	25
Length of micronucleus	4	10	5	4	1.71	16
	5	13	7.5	6	2.33	25
Length of extrusomes	7	10	8.1	8	1	12
	5	7	5.6	6	0.58	25

* Perioral kineties 2, 3 included; ** Perioral kinety 1 included.

value in separating species of the genus *Loxophyllum* (Dragesco 1960, 1965, 1966; Foissner 1984; Song and Wilbert 1989). Up to now, over 40 *Loxophyllum*-like morphotypes have been reported, most of which (>30) were found in marine biotopes (Kahl 1931; Dragesco 1960, 1965; Carey 1991).

Based on the general body shape and the number of macronuclear nodules, at least 8 nominal marine *Loxophyllum* spp. should be compared with *L. jini*. Because none of these have been investigated using modern methods, our comparison is mainly based on their morphological characters in life (Sauerbrey 1928; Kahl 1931; Shigematsu 1953; Dragesco 1960, 1965) (Table 2).

Like *Loxophyllum jini*, *L. acutum* Dragesco, 1965 possesses many contractile vacuoles. However, *L. jini* can be distinguished from the latter by: (i) its larger body size (300-600 *vs. ca* 260 μm long); (ii) the broadly rounded posterior end of the body (*vs.* pointed tail-like posterior end in *L. acutum*); (iii) the absence of warts (*vs.* warts present along the dorsal margin in *L. acutum*).

The smaller sized *Loxophyllum compressum* Dragesco, 1965 differs from *L. jini* mainly in the distribution of contractile vacuoles (along both margins *vs.* along dorsal margin only in *L. jini*).

At least five congeners posses multiple macronuclear nodules like *Loxophyllum jini*, namely: *L. vermiforme* Sauerbrey, 1928, *L. meleagris* Dujardin, 1841, *L. multinucleatum* Kahl, 1928, *L. ozakii* Shigematsu, 1953 and *L. levigatum* Sauerbrey, 1928. All of these can easily be distinguished from *L. jini* by the number and positions of the contractile vacuoles (Table 2).

It is noteworthy that *Loxophyllum levigatum* was originally described as having a single contractile vacuole and the extrusomes are arranged along the ventral margin only (Sauerbrey 1928). Dragesco (1960), however, described another population (incorrectly spelt as *L. laevigatum*) with two CVs and extrusomes distributed along both the ventral and dorsal margins (Table 2). A re-investigation of both morphotypes is required in order to determine whether they are conspecific.

Loxophyllum qiuianum sp. n. (Figs 4, 5; Table 1)

Diagnosis: Medium-sized marine *Loxophyllum*, about 150-350 μm long *in vivo* with leaf-shaped body; mostly with 2 macronuclear nodules and 1 micronucleus;

Table 2. Morphological comparison of *Loxophyllum jini* sp. n. with some marine *Loxophyllum* spp. that contain multiple macronuclear nodules. All measurements in μm. CV - contractile vacuoles, Ex - extrusomes, Ma - macronuclear nodules, No. - number, RK/LK - right/left kineties.

	Body length	No. and position of CV	No. of Ma	No. of RK/LK	Distribution of Ex	Warts	Data source
L. jini	300-600	3-7; along dorsal margin	8-17	13-20 / 9-14	along both margins	absent	present work
L. acutum	*ca* 260	many; along dorsal margin	10-14	11-12 / -	along both margins	present	Dragesco (1965)
L. compressum	280	4-5; along both margins	6-7	14-16 / *ca* 10	along both margins	absent	Dragesco (1965)
L. meleagris	300-700	1; subterminal, dorsally	many	-	along both margins	present	Kahl (1931)
L. multinucleatum	140-160	3; along ventral margin	many	10 / -	along both margins	absent	Kahl (1931)
L. vermiforme	*ca* 1600	1; subterminal	many	60 / -	along both margins	absent	Sauerbrey (1928)
L. ozakii	170-230	1-3; along dorsal margin	170-200	-	along both margins	absent	Shigematsu (1953)
L. levigatum	*ca* 270	1; subterminal	7-15	40 / -	along ventral margin	absent	Sauerbrey (1928)
L. laevigatum	*ca* 270	2; along dorsal margin	many	-	along both margins	absent	Dragesco (1960)

- data not available.

Table 3. Morphological comparison of *Loxophyllum qiuianum* sp. n. with some marine *Loxophyllum* spp. that possess two macronuclear nodules. All measurements in μm. CV - contractile vacuoles, Ex - extrusomes, No. - number, RK/LK - right/left kineties.

	Body length	No. and position of CV	No. of RK/LK	Furrows	Distribution of Ex	Warts	Data source
L. qiuianum	150-350	1, terminal	19-30 / 8-13	absent	no Ex along oral slit	present	present work
L. rostratum	150-250	1, subterminal	16-18 / 6-7	present	along both margins	present	Song (1993)
L. chaetonotum	70-120	1, subterminal	7 / -	present	along both margins	present	Borror (1965)
L. perihoplophorum	*ca* 400	several, dorsal margin	20 / -	-	along both margins	present	Buddenbrock (1920)
L. multiverrucosum	400	several, dorsal margin	- / -	-	along both margins	present	Kahl (1933)
L. elegans	*ca* 170	1, terminal	18 / -	absent	mainly along oral slit	absent	Kattar (1970)
L. vitraeum	80-100	1, subterminal	8 / -	-	mainly along oral slit	absent	Dragesco (1965)
L. fasciolatus	*ca* 130	1, terminal	12 / -	present	along ventral margin	absent	Dragesco (1966)
L. multiplicatum	250	1, terminal	*ca* 15 / -	-	only along oral slit	absent	Kahl (1931)

- data not available.

about 8-13 left and 19-30 right kineties; 1 terminally located contractile vacuole; extrusomes bar-shaped, evenly spaced along ventral margin but clustered along dorsal margin to form about 6-12 inconspicuous warts.

Type locality and ecological features: Found in a shrimp-farming pond (14 October 2002) in coastal waters of the Yellow Sea near Qingdao (Tsingtao), China, 36°08'N; 120°43'E. Water temperature *ca* 23°C, salinity *ca* 22‰, pH *ca* 8.1.

Type slides: One permanent slide of protargol impregnated specimens is deposited as a holotype in the Natural History Museum, London, UK with registration No. 2005:2:2:2. One paratype slide (No. Lin-02-10-14) is deposited in the Laboratory of Protozoology, OUC, China.

Dedication: We dedicate this new species to our distinguished colleague, Prof. Zijian Qiu, Harbin Normal University, Harbin, China, in recognition of his academic contributions to protozoology.

Morphology and infraciliature: Size highly variable among individuals, 150-350 × 70-120 μm *in vivo* (for morphometrics see Table 1). Cell very flexible, from slender leaf-shaped when fully extended to broadly rounded when contracted (Figs 4C, 5A-C); with short (about 20-25% of cell length) neck region at anterior end of cell and a short tail-like region at posterior end (arrows in Figs 4C; 5A-C, F). Laterally compressed about 3:1, right side flat, left slightly to distinctly vaulted, mostly due to the presence of ingested food material (Figs 5A-C). Right side densely ciliated, cilia about 5 μm long; left side sparsely ciliated, cilia difficult to detect in life; the ciliary rows on both sides are marked by the presence of conspicuous, longitudinal, shallow grooves that appear as white lines on the cell surface (Figs 4B, 5D).

Cytoplasm grayish, often with numerous tiny (3-8 μm across) greasily shining globules and short (*ca* 1 μm long) rod-like greenish crystals (Fig. 4E), that render the main part of the body opaque (Figs 5A-C). Extrusomes bar-shaped, slender, straight to slightly curved, 5-7 μm long (Fig. 4D); evenly arranged along the ventral margin with the exception of the buccal area, and clustered together to form 6-12 inconspicuous warts (Wa) along the dorsal margin although absent from the anterior neck-like region; some extrusomes scattered in cytoplasm (Figs 4F, 5H). Food vacuoles few in number, each 10-15 μm across. One small contractile vacuole (CV), 5-10 μm in diameter, terminally located, pulsating infrequently (Fig. 4A, arrow).

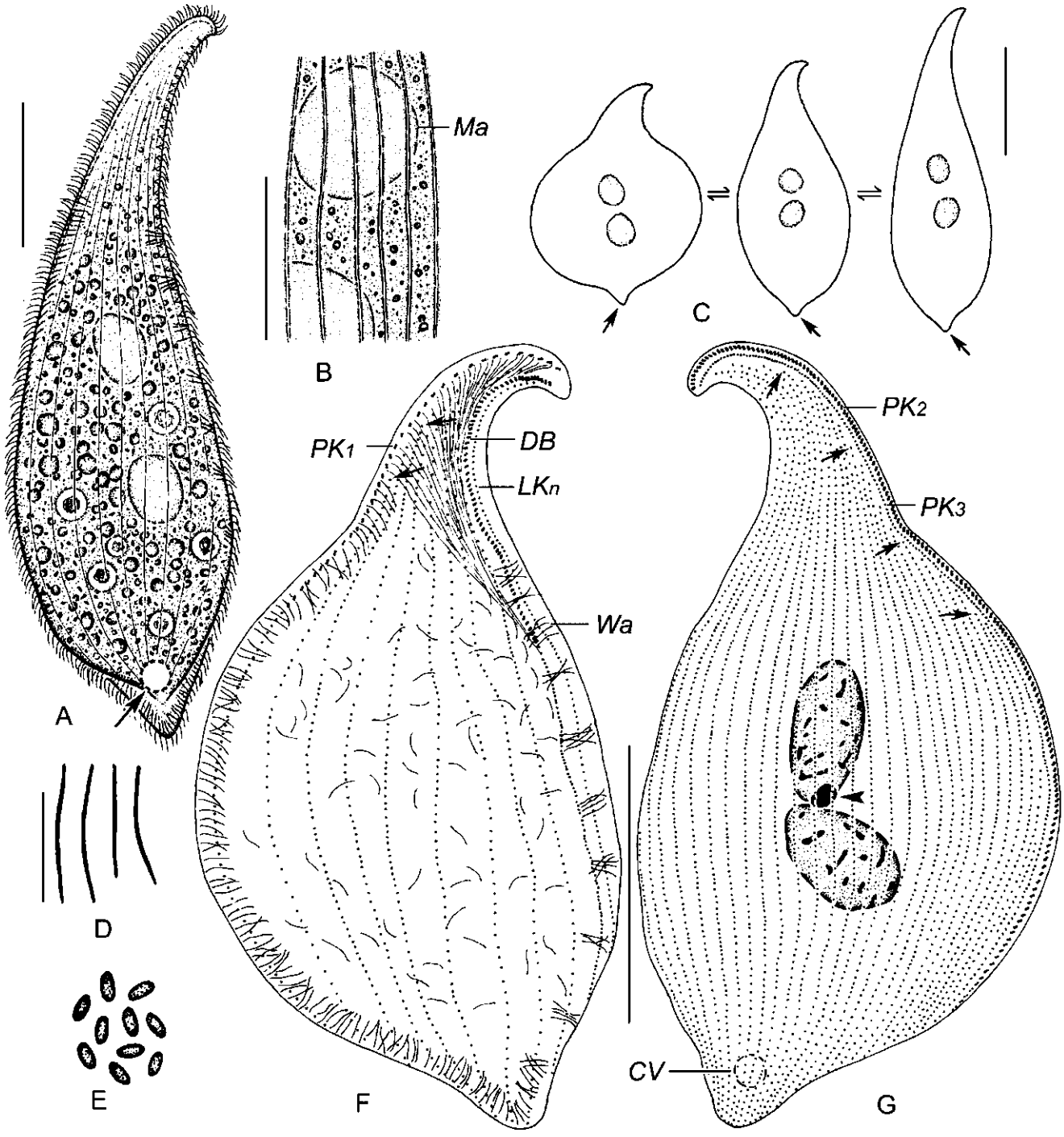
Usually with two macronuclear nodules, 28-72 × 18-32 μm in size after fixation, ovoid to ellipsoid, located in mid-body region, and appear as two large transparent areas *in vivo* (Figs 5A-C). Single micronucleus, 5-13 μm in length, located between macronuclear nodules (Fig. 4G, arrowhead).

Generally insensitive to disturbance. Usually gliding extremely slowly on substrate, rarely swimming through the water.

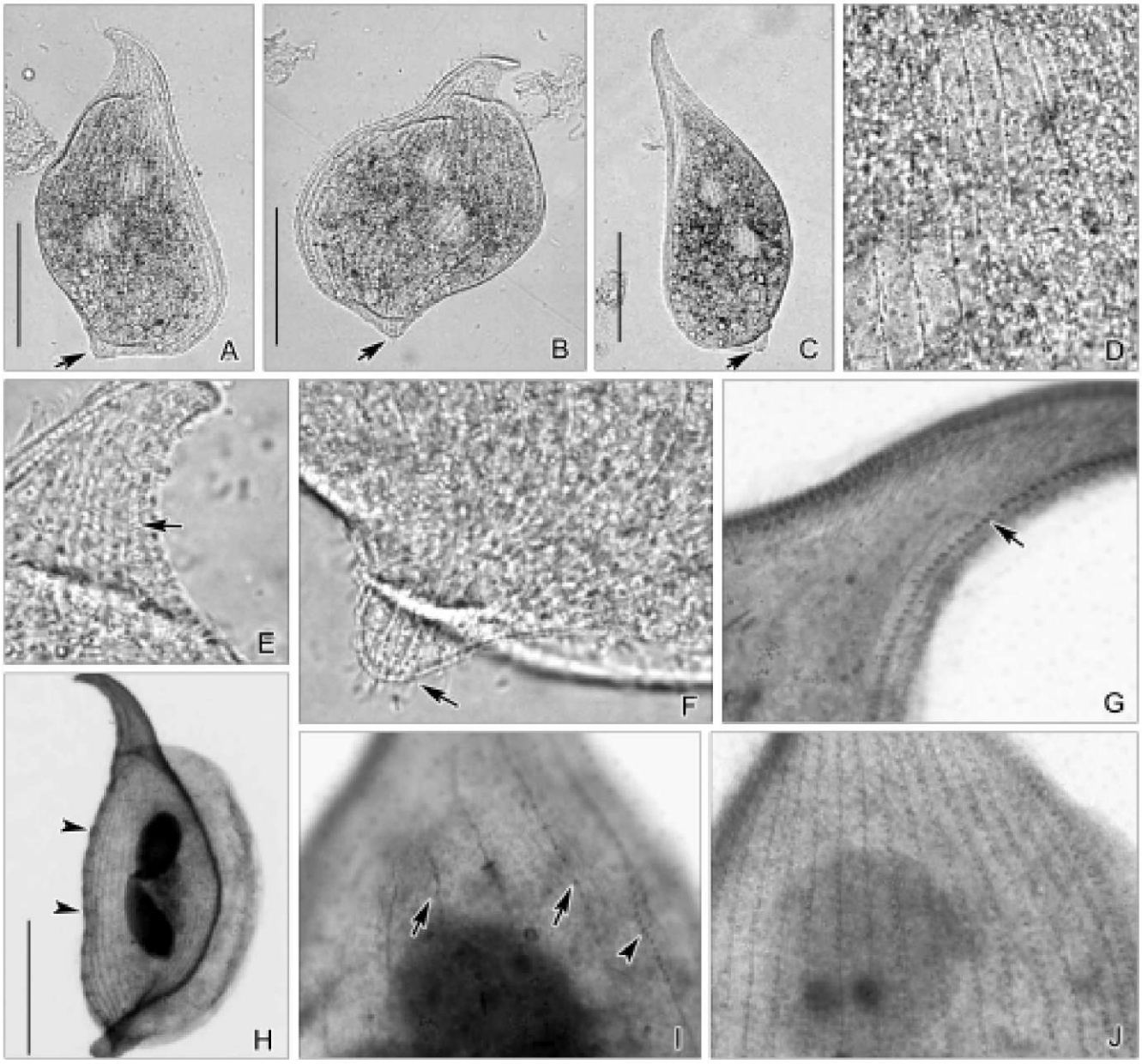
Infraciliature as shown in Figs 4F, G; 5G-J. Perioral kineties 2 and 3 ($PK_{2,3}$) right of cytostome and extend 80% of cell length, terminating in posterior 1/5 of body; PK_2 consists of closely spaced kinetosome pairs (i.e. dikinetids), PK_3 comprises a row of close-set monokinetids (Fig. 4G). Perioral kinety 1 (PK_1) left of oral slit, with loosely spaced basal body pairs in anterior 1/3 and continues posteriorly as a row of monokinetids (Fig. 4F). Right side with 19-30 (mean 23, including $PK_{2,3}$) closely spaced kineties (Fig. 5J), somatic kineties terminating anteriorly along PK_3 (arrows in Fig. 4G). Left side loosely ciliated with 8-13 kineties (including PK_1 and the dorsal brush kinety) (Fig. 5I); somatic kineties terminating anteriorly along PK_1 (Fig. 4F, arrows). Dorsal brush kinety (DB) composed of densely spaced basal body pairs in anterior 1/3 and continues posteriorly as a row of densely spaced monokinetids (Figs 4F; 5E, G, I). Nematodesmata well developed (Fig. 4F).

Comparison: Among the known marine *Loxophyllum* species, our new form is most similar to *L. rostratum* Cohn, 1866, which was investigated by Song (1993) using modern methods (Table 3). Compared with *L. rostratum*, *L. qiuianum* is identified by: (i) the absence of extrusomes along the oral slit (*vs.* extrusomes present along the entire ventral margin in *L. rostratum*); (ii) the terminal position of the contractile vacuole (*vs.* subterminally located); (iii) the absence of furrows on the left side (*vs.* with 5 distinct furrows); (iv) the shape of the posterior end (constantly with a short tail-like region *vs.* shape highly variable in *L. rostratum*); (v) the possession of greater numbers of somatic kineties on both the right and left sides (19-30 and 8-13 *vs.* 16-18 and 6-7 in *L. rostratum*).

Among other related morphotypes, *Loxophyllum qiuianum* is most similar to *L. chaetonotum* Borror, 1965 (Table 3). Apart from its smaller body size (70-120 *vs.* 150-350 μm long for *L. qiuianum*) and its large subterminal contractile vacuole (*vs.* small, terminal CV in *L. qiuianum*), *L. chaetonotum* also differs from



Figs 4 A-G. Morphology and infraciliature of *Loxophyllum qiuianum* sp. n., from life (A-E) and after protargol impregnation (F, G). **A** - left view of a typical individual, arrow depicts the contractile vacuole; **B** - right side view, showing the ciliary rows marked by the position of shallow grooves that typically appear as longitudinal white lines; **C** - shape variations of the same individual, noting the short tail-like posterior end (arrows); **D** - extrusomes; **E** - greenish crystals in the cytoplasm; **F, G** - infraciliature of right (F) and left (G) sides of the same specimen, arrows mark the somatic kineties terminating along the perioral kineties, arrowhead marks the micronucleus in (G), note the warts (Wa) along the dorsal margin in (F). CV - contractile vacuole, DB - dorsal brush kinety, LKn - the right-most kinety on the left side, Ma - macronucleus, PK_1 - perioral kinety 1, PK_2 - perioral kinety 2, PK_3 - perioral kinety 3, Wa - wart. Scale bars 5 μm (D); 50 μm (A, B); 80 μm (E); 100 μm (C).



Figs 5 A-J. Photomicrographs of *Loxophyllum qiuianum* sp. n. from life (A-F) and after protargol impregnation (G-J). **A-C** - different body shapes when the cell contracts and extends, noting the constant tail-like posterior end (arrows); **D** - right side view, showing the shallow grooves that appear as longitudinal white line marking the positions of the ciliary rows; **E** - anterior part of left side, arrow marks the dorsal brush; **F** - posterior region, showing the short tail-like end (arrow); **G** - left side view, arrow indicates the dorsal brush kinety; **H** - infraciliature of right side, arrowheads mark the warts; **I, J** - somatic kineties on left (I, arrows) and right (J) sides, arrowhead in (I) marks the dorsal brush kinety. Scale bars 100 μm.

L. qiuianum in having fewer kineties on the right side (7 *vs.* 19-30) and the presence of extrusomes along the oral slit (*vs.* extrusomes absent along oral slit in *L. qiuianum*).

Loxophyllum perihoplophorum Buddenbrock, 1920 and *L. multiverrucosum* (Kahl, 1933) Carey, 1991 are two similar forms that can be separated from *L. qiuianum* by the presence of warts in the anterior dorsal region (*vs.* warts absent from this region in *L. qiuianum*) and the possession of several contractile vacuoles distributed along the dorsal margin (*vs.* a single, terminal CV in *L. qiuianum*) (Table 3) (Buddenbrock 1920, Kahl 1933).

Other *Loxophyllum* spp. that possess two macronuclear nodules and a single CV include: *L. elegans*

Wenzel, 1961, *L. fasciolatus* Dragesco, 1966 and *L. vitraeum* Dragesco, 1965. *Loxophyllum qiuianum* can be separated from these by its possession of warts (*vs.* warts absent in each of these three) (Table 3).

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Descriptions of Two New Species of the Genus *Trichodina* Ehrenberg, 1838 (Protozoa: Ciliophora: Peritrichida) from Indian Fresh Water Fishes

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Summary. Two new species of the genus *Trichodina* Ehrenberg, 1838 were obtained during surveys of trichodinid ciliophorans in the Churni River system and adjacent water bodies in West Bengal, India. These are *T. giurusi* sp. n. from *Glossogobius giuris* (Hamilton-Buchanan) and *T. molae* sp. n. from *Amblypharyngodon mola* (Hamilton-Buchanan). This papers deals with taxonomic descriptions of the two new species based on wet silver nitrate impregnated specimens along with prevalence and morphometric comparisons with closely related species.

Key words: Ciliophora, fish ectoparasites, India, *Trichodina giurusi* sp. n., *T. molae* sp. n., Trichodinidae.

INTRODUCTION

Since the work of Annandale (1912) who first reported the occurrence of *Trichodina pediculus* Ehrenberg, 1838 from the lymnocnidid medusa, *Lymnocnida indica* in Bombay Presidency of British India no serious work on this complex group had been carried out in India for a long time. Information on the taxonomy of trichodinids and other groups of ciliophorans is available from early 80's. As a result, more than twenty species of trichodinids and one species of chilodonellid ciliophorans representing the genera *Trichodina* Ehrenberg, 1838; *Paratrichodina* Lom, 1963; *Tripartiella* Lom, 1959; *Trichodinella* Šramek-Hušek, 1953 and *Chilodonella* Strand, 1926 were identified from different estuarine and freshwater fishes (Hagargi and Amoji 1979; Mukherjee and Haldar 1982; Das and Haldar 1987; Das *et.al.* 1987; Mishra and Das 1993; Saha *et. al.* 1995 a, b; Saha and Haldar 1996, 1997; Asmat and Haldar 1998; Basu and Haldar 1998; Asmat 2000a, b; 2001a, b, c, d; 2002a, b, c; Basu *et al.* 2003; Mitra and Haldar 2003, 2004 a, b). An expedition in search of trichodinid ciliophorans in freshwater fishes of the river Churni revealed the occurrence of two new species of trichodinid ectoparasites belonging to the genus *Trichodina* Ehrenberg, 1838. These are *T. giurusi* sp. n. and *T. molae* sp. n. The present communication reports on the taxonomy, systematics and prevalence of

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these two new species based on silver nitrate impregnated preparations.

MATERIALS AND METHODS

River Churni is one of the many tributaries of the river Ganges and flows through the district of Nadia in West Bengal (23°E, 88.5°W). It is a small and docile river and provides a complete fresh water environment. Samplings were carried out to collect host fishes from the River Churni and adjacent water bodies. Host fishes were brought to the laboratory in living conditions and gill and skin smears were made on grease free slides. Slides containing trichodinid ciliophorans were impregnated using Klein's dry silver impregnation technique (Klein 1958). Examinations of preparations were made under an Olympus phase contrast microscope at ×100 magnifications with an oil immersion lens and photographs were taken with an Olympus camera. All measurements are in micrometers and follow the uniform specific characteristics as proposed by Lom (1958), Wellborn (1967) and Arthur and Lom (1984). In each case minimum and maximum values are given, followed in parentheses by arithmetic mean and standard deviation. In the case of denticles and radial pins, the mode is given instead of the arithmetic mean. The span of the denticle is measured from the tip of the blade to the tip of the ray. Body diameter is measured as the adhesive disc plus border membrane. The description of denticle elements follows the guidelines of Van As and Basson (1989). Sequence and method of the description of denticle elements follows the recommendations of Van As and Basson (1992).

RESULTS AND DISCUSSION

Two new species of trichodinid ciliophorans belonging to the genus *Trichodina* Ehrenberg, 1838 were obtained from the collected fishes. These are *T. giurusi* sp. n. and *T. molae* sp. n. Descriptions of these are provided below.

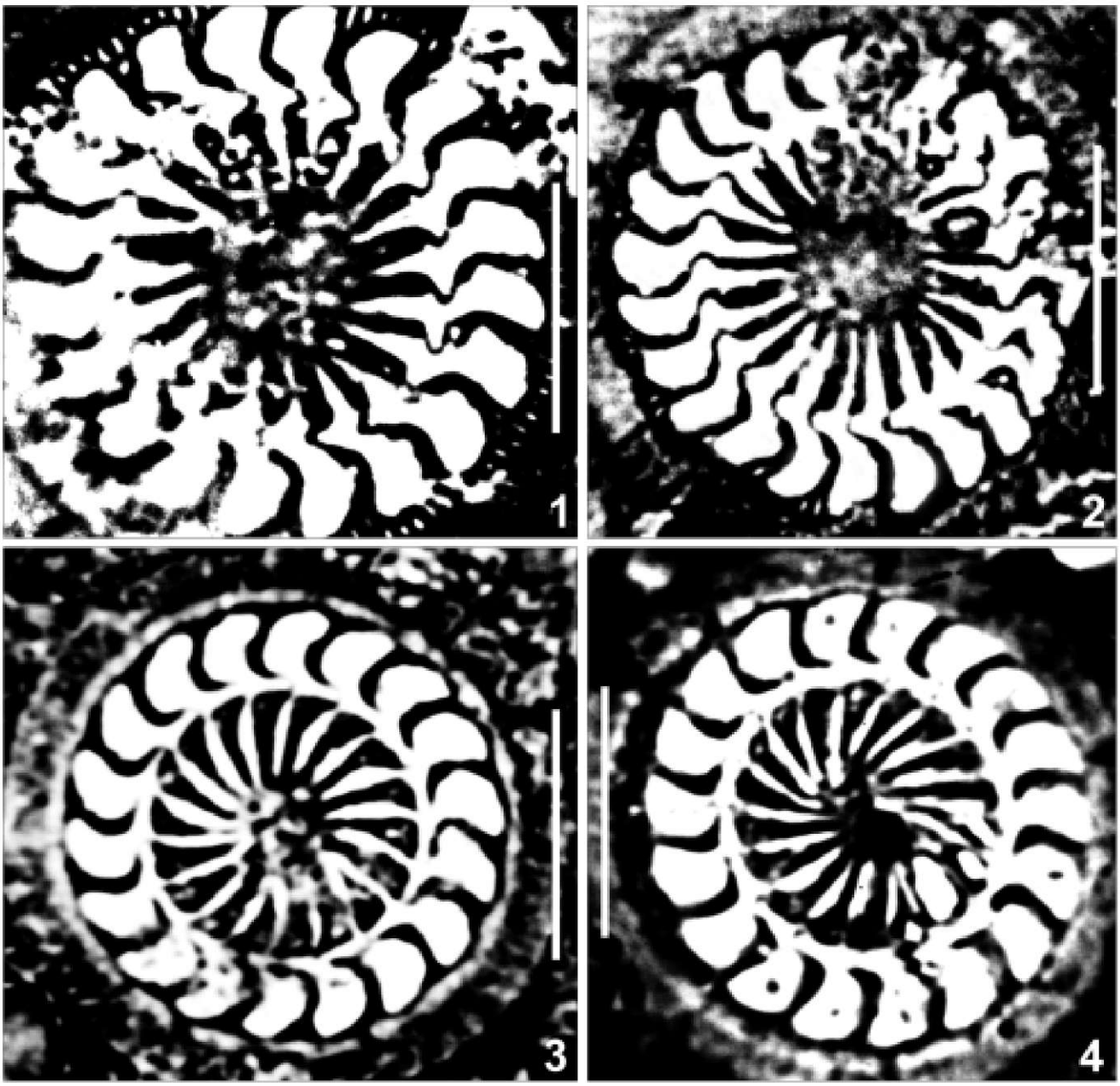
Trichodina giurusi sp. n. (Figs 1, 2, 5; Table 1)

Small-sized trichodinid. Denticle consisting of broad blade, occupies most area between y-axes. Distal surface of blade slightly rounded, parallel to border membrane. Tangent point flat, like small line rather than point and situated lower than distal surface. Differentiation between distal surface and anterior surface not significant. Anterior surface sloping down backward to form distinct apex, reaches or even extends beyond y+1-axis. Blade apophysis extremely prominent, consisting of 1-2 notches. Anterior and posterior surfaces of blade almost parallel. Posterior margin of blade takes slightly angular curve to form shallow semilunar curve, deepest point of which remains at same level as apex. Blade connection robust. Differentiation between blade and central part not distinct. Central part delicate in comparison to blade, and spine-shaped in most specimens, fits tightly into preceding denticle and extends almost halfway between y-axes. Sections of central part above and below x-axis similar. Indentation in lower half of central part opposite to ray apophysis of preceding denticle not present. Ray connection broad. Ray apophysis prominent and anteriorly directed. Ray apophysis situated at middle of y-axes. Well-developed ray gradually tapers to sharply pointed end. Rays directed towards y-1 axis. Ratio between the length of the blade and ray is greater than 1. Macronucleus horseshoe shaped but micronucleus could not be detected.

Taxonomic summary

Type host: *Glossogobius giuris* (Hamilton-Buchanan)
Host family: Gobiidae
Type locality: Ranaghat, W. Bengal, India
Location: Gills
Prevalence: 3/56 (5.3 %)
Etymology: The species is named after the specific epithet of the type host *Glossogobius giuris* (Hamilton-Buchanan).
Reference material: Holotype, slide GG-3/2001, and paratype slide GG-2/2002 in the collection of the Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India and slide GG-1/2002 bearing some paratype materials in the collection of the Harold W. Manter Laboratory of Parasitology, Lincoln, Nebraska, USA (Accession No. HWML 45700).
Remarks: The denticle shape of *Trichodina giurusi* sp. n. obtained from the freshwater fish *Glossogobius giuris* resembles to some extent only two marine species, i.e., *Trichodina jarmilae* Lom *et* Laird, 1969 and *Trichodina parvula* Lom, 1970.

Trichodina giurusi is comparatively smaller in size than *T. jarmilae* reported by Lom and Laird (1969) from gills of sea raven *Hemitripterus americanus* in Canada. The blade apophysis is extremely prominent in the new species, which is not significant in *T. jarmilae*. In the new species under discussion the distal surface of the blade and anterior blade surface can easily be differentiated. But in *T. jarmilae* this differentiation is not well visible. Extremely prominent apex of the blade of the new species touches or even extends beyond y+1 axis, but in *T. jarmilae* it never touches y+1 axis. The tangent point of the blade is situated almost at the same level as distal point of distal surface in the specimens obtained from *Glossogobius giuris*. In *T. jarmilae* tangent point



Figs 1-4. Photomicrographs of silver nitrate impregnated adhesive discs of trichodinid ciliophorans. **1, 2** - *Trichodina giurusi* sp. n. obtained from the gills of *Glossogobius giuris* (Hamilton-Buchanan); **3, 4** - *Trichodina molae* sp. n. obtained from the gills of *Amblypharyngodon mola* (Hamilton- Buchanan). Scale bars 20 μm.

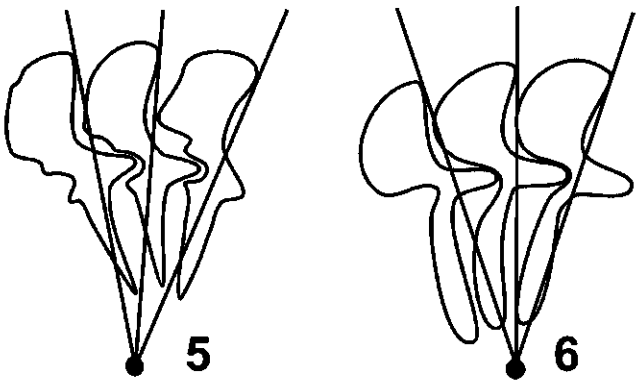
of the denticle is situated enough below the distal point of distal surface of the blade. The posterior surface of the blade of *T. jarmilae* is comparatively deeper than that of *T. giurusi*. In *T. jarmilae* the deepest point of the semilunar curve formed by the posterior blade surface remains slightly upper in position than the apex, but in the new species, deepest point of the semilunar curve remains at the same level as apex. The central part of the denticle of *T. giurusi* is delicate, but that of *T. jarmilae* is elongated and conical. The ray of the new species contains prominent and anteriorly directed ray apophysis. The ray apophysis is completely absent in

Table 1. Morphometric comparison of *Trichodina giurusi* sp. n. with *T. jarnilae* Lom *et* Laird, 1969 and *T. parvula* Lom, 1970. Measurements in μm.

Species	*T. giurusi* sp. n.	*T. jarnilae*	*T. parvula*
Host fish	*Glossogobius giuris*	*Hemitripterus americanus*	*Dasycottus setiger*
Locality	India	Canada	USA
Location	gills	gills	gills
Reference	present study	Lom and Laird (1969)	Lom (1970)
Diameter of			
body	24.4-34.8 (29.7 ± 2.7, 20)	42 (37-45)	35 (30-39)
adhesive disc	20.4-28.6 (24.6 ± 2.2, 20)	26 (23-30)	27 (23-30)
Dimension of body			
denticulate ring	11.7-17.3 (14.9 ± 1.7, 20)	13 (12-16)	14 (13-15)
central area	4.1-7.1 (5.2 ± 0.9, 20)	-	-
Width of border membrane	2.0-3.6 (2.5 ± 0.5, 20)	5	3
Number of			
denticles	20-23 (21, 20)	21 (19-23)	21 (20-23)
radial pins/denticle	5-8 (6, 20)	7 (6)	6-7
Dimension of denticle			
span	7.2-10.8 (8.9 ± 0.9, 20)	-	-
length	3.1-6.6 (4.2 ± 0.9, 20)	5	5
Dimension of denticle components			
length of ray	2.6-4.6 (3.5 ± 0.6, 20)	2-3	4
length of blade	3.6-5.1 (3.8 ± 0.5, 20)	3-4	2.5
width of central part	1.2-2.0 (1.5 ± 0.2, 20)	1-1.5	1.5
Adoral ciliary spiral	390-400°	-	-

Table 2. Morphometric comparison of *Trichodina molae* sp. n. with *T. tenuidens* Fauré-Frémiet, 1943 and *T. puytoraci* Lom, 1962. Measurements in μm.

Species	*T. molae* sp. n.	*T. tenuidens*	*T. puytoraci*
Host fish	*Amblypharyngodon mola*	*Gasterosteus aculeatus*	*Alburnus alburnus*
Locality	India	Poland	Bulgaria
Location	gills	gills	gills
Reference	present study	Lom and Stein (1966)	Grupcheva (1993)
Diameter of			
body	35.7-42.8 (38.2 ± 3.0, 20)	45-69	45 (37-62)
adhesive disc	28.6-34.7 (31.1 ± 2.2, 20)	50 (40-62)	32 (27-38)
Dimension of body			
denticulate ring	12.2-24.5 (19.0 ± 4.6, 20)	31 (25-40)	20 (17-25)
central area	2.6-4.2 (3.3 ± 0.6, 20)	-	-
Width of border membrane	2.9–4.6 (3.6 ± 0.6, 20)	-	3.5 (2.9-4.1)
Number of			
denticles	18-20 (20, 20)	28 (25-33)	24 (20-27)
radial pins/denticle	6-7 (6.3 ± 0.5, 20)	8 (9)	7 (6-8)
Dimension of denticle			
span	8.7-10.1 (9.8 ± 0.8, 20)	-	8.8 (7.6-10.4)
length	3.3-5.1 (4.2 ± 0.6, 20)	7-9	4.5 (2.9-5.3)
Dimension of denticle components			
length of ray	4.1-5.1 (4.4 ± 0.3, 20)	5-7	3.4 (2.9-4.6)
length of blade	3.2-4.6 (3.7 ± 0.5, 20)	4.5-7	3.9 (3.4-4.8)
width of central part	1.1-3.5 (1.7 ± 0.7, 20)	2-2.5	1.4 (1-2)
Adoral ciliary spiral	400-410°	-	-



Figs 5, 6. Diagrammatic drawings of the denticles of trichodinid ciliophorans. **5** - *Trichodina giurusi* sp. n. obtained from the gills of *Glossogobius giuris* (Hamilton-Buchanan); **6** - *Trichodina molae* sp. n. obtained from the gills of *Amblypharyngodon mola* (Hamilton-Buchanan).

T. jarmilae. In *T. giurusi* the ray gradually tapers to a sharp end, but in *T. jarmilae* the width of the ray is almost equal along the entire length and ends with a bluntly rounded tip.

Trichodina giurusi is also smaller than *T. parvula* in its body dimensions reported by Lom (1970) from the gills of marine fish *Dasycottus setiger* and *Radulinus asprellus* in the Pacific Coast of USA. The main differences between these two species lie in the structures of the blade and the ratio of blade: central part. The blade of *T. parvula* is angular in orientation to the y-axes, while that of the new species is straight. Both prominent apex and blade apophysis are present in *T. giurusi*. However, these are not present in *T. parvula*. The anterior and posterior margins of the blade of *T. giurusi* are parallel but these are not so in *T. parvula*. The anterior margin touches, or even extends beyond the y+1 axis, but in case of *T. parvula* the anterior margin never crosses the y+1 axis. The ratio between the blade and ray is greater than 1 in the species under discussion, but in both the populations of *T. parvula* reported from *Dasycottus setiger* and *Radulinus asprellus* this is much lesser than 1.

There are also habitat differences between *T. giurusi* and these trichodinids since *T. giurusi* is a freshwater species whereas *T. jarmilae* and *T. parvula* are marine. The superficial resemblance in the blade, central part and ray structures notwithstanding, we propose to designate the trichodinid ciliophoran as a new species. Morphometric comparison between *Trichodina giurusi* sp. n. and the other two closely related species is presented in Table 1.

Trichodina molae sp. n. (Figs 3, 4, 6; Table 2)

Medium-sized trichodinid. Concave adhesive disc bears dark central area. The denticulate ring consists of uniquely shaped denticles. Blade broad, almost semilunar, occupies most area between y-axes. Distal margin of blade flat; slightly rounded and remains in close proximity to border membrane. Tangent point rounded, situated lower than distal point of distal surface. Anterior margin slopes down gradually to form inconspicuous apex that almost touches and rarely extends beyond y+1 axis. Blade apophysis not visible. Posterior margin of blade forms shallow curve, deepest point of which remains at same level or slightly lower than apex. Blade connection thick. Posterior blade projection not observed. Central part slender, conical, extends halfway past y-1 axis in most cases, and fits tightly into preceding denticle. Sections above and below x-axis similar in shape. Ray apophysis absent. Ray connection narrow. After origin, ray gradually flattens laterally and then terminates with bluntly rounded end to take a spatula shape. Rays directed slightly anteriorly towards y+1 direction. Macronucleus horseshoe shaped. Micronucleus could not be detected.

Taxonomic summary

Type host: *Amblypharyngodon mola* (Hamilton-Buchanan).

Host family: Cyprinidae.

Type locality: Ranaghat, W. Bengal, India

Location: Gills.

Prevalence: 17/285 (5.9 %).

Etymology: The species is named after the specific epithet of the type host *Amblypharyngodon mola* (Hamilton-Buchanan).

Reference material: Holotype, slide AM-14/2001, and paratype slide AM-12/2001 in the collection of the Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India and slide AM-2/2001 bearing some paratype materials in the collection of the Harold W. Manter Laboratory of Parasitology, Lincoln, Nebraska, USA (Accession No. HWML 16742).

Remarks: The present trichodinid species is characterized by a broad blade, elongated conical central part, a narrow junction between the central part and ray and especially the depressed spatulate ray with bluntly rounded tip and, as such, differs significantly from other known trichodinid species. It shows some resemblance with *Trichodina tenuidens* Fauré-Frémiet, 1943 (Lom and

Stein 1966) and *Trichodina puytoraci* Lom, 1962 (Grupcheva 1993) only if the blade and ray structures are considered.

Trichodina tenuidens and *T. puytoraci* resemble our specimen in the blade and ray structures, but differ mainly in having a central subdivided clear area in the form of non-impregnable granulation, which is totally absent in the specimen under discussion. Instead, the central area of the adhesive disc is totally dark in *T. molae*. Both *T. molae* and *T. tenuidens* have spatulate rays, but their blade structure is completely different. The blade of *T. tenuidens* is elongated, while this is broad and slightly rectangular in the specimen under discussion. The number of denticles as reported by Lom and Stein (1966) in *T. tenuidens* is also higher (25-33 in Polish and 23-32 in Russian populations (*vs.* 18-20 in *T. molae*). *T. puytoraci* resembles the trichodinid specimen obtained from *Amblypharyngodon mola* when blade, central part and ray structures are compared. The blade is comparatively broader in *T. molae*. The tip of the ray is swollen in *T. puytoraci* (Grupcheva 1993), but rather spatulate in the new species. Considering all these differences we propose the trichodinid ciliophoran obtained from gills of the freshwater fish *Amblyphryngodon mola* a distinct one and designate in this paper as *Trichodina molae* sp. n. Morphometric comparison of *Trichodina molae* sp. n. obtained in the present study with closely related species is presented in Table 2.

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Eimeria fragilis and *E. wambaensis*, Two New Species of *Eimeria* Schneider (Apicomplexa: Eimeriidae) from African Anurans

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Summary. Two new species of coccidia (Apicomplexa: Eimeriidae) are described from Kenyan frogs. *Eimeria fragilis* sp. n., described from *Chiromantis petersii,* has ellipsoidal oocysts, 18.5 (17-19.5) × 15.2 (14.5-16) μm; lacking micropyle, oocyst residuum and polar granule. Sporocysts are dizoic, navicular, 10.6 (9.5-12) × 6.8 (6-7) μm. Oocysts of *Eimeria wambaensis* sp. n. found in *Hyperolius viridiflavus* are ellipsoidal to ovoidal, 17.0 (15.0-18.5) × 13.0 (11.0-14.0) μm; without micropyle, oocyst residuum and polar granule. Sporocysts are dizoic, navicular, 8.7 (8.0-10.5) × 6.0 (5.5-7.0) μm. Described species are the first *Eimeria* reported from African anurans.

Key words: Africa, anura, Apicomplexa, *Chiromantis petersii*, coccidia, *Eimeria fragilis* sp. n., *E. wambaensis* sp. n., *Hyperolius viridiflavus*, intranuclear development, Kenya.

INTRODUCTION

To date, there are 30 valid species of anuran coccidia, described within four genera: *Eimeria* Schneider, 1875 (16 species), *Goussia* Labbé, 1896 (2 species), *Hyaloklossia* Labbé, 1896 (1 species) and *Isospora* Schneider, 1881 (11 species) (Upton and McAllister 1988, Chen and Desser 1989, McAllister *et al.* 1995, Molnár 1995, Paperna and Lainson 1995, Paperna *et al.* 1997, Modrý *et al.* 2001, Bolek *et al.* 2003). Most named species originate from the holarctic region (Upton and McAllister 1988), with only a single species being described from anurans from the African continent. *Goussia hyperolisi* Paperna, Ogara *et* Schein, 1997 has been reported from central Kenya, parasitizes the digestive tract of tadpoles of the Common reed frog *Hyperolius viridiflavus* (Duméril *et* Bibron, 1841) (Paperna *et al.* 1997).

The coccidians described in this report represent first members of the genus *Eimeria* described from African anurans. Peters' foam-nest treefrog, *Chiromantis petersii kelleri* Boettger, 1893, and Common reed frog, *Hyperolius viridiflavus,* are African anurans of the families Rhacophoridae and Hyperoliidae, respectively. *Chiromantis p. kelleri* is restricted in its distribution to

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xeric areas covered by dry savannah from northern Kenya through Ethiopia to northern Somalia. In contrary, *H. viridiflavus* is widely distributed throughout the tropical savannah from Senegal to the Indian Ocean coast (Schiøtz 1999).

MATERIALS AND METHODS

Animals were collected during two field trips to Kenya in 2003 and 2004. Forty adult *Hyperolius viridiflavus* were collected in September 2003 by hand at night from a pond, approximately 0.5 km from Udo's camp site in Kakamega forest reserve in western Kenya (Western province, 00°20'55.7" N, 34°51'56.2" E). Two more adults of *H. viridiflavus* were captured in February 2004, by hand at night in a creek in the vicinity of Wamba (Rift Valley province, 00°56'58.4" N, 37°20'56.9" E). A single adult *Chiromantis petersii kelleri* was collected in February 2004, by local people in Kula Mawe (Eastern province, 00°34'11.1" N, 38°11'56.3" E). Frogs were identified according to Schiøtz (1999). Animals were housed for several hours in individual plastic boxes until they defecated, afterwards they were euthanized by overdosing with barbiturates (Thiopental® Spofa), dissected, and processed for following standard parasitological protocol.

Equidistantly spaced portions of the gastrointestinal tract, liver, kidney and muscle samples were fixed in 10% buffered formalin, processed routinely for histology, stained with haematoxylin and eosin, and examined for the presence of endogenous stages of coccidia. Measurements of endogenous stages were made on 10-15 individuals of each stage.

Faecal samples were placed immediately in 2.5% potassium dichromate, stored at room temperature for 4 weeks, transported to the laboratory in the Czech Republic, stored for next 3 months in 6-7°C, and examined for the presence of coccidian oocysts. Oocysts were concentrated by flotation method, using modified Sheather's sugar solution (specific gravity 1.30), and examined using Nomarski interference contrast optics (NIC).

Measurements were made on 20 oocysts, using an Olympus AX 70 microscope equipped with a calibrated ocular micrometer and are reported in micrometers (μm), usually as the mean, followed by range in parentheses.

RESULTS

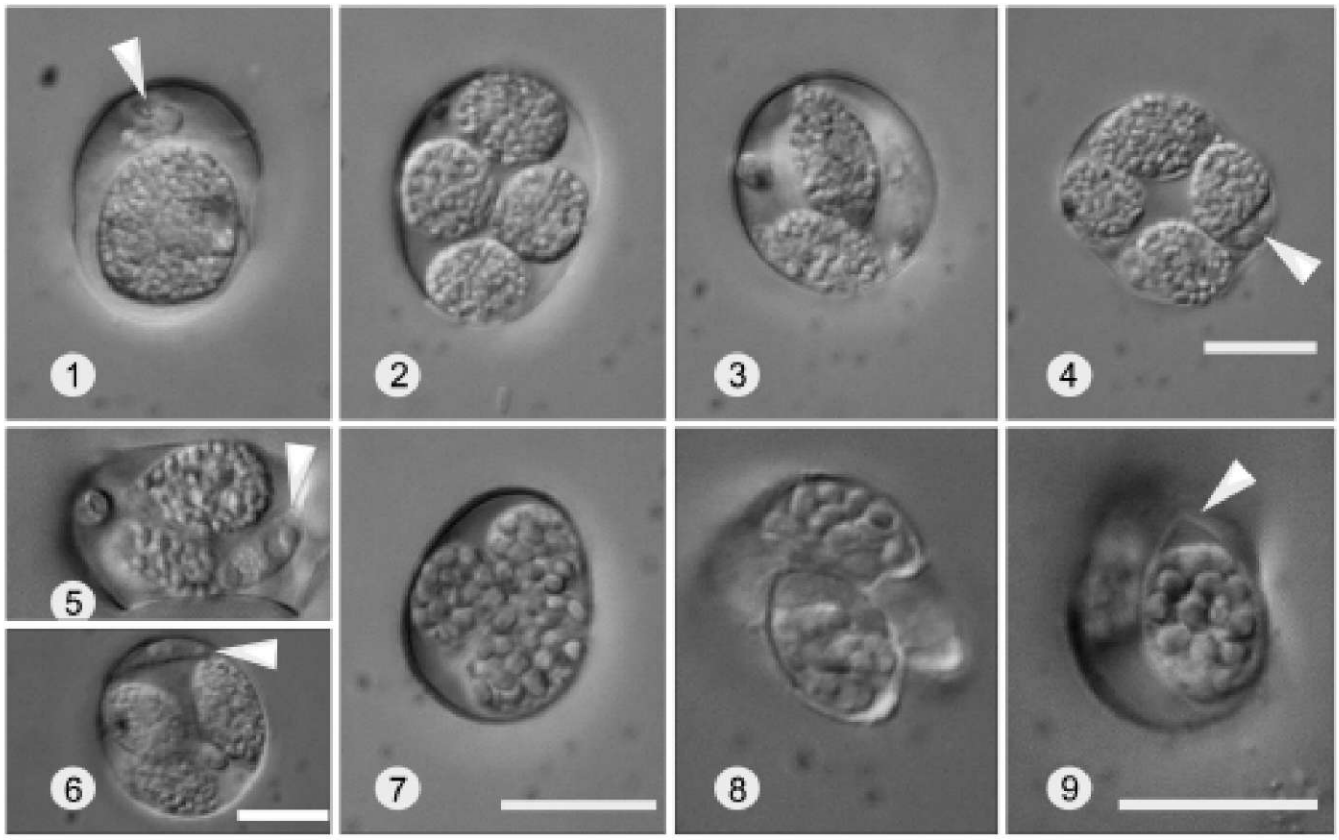
Adult *Chiromantis petersii kelleri* from Kula Mawe and a single adult *Hyperolius viridiflavus* from Wamba shed oocysts of 2 different, previously undescribed coccidia, both belonging to the genus *Eimeria*. No coccidian infection was detected in the 40 of *H. viridiflavus* from the Kakamega forest reserve. All *H. viridiflavus* from both Wamba and Kakamega were breeding individuals. Breeding status of *C. p. kelleri* was not recognized.

Eimeria fragilis sp. n. (Figs 1-6, 10, 12-16)

Description of oocysts: Unsporulated oocysts (Fig. 1) contain a subspherical sporont, approximately 13 × 11, composed of fine granules, 0.5-1 in diameter, containing vacuolised area (probably nucleus) ~2.5 in diameter. Outside this granular mass, usually a few larger granules, 1.5-3.5 in diameter, are present near one end of the oocyst. In sporulating oocysts (Fig. 2), 4 subspherical sporoblasts are present, measuring approximately 8.5 × 7. Fully sporulated oocysts (Figs 3-6, 10) are broadly ellipsoidal, 18.5 (17-19.5) × 15.2 (14.5-16); shape index (length: width ratio, SI) 1.2 (1.1-1.3). Micropyle, oocyst residuum and polar granule are absent. Oocyst wall is smooth, colorless, appearing as single-layered in light microscopy, ~0.5 thick. Sporocysts are dizoic, navicular (Fig. 3), 10.6 (9.5-12) × 6.8 (6-7), SI 1.57 (1.46-1.71), with smooth, colourless and single-layered sporocyst wall. One pole of each sporocyst is slightly thickened, with a barely distinct Stieda body. Sporocyst residuum present, usually as a mass composed of fine granules ~1 in diameter. Sporocyst residuum (even in completely sporulated oocysts) almost completely fills the sporocyst, leaving only small parts of the sporozoites visible (Fig. 4). Sporozoites are elongated, 10 × 2, arranged head to tail within sporocyst. Each sporozoite possesses two spherical refractile bodies 1-1.5 in diameter, located on the opposite ends of the zoite, and a centrally located nucleus, 1.5 in diameter. Sporozoite cytoplasm is finely granulated (Fig. 5).

One of the most distinctive features of *E. fragilis* sp. n. is its fragility. There is a tendency of sporocysts in sporulated oocysts to disintegrate and release free sporozoites into the oocyst's content (Figs 5, 6). After four months of storage, 80% of sporulated oocysts contained free zoites. Additionally, oocysts start to break down immediately in hypertonic conditions of Sheather's solution, and after ~15-20 min, oocyst walls are completely broken, leaving only collapsed sporocysts.

Morphology of endogenous stages: All endogenous stages putatively identified as *E. fragilis* are surrounded by a parasitophorous vacuoles and develop within nuclei of epithelial cells, in the small intestine. Occasionally, we observed more than one trophozoite in some nuclei of host cells. Additionally, when multiple developmental stages of *E. fragilis* were present within an individual host cell nucleus, each stage evidently possessed its own parasitophorous vacuole. Early trophozoites (Fig. 12), 3 × 2, were located within their vacuole (5 × 3.5-4) inside a host cell nucleus. Meronts



Figs 1-6 - Nomarski interference contrast micrographs of oocysts of *Eimeria fragilis* sp. n. in various stage of sporulation. **1** - oocyst with concentrated sporont. Note free granules near the pole (arrowhead); **2** - oocyst with four subspherical sporoblasts; **3** - sporulated oocyst. Note typical shape of the sporocyst; **4** - sporulated oocyst with collapsing oocyst wall. Arrowhead indicates the only visible part of sporozoite, almost entirely obscured by sporocyst residuum; **5, 6** - collapsed oocysts, containing free sporozoites (arrowheads). Scale bar 10 μm. Figs 1-5 are in the same scale.
Figs 7-9 - Nomarski interference contrast micrographs of oocysts of *E. wambaensis* sp. n. in various stage of sporulation. **7** - oocyst containing four (only three visible) subspherical sporoblasts; **8** - collapsed oocyst, showing typical shape of sporocysts; **9** - sporulated oocyst. Note typically pointed pole of sporocyst (arrowhead). Scale bars 10 μm. Figs 8, 9 are in the same scale.

(Fig. 13) containing approximately 5 merozoites measuring 5 × 1, are 6-7 × 4-5. Mature microgamonts (Fig. 14) of irregular shape, measured approximately 10-15 × 7.5-14. Macrogamonts (Figs 15, 16) in various stages of maturity measured 14-17 × 11-17 and contained a large nucleus and numerous eosinophilic granules, 0.5-1 in diameter.

Type host: *Chiromantis petersii kelleri* Boettger, 1893, (Anura: Rhacophoridae), Peters' foam-nest treefrog or Central foam-nest tree frog (common names according to Frost (2004)).

Type material: Photosyntypes of sporulated oocysts and histological sections with endogenous stages are deposited under collection number R 60/04 in the collection of Department of Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. Voucher specimen of *C. p. kelleri* [a symbiotype sensu Frey *et al.* (1992)] is deposited in the herpetological collection of the National Museums of Kenya, Nairobi under collection number A/4138.

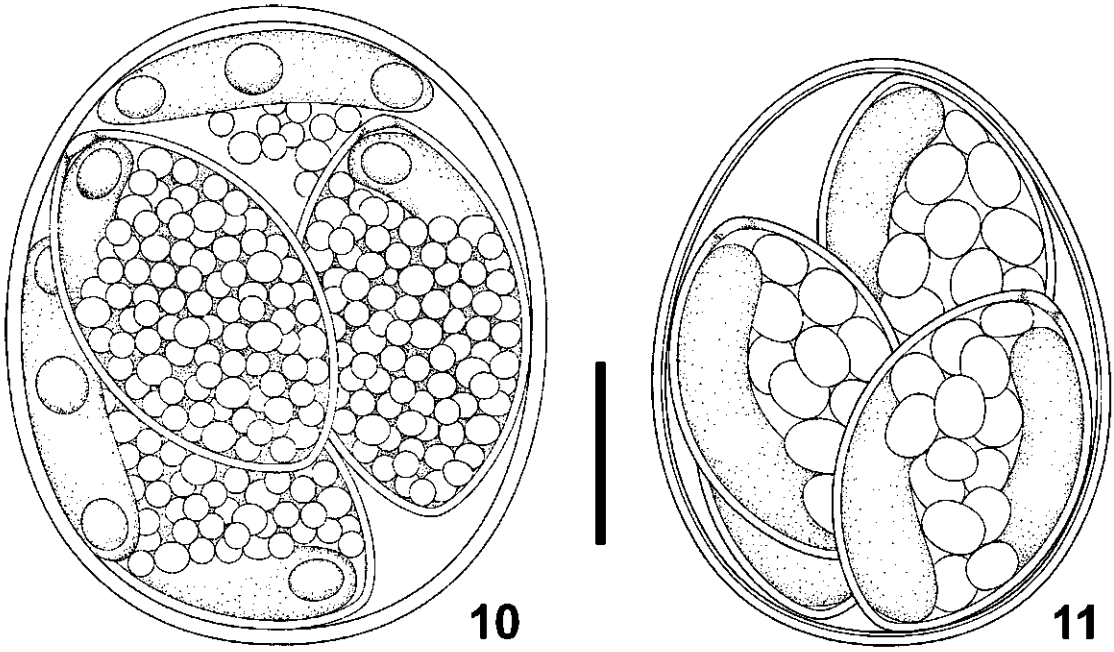
Type locality: Kula Mawe, Kenya (Eastern province, 00°34'11.1" N, 38°11'56.3" E).

Prevalence: Only a single animal was examined.

Sporulation and sporulation time: Unknown, oocysts obtained from faeces preserved in 2.5% potassium dichromate.

Site of infection: Intranuclear in epithelial cells of small intestine.

Etymology: Specific epithet refers to very fragile oocyst and sporocyst walls and tendency of sporocysts to disintegrate and release free sporozoites in to the lumen of oocyst.



Figs 10, 11. Composite line drawings of sporulated oocysts, both in the same scale. **10** - *Eimeria fragilis* sp. n. containing free sporozoites and three sporocysts; **11** - *Eimeria wambaensis* sp. n. Scale bar 5 μm.

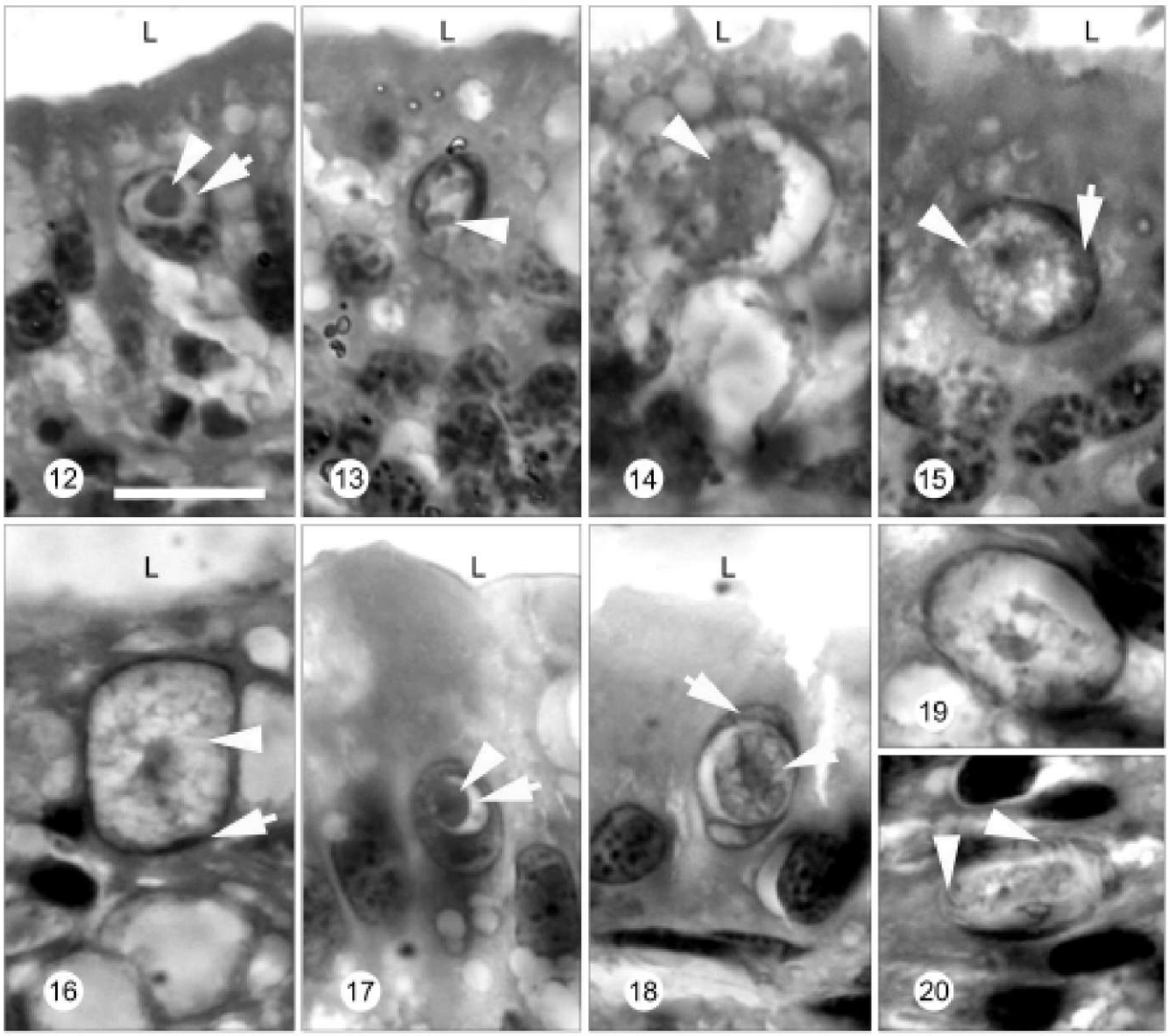
Eimeria wambaensis sp. n. (Figs 7-9, 11, 17-20)

Description of oocysts: Oocysts (Figs 7-9, 11) are ellipsoidal to ovoidal, 17.0 (15.0-18.5) × 13.0 (11.0-14.0); SI 1.3 (1.15-1.55). Micropyle, oocyst residuum and polar granule absent. Oocyst wall is smooth, appearing bilayered in light microscopy, outer layer approximately 0.5-0.7 thick, inner layer ~ 0.1-0.2 thin. Sporocysts are dizoic, navicular, 8.7 (8.0-10.5) × 6.0 (5.5-7.0), SI 1.4 (1.2-1.6), with smooth, colourless and single-layered sporocyst wall. One pole of sporocyst is slightly thickened, with a barely distinct Stieda body. Sporocyst pole opposite to Stieda body pointed (Fig. 9). Oocyst wall encloses sporocysts quite tightly and sporocysts sometimes seem a little bit deformed. Sporocyst residuum almost completely fills the sporocyst, composed of granules of irregular shape, 1.5-2 in diameter, leaving only small parts of the sporozoites visible. Sporozoites are arranged head to tail within the sporocyst. Each sporozoite possesses 1 spherical body (probably nucleus), 1.5 in diameter, located in the central part of the zoite. Sporozoite cytoplasm is finely granulated.

Morphology of endogenous stages: All endogenous stages putatively identified as *E. wambaensis* are surrounded by a parasitophorous vacuoles and develop within nuclei of epithelial cells, of the small and large intestine, with most of the infection occurring in the anterior and central part of small intestine. Early trophozoites (Fig. 17) are 3.5 × 2-3, located within a vacuole (5 × 4) inside the host cell nucleus. Mature microgamonts (Fig. 20) are spherical to elongately ellipsoidal, approximately 10-15 × 8-10. Macrogamonts, in various stages of maturity, (Figs 18, 19) were 11-16 × 7-12 and contained large nucleus and numerous eosinophilic granules resembling wall forming bodies, up to 1 in diameter. No meronts were observed.

Type host: *Hyperolius viridiflavus* (Duméril *et* Bibron, 1841), (Anura: Hyperoliidae), Common reed frog or Kenyan reed frog (common names according to Frost (2004)).

Type material: Photosyntypes of sporulated oocysts and histological sections with endogenous stages are deposited in the collection of the Department of Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic, R 65/04. Voucher specimen of *Hyperolius viridiflavus* (a symbiotype) is deposited in the herpetological collection of National Museums of Kenya, Nairobi, A/4130.



Figs 12-20. Micrographs of endogenous stages. **12** - early trophozoite of *Eimeria fragilis* sp. n. (arrowhead), surrounded by distinct parasitophorous vacuole (arrow) located within host cell nucleus; **13** - meront containing merozoites (arrowhead); **14** - nearly mature microgamont of *E. fragilis* (arrowhead); **15** - immature macrogamont of *E. fragilis* (arrowhead) surrounded by residuum of host cell nucleus (arrow); **16** - mature macrogamont of *E. fragilis* sp. n. (arrowhead). Note that host cell nucleus surrounds macrogamont tightly, forming thin layer on its surface (arrow); **17** - early trophozoite of *E. wambaensis* (arrowhead), surrounded by parasitophorous vacuole (arrow), located within the host cell nucleus; **18** - immature macrogamont of *E. wambaensis* (arrowhead). Note deformed and enlarged host cell nucleus (arrow) containing distinct parasitophorous vacuole; **19** - mature intranuclear macrogamont of *E. wambaensis*; **20** - mature microgamont of *E. wambaensis* containing fully developed microgametes (arrowheads). L - intestinal lumen. Scale bar 10 µm.

Type locality: Kenya, Wamba, (Rift Valley province, 00°56'58.4" N, 37°20'56.9" E).

Prevalence: 1/2 (50%) frogs from Wamba was infected; all 40 specimens from Kakamega forest were coccidia free.

Sporulation and sporulation time: Unknown, oocysts obtained from faeces preserved in 2.5% potassium dichromate.

Site of infection: Intranuclear in epithelial cells of both small and large intestine.

Etymology: Specific name *wambaensis* is derived from the Wamba, a village in northern Kenya, which is the type locality.

DISCUSSION

Both species of coccidia described in this study differ clearly from each other in oocyst and sporocyst shape and size. Oocysts of *E. wambaensis* are smaller and often have an ovoidal shape, with one end thickened, compared to ellipsoidal oocysts of *E. fragilis*. Sporocysts of the two described species differ both in their dimensions, and also in shape. Sporocysts of *E. fragilis* are longer, with SI exceeding 1.5, containing sporocyst residuum composed of remarkably finer granules not exceeding 1 μm in diameter even in oocysts of different age, in contrary to large sporocyst residuum granules measuring 1.5-2 in diameter, as typical for *E. wambaensis*. Additionally, the tendency of sporocysts of *E. fragilis* to disintegrate and release free sporozoites into the oocyst content is another distinguishing feature. However, oocysts of *E. fragilis* were first examined after four months of storage, when 80% of sporulated oocysts contained free sporozoites. Thus, it is possible, that free sporozoites do not occur in freshly sporulated oocysts. Among all anuran coccidia, presence of free sporozoites in oocysts is reported only in *Eimeria prevoti* (Laveran *et* Mesnil, 1902) from *Rana* kl. *esculenta* Linnaeus, 1758 from Europe, but this species differs in having distinct Stieda body and large oocyst residuum (Laveran and Mesnil 1902, Boulard 1975). Both new species of *Eimeria* described in this study differ from all other known anuran coccidia in oocyst or sporocyst dimensions and other morphological features. Moreover, the geographical origin and host phylogenetic distance make the conspecificity with other described species unlikely.

Recent studies on excystation structures of coccidia from poikilothermic hosts (Jirků *et al.* 2002) pointed out the importance of these structures for the classification of *Eimeria*-like coccidia. Although particular attention was given to describe the excystation structures on sporocysts of *E. fragilis* and *E. wambaensis*, we were unable to detect longitudinal sutures on the sporocysts of both *E. fragilis* and *E. wambaensis*. However, longitudinal sutures typical for sporocysts of numerous coccidia from poikilotherms usually are visible using light microscopy (pers. obs.). More importantly, both *E. fragilis* and *E. wambaensis* possessed barely distinct Stieda body on one pole of each sporocyst, and we believe these represent true Stieda bodies, clearly indicating that both of these coccidians are representatives of the genus *Eimeria*. Barely visible Stieda body also was reported in other anuran coccidia, for example in North American *E. flexuosa* Upton *et* McAllister, 1988; *E. streckeri* Upton *et* McAllister, 1988; *E. fitchi* McAllister, Upton, Trauth *et* Bursey, 1995 and South American *E. bufomarini* Paperna *et* Lainson, 1995 (Upton and McAllister 1988, McAllister *et al.* 1995, Paperna and Lainson 1995, Bolek *et al.* 2003).

Intranuclear localisation of endogenous stages observed in both *Eimeria* species described in this study was previously reported in several eimerian coccidia of the genera *Isospora*, *Cyclospora*, *Tyzzeria* and *Eimeria*, parasitizing higher vertebrates (Pellérdy 1974, Shibalova and Morozova 1979, Entzeroth and Scholtyseck 1984, Mohamed and Molyneux 1990, Paperna and Finkelman 1998, Pakandl *et al.* 2002). Among species parasitizing amphibians, intranuclear localization was reported in *E. ranarum* (Labbé, 1894), from *Rana* kl. *esculenta* and *R. temporaria* Linnaeus, 1758, and *E. salamandrae* (Steinhaus, 1889) from *Salamandra salamandra* (Linnaeus, 1758) (Steinhaus, 1889; Labbé, 1894; Pellérdy 1974). However, no comparable micrographs and adequate description of endogenous stages morphology were provided by authors, and intranuclear development of these two taxa must be confirmed by further studies.

The diversity of African Anura sharply contrasts with low number of described coccidian parasites. It is evident, that apicomplexan parasitic organisms represent a neglected part of African biodiversity and as such require further studies.

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Ultrastructual Study and Description of *Paramyxoides nephtys* gen. n., sp. n. a Parasite of *Nephtys caeca* (Fabricius, 1780) (Polychaeta: Nephtyidae)

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Summary. The new paramyxean *Paramyxoides nephtys* gen. n., sp. n. is described based on cytological characters. The organism reproduces by endogenous budding, and sporogony is the only sequence of reproduction observed. A primary cell with narrow haplosporosomes gives rise to two or four secondary cells (sporonts), each yielding four tetracellular spores in which the cells are situated one within the other. Spores fusiform, about 10 µm long, arranged with the long axes in parallel to a fusiform to globular body with terminal, striated projections. Nuclei are arranged in a line. Two of the spore cells (cells 4-5) have haplosporosomes. Haplosporosomes of cell 4 are spherical, measuring 80-300 nm in diameter, of cell 5 rod-like 70-100 nm wide. The host is the polychaete *Nephtys caeca*, and the parasite develops intracellularly in the gut epithelium in direct contact with the cytoplasm of the host cell. About 3% of studied hosts harboured the parasite. This is the first report of Paramyxea from Scandinavia.

Key Words: *Nephtys caeca*, Øresund, Paramyxea, *Paramyxoides nephtys* gen. n., sp. n., polychaete parasite, sporogony.

Abbreviations: C1-C6 - cells of generations 1-6, CE - centriole, F - fibrils, H - haplosporosomes, HN - host cell nucleus, M - mitochondrion, N1-N6 - nuclei of generations 1-6, P - primordia of vacuoles and plasma membrane, R - ribosomes.

INTRODUCTION

Paramyxea comprises a small number of parasites restricted to the marine environment. Polychaetes, crustaceans and molluscs are the known groups of hosts, and at least as parasites of bivalves paramyxeans might be of considerable economic importance. The species are inter- or intracellular parasites, and most tissues can be invaded. Gills, gut epithelium, hepatopancreas, ovocytes and testes are the preferred sites.

The systematic position of the phylum Paramyxea Desportes *et* Perkins, 1990, has been debated (Desportes and Perkins 1990), but molecular evidence now supports the cytological arguments for the ranking as a separate phylum (Berthé *et al.* 2000).

The first species of Paramyxea, *Paramyxa paradoxa* Chatton, 1911, was described from an unidentified pelagic polychaete larva, collected at Banyuls-sur-Mer at the Mediterranean coast of France (Chatton 1911). At present time, nearly hundred years later, the phylum is still very poor in species. The total number has grown to nine, distributed into four genera. Two genera are para-

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sites of molluscs: *Marteilia*, with the species *M. refringens* Grizel, Comps, Bonami, Cousserans, Duthoit *et* Le Pennec, 1974, *M. sydneyi* Perkins *et* Wolf, 1976, *M. lengehi* Comps, 1977, *M. maurini* Comps, Pichot *et* Papagianni, 1982, and *M. christenseni* Comps, 1983, and *Marteilioides*, with the species *M. chungmuensis* Comps, Park *et* Desportes, 1986, and *M. branchialis* Anderson *et* Lester, 1992. *Paramarteilia orchestiae* Ginsburger-Vogel *et* Desportes, 1979 was found in crustaceans, and *Paramyxa paradoxa* Chatton, 1911 in polychaetes. *Paramyxa* and *Paramarteilia* have been recorded from France, *Marteilia* from France, Spain and Australia, and *Marteilioides* from Australia, Korea and Japan. No Paramyxea species have previously been reported from Scandinavia.

A paramyxean parasite of the polychaete *Nephtys caeca*, was recently collected in the northern part of Øresund, the narrow water separating Denmark and Sweden. The reproduction is similar to that of *Paramyxa paradoxa*, the only previous paramyxean of polychaetes, but the spore shape and cytology is different. The species, which is new to science is described herein and a new genus is established.

MATERIALS AND METHODS

Specimens of the polychaete *Nephtys caeca* (Fabricius, 1780) were collected in the northern Øresund, at 14-16 m depth, on 1st July, 2002, and 3rd April, 2003. About 200 specimens were studied. The polychaetes are fragile, making exact count difficult. Six specimens harboured the parasite.

For transmision electron microscopy (TEM) whole segments were fixed in 2.5 % (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4°C, and postfixed in 1% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C. After washing and dehydrating in a series of buffer-acetone solutions to absolute acetone, specimens were embedded in Epon.

For light microscopy small pieces of polychaete segments, fixed in glutaraldehyde, were squashed on microscope slides and stained using Giemsa solution or Heidenhain's iron haematoxylin (Romeis 1968). Alcohol-fixed segments were embedded in paraffin, applying routine protocols, and sectioned. Permanent preparations were mounted in D.P.X. (BDH Chemicals Ltd., Poole, UK).

RESULTS

Influence on the host

The infection was restricted to the gut epithelium and initially no particular pathogenic action was observed. The parasite developed intracellularly and in direct contact with the cytoplasm of the host cell. In the early development no interfacial envelopes separated the parasite from the cytoplasm (Fig. 1), but the mature spores were enclosed in individual sacs produced by the parasite. When the sporogony was nearly completed host cell lysis was observed and mature spores were shed into the gut lumen.

Life cycle

The only reproduction observed was sporogony, and, like the typical for Paramyxea, the spores were formed by endogenous budding (Fig. 2). A primary cell (C1) [alternative terminology: plasmodium (Perkins 1976), cellule souche (Ginsburger-Vogel and Desportes 1979), primary stem cell (Desportes 1984)] generated 2 or 4 daughter cells, secondary cells (C2) [alternative terminology: sporangia (Perkins 1976), pansporoblastes (Ginsburger-Vogel and Desportes 1979), sporontes (Desportes 1981)]. Each secondary cell produced four spores, initially as four tertiary cells (C3). Budding of the tertiary cells generated three more generations of cells (C4-C6) which finally made each spore tetracellular (C3-C6). The mature spore was fusiform and remained in an individual envelope produced by the tertiary cell. Mature spores were arranged with the longitudinal axes in parallel and with the poles orientated in the same or opposite way. The four spore sacs were connected at the poles to form a fusiform body with polar prolongations (Fig. 3). In smears, with all spores in the same plane, the spore groups appeared spherical (Fig. 3).

Cytology of the developmental stages

Cell 1: The primary cell. The primary cell (Fig. 4), like the following generations of cells, was delimited by *ca* 9 nm thick unit membrane. The cytoplasm was more lucent than the cytoplasm of the daughter cells. Free ribosomes were uniformly dispersed and occurred together with a unique type of haplosporosomes, appearing as short membrane-lined tubules, approximately 55-90 nm wide. Mitochondria were not observed in primary cells.

The cytology of the nucleus was identical in all generations of cells. Initially the nucleus was an almost spherical body. It was uniformly granular with a large eccentric nucleolus. Characteristic electron-dense bodies, mostly in the neighbourhood of the nucleus, were seen in cells of all generations (cells C1-C6).

During the sporogony the nucleus of the primary cell was forced to the cellular periphery and the shape changed to appear triangular in sections. The primary

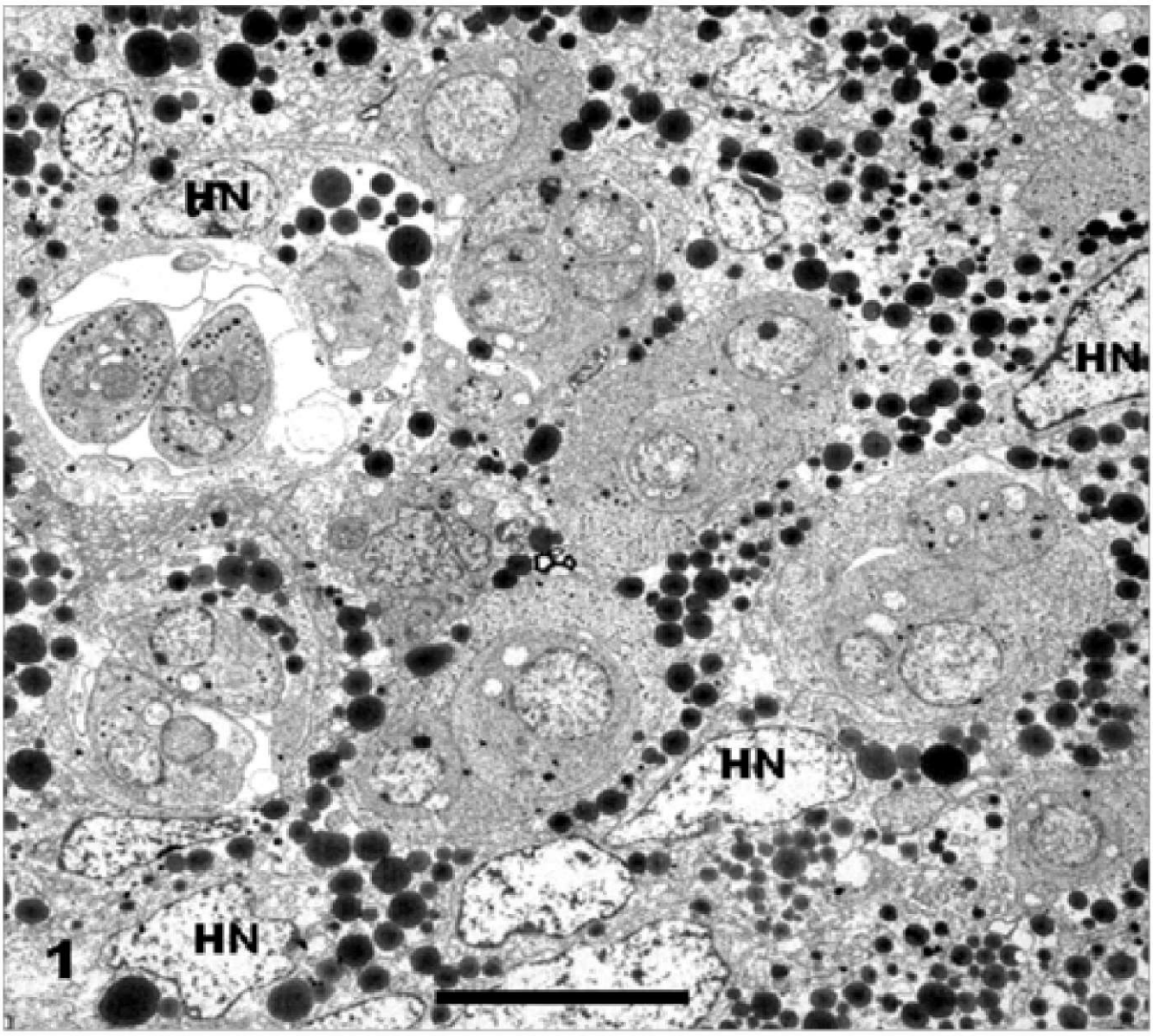


Fig. 1. The paramyxean *Paramyxoides nephtys* gen. n., sp. n. in various stages of sporogony in the gut epithelium of the polychaete *Nephtys caeca*. The parasite develops intracellularly and in close contact with the cytoplasm of the host cell. Dark grains are inclusions of the host cells. HN - nucleus of the host cell. TEM. Scale bar 10 μm.

cell degenerated successively and when the spores were mature no traces remained.

Cell 2: The sporont. Secondary cells were initially spherical (Figs 4, 5). Like the following generations of cells, they had a uniform, fairly dense cytoplasm with numerous free ribosomes, and large vesicular mitochondria. The plasma membrane originated together with the membrane of the surrounding vacuole from vesicles in the cytoplasm of the mother cell. These vesicles initially appeared in ring- or chain-like arrangements (Figs 6, 6A). Addition of more vesicle material built a vacuole envelope with several layers of membranes (Fig. 6).

The nucleus was of the general type for all cells. Centrioles connecting the nuclear envelope with the plasma membrane were only seen in stage C2 (Fig. 6), but were probably present also in other cells. Typical for

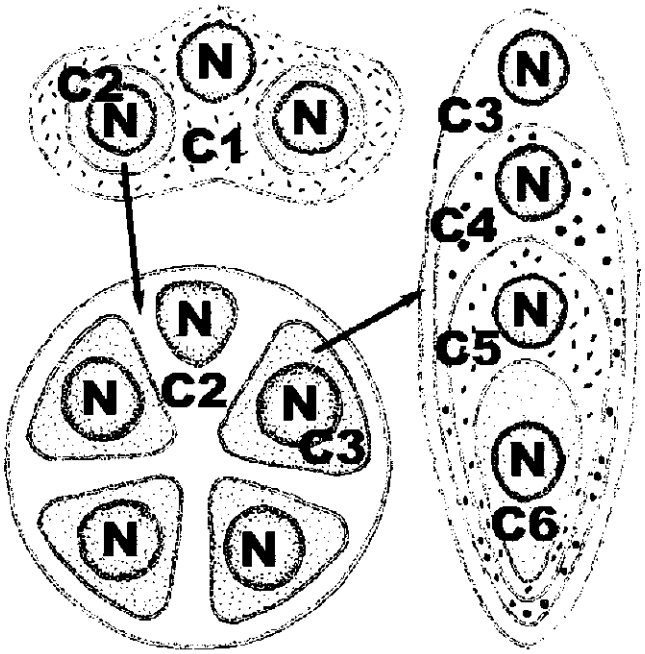


Fig. 2. Diagrammatic representation of the life cycle of *Paramyxoides nephtys* gen. n., sp. n. observed in the polychaete *Nepthys caeca*, showing the primary cell (C1), the sporont (C2) and the spore (C3-C6) (C1-C6) cell generations 1-6. N - nucleus.

the secondary cell were strains of electron-dense material in the cytoplasm, closely following the outline of the nuclear envelope (Figs 6, 7).

The nucleus, like the nucleus of cell 1, was forced to the cellular periphery when the spores developed, and the shape was compressed to triangular in sections (Fig. 8). Also the secondary cell successively disintegrated during the sporogony.

Cells 3-6: The spore. Cytologically these cells were identical to cell 2 when young (Fig. 8). One cell was formed in each generation and all cells remained in the mother cells where they originated. Newly formed cells were spherical, but with increasing generations of cells the external cells were extended to oval and finally fusiform shape, and the nuclei became lined (Figs 3, 8-10). The mitochondria were voluminous bodies when newly formed, but the size was successively reduced. Only in the last formed cell they remained large (Fig. 10).

With the appearance of cell 4, the plasma membrane of cell 3 was reinforced with electron-dense material, arranged as 35-40 nm wide parallel strands, separated by a distance of similar dimensions, on the surface (Figs 8-12). When the budding was finished, and the spore had four cells, the nucleus of the external cell (cell 3) was located to the anterior pole of the spore. In sections the shape was characteristically trapezoid (Fig. 10). Tetrasporous groups of mature spores measured up to 13.3 µm long (fixed and stained). The individual spore was up to 9.8 µm long (fixed and stained).

Maturation of the spore

Apart from change of shape from oval to fusiform, two events occurred during the maturation process: an external sac formed around each spore and haplosporosomes were produced.

When the four spores matured, the two early cells (1 and 2) were disintegrated leaving the spores free in the cytoplasm of the gut cell of the polychaete (Fig. 11). The electron dense regularly arranged ridges on the surface of cell 3 released material from which an external sheath was formed (Fig. 12). Successively it developed into a sac surrounding the spore (Fig. 13). Initially the sac appeared rounded in transverse sections and it was empty except for the central spore (Fig. 13). With increasing maturation the volume shrinked and the space between the envelope and the spore was traversed by distinct fibrous material (Figs 14, 15). At the time the sacs joined electron-dense spots appeared externally to the points of connection (Fig. 13). These points merged to become the poles of the mature spores, and generated striated projections (Figs 13-16). The striated material was probably not restricted to the terminal projections, but probably also formed longitudinal ridges at least a short distance along the connection between the spore sacs (Figs 3, 15). The material cemented the spore sacs together. No internal coupling devices were seen and longitudinal sections revealed that the spore sacs were only coupled at the poles (Fig. 15).

Haplosporosomes were formed in cells 4 and 5 (Figs 17, 18). They originated as vacuole-like bodies, which successively were filled with electron-dense material (Fig. 19). The mature haplosporosome had a membraneous coat and an electron-dense centre. The haplosporosomes of cell 4 were almost spherical, measuring 80-300 nm in diameter (Figs 17, 18). The material had two electron-densities, with the more dense material in an intermediate zone. A membrane-like layer separating peripheral and central material was only occasionally seen. Haplosporosomes of cell 5 were more narrow, 70-100 nm in diameter, and appeared as short, dense rods (Fig. 18).

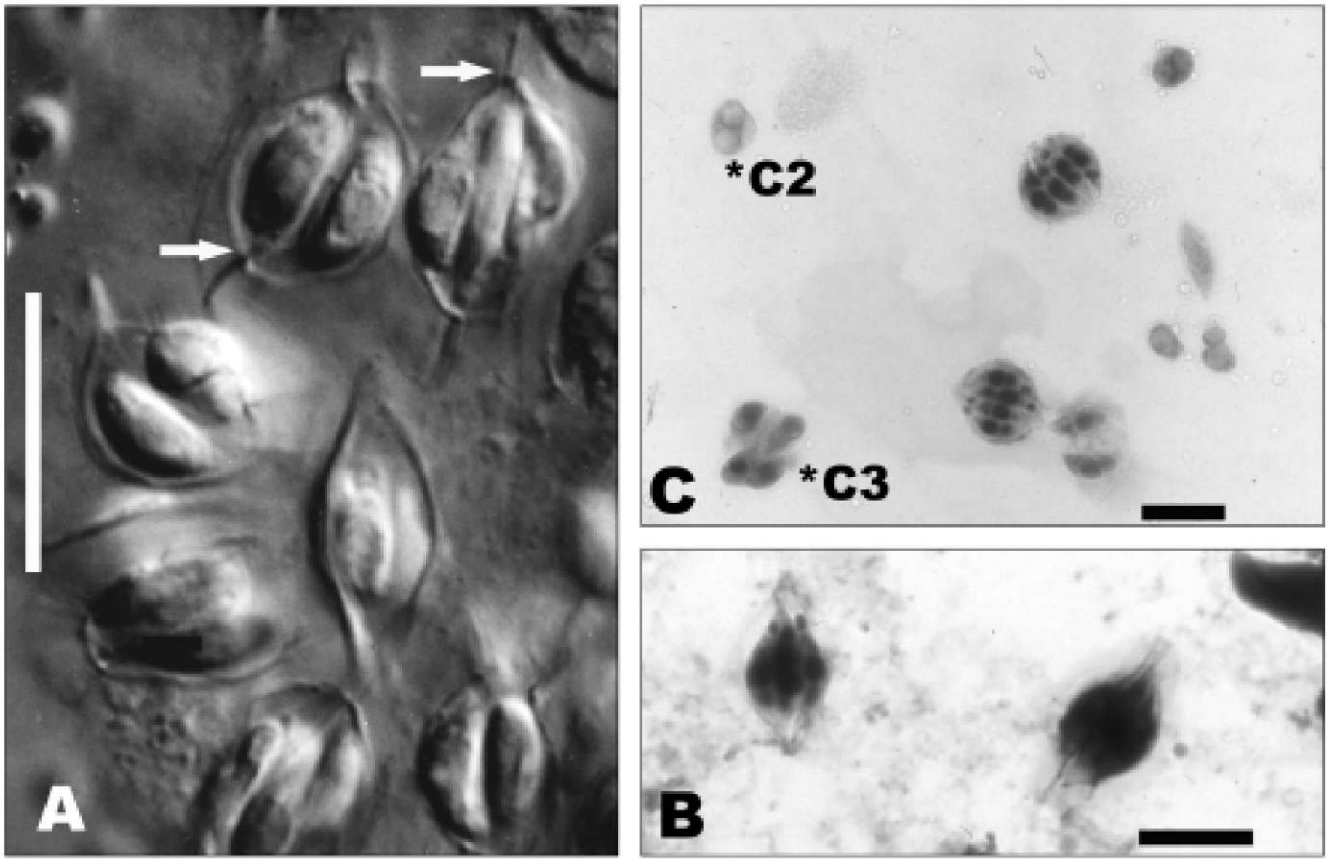


Fig. 3. Light microscopic aspect of *Paramyxoides nephtys* gen. n., sp. n. sporogony and mature spores. Groups of mature spores with terminal projections (**A** - fresh squash preparation, interference phase contrast; **B** - Giemsa stain; arrows point at positions where the spore sacs are connected); **C** - four stages of sporogony (production of sporonts *C2, production of spores *C3, and groups of mature spores; Giemsa stain). Scale bars 10 μm.

Diagnosis

Genus: *Paramyxoides* gen. n.

Sporogony by endogenous budding. The primary cell yields 2 or 4 secondary cells (sporonts), each of which generates four tetracellular and fusiform spores with smooth spore wall and pointed ends, lacking terminal plugs. Each spore is enclosed in an individual sac, produced by the external spore cell (C 3). The sac surrounding the mature spore is filled with a dense fibrous material. The four sacs are joint to a fusiform body with terminal, striated projections. Haplosporosomes of three types are present in cells 1, 4 and 5.

Paramyxoides nephtys sp. n.

Spore groups: Fusiform to spherical (stained smears) with polar projections, measuring up to 13.3 μm long in fixed and stained preparations. The four spores are arranged with the longitudinal axes in parallel, with their poles directed in the same or opposite way.

Spore: Fusiform, measuring up to 9.8 μm long (fixed and stained). Haplosporosomes in cell 1 (tubular, 55-90 nm wide), cell 4 (spherical, 80-300 nm wide), and cell 5 (short tububular, 70-100 nm wide).

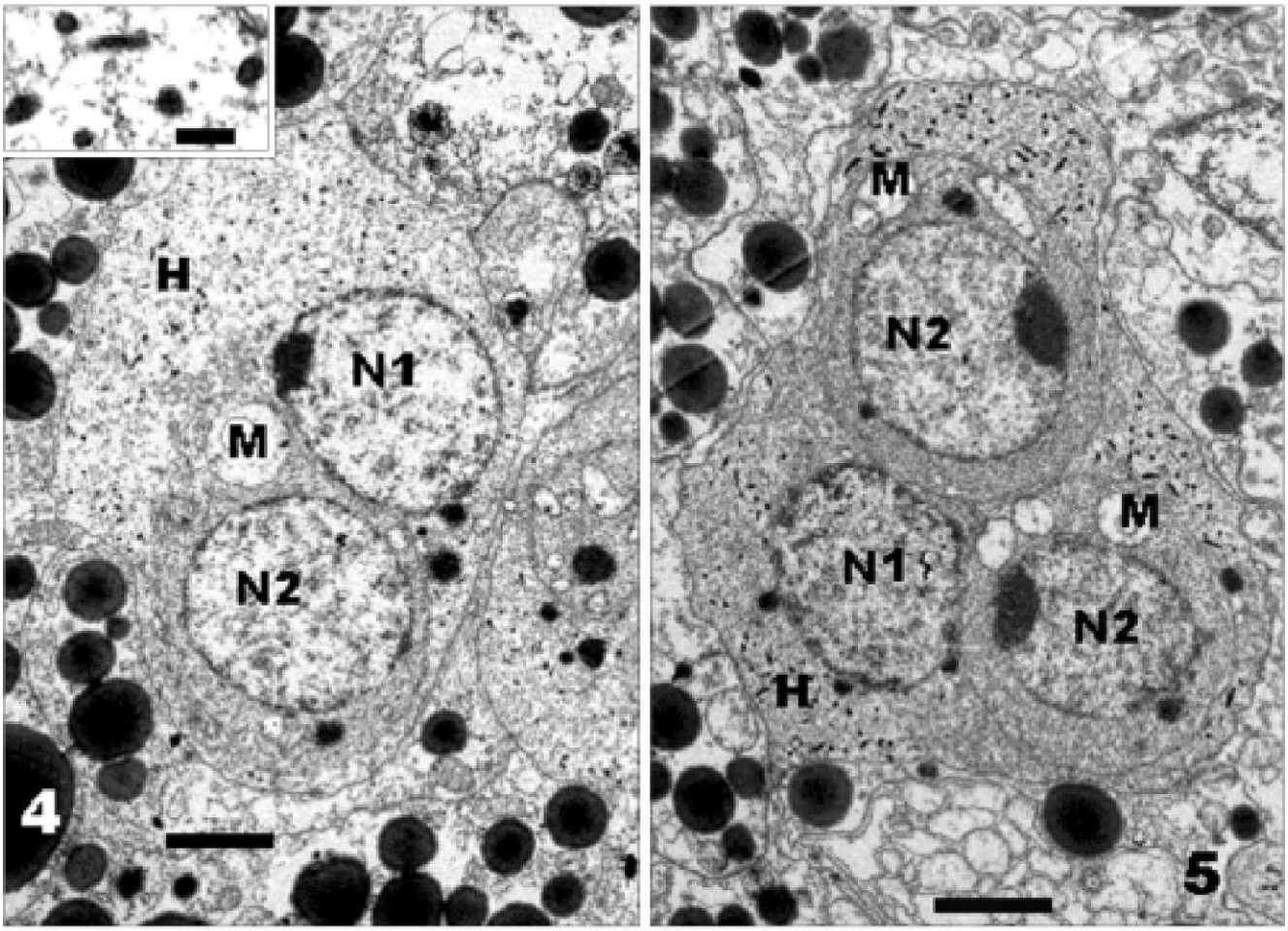
Type host: *Nephtys caeca* (Fabricius, 1780) (Polychaeta: Nephtyidae).

Infection site: Gut epithelium. Prior to the production of spore sacs no interfacial envelopes separate the parasite from the cytoplasm of the host cell. Prevalence of infection about 3%.

Type locality: Northern Øresund, depth 14-16 m.

Type material: Syntypes on slides 030502-A.

Deposition of type material: In the International Protozoan Type Slide Collection at Smithsonian Institution, Washington D. C., U. S. A., and in the collection of R. L.

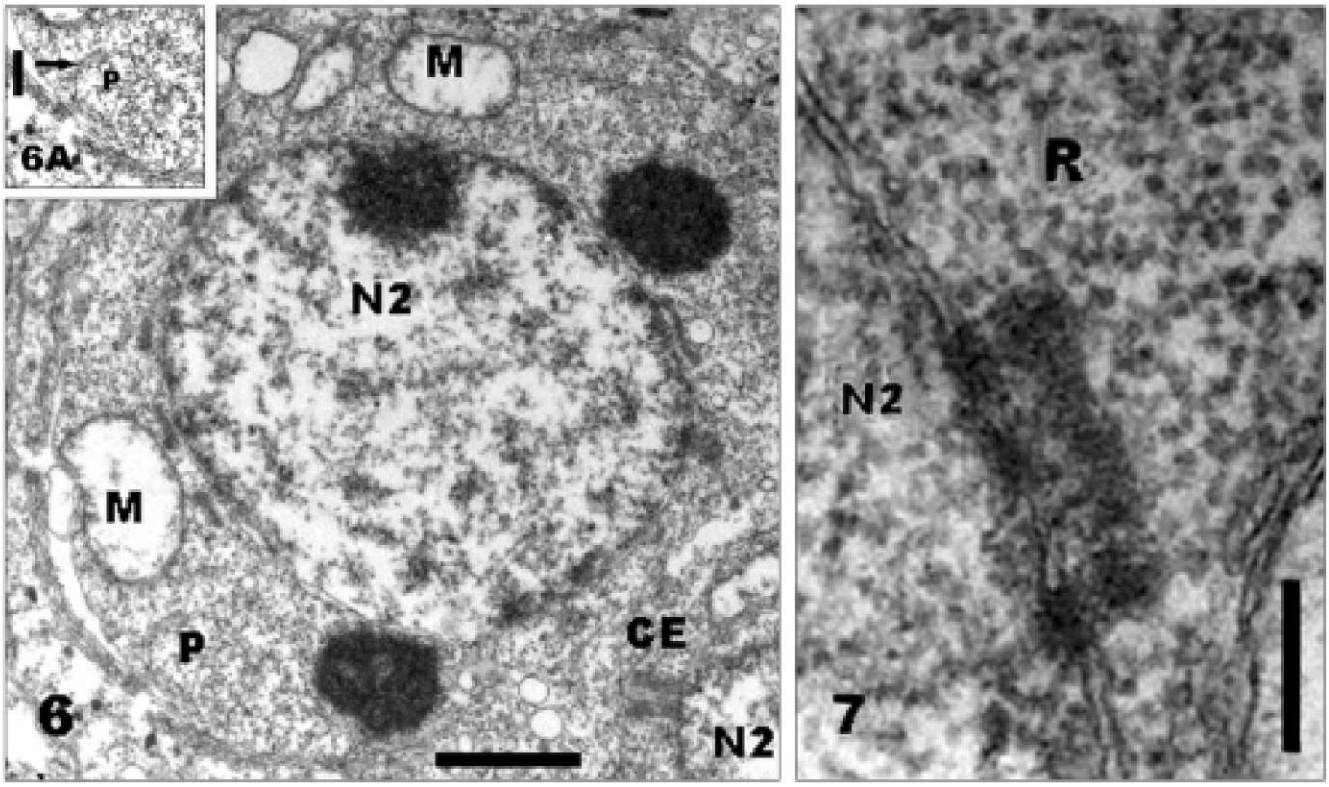


Figs 4, 5. Primary and secondary cells of *Paramyxoides nephtys* gen. n., sp. n. **4** - one primary cell with one secondary cell. Both cells have nuclei of identical cytology. Primary cell with elongated narrow haplosporosomes (H); inset shows haplosporosomes at greater magnification. The cytoplasm of the secondary cell is denser from free ribosomes. Secondary cell with vesicular mitochondria (M). Both cells have electron dense bodies adjacent to the nuclei. The close contact with the host cytoplasm is apparent; **5** - primary cell with two secondary cells. No mitochondria are visible in the primary cell. N1, N2 - nuclei of primary and secondary cells. TEM. Scale bars 200 nm (inset on 4); 2 μm (4-5).

Etymology: Names derived from the similarity with the genus *Paramyxa* Chatton, 1911 and from the type host.

DISCUSSION

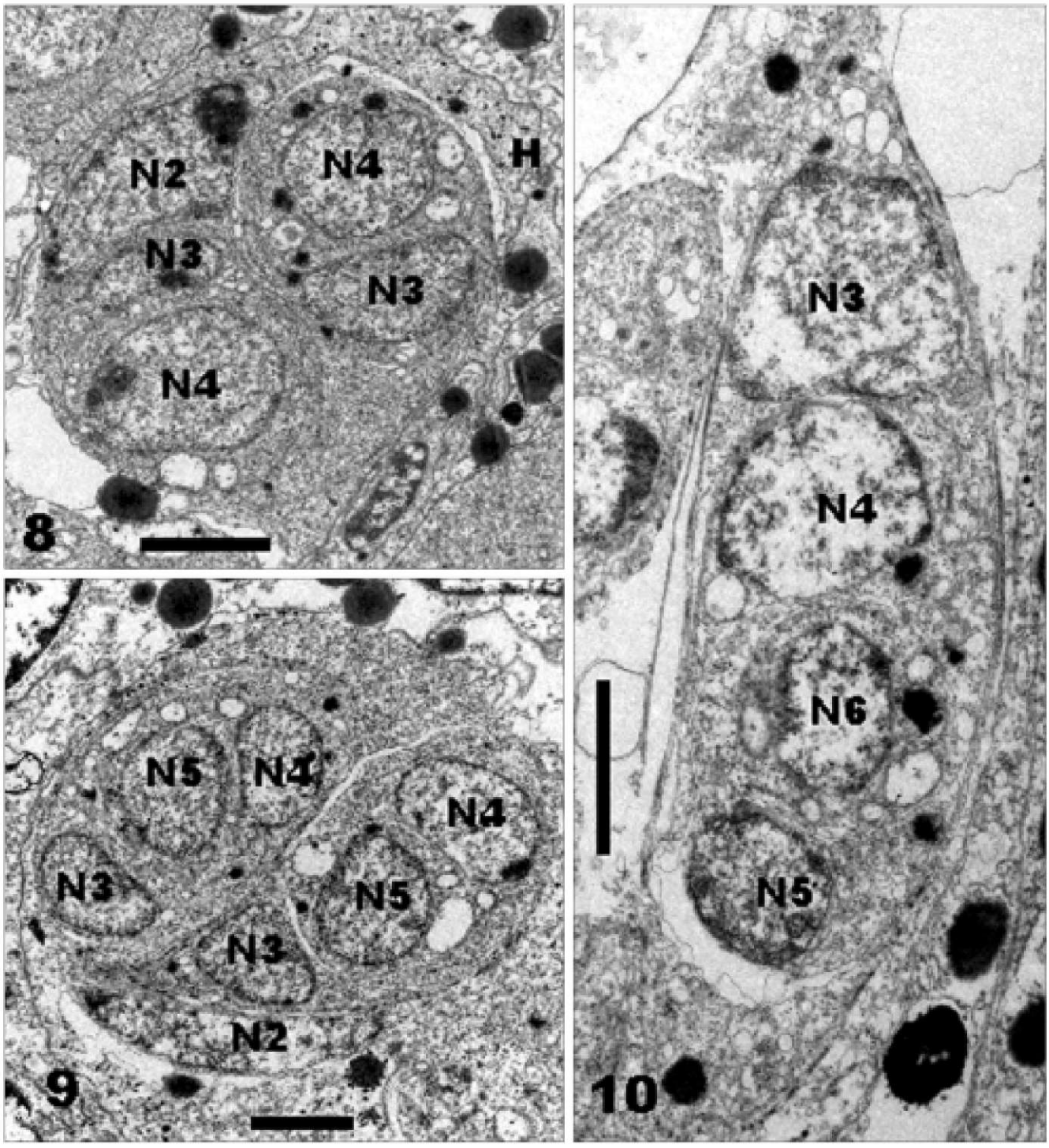
All four genera of paramyxeans have been studied at the ultrastructural level. *Marteilia* was treated by Grizel *et al.* (1974), Perkins (1976) and Perkins and Wolf (1976), *Marteilioides* by Comps *et al.* (1986), Anderson and Lester (1992) and Itoh *et al.* (2004), *Paramarteilia* by Ginsburger-Vogel and Desportes (1979), and *Paramyxa* by Desportes (1981). The cytology of the four genera is more or less identical, and also basically identical to the species treated herein. In all genera the primary cell has narrow haplosporosomes, the secondary cell lacks haplosporosomes, while one or more spore cells have haplosporosomes of a wider type. The external spore cell (C 3) is the cell with haplosporosomes in the genera *Marteilia*, *Marteilioides* and *Paramarteilia*. In *Paramyxa* haplosporosomes are found in cell 4. In the species treated by us, cells 4 and 5 have haplosporosomes of different size.



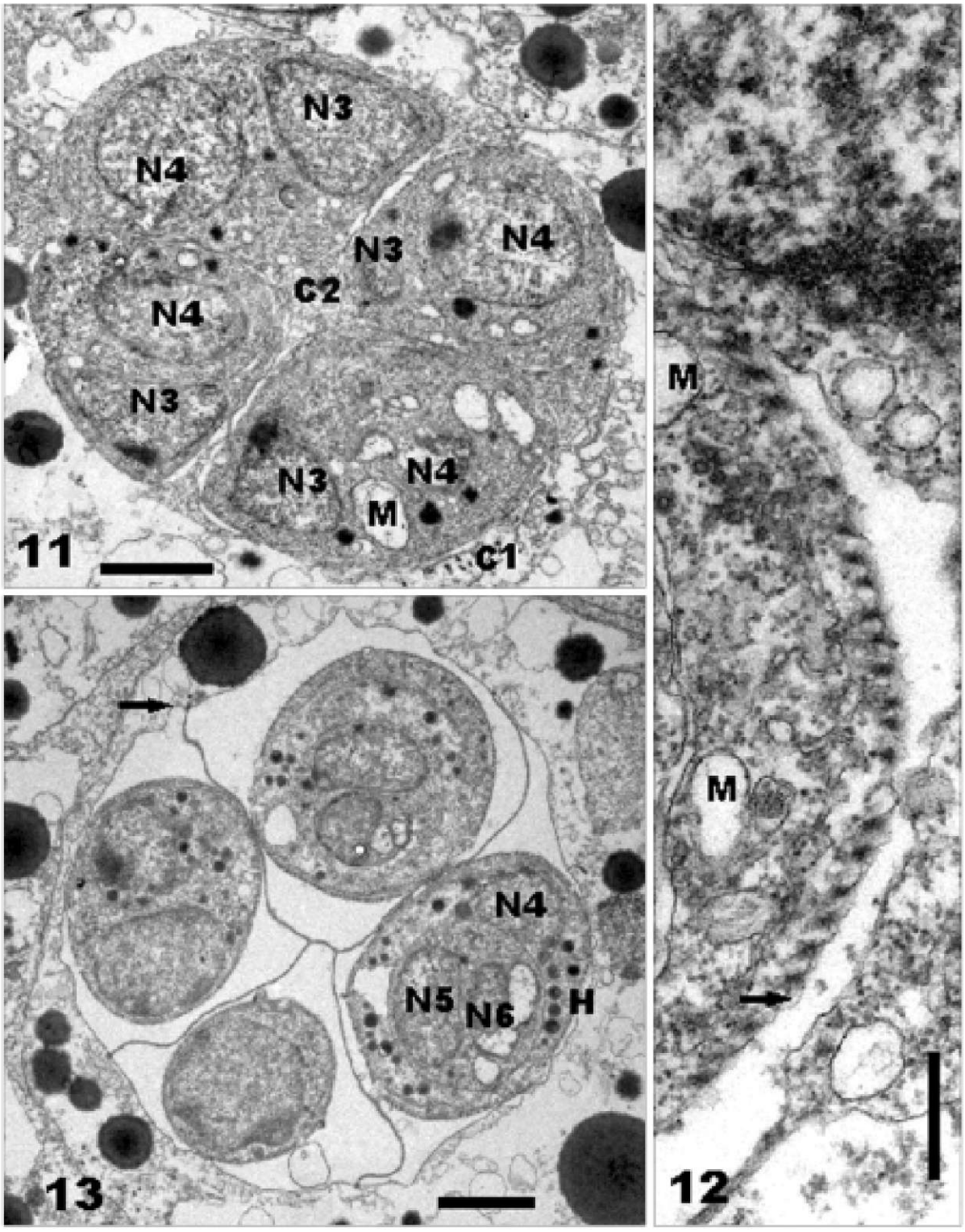
Figs 6, 7. Two aspects of *Paramyxoides nephtys* gen. n., sp. n. secondary cells. **6** - one secondary cell with strands of electron-dense material close to the nuclear periphery. A centriole (CE) is visible in the cell to the right. Lined vacuoles are the primordia of the plasma membrane and the surrounding vacuole of cell 3 (P, shown at greater magnification and indicated by arrow in 6 A). Several layers of vacuole membranes are visible externally; **7** - detail of the nuclear envelope and the external dense material. M - mitochondrion, N2 - nucleus of cell 2, R - ribosomes. TEM. Scale bars 0.2 μm (7); 0,5 μm (6 A); 1 μm (6).

Haplosporosomes are electron-dense spherical or tubular membrane-lined organelles reported from three groups or organisms: Haplosporidia (protists, now often classified in the phylum Alveolata), Myxozoa (a phylum of pluricellular organisms related with coelenterates or bilaterians) and Paramyxea. As these three groups are not likely to be a monophyletric unit, the term "haplosporosome" obviously denotes different organelles in the three groups. Typical haplosporosomes of Paramyxea have a more electron-dense surface layer (cortex), a less dense centre (medulla), and between them a membrane-like layer. Sometimes haplosporosomes appear uniformly electron-dense and the internal membrane-like component in not visible. It cannot be excluded that the fixation procedure influences whether the internal membrane-component is preserved or not. Most publications on *Marteilia refringens* show the internal membrane (e. g. Perkins 1976, Robledo and Figueras 1995). In the description of *Marteilioides chungmuensis* haplosporosomes appear uniformly electron-dense (Comps *et al.* 1986). In haplosporosomes of *Paramarteilia orchestiae* the internal membrane is distinct (Ginsburger-Vogel and Desportes 1979), while haplosporosomes of *Paramyxa paradoxa* appear to lack the membrane component (Desportes 1981). In the organism described herein only haplosporosomes of cell 4 sometimes reveal an internal membrane-like layer (Fig. 19).

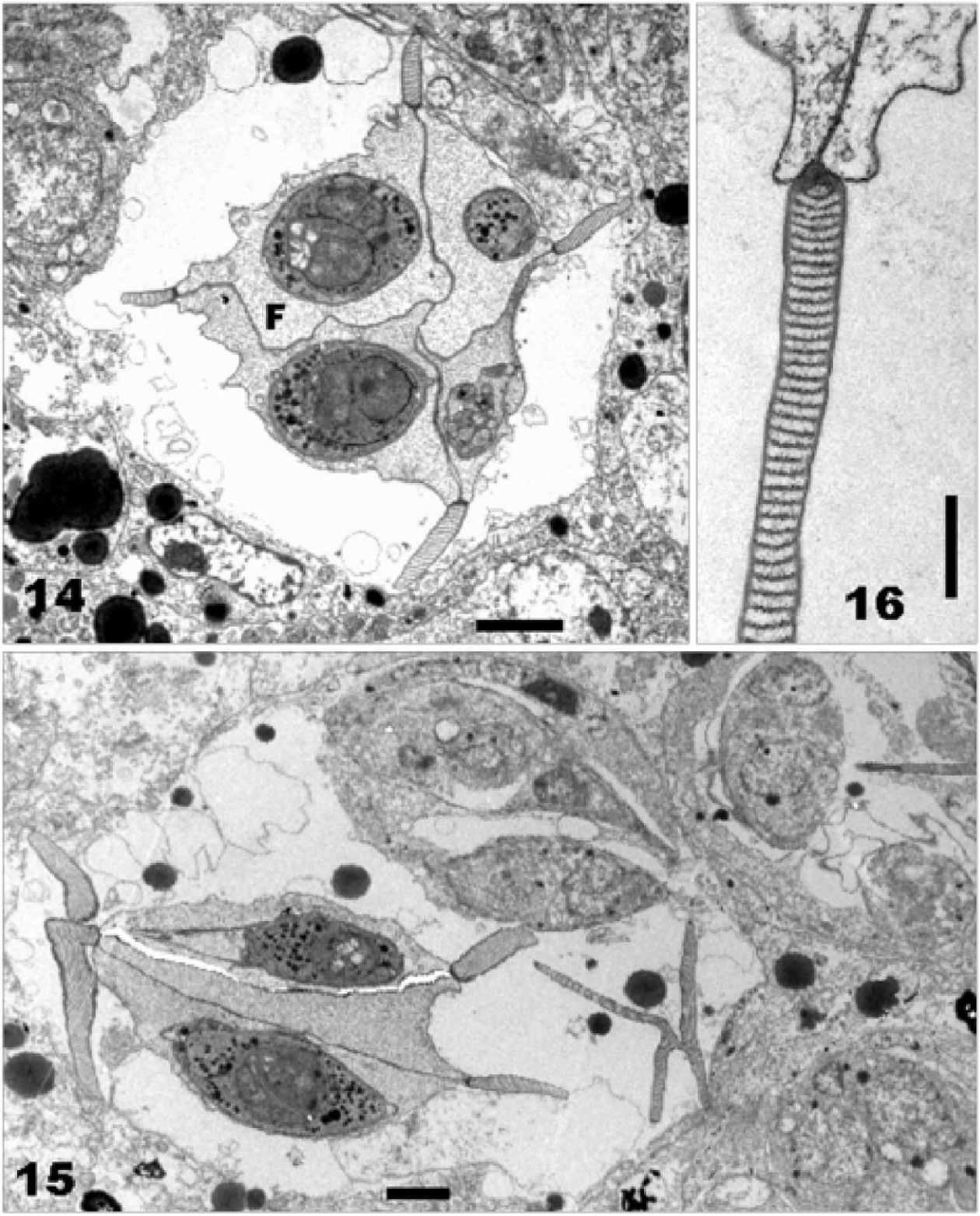
Principal distinguishing characters at the genus level are the number of spores per secondary cell (sporont), the number of cells per spore, and the shape of the spores. Three of the genera, *Marteilia*, *Marteilioides* and *Paramarteilia*, produce rounded spores, while the spores of *Paramyxa* are rod-shaped. Each sporont of *Marteilia* gives rise to 2-4 spores. *Marteilioides* species generate a single spore, while *Paramarteilia* pro-



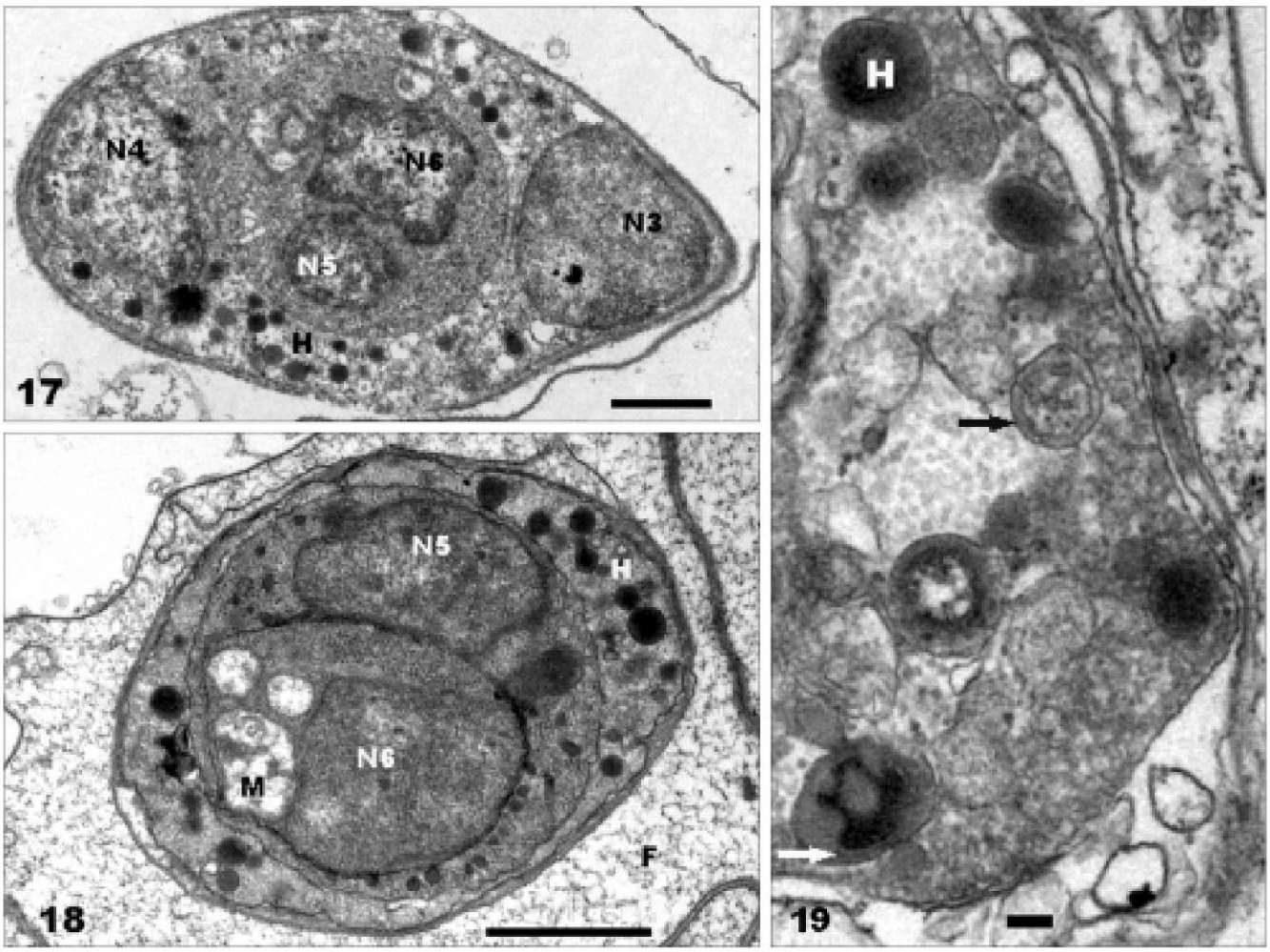
Figs 8-10. Sporogony of *Paramyxoides nephtys* gen. n., sp. n. **8** - periphery of a primary cell with one secondary cell (triangular nucleus at the periphery) containing two spore primordia (cells 3-4); **9** - primary cell containing one secondary cell with two immature spores consisting of cells 3-5. Nucleus of cell 2 pushed to the side. The surface of the tertiary cell with beginning production of electron-dense material in regular arrangement; **10** - immature spore with cells 3-6. Nucleus of cell 3 has trapezoid shape and is pushed to the anterior pole. All nuclei are lined. H - haplosporosomes of cell 1, N2-N6 - nuclei of generations 2-6. TEM. Scale bars 2 μm.



Figs 11-13. Transversely sectioned *Paramyxoides nephtys* gen. n., sp. n. sporonts (C2). **11** - C2 (nucleus not visible) with four primordial spore cells (cells 3-4). Areas of regularly arranged dense material present at the surface of the tertiary cells. Remainders of the disintegrating primary cell (C1) are visible at the surface; **12** - cell surface of a tertiary cell showing dense surface material from which the individual spore sac is released (arrow points at primordium); **13** - transverse section through four developing spores. Cells 1-2 are disintegrated and the spores lie directly in the cytoplasm of the host cell. Sacs surrounding spores appear empty. Electron-dense spots (arrow) are present at the positions where the spore sacs join. Haplosporosomes (H) are visible in cell 4. M - mitochondrion, N3-N6 - nuclei. TEM. Scale bars 0.5 μm (12); 2 μm (11, 13).



Figs 14-16. Mature *Paramyxoides nephtys* gen. n., sp. n. spore sacs. **14** - transverse section of four coupled spores. Fibrous material (F) fills the spore sacs, striated projections have developed at the points where the sacs join. Primary and secondary cells are completely disintegrated and the spores lie free in a host cell showing signs of beginning lysis; **15** - longitudinal sections of mature spores. Spore sacs are only coupled at the poles. The striated projections to the right cover the pole of another spore; **16** - detail of a striated projection. No internal couplings are visible between the two spore sacs. TEM. Scale bars 0.5 μm (16); 2 μm (14, 15).



Figs 17, 18. Aspects of maturing *Paramyxoides nephtys* gen. n., sp. n. spores. **17** - cell 3 with anterior nucleus (N3) and cytoplasm lacking haplosporosomes, cell 4 with wide haplosporosomes (H) and posterior nucleus (N4), cell 5 with two nuclei (N5 and N6) and no haplosporosomes (stage with spore sac devoid of fibrils); **18** - periphery of cell 3 is visible externally; cytoplasm of cell 4 with wide haplosporosomes, cell 5 with narrow haplosporosomes, and cell 6 lacking haplosporosomes; stage with fibrils (F) in the spore sac. M - mitochondrion. TEM. Scale bars 1 μm.
Fig. 19. Development of *Paramyxoides nephtys* gen. n., sp. n. haplosporosomes (H), from vacuoles to spherical bodies filled with dense material, in cell 4 (stage with beginning production of fibrils in the spore sac); arrows point at positions where internal membrane-like layer is visible. TEM. Scale bar 100 nm.

duces two spores. *Paramyxa*, like the present species, generates four spores per sporont. Spores of *Marteilia* are tricellular. *Marteiliodes chungmuensis* produces tricellular spores (Comps *et al.* 1986), but spores of *Marteilioides branchialis* are bicellular (Anderson and Lester 1992). *Paramarteilia* spores are bicellular, while the spores of *Paramyxa*, like of the organism described herein, are tetracellular.

The parasite described by us resembles *Paramyxa paradoxa* in life cycle characteristics and the location in the gut epithelium of the host (Desportes 1981), but there are also obvious differences. The size of the spore (*ca* 10 μm) is about half the size of *Paramyxa* spores (*ca* 20 μm). The fusiform shape differs from the rod shape of *Paramyxa*. Two spore cells (C4 and C5) contain haplosporosomes - one cell (C4) in *Paramyxa*. The spore coat has conspicuous striated projections, while spores of *Paramyxa* lack projections. Further there are two characteristics of *P. paradoxa* spores lacking in the organism treated by us. Spores of *P. paradoxa* have longitudinal ridges making transverse sections appear star-like, and there are distinct plugs at each end of the *Paramyxa* spore. *P. paradoxa* has distinct pathogenic influence on the host cell, causing

hypertrophism in the nucleus and mitochondria. The only effect on the host noticed by us is lysis when the cell contains mature spores. The spore size, and probably also the less severe effect on the host, tell that the species described herein differs from *P. paradoxa*.

In a group like Paramyxea, with a very limited number of species, it is not easy to judge which chararacters are distinctive for the discrimination of the genus, and which are significant at supergeneric levels. The number of cells per spore has been used for the distinction of genera, even if the two species of the genus *Marteilioides* differ in the number of spore cells: *M. chungmuensis* has three (Comps *et al.* 1986), *M. branchialis* only two spore cells (Anderson and Lester 1992). Comparing with other protists, or former protists, the number of cells produced in the sporogony could equally be a family character. In microsporidia, for example, there are numerous genera producing uninucleate octosporoblastic spores (i. e. each sporont produces eight cells) (Sprague 1977). Hazard and Oldacre used this combination of characters for the establishment of the new family Thelohaniidae (Hazard and Oldacre 1975). With increased knowledge of the cytology and molecular biology of microsporidia it became apparent that a combination of these characters not even is distinctive at the family level any longer. In Myxozoa genera are often distinguished only by minute cytological differences, for example the shape of spore projections (cf. for example the genera *Alatospora* and *Pseudoalatospora*) (Kent *et al.* 2000).

The species treated herein differs from *Paramyxa paradoxa* in some characters which in other protists clearly would have indicated superspecific differences. *P. paradoxa* has rod-shaped spores and distinct plugs at each end of the spore, clearly described ("le germe…est cylindrique, arrondi aux deux extrémités qui sont coiffées par deux calottes résiduelles") and illustrated in Figs 9, 10 by Chatton (1911) and, at the ultrastructual level, in Figs 15A and 17 by Desportes (1981), while the paramyxean of *Nephtys* lacks terminal plugs and the spores are fusiform with pointed ends. The longitudinal ridges of *P. paradoxa*, proven in electron micrographs by Desportes (1981; Figs 12, 13), and described at the light microscopic level ("Sa surface présente deux striations obliquement croisées") and drawn in Fig. 10 by Chatton (1911), are absent from the organism studied by us. However, this species has striated terminal projections not present in *P. paradoxa*. It is obvious that the organism studied by us is related to the genus *Paramyxa*, but it cannot be included in this genus. Consequently a new genus is established for the new species parasitic in *Nephtys caeca*.

The only phase of reproduction observed by us is the sporogony. However Itoh and colleagues have proven that the development of *M. chungmuensis* involves reproduction by binary fission prior to sporogony (Itoh *et al.* 2004). As transmission experiments with Paramyxea species has failed, it has been suspected that a part of the life cycle takes place in an intermediate host. It has recently been proven, by molecular methods and transmission experiments, that *Marteilia refringens* alternates between bivalves and copepods (Audemard *et al.* 2002). It cannot be excluded that also the organism treated herein needs one more host to complete the development.

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