

Euplotespora binucleata n. gen., n. sp. (Protozoa: Microsporidia), a Parasite Infecting the Hypotrichous Ciliate *Euplotes woodruffi*, with Observations on Microsporidian Infections in Ciliophora

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ABSTRACT. A new microsporidian species, *Euplotespora binucleata* n. gen., n. sp., from the brackish-water ciliate *Euplotes woodruffi* is described and defined on the basis of life history characteristics, light and electron microscopic features, and small subunit (SSU) ribosomal DNA (rDNA) sequencing. The life cycle of *E. binucleata* n. sp. probably has rather short merogonic and relatively long sporogonic phases. Some uninuclear meronts and sporonts, along with diplokaryotic sporoblasts and spores, were found in experimentally infected host cells. Such a peculiar life cycle has been induced experimentally in *Euplotes eurystromus* and constitutively microsporidian-free stocks of *E. woodruffi*. Spores of *E. binucleata* n. sp. are monomorphic, ovoid-cylindrical in shape, $3.44 \pm 0.17 \times 1.65 \pm 0.22 \mu\text{m}$ in size, and characterized by a diplokaryotic condition and a large posterior vacuole. The polar tube is isofilar, $4.5-5.5 \mu\text{m}$ in length when ejected, and lacking a distinctive coiled region (half-coiled). The polaroplast is divided into two regions: the anterior part has a few lamellae close to the anchoring disc; and the posterior part is a rounded body (sack), about one-quarter of the spore length. Spores do not appear to cluster together as a group. Each spore is surrounded by a sporophorous membrane closely adjacent to the exospore layer. A phylogenetic analysis of SSU rDNA sequences by different methods placed *E. binucleata* n. sp. in a clade with representatives of the microsporidian genera *Cystosporogenes* and *Vittaforma*. Observations of microsporidia in several other ciliates are discussed in view of the microsporidian infection frequency in the phylum Ciliophora.

Key Words. *Euplotes*, phylogeny, rDNA, taxonomy.

THE phylum Microsporidia is a large and diverse group of obligate intracellular parasites. Microsporidian infections of invertebrates and vertebrates are common events, but rarely occur in protists (Canning and Vavra 2000; Foissner and Foissner 1995; Issi 1986; Issi and Voronin 2007; Larsson 1999; Viver 1975). There are some examples of microsporidiosis in Gregarinina, but in Ciliophora, just a few microsporidians have been recorded so far (Foissner and Foissner 1995; Görtz 1987; Hovasse 1950; Krüger 1956; Lutz and Splendore 1908).

This type of parasitism is probably uncommon in ciliates. Thus, any new cases of the infection are of special interest. Up to now, a total of four free-living ciliate species have been found infected, but only the microsporidian infection of *Platyophrya terricola* has been thoroughly investigated from a morphological point of view (Foissner and Foissner 1995). The microsporidian parasite of the other two ciliates, *Stentor roeseli* and *Stentor polymorphus*, was reported by Görtz (1987). It seems likely that most microsporidia infecting different species of ciliates belong to different genera.

Despite vast data on small subunit (SSU) ribosomal gene (rDNA) sequences of various microsporidia (Baker et al. 1995; Canning et al. 2001, 2002; Hester et al. 2000; Morris, Terry, and Adams 2005), no molecular data are available yet for establishing phylogenetic relationships of those infecting ciliates.

In this report we present detailed information about a new case of microsporidiosis in Ciliophora and some observations on the frequency of this phenomenon in the phylum. Further investigations of microsporidiosis in ciliates could help to understand why these otherwise successful intracellular parasites seemingly underutilize such a suitable and diverse econiche as a large cell of a ciliated protist, which is frequently colonized by different prokaryotic and eukaryotic symbionts showing various types of functional relationships.

MATERIALS AND METHODS

Ciliate host stocks and cultivation. A population of *Euplotes* (CoMa) was collected from a brackish lagoon (salinity 4‰) near

Comacchio in Northeastern Italy ($N 44^{\circ}42' E 12^{\circ}10'$), an area particularly rich in aquacultures of various fish species. Using the classical ciliate taxonomic techniques of producing nuclear and cortical preparations (Fig. 1, 2), the CoMa population was assigned to *Euplotes woodruffi*, a ciliate morphospecies occurring in fresh-, brackish- and seawater bodies (Kosaka 1973). The CoMa population was found to possess a permanent endocytobiotic relationship with an unknown microsporidium. Twelve single *Euplotes* were isolated separately from the two collecting vessels drawn at the same location. Among the 12 CoMa stocks established from these, three representatives, CoMa1, CoMa3, and CoMa6, derived from different vessels, were selected to be retained in the collection at the University of Pisa. Stock CoMa6 was chosen, at random, for further detailed studies (Fig. 3, 4).

Different stocks of *E. woodruffi*, derived from different geographic locations and maintained in the University of Pisa collection, were used in a comparative taxonomic study of the CoMa population (Table 1). In experimental infections, stock cultures C6 of *Paramecium multimicronucleatum*, Ano3 of *Euplotes eurystromus*, and the originally microsporidium-free MS-3 of *E. woodruffi* (Table 1 and Fig. 1, 2) were used as testers.

In the University of Pisa collection, original stocks of *Euplotes* are regularly maintained in duplicate at 18°C and fed separately with either micro-algae or bacteria. The freshwater micro-alga *Chlorogonium* sp. was used as the main food organism for growing *Euplotes* cultures, because they had a higher growth rate with this micro-alga than with bacterial diets, and *Chlorogonium* was able to tolerate even the low culture medium salinity of the brackish-water required by these *E. woodruffi* stocks. The basic freshwater microalgal medium SMC (0.5 μM MnCl₂, 9.0 μM FeCl₃, 1.25 mM NH₄NO₃) was used (Miyake and Beyer 1973).

The *Euplotes* stocks with the micro-alga were incubated at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a 12-h light/dark regimen for at least 10 days inside a daylight- (Osram Daylight lamp, 36W/10; Osram, Germany) and fluorescent-illuminated incubator system (Osram Fluora lamp, 40W/77, Osram) before use. In some instances, experimental procedures involved the use of bacterial food. Bacterial cultures were grown monoxenically either with 0.05% (w/v) proteose-peptone in distilled water for freshwater *Euplotes* or in salt water at 4‰ for brackish-water *Euplotes*, sterilized by autoclaving, bacterized with *Enterobacter aerogenes* (= *Klebsiella*

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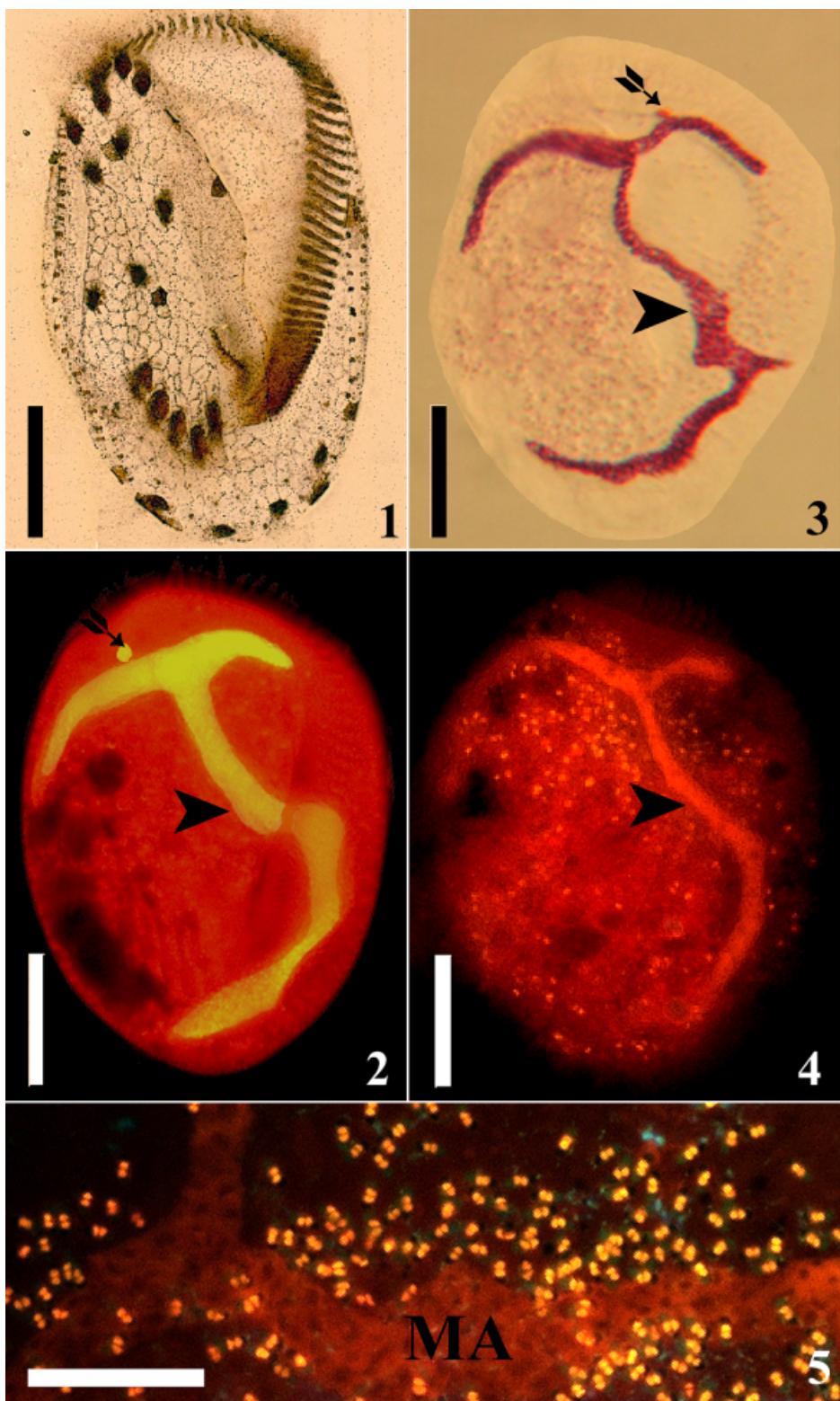


Fig. 1–5. Ventral view of *Euplates woodruffi*, uninfected and infected by the microsporidium *Euplatespora binucleata* n. gen., n. sp. 1. Silver nitrate impregnation 3. Feulgen reaction. 2, 4, 5. Fluorescence microscopy following ethidium bromide staining. 1–4. General view of uninjected (MS-3) (1, 2) and infected (CoMa6) (3, 4) specimens of *E. woodruffi*. Scale bar = 30 µm. 5. Part of an infected *E. woodruffi*. All microsporidia in the cytoplasm are binuclear spores. Arrows point to the micronucleus. Arrowheads point to the macronucleus (MA). Scale bar = 10 µm.

Table 1. Characterization of *Euplotes* and *Paramecium* stocks used in the present study.

Morphospecies	Stock	Origin	Habitat	Collector (year)	GenBank/EMBL databases Accession No. of the SSU rDNA sequence (G. Di Giuseppe & F. Dini)
<i>E. woodruffi</i>	MS-3	Yamagata Prefecture, Japan	Freshwater	T. Kosaka (1999)	EF193245
<i>E. woodruffi</i>	MY-32	Hiroshima Prefecture, Japan	Brackish-water	T. Kosaka (2001)	EF193246
<i>E. woodruffi</i>	Fane1	Corsica, France	Freshwater	F. Dini (2002)	EF193247
<i>E. woodruffi</i>	SydEu6	Newcastle, Australia	Freshwater	S. Fokin (2003)	EF193248
<i>E. woodruffi</i>	CoMa6	Comacchio, Italy	Brackish-water	M. Barbieri (2005)	EF193244
<i>E. woodruffi</i>	Sibn	Rosignano Solvay, Italy	Brackish-water	F. Dini (2005)	EF193249
<i>E. eurystomus</i>	Ano3	Pisa, Italy	Freshwater	F. Dini (2003)	EF193250
<i>P. multimicronucleatum</i>	C6	Sicilia, Italy	Freshwater	S. Fokin (2004)	Sequence not determined

(*pneumoniae*), and incubated in the dark at 37 °C for 24 h. For the *Paramecium* stock, a freshwater lettuce medium was inoculated with the same bacterium (Wichterman 1986).

Light microscopy. Investigations dealing with the microsporidian infection and morphological details of spores were carried out using an Orthoplan Leitz microscope (Leitz, Germany) equipped with differential interference contrast (DIC) microscopy, as well as a Leica DMR microscope at 300–1,250X magnification. In order to reduce cell movements and decrease the thickness of the optical field, observations were made with a compression slide device (Skovorodkin 1990). The Leica DMR microscope (Leica, Switzerland) was also used to observe native and squashed, unfixed infected cells, stained with ethidium bromide and illuminated with ultraviolet(UV) light.

Infection experiments. Experimental infection was established by feeding uninfected cells with a homogenate of infected ones, following the protocol described by Preer (1969). Briefly, the homogenate of microsporidian-harboring *E. woodruffi* cultures was concentrated by a hand-operated centrifuge and then added to microsporidian-free ciliate cultures in spent culture medium. The concentration procedure produced homogenates comprising up to 2–2.5 × 10³ microsporidian spores per milliliter, as scored by using a Bürken camera. In experiments with various uninfected representatives of *P. multimicronucleatum*, *E. eurystomus*, and *E. woodruffi*, each ciliate cell was typically infected by roughly 50–250 microsporidian spores. Experimentally infected cells were examined 12, 24, 48, and 72 h following the treatment with the spore-containing homogenate, and then at 1 and 2 wk.

Fixation, staining, and electron microscopy. The Feulgen staining procedure after fixation in Bouin's fluid was used to reveal the nuclear apparatus of *E. woodruffi* and its microsporidian symbionts using previous protocols (Fokin 1989). The microsporidian infection in living cells was revealed using ethidium bromide staining (0.005%) followed by fluorescence microscopy. Silver nitrate impregnation for identification of *Euplotes* spp. was performed after cells were fixed with Champy's fluid, according to the classical procedure (Corliss 1953).

In order to reveal the dynamics of the constitutive, endocytoblastic bacterial population in microsporidian-infected ciliate cells, an in situ hybridization method used the FITC-labelled (fluorescein) β -proteobacterial, symbiont-specific probe Poly_862 (5'-GGCTGACTTCACCGCGTTA-3') was used (Petroni et al. 2003). Briefly, cells were fixed for 2 h with 4% (w/v) formaldehyde in phosphate-buffered saline solution (PBS), pH 7.2, in a reaction vial, and then washed with PBS. Cells were incubated

with oligonucleotide probes in hybridization buffer (750 mM NaCl, 75 mM sodium citrate, 0.1% sodium dodecylsulfate, pH 7.2 without formamide) at 46 °C for 1.5 h. Then specimens were washed twice with the same buffer at 48 °C. Cells were mounted on slides with Citifluor (PLANO, Heidelberg, Germany) and probed.

Specimens were observed using a Leica DMR microscope with a planoneofluar 100 oil immersion objective (N.A.1.3). For detection of FITC, an appropriate emission filter, BP 510–525 nm, was used. Photomicrographs were taken using a telecamera (Leica DC 200) and automatically saved as TIFF files. For electron microscopy, cells were processed as described elsewhere (Fokin and Görtz 1993).

Measurements and recording. Living and fixed microsporidians were measured using computer images of 500–1,250X magnified specimens, obtained using a digital camera (Canon S45, Canon, New York) or a telecamera (Leica DC 200) and saving the selected images as either JPG or TIFF files, respectively.

DNA isolation, PCR amplification, and DNA sequencing. The DNAs of both microsporidian and *E. woodruffi* were isolated following the standard protocol of Sambrook, Fritsch, and Maniatis (1989), modified and optimized for the genomic DNA isolation from protists. Individuals of the symbiont-harboring *E. woodruffi* were pelleted by centrifugation, washed several times in distilled water, incubated in lysis buffer (0.5 M EDTA, 1% SDS, 10 mM Tris-HCl, pH 9.5) at 55 °C for 12–15 h, and finally, the DNA was extracted using phenol/chloroform treatments. The DNA content was spectrophotometrically determined with a DU 640 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The microsporidian SSU rDNA was PCR-amplified from the isolated mixture of symbiont and host DNAs using the microsporidian-specific forward primer V1 (5'-CAC CAG GTT GAT TCT GCC TGA C-3') (Franzen et al. 2005) and the R1665 reverse primer (5'-GYW RCC WTG YTA CGA CTT-3'). This latter primer was designed on the basis of conserved SSU rDNA regions of all microsporidian species so far reported in the GenBank/EMBL databases. As for the SSU rDNA of the symbiont-harboring host, *E. woodruffi*, it was PCR-amplified from the same mixture of symbiont and host DNAs, but using the universal eukaryotic 18S F9 forward primer 5'-CTG GTT GAT CCT GCC AG-3' (Medlin et al. 1988) and the 18S R1513 Hypo reverse one, 5'-TGA TCC TTC YGC AGG TTC-3' (Petroni et al. 2002). These latter primers also worked for the uninfected *E. eurystomus* tester stock. The PCR amplification was performed in a total volume of 50 µl, including 2 mM MgCl₂, 0.2 µM of each primer, 250 µM of each dNTP, one unit of Taq DNA polymerase (Polymed, Florence,

Italy), and 20 ng of DNA. Reactions were accomplished using a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA) that was programmed with an amplification profile of 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 120 s at 72 °C, followed by 5 min at 72 °C for final extension. The PCR products were purified using Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA). We chose the direct sequencing procedure for both DNA strands of the PCR products, because the reciprocal comparison of the two generated sequences allowed discrimination between PCR-introduced modifications and occurrence of an actual polymorphism. In order to obtain a reference sequence of the new microsporidian species to be used as template for the designation of internal primers for the direct sequencing procedure, the PCR-amplified product was cloned into *Escherichia coli* using the TOPO TA Cloning Kit, following the instructions of the manufacturer (Invitrogen, Carlsbad, CA). One randomly selected clone was analyzed using the vector-specific primers, M13 forward (5'-GTA AAA CGA CGG CCA G-3') and M13 reverse (5'-CAG GAA ACC AGC CCT AAT TTG GAC C-3'). On the basis of the produced sequence, two internal primers were designated: the F691 forward (5'-CGG AGC TAC ACC ACA AGG AGT GG-3') and the R838 reverse (5'-GCA TTT AAG CCC AAA GTC AAC CC-3'). One forward [F783 (5'-GAC GAA ATC AAA GAA ATA CCG TC-3')] as well as two reverse [R536 (5'-CTG GAA TTA CCG CCG GCT G-3') and R1052 (5'-AAC CTT AAG GAA CCC CCG GCC ATG GCA A-3')] internal primers (Petroni et al. 2002) were used for the direct sequencing of the *Euplates* SSU rDNA. Sequencing of both PCR products and the plasmid DNA was performed with an ABI Prism 310 Automated DNA Sequencer (Applied Biosystems, Foster City, CA) using dye terminator and Taq FS.

Phylogenetic analysis. To establish the phylogenetic relationships of the new microsporidian species, a comparative analysis of its SSU rDNA sequence to those most closely similar recorded in GenBank/EMBL databases was carried out using a BLAST search. The selected sequences were automatically aligned on a personal computer employing CLUSTAL X, version 1.81 (Thompson et al. 1997) and the default parameter settings. Finally, the sequences were edited visually using BioEdit 7.0.0 (Hall 1999). The phylogenetic analysis of the selected sequences was carried out by several different algorithms (neighbor joining, NJ; maximum likelihood, ML; and maximum parsimony, MP) using PAUP version 4.0b10 (Swofford 2003). The Modeltest 3.6 software (Posada and Crandall 1998) was employed to select the most appropriate model of substitution, warranting a reliable application of NJ and ML methods. The Tamura–Nei model (TrN; Tamura and Nei 1993) of substitution, which considers unequal base frequencies, associated with a rate variation among sites (+G), was the most appropriate ($\ln L = -4042.6597$). Settings for the TrN+G model produced these base frequencies: 0.2614 (A), 0.2025 (C), 0.3252 (G), and 0.2109 (T), and a γ distribution shape parameter (G) of 0.2734. Phylogenetic analysis was performed using heuristic search with the Tree Bisection-Reconnection (TBR) branch swapping algorithm and random addition of taxa (10 replicates). The bootstrap method (Felsenstein 1988) with 1,000 replicates was used in assessing the reliability of internal branches.

RESULTS

The reliability of the morphological identification made for the CoMa6 stock was supported by a molecular phylogenetic approach, which entailed a comparative analysis of the SSU rDNA (1,832 base pairs) of the ciliate with that of other *E. woodruffi* stocks in the Pisa collection (Table 1), whose typologically defined morphospecific status has been affirmed by different au-

thors. The SSU rDNA of the CoMa6 showed 100% sequence identity with the con-morphospecific stocks of the Pisa collection, some collected from distant geographic regions (data are not presented).

Nevertheless, it is worth mentioning that the GenBank/EMBL databases record a two-mutation difference among the SSU rDNA sequences of the above-mentioned stocks and the con-morphospecific strain XXH (Accession no. AF452710, Chen and Song, unpubl.); differences which increase up to 9–10 when the stock BHY of *E. woodruffi* is considered (Accession no. AF492707, Chen and Song, unpubl.).

Description of *Euplatespora binucleata* n. sp.

Host, prevalence and possible pathology. The host, *E. woodruffi* Gaw, 1939, is a large and rather common euryhaline morphospecies with nine fronto-ventral, five transverse and four caudal cirri. It shows a unique T- or Y-shaped macronucleus (Ma), anteriorly flanked by a single micronucleus (Curds 1975; Song and Bradbury 1997) (Fig. 1–4). The infected population of *E. woodruffi* was collected from the large brackish (4‰ salinity) marsh of Comacchio, along the western coast of the Adriatic Sea. Each of the 38 simultaneously examined ciliates of the CoMa6 stock were infected by about 25–100 microsporidian spores (Fig. 4, 5). In some specimens the infection was even higher (Fig. 6, 7). The parasites were typically distributed along the macronucleus in an area between the right arm of the nucleus and its posterior end, but they could also be found in all of the central parts of the cytoplasm (Fig. 3, 4–7). The bulk of the symbiont population was represented by mature binuclear spores (Fig. 5–8, 11, 12), with some uninucleate forms (sporonts) (Fig. 8, 13).

Infected cells did not show changes in their motility and general morphology. No significant differences in cellular fission rate or feeding activity were recorded when representatives of microsporidian-infected (CoMa6) and uninfected (MS-3) stocks were compared. Cytological observations with fluorescent microscopy on ethidium bromide- and FISH-stained cells showed that microsporidian-infected cells also harbored a number of long, rod-like bacteria (Fig. 8, 12), randomly distributed in the cytoplasm, very often close to microsporidians. Their *Polynucleobacter* nature was confirmed by FISH-specific staining (data not presented). No competition was apparent between bacteria and microsporidians in the cytoplasm of *E. woodruffi*.

Life-cycle stage morphology. Clear merogonial stages were never observed in the original and naturally infected ciliate cultures, but they were present in experimentally infected cells. In the latter, at 24 h, the majority of the parasite's population had a nucleus about 1.0–1.1 μm in diam., but a minority had nuclei about 0.6 μm in diam. (Fig. 20). The latter was a merogonial stage, which might have been completed by 24 h after infection. The majority of the parasites after 24 h had similar cytoplasmic structures and similar thickness of their plasma membranes (Fig. 23). Some of them, judging by both the cytoplasmic structure (i.e. fairly dense, containing numerous free ribosomes, and ER cisternae) and large nucleus could be sporonts, whereas the others with more homogeneous cytoplasm and smaller nuclei could be meronts (Fig. 20, 23). Very few cells were binuclear, possibly sporoblasts (Fig. 24). Sporoblasts were found in the native CoMa6 stock (Fig. 18) and became rather common between 48 and 72 h after experimental infection (Fig. 22, 25–27). These cells were irregularly shaped, about 1.5–2.0 \times 2.5–4.5 μm in size, with two nuclei about 0.8–0.9 μm in diam., and a less thick cell wall (Fig. 16, 18, 24–26). These ripened into spores without further division (Fig. 18, 19, 26, 27). This suggests an alternation of the uninucleate and binucleate stages in the life cycle of the microsporidium infecting stock CoMa6 of *E. woodruffi*, as well as during the experimental infection of the con-morphospecific stock MS-3.

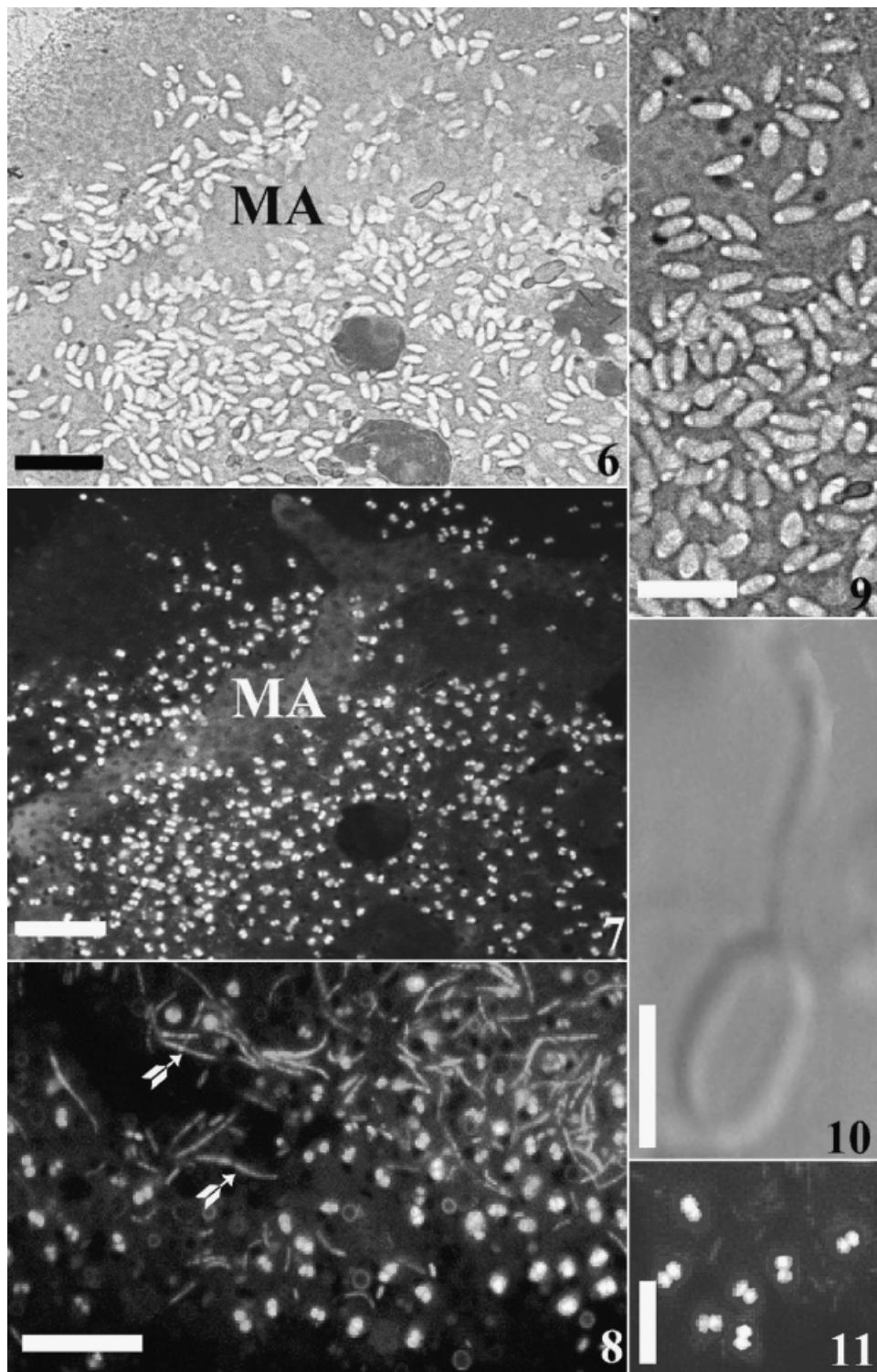


Fig. 6–11. Parasite distribution in the native infected ciliate, *Euplotes woodruffi* and some peculiarities of spores of *Euplotespora binucleata* n. gen., n. sp. **6, 9, 10.** Differential interference contrast light microscopy. **7, 8, 11.** Fluorescence light microscopy following ethidium bromide staining. **6.** Numerous spores are located along the macronucleus (MA) of the host ciliate. Scale bar = 10 µm. **7.** The same host cell treated with ethidium bromide. All spores are binucleate (diplokarya). Scale bar = 10 µm. **8.** Higher magnification of binucleate microsporidian spores, some uninucleate sporonts and interspersed endocytobiotic bacteria (arrows) comprising the genus *Polynucleobacter*. Scale bar = 6 µm. **9.** Mature spores with the large posterior vacule. Scale bar = 6 µm. **10.** A spore with extruded polar tube. Scale bar = 2 µm. **11.** Mature binucleate spores treated with ethidium bromide. Scale bar = 3 µm.

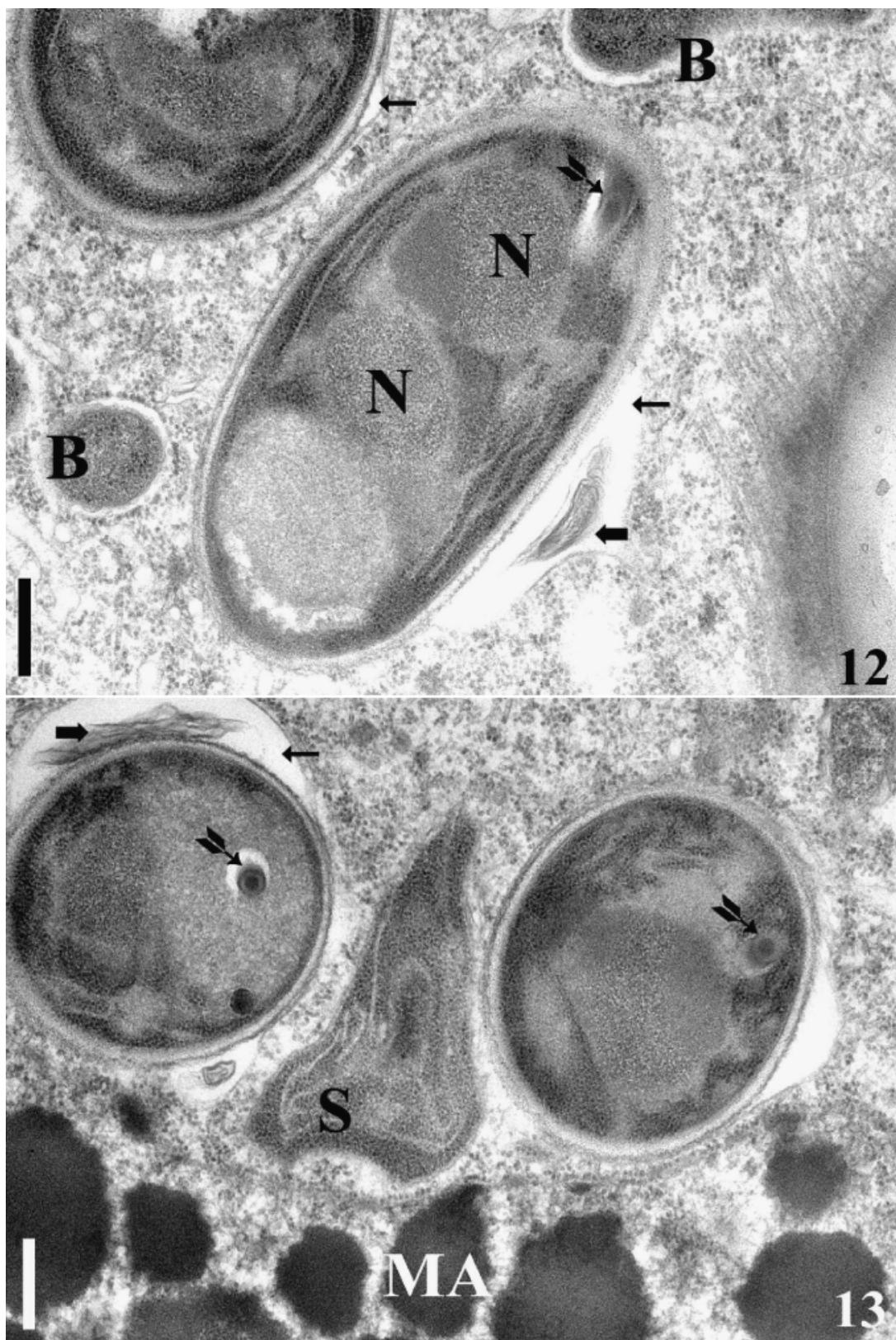


Fig. 12,13. Transmission electron micrographs of mature spores and sporont of *Euplotespora binucleata* n. gen., n. sp. 12. Longitudinal section of a binucleate (N) spore and interspersed bacteria (B). 13. Cross section of spores showing a polar tube (arrows) and a uninucleate sporont (S) adjacent to the host ciliate macronucleus (MA). Fine arrows—sporophorous vesicles; thick arrows—sporophorous vesicle inclusions. Scale bar = 0.5 μ m.

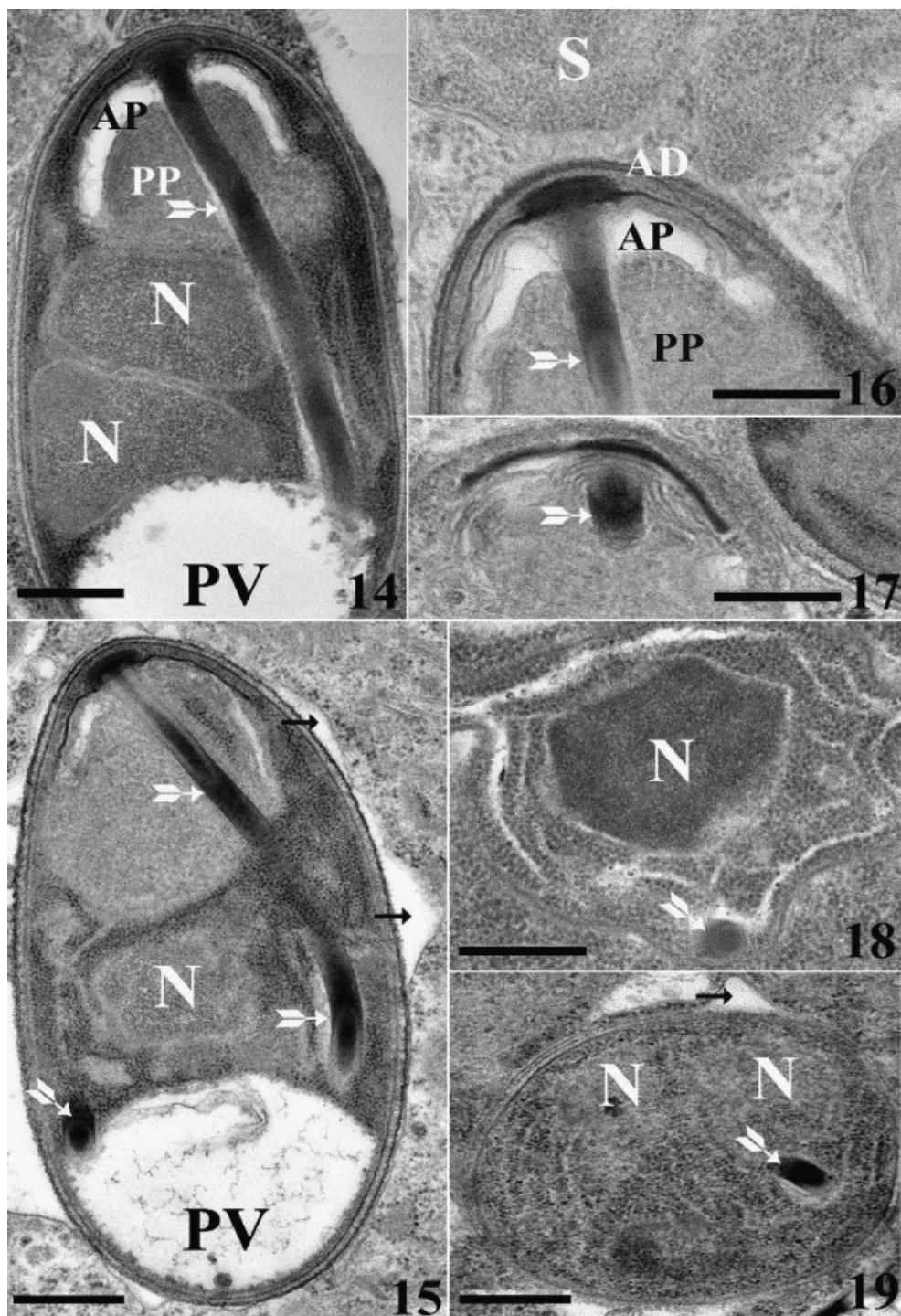


Fig. 14–19. Transmission electron microscopy of *Euplotespora binucleata* n. gen., n. sp. from the native infected stock CoMa6 of the ciliate *Euplates woodruffi*, showing salient features of the life-cycle stages. **14.** A mature binucleate (N) spore with an uncoiled polar tube (arrow), a bipartite polaroplast, and large posterior vacuole (PV). **15.** View of a mature, posteriorly vacuolated (PV) spore showing the uncoiled polar tube (arrows) attached to the apically located anchoring disc. **16.** A typical anchoring disc (AD) located in the apical part of a spore; a sporont (S) is visible near by. Two different parts of polaroplast: anterior-lamellar (AP) and posterior-homogeneous (PP) are visible. Empty space in the anterior part of polaroplast is a result of the cutting procedure: lamellae were partly destroyed. **17.** Stemming point of polar tube (arrow) and the anterior polaroplast region; a few lamellae of the polaroplast are visible just beneath the anchoring disc. **18.** Sporoblast and its uncoiled polar tube (arrow). Only one nucleus (N) is visible. **19.** Immature binucleate spore with the polar filament. Scale bar = 0.6 μ m.

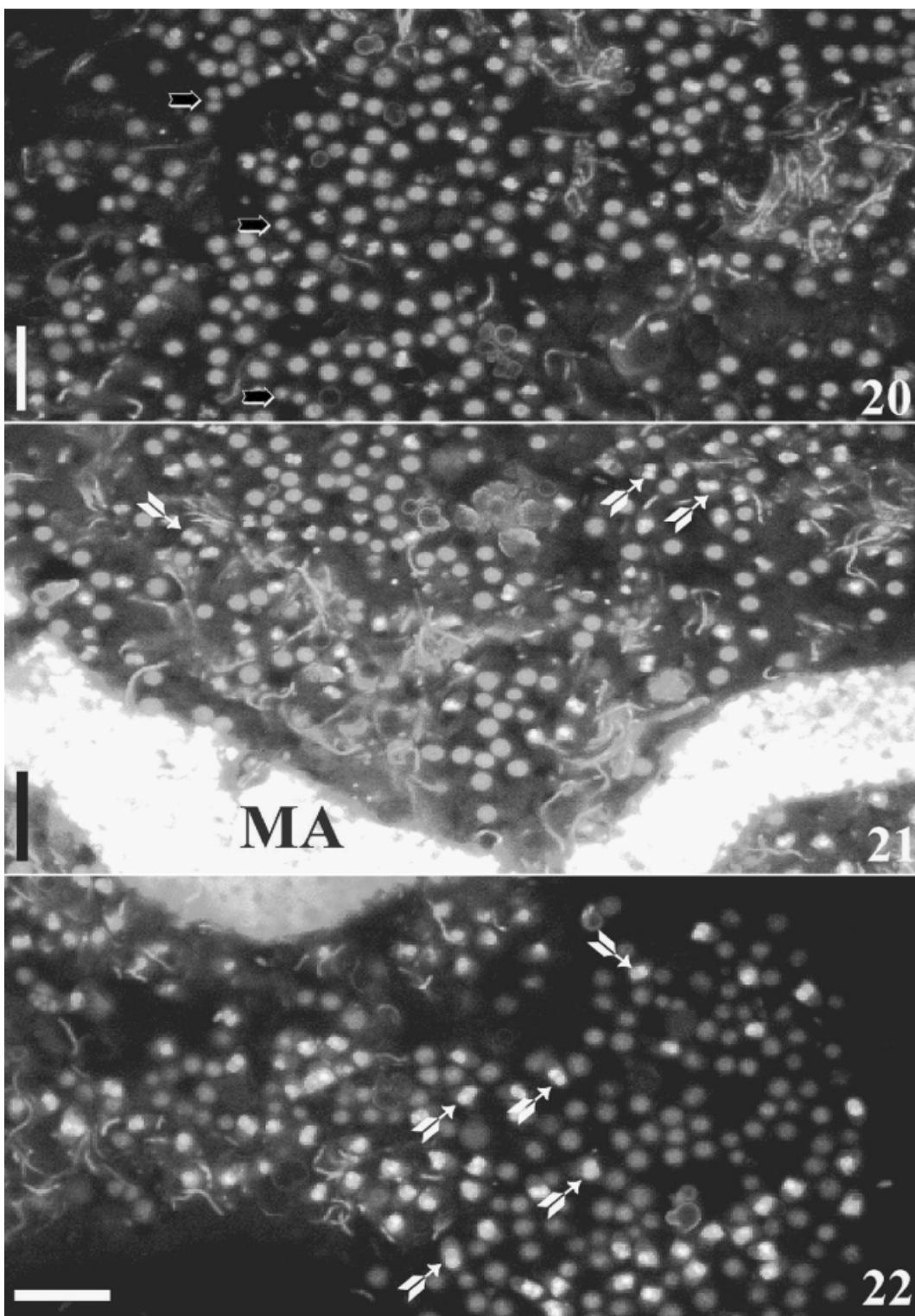


Fig. 20–22. Photomicrographs of the developing experimental infection of *Euplatespora binucleata* n. gen., n. sp. in the ciliate *Euplates woodruffi*, stock MS-3 using fluorescence light microscopy following ethidium bromide staining. 20. Uninucleate meronts and sporonts in the host cytoplasm, 24 h after infection; Crashed host cell. Meronts with small nuclei indicated by arrows. 21. Uninucleate sporonts and binucleate (arrows) sporoblasts (spores?), 48 h after infection, occurring in a cytoplasmic area in the region of the host macronucleus (MA) and *Polynucleobacter* endocytobacteria. 22. Some uninucleate sporonts and binucleate sporoblasts together with binucleate spores, appearing alongside double particles (arrows), 72 h after infection. Scale bar = 4 μ m.

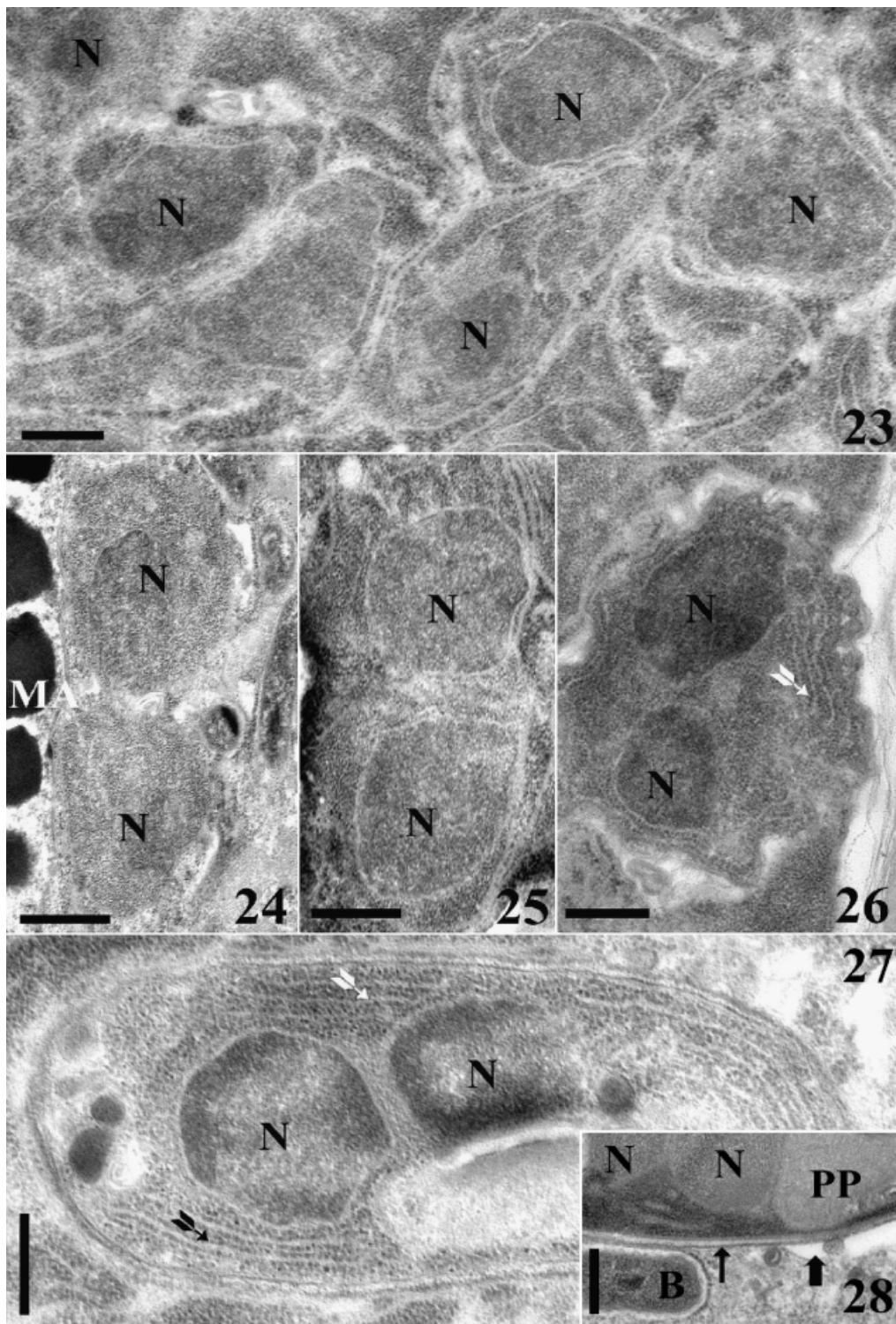


Fig. 23–28. Ultrastructure of developing life-cycle stages of *Euplotespora binucleata* n. gen., n. sp., following the experimental infection of the constitutively, microsporidian-free ciliate *Euplotes woodruffi*, stock MS-3. **23.** Population of uninuclear meronts (small nucleus—N) and sporonts (large N), 24 h after the start of the infection. **24.** Dividing nucleus of sporont in the proximity of the host ciliate's macronucleus (M), 24 h after the start of the infection. **25.** Binucleate sporoblast, 24 h after the infection's start. **26.** Binucleate sporoblast, 48 h after the infection's start; arrow points to the rough endoplasmic reticulum in this as in the next figure. **27.** Immature spore, 72 h after the start of the infection. Scale bar = 0.6 μ m. **28.** Part of a mature spore with the surrounding host cytoplasm. B—bacterium; PP—posterior part of polaroplast; fine arrow—exospore layer of the spore wall; thick arrow—a sporophorous vesicle. Scale bar = 0.5 μ m.

Table 2. List of microsporidia produced from the BLAST search analysis.

Species	GenBank/EMBL Accession No.	BLAST search total score	Reference
<i>Cystosporogenes</i> sp. CRV-2004	AY566237	1364	van Frankenhuyzen et al. (2004)
<i>Cystosporogenes legeri</i>	AY233131	1364	Kleespies et al. (2003)
<i>Vittaforma corneae</i>	L39112	1265	Baker et al. (1995)
<i>Endoreticulatus schubergi</i>	L39109	1158	Baker et al. (1995)
<i>Endoreticulatus</i> sp. CHW-2004 Bulgaria	AY502945	1110	Wang et al. (2005)
<i>Endoreticulatus</i> sp. CHW-2004 Taiwan	AY502944	1102	Wang et al. (2005)
<i>Orthosomella operophterae</i>	AJ302316	808	Unpublished

Ovoid–cylindrical mature spores, which never produced definite groups, were monomorphic, ranging from 3.2×1.4 – $3.7 \times 1.9 \mu\text{m}$ (fresh spore, mean $3.44 \pm 0.17 \times 1.65 \pm 0.22 \mu\text{m}$; Fig. 6, 9, 14, 15). The polaroplast is divided into two regions: the anterior part has a few densely packed lamellae located just beneath the anchoring disc; the posterior part is a rounded body (sack), about one-quarter of the spore length, lacking ribosomes and any special infrastructure. Insertion of the isofilar polar tube (polar filament) into the anchoring disc mimicked the situation occurring in the majority of microsporidians; the manubrial region was slightly wider than the rest of the tube (Fig. 14–17). The quite short (i.e. 4.5–5.5 μm), uncoiled (or half-coiled) polar tubes had a typical structure (Fig. 10, 15). At the posterior end of spores, a large vacuole was observed by both bright field microscopy and electron microscopy (Fig. 9, 12, 14, 15). The mature spore showed a three-layered cell wall, 0.08 μm thick: the plasma membrane plus endospore and exospore coats, separated from the host cell by a uniform sporophorous vacuole closely associated with the exospore layer (Fig. 12–16, 28). Quite often some membranous material (metabolic products?) was present in sporophorous vacuoles (Fig. 12, 13, 28).

Sequence availability and phylogenetic analysis. The PCR amplification products of the SSU rDNA of the new microsporidian species produced a 1.15-kb DNA band. Sequencing of this genomic region provided a 1,146-bp DNA sequence with a GC content of 41.1%. A BLAST search of GenBank/EMBL databases using the SSU rDNA sequence of *E. binucleata* n. sp. produced a series of microsporidian SSU rDNA sequences, ranked by sequence identity (Table 2). In order to avoid the problems associated with the sequence errors in the unpublished sequences accessible through GenBank, we included only sequences published in peer-reviewed journals (i.e. sequences with proven identification). Among these, species of the genus *Cystosporogenes* and *Vittaforma corneum* showed the highest total scores, corresponding to 84.6% and 84.1% sequence identity, respectively. The selected sequences were aligned with that of *E. binucleata* and analyzed. The microsporidian species, *Orthosomella operophterae*, was used as an outgroup to polarize the inferred phylogenetic trees. This taxon was chosen because it has the lowest homology among the selected full-length sequences and greater positional reliability in published phylogenetic trees involving the taxa identified by the BLAST search (Kleespies et al. 2003; Van Frankenhuyzen et al. 2004).

All methods applied revealed the same general tree topology, clustering *E. binucleata* n. sp. together with species representative of the genus *Cystosporogenes*, as well as with those of the genus *Vittaforma* (Fig. 33). In all of the trees, the bootstrap values supporting the upper branch of this clade were high: NJ (94%), MP (97%), and ML (98%); substantial reliability thus exists for this taxonomic grouping. Two topologies were observed for relationships inside this monophyletic group: one topology, supported by bootstrap values related to the NJ (82%) and MP (51%) algorithms, showed *E. binucleata* as a sister taxon of the *Cystosporogenes* spp. (Fig. 33A), whereas the alternative topology showed *E. binucleata* grouped as a sister taxon of both *Cystosporogenes* and *Vittaforma* genera (Fig. 33B).

Microsporidian biodiversity in ciliophora. Nearly 2,000 samples were collected from many locations throughout Europe, Northern America and Asia, mainly in Russia, Germany, Italy, and Japan (SF., unpubl. data). More than 40 relatively common ciliate morphospecies from these areas were repeatedly checked for the occurrence of any endocytobiosis, preferably of prokaryotic nature. In addition, 62 ciliates were checked for endocytobiosis just once. Notwithstanding such an extensive survey, only four instances of microsporidian infection were disclosed: twice in *Frontonia leucas* (Russia, St. Petersburg district, and Japan, Yamaguchi district) and once in *Vorticella* sp. (Japan, Yamaguchi district), as well as in *Sonderia vorax* (Italy, Pisa district). Although these ciliate-infecting microsporidians have not been investigated thoroughly, they appear quite different from each other and are therefore likely to represent separate genera within the phylum Microsporidia (Fig. 29–32).

DISCUSSION

Together, features such as host specificity and habitat, general and developmental morphology, and phylogeny render us confident in conferring to *E. binucleata* the status of new species of a new genus in the Microsporidia phylum. Some microsporidians (e.g. *Ciliatosporidium platyophryae*) show a short uncoiled polar tube inside spores (Foissner and Foissner 1995) similar to *E. binucleata* n. sp. Other taxa (e.g. *Rectispora* or *Coccospora*; Canning and Vavra 2000) share with *E. binucleata* n. sp. a rather small lamellar anterior part of the polaroplast; however, the posterior part of the structure differs in these species. All together, these characters associated with a binucleate (diplokaryotic) spore state but uninucleate meronts in *E. binucleata* n. sp. represent a novelty in Microsporidia.

The life cycle of *E. binucleata* is unique, consisting of an unusual succession of exclusive states, such as unikaryotic presporal stages but diplokaryotic sporoblasts and spores. To our knowledge, such sequence shifting from uni- to binucleate stages has never been recorded in Microsporidia (Canning and Vavra 2000; Larsson 1999). On the contrary, the succession from diplokaryotic sporonts to unikaryotic spores or stable unikaryotic or diplokaryotic stages for both merogony and sporogony are the common patterns (Issi and Voronin 2007; Larsson 1986, 1999).

The life cycle of *E. binucleata* n. sp. is somewhat reminiscent of that exhibited by *Amblyospora* spp. (Canning and Vavra 2000). However, species in *Amblyospora* genus have two hosts—mosquitos and copepods. In this genus alternation from unikaryotic meronts to diplokaryotic sporonts occurs via small unikaryotic gametes but not by means of karyogamy within sporonts as *Eupletespora* manifests.

It is known that in different hosts, the life cycle of parasites can be different, for example, with and without merogony (Avery and Undeen 1990). At least for 20%–25% of microsporidium genera, merogony simply has not yet been found (Canning and Vavra 2000; Issi and Voronin 2007). However, in some of those genera

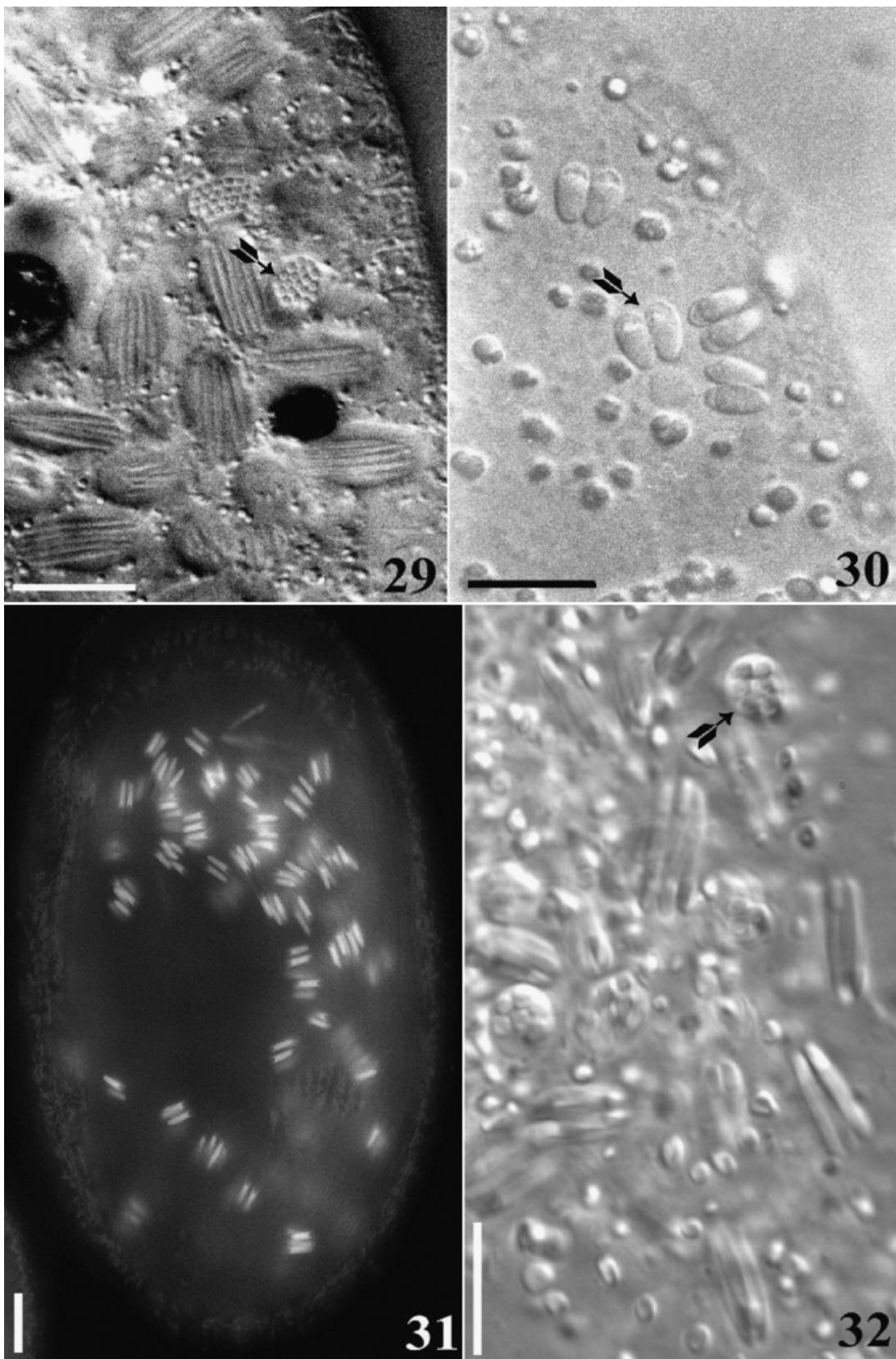


Fig. 29–32. Newly found microsporidian infections in ciliates. 29, 30, 32. Differential interference contrast of living cells. 31. Fluorescence microscopy. 29. Microsporidia in the cytoplasm of *Frontonia leucas*. Scale bar = 15 μm . 30. Microsporidia in *Vorticella* sp. 31, 32. Microsporidia in *Sonderia vorax*; arrow point to the number of spores in one sporophorous vesicle. Scale bar = 10 μm .

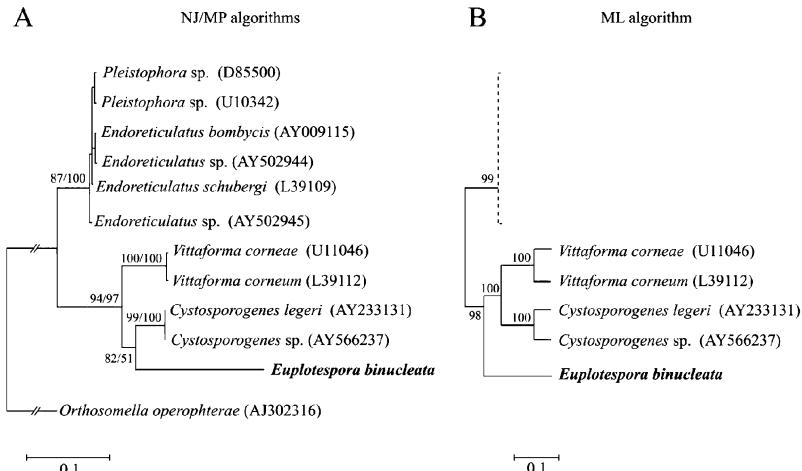


Fig. 33. Phylogenetic trees based on the small subunit (SSU) rDNA sequence of eight species of microsporidians. Both neighbor joining (NJ) and maximum parsimony (MP) methods produced the same tree, as shown on the left (A). On the right (B) is shown the alternative tree topology produced by the maximum likelihood (ML) algorithm. The numbers at the nodes represent the bootstrap percentages from 1,000 replicates (values below 50% not shown). *Orthosomella operophterae* was used as the outgroup taxon. The scale bars correspond to a distance of 10 substitutions per 100 nucleotide positions.

the reason could be connected with incomplete investigation, especially because in field-collected material reproduction often has proceeded beyond merogony (Larsson 1999).

It was rather difficult for us to determine whether *Euplotespora* has merogony or whether this stage is absent in the life cycle. We did not find any presporal cells of *Euplotespora*, defined as having less dense material on the surface than existed in definitive sporogonial stages. However, in our material, even mature spores had an exospore layer of almost the same thickness as presporal cells. We presume that a more distinctive difference between meronts and sporonts in our material could be the size of their nuclei. The better feature for definition of the sporoblastic stage in *Euplotespora* populations is, probably, its binucleate state.

Two of the specialists in this field who reviewed our experimental material (Prof. R. Larsson, Dr. Y. Sokolova, pers. commun.) have found some of features of meronts in it, but one anonymous reviewer did not concur. In our naturally infected material from stock CoMa6, meronts probably were absent. It seems that the difference between surface structure of merogonial and sporogonial stages in some cases is not as large as is usually indicated in the literature (Larsson 1999). For instance, Foissner and Foissner (1995) indicated sporonts as the earliest presporal stages, which they have found for *Ciliostoridium*, but Canning and Vavra (2000) indicated merogony for this species based on some figures in the Foissners' paper.

The results of our phylogenetic analysis, regardless of the reconstruction method pursued, provided strong support for establishing quite a close phylogenetic relationship of *E. binucleata* with representatives of the microsporidian genera *Vittaforma* and *Cystosporogenes*, alternatives as to the definition of the sister taxon relationships notwithstanding. The relatedness of these last two genera had been previously suggested by Kleespies et al. (2003) and Van Frankenhuyzen et al. (2004), applying molecular methods. The phylogenetic clustering *Cystosporogenes*—*Vittaforma*—*Euplotespora*, including the sister taxon relationships, are supported by other cladistic approaches (Canning et al. 2002; Fries et al. 1999). However, according to morpho-biological features (Canning et al. 1985; Kleespies et al. 2003; Silveira and Canning 1995) these genera are located in different subclasses of the class Microsporea (Issi and Voronin 2007).

Vittaforma, comprising the synonymous *Nosema cormeum* as a type species, infects *Homo sapiens* and has Primates as a type host (Silveira and Canning 1995). This microsporidium is monomorphic and diplokaryotic throughout all its life cycle. Merogony is accomplished through binary fission of diplokaryotic forms. Spores are $3.7 \times 1.0 \mu\text{m}$ in size and cylindrical in shape; the polaroplast is composed of tightly packed lamellae and the polar tube has five–seven coils. Sporogony produces eight diplokarya (Table 3). The degree of diversity in such general and developmental morphological features quite slightly supports the possibility that *Euplotespora* may be a relative to *Vittaforma* (Table 3).

On the other hand, *Cystosporogenes* infects some butterflies (type host, *Operophtera brumata*, Lepidoptera) (Canning et al. 1985; Kleespies et al. 2003). It is monomorphic and monokaryotic at the spore stage. Merogony produces small plasmodia with several isolated nuclei, and multinucleated plasmodia are still generated following sporogony. Spores are uninucleate; the polaroplast has never been described for the type species, but in *C. legeri* remarkable binary lamellar polaroplast was mentioned (Kleespies et al. 2003). The polar tube shows 10–11 coils. Various numbers of ellipsoid-shaped spores were often observed in multiples of 8 (8, 16, and 32) and were grouped in sporophorous vesicles (Table 3).

Thus, meronts are bi- or polynucleate in genera *Vittaforma* and *Cystosporogenes* but uninucleate in *Euplotespora*. Representatives of *Vittaforma* and *Cystosporogenes* have long (not $<20 \mu\text{m}$) coiled polar tube but *Euplotespora* has an uncoiled short one (about $5 \mu\text{m}$ only). At the same time *Vittaforma* and *Euplotespora* have diplokaryotic spores which are never packeted in groups but *Cystosporogenes* has uninucleate spores in groups of 4–60 (Table 3). The organization of the polaroplast is also different among the three genera, especially in the case of *Euplotespora* and *Cystosporogenes*. It means differences in general and developmental morphological features between *Cystosporogenes* and *Euplotespora* appear even larger than those occurring between the latter and *Vittaforma*. However, *Cystosporogenes* and *Vittaforma*, in their turn, are not similar to each other at the morpho-biological level either (Table 3; Canning and Vavra 2000; Issi and Voronin 2007).

It is well-known that trees constructed according to sequences of the 16S rRNA do not fit dramatically to taxonomical systems of

Table 3. Comparative characteristics of microsporidia belonging to the genera *Vittaforma*, *Cystosporogenes*, and *Euplotespora*.

Features	Microsporidian genus		
	<i>Vittaforma</i>	<i>Cystosporogenes</i>	<i>Euplotespora</i>
Host organisms	Primates	Insecta	Ciliophora
Number of nuclei in meront	2 or 2 × 2	1–3	1
Number of nuclei in sporont	2 × 4–8	1	2
Number of nuclei in spore	2	1	2
Size of spore in μm	3.7 × 1.0	2.5 × 1.2	3.4 × 1.6
Spores in distinct groups	—	+	—
Number of spores per group	—	4–16 ^a	—
Polar tube type	Isofilar, coiled	Isofilar, coiled	Isofilar, uncoiled
Number of polar tube coils	5–7	10–11	0
Construction of the polaroplast	Uniform lamellar ^b	Uniform lamellar ^c	Bipartite lamellar/sack

^aAccording to the review (Larsson 1999), in the original description of the genus (Canning et al. 1985), the number of spores per group indicated as four to about 60.

^bIn the original description (Silveira and Canning 1995), different parts of the polaroplast were not mentioned and could not be seen in figures, but in the review (Canning and Vavra 2000) posterior lamellae in group of four were indicated.

^cThe polaroplast was not described for the type species, but has been presented (Canning and Vavra 2000) as a uniform lamellar one according to only original picture (Canning et al. 1985). For the related species *C. legeri* the remarkable binary polaroplast was described, but it is not similar with those in *Euplotespora* (Kleespies et al. 2003).

the phylum Microsporidia, which are based on morphology (Issi and Voronin 2007). From a morphological point of view, the authors refrain so far from suggesting a placement of *Euplotespora* into any of the six families of the order Dissociodiaphlophasea.

Microsporidian taxonomy suffers gaps in comparing molecular neotypes of type species or of correctly identified species, which have not often been collected from the same geographic area of the type material. Consequently, inferring phylogenetic relationships of *E. binucleata*, we referred to the most reliable literature records of gene sequences produced by microsporidian species, which met the above-mentioned taxonomic attributes as strictly as possible. Living *E. woodruffi* strains harboring the *E. binucleata* type species and their frozen DNA will be available indefinitely.

Phylum Microsporidia Balbiani, 1882

Class Microsporea Corliss & Levine, 1963

Subclass Dihaplophasea Sprague, Becnel & Hazard, 1992

Order Dissociodiaphlophasea Sprague, Becnel & Hazard, 1992

Euplotespora n. gen. taxon inserta sedis within the order

Diagnosis. Monotypic genus. Only member monomorphic. Occurrence of life cycle changes characterized by variation in number of nuclei, from unikaryotic status in meronts and sporonts to a diplokaryotic condition in sporoblasts, and then in immature and mature spores. Meiosis has not been detected. Sporonts develop individually into sporoblasts and then into ovoid–cylindrical spores. The polar tube is isofilar and short, and it does not produce even one complete coil. The polaroplast is divided into two regions: the anterior part is relatively small with a few lamellae; and the posterior part is a rounded body (sack) with homogeneous contents, about one-quarter of the spore

length. Large posterior vacuole always presented in the mature spores.

Euplotespora binucleata n. sp.

Etymology. The genus name refers to finding this species in ciliates of the genus *Euplotes*.

Euplotespora binucleata n. sp.

Diagnosis. With characters of the genus as modified below.

Meronts. Few examples of this stage were found, and only in experimentally infected cells. They have an irregularly roundish shape, about 1.5–2.0 μm in diam. with a nucleus about 0.6 μm in diam. This stage appears to be short, and is usually terminated 24 h after infection. The difference between surface structure of meront and sporont in the species is not very distinctive.

Sporonts. These are also irregularly roundish in shape, about 1.5–2.0 μm in diam., with one large nucleus (1.0–1.1 μm). At a certain stage of the life cycle (between 24 and 48 h after infection), the uniform uninuclear sporont population starts becoming heteromorphic with the appearance of diplokaryotic sporoblasts about 1.5–2.0 × 2.5–4.5 μm in size, which developed directly into spores.

Spores. These are ovoid–cylindrical, monomorphic, 3.44 ± 0.17 × 1.65 ± 0.22 μm (fresh spores), binucleated (diplokaryotic) throughout the life stage, with a large posterior vacuole and a three-layered wall about 0.08 μm in thickness. The polar filament (tube) is uniform in thickness (isofilar) and short, 4.5–5.5 μm in the ejected status (fresh spores, under pressure), and apparently lacking a distinctive coiled region. The polaroplast is divided into two regions. The anterior part is relatively small with a few lamellae close to the anchoring disc; the posterior has a rounded body (sack), about one-quarter of the spore length. Insertion of the polar tube into the anchoring disc is organized as in the majority of Microsporidia. Spores do not appear to cluster together as a group, and an individual sporophorous vesicle closely encircles the spore's surface.

Location in the host. There is a general intracytoplasmic distribution, with some preference toward the endoplasmic region, alongside the macronucleus.

Type host. Stock CoMa6 of *E. woodruffi*, Ciliophora, Spirotrichaea. Constitutively microsporidium-free *E. woodruffi*, as well as *E. eurystomus*, could be experimentally infected, but the latter one lost microsporidians within a short time span or died.

Prevalence. Natural infection rate is 100%, based on 38 examined specimens.

Type material. Permanent Feulgen staining preparations (slide No 10) and Epon-embedded material from electron microscopic investigation, registered in the collection of the Museo di Storia Naturale e del Territorio dell'Università di Pisa, Calci (PI), Italy. The living CoMa6 stock of symbiont-harboring *E. woodruffi* was deposited at the Culture Collection of Algae and Protozoa (<http://www.ccap.ac.uk/index.htm>) under the CCAP number 1624/24. Frozen DNAs of both microsporidian and *Euplotes* representatives are available at the Dipartimento di Biologia of the Università di Pisa.

Molecular sequence. The SSU rDNA sequence of the new microsporidian species, named *Euplotespora binucleata*, is available from the GenBank/EMBL databases under Accession no. DQ675604.

Etymology. The species name refers to a characteristic trait of the microsporidian spore.

As stressed above in the Results section, the up-to-date experience amassed with ciliate screening for endocytobiosis would seem to bolster the low frequency of infection mediated by microsporidians in Ciliophora. The question may be raised as to the sampling density of ciliate biodiversity. Parsimonious assessments report the occurrence of 8,000–9,000 species of extant, free-living ciliates (Lynn and Corliss 1991). Other calculations

raise this number up to 20,000–30,000 species (Fokin 2004, 2007). Nevertheless, it is worth noticing that the modern ciliatology records one new microsporidian infection more or less every 13 yr (Foissner and Foissner 1995; Götz 1987; Krüger 1956). The four new microsporidian species described since 1999 actually enhance the recording rate, but only slightly alter the notion of a scanty occurrence of microsporidian–ciliate symbioses. This state of affairs changes drastically when invertebrates are considered: various new microsporidian species are described each year in this taxon. In a comparative view, there are no apparent large differences in terms of specific ecological niches and global geographical distribution between ciliates and insects; the latter are likely the most “preferred” hosts of microsporidians.

Still, the well-documented cases of microsporidiosis in ciliates suggest no biological constraint on the establishment of such infections. Moreover, hundreds of prokaryotic endocytobionts can occupy almost all cellular compartments of ciliated protists, some bacteria being much larger than the majority of microsporidians (Fokin 2004). Hence, the relatively low frequency of microsporidian infections in Ciliophora is a vexing problem. Auxiliary mechanisms with peculiar principles of operation could be involved: for example, the establishment of peculiar cytoplasmatic environmental conditions in the ciliate could upset the balance between the prokaryotic and microsporidian endocytobionts, leading to the loss of the latter. The loss of bacteria might be much more critical insofar as it is a well-established notion that ciliate survival and/or fitness often depends upon the benefits provided by the prokaryotic endocytobionts.

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