

## Light and Electron Microscopic Cytology of *Trichotuzetia guttata* gen. et sp. n. (Microspora, Tuzetiidae), a Microsporidian Parasite of *Cyclops vicinus* ULJANIN, 1875 (Crustacea, Copepoda)

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**Summary:** The microsporidium *Trichotuzetia guttata* gen. et sp. n., a parasite of the copepod *Cyclops vicinus* in the Czech Republic, is described based on light microscopic and ultrastructural characteristics. All life cycle stages have isolated nuclei. In the merogonial reproduction multinucleate plasmodia divide by plasmotomy, yielding uninucleate merozoites. Sporonts develop into multinucleate plasmodia, which split in a rosette-like manner, initially into wide lobes with large nuclei, finally into narrow lobes with small nuclei. Sporoblasts are formed in individual sporophorous vesicles generated by the sporogonial plasmodium. Immature sporogonial stages are covered by fibrillar projections uniting the exospore with the envelope of the sporophorous vesicle. Mature spores, which are pyriform with pointed anterior end, normally lack projections. Unfixed spores measure 4.6–5.7×2.7–3.3 µm. The exospore is four-layered. The anterior lamellae of the polaroplast are wide. The posterior ones (present in a short zone) are narrow close to the filament, wider at the periphery. The polar filament is isofilar with 9–10, 102–131 nm wide, coils in a single layer close to the spore wall in posterior half of the spore. The angle of tilt is 60–65°. Isometric, 21–25 nm wide virus-like particles were observed in the nuclei of mature spores. Experimental transmission of the microsporidium per os has failed. The small subunit rRNA gene has been sequenced and the data have been used in a phylogenetic analysis (PAUP). The species is compared to previously described microsporidia of copepods, and the taxonomy is discussed.

**Key Words:** *Trichotuzetia guttata* gen. et sp. n.; *Cyclops vicinus*; Ultrastructure; Molecular phylogeny; Taxonomy; Microsporidia; Copepoda.

### Introduction

Copepods (Crustacea, Copepoda) harbour a rich microsporidian fauna (SPRAGUE 1977; VORONIN 1986). Moreover, recently some microsporidia parasitizing copepods have been known as intermediate hosts of some microsporidia the definitive hosts of which are mosquitoes (ANDREADIS 1985; SWEENEY et al. 1985). Thus the study of new and undescribed microsporidia of copepods not only increases our knowledge of microsporidian distribution and taxonomy, but also

contributes to our understanding of microsporidian life cycles. The present paper treats a new species of microsporidia which occurs frequently in *Cyclops vicinus*, an abundant copepod in ponds rich in organic nutrients, in the Czech Republic. The light and electron microscopic cytology and the life cycle are described, the taxonomic position, the molecular phylogeny, the possibly polymorphic life cycle, and the arguments for establishing a new genus are discussed.

## Material and Methods

Infected specimens of *Cyclops vicinus* ULJANIN, 1875 (Crustacea, Copepoda, Cyclopidae) were collected in several small and medium sized carp ponds in the Czech Republic. A small pond (around 500 m<sup>2</sup>) in the village of Biskoupy, near Zbiroh (County Rokycany) was selected as the type locality, because of the regular presence of this microsporidium in the copepod host.

Standard techniques for microsporidia were applied to the material (VÁVRA & MADDOX 1976). Infected copepods were smeared and the dry smears were stained using Giemsa's stain with or without hydrolysis in 1 N hydrochloric acid. The outline of the microsporidia was made more visible in smears by rapid treatment of the smear with a solution of nigrosine [the Giemsa-Burri ink method of VÁVRA (1978)]. Spores immobilized on an agar layer (VÁVRA 1964) were measured using A.E.I. Vickers Image Splitting Eyepiece.

For transmission electron microscopy copepods were fixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.3) or with 0.2 M sodium cacodylate buffer (pH 7.2). They were then washed in buffer, post fixed for 1 hr in 2% (w/v) osmium tetroxide in the same buffer as was used for the glutaraldehyde fixation, dehydrated and embedded in Spurr's low viscosity resin or in epon. Ultrathin sections were stained with uranyl acetate and lead citrate (REYNOLDS 1963).

For scanning electron microscopy a spore suspension was flooded on circular cover glasses, air dried lightly and fixed in 2.5% glutaraldehyde in cacodylate buffer at 4 °C. After washing in buffer and critical point drying, smears were covered with gold and palladium.

Infection experiments were performed by adding suspensions of spores, isolated from copepods, to copepodite and adult stages of the same host and from the same habitat. Fresh spores or spores stored in pond water in a refrigerator for various periods of time were used.

For establishing the molecular phylogeny, DNA was sequenced and amplified according to VOSSBRINCK et al. (1993) and BAKER et al. (1995). The primers 530f, 1047R, 1061F and 1492R (BAKER et al. 1995) were used to sequence the 3' two-thirds of the small subunit ribosomal RNA gene. Sequences were aligned visually and phylogenetic analyses were performed on a Macintosh Performa 6214CD using PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1 (SWOFFORD 1993). Only those portions of the sequence which could be unambiguously aligned (approximately 690 base pairs) were used in this study. Trees were found using the heuristic option employing random addition of taxa with 5 replicates and the tree bisection reconnection (TBR) algorithm. Spores of *Hazardia* sp. were supplied by Dr. A. H. Undeen, Gainesville, U.S.A.

## Abbreviations

A	anchoring disc
E	endospore
ER	endoplasmic reticulum
EX	exospore
F	polar filament
G	Golgi apparatus
N	nucleus
P	plasma membrane
PA	anterior polaroplast
PP	posterior polaroplast
PR	polyribosomes
PS	polar sac
S	sporophorous vesicle
SC	scindosome
V	posterior vacuole

## Results

### Prevalence and pathogenicity

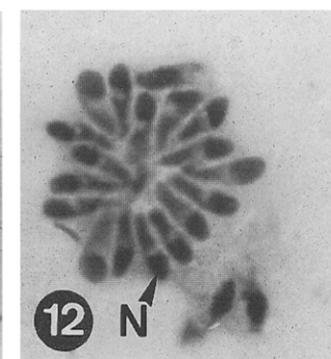
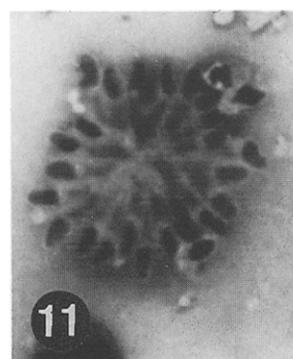
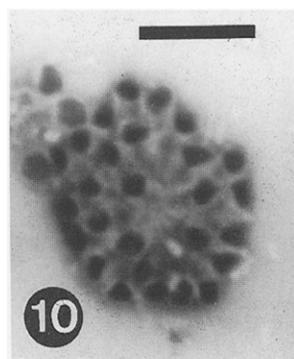
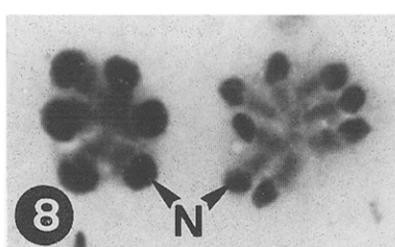
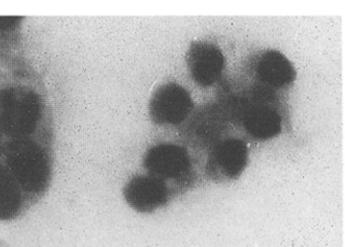
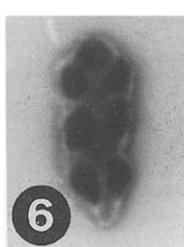
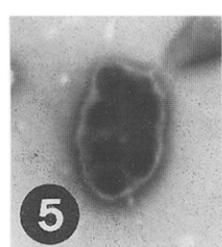
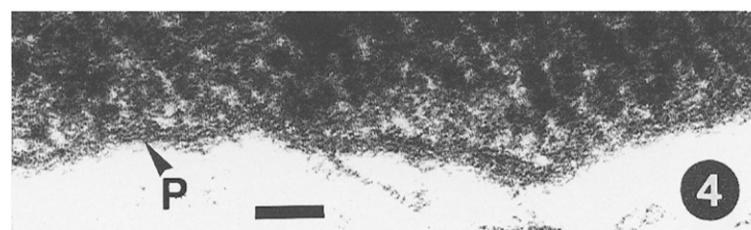
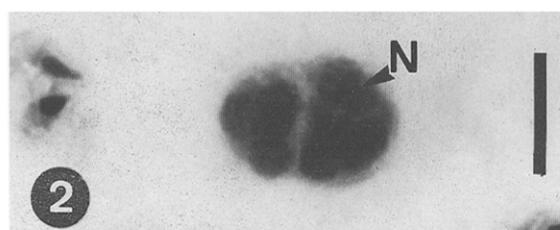
The microsporidian was found specifically in the last copepodite stage or in adults of both sexes of *Cyclops vicinus*. Even if infected hosts were present in the ponds all over the year, massive infections, involving up to 80% of the population, occurred only for about two weeks during the late spring or early summer (end of April, May, or early June in different years). This seasonality of infection was very regular from year to year during five years of observation.

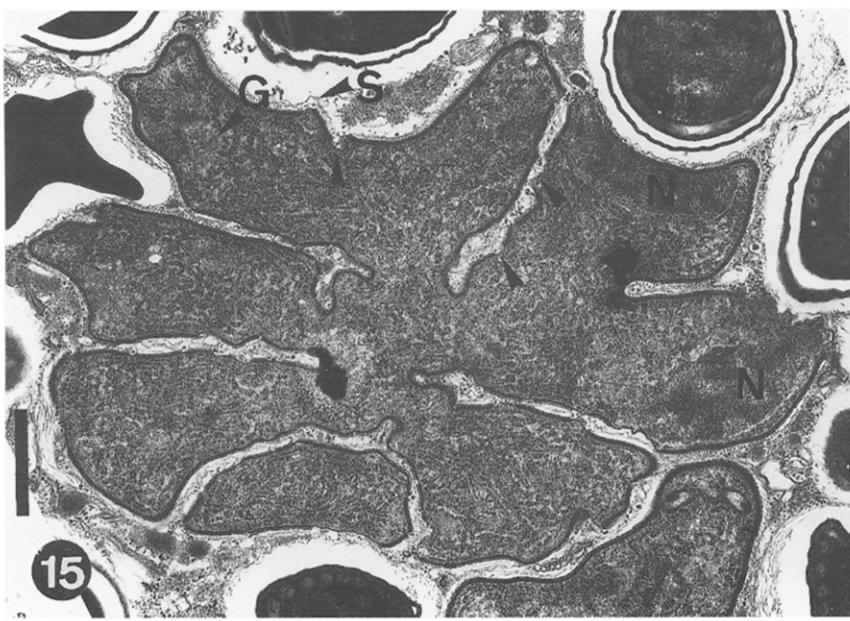
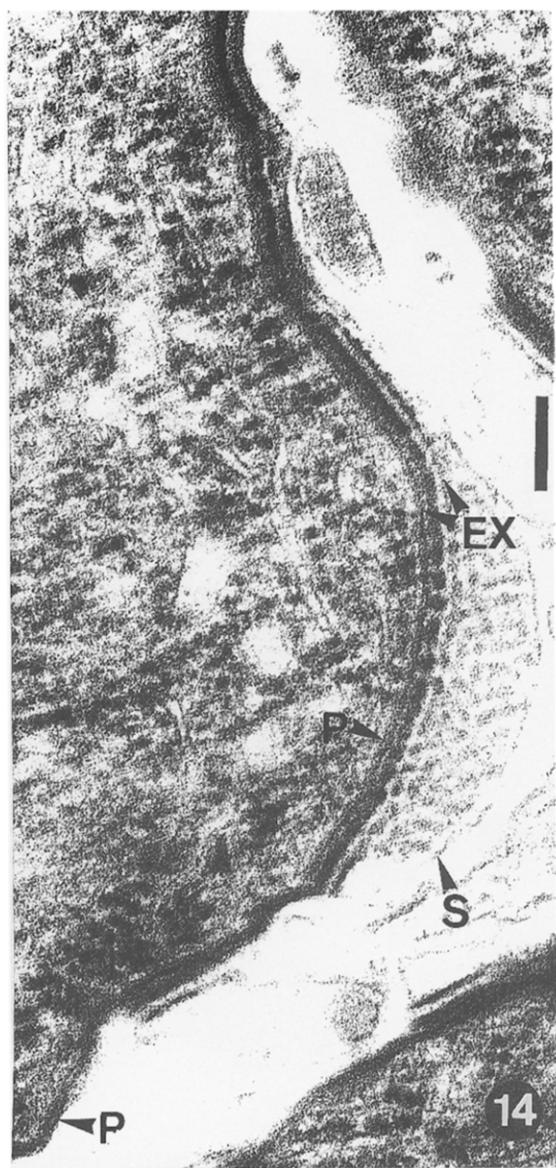
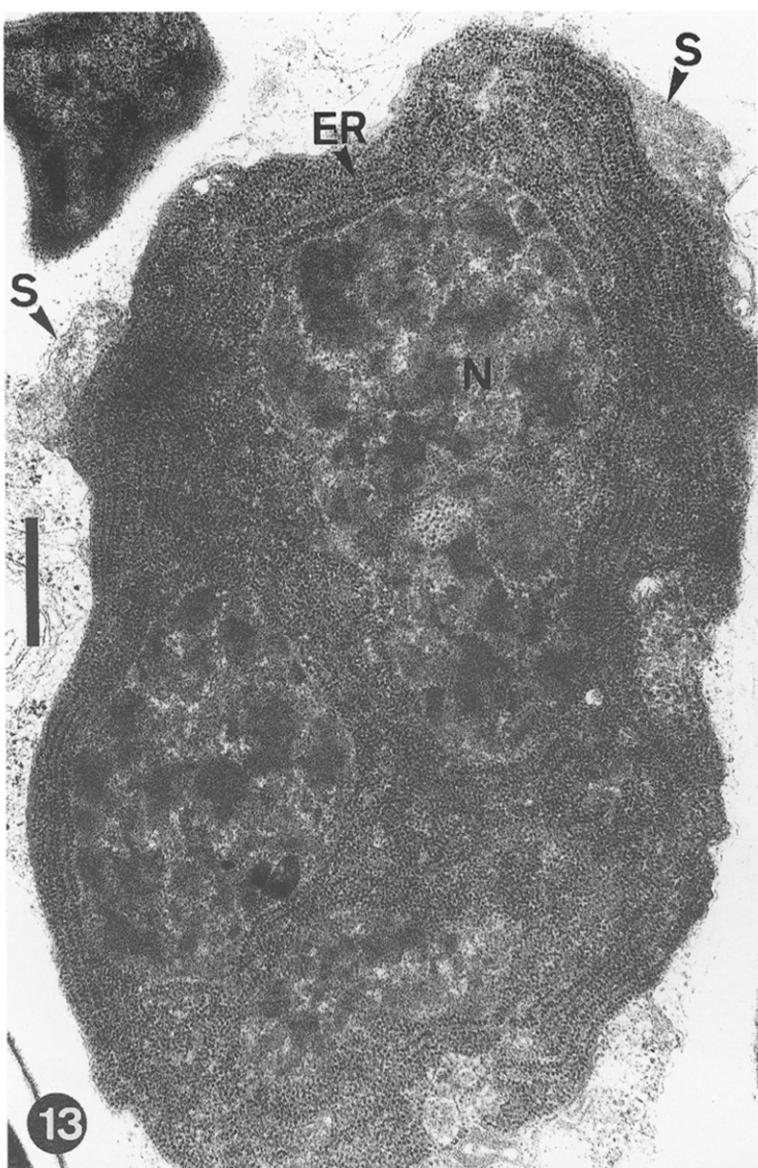
Oocytes were the primary site of infection in females, testes in males. From these sites the infection spread to involve practically all tissues, except for the gut epithelium and the musculature (Fig. 1). Such hosts were conspicuously white caused by total reflection of light from the spore loaded tissues.

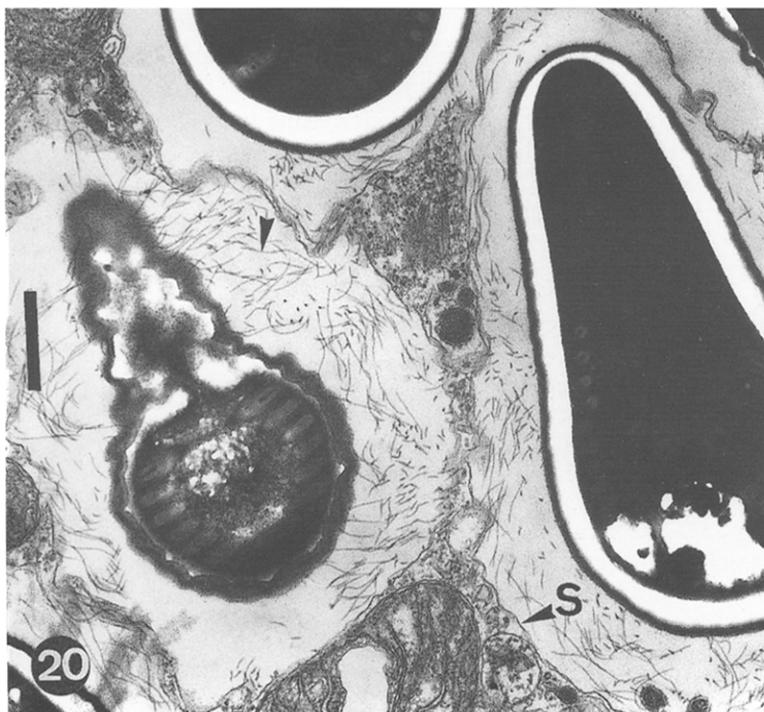
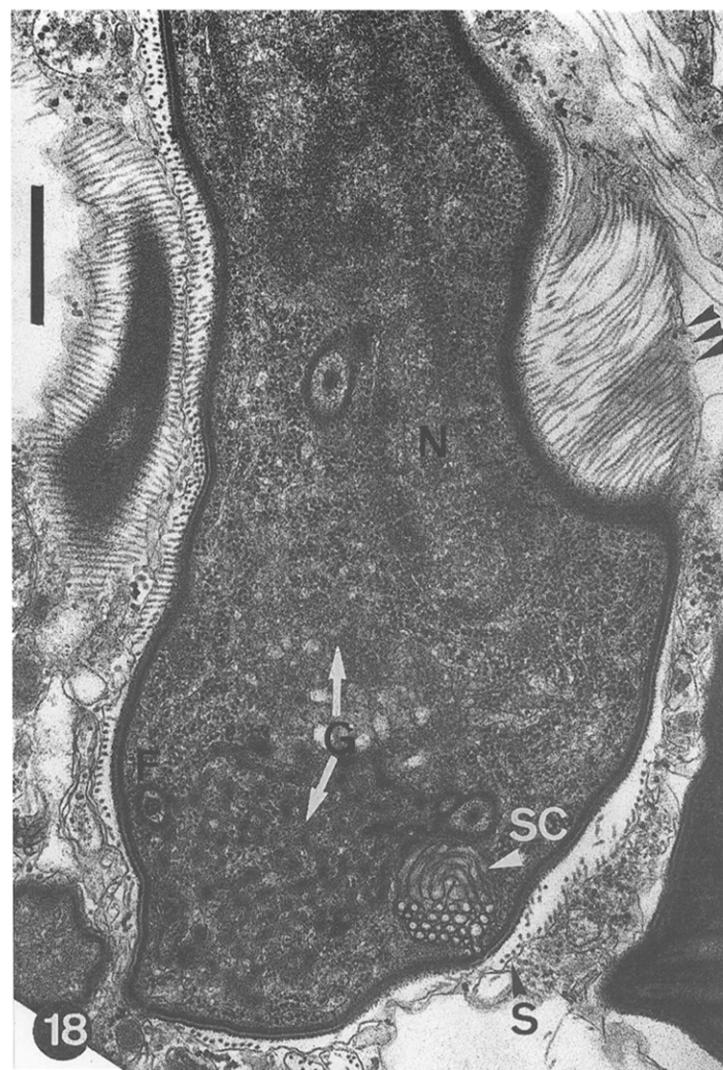
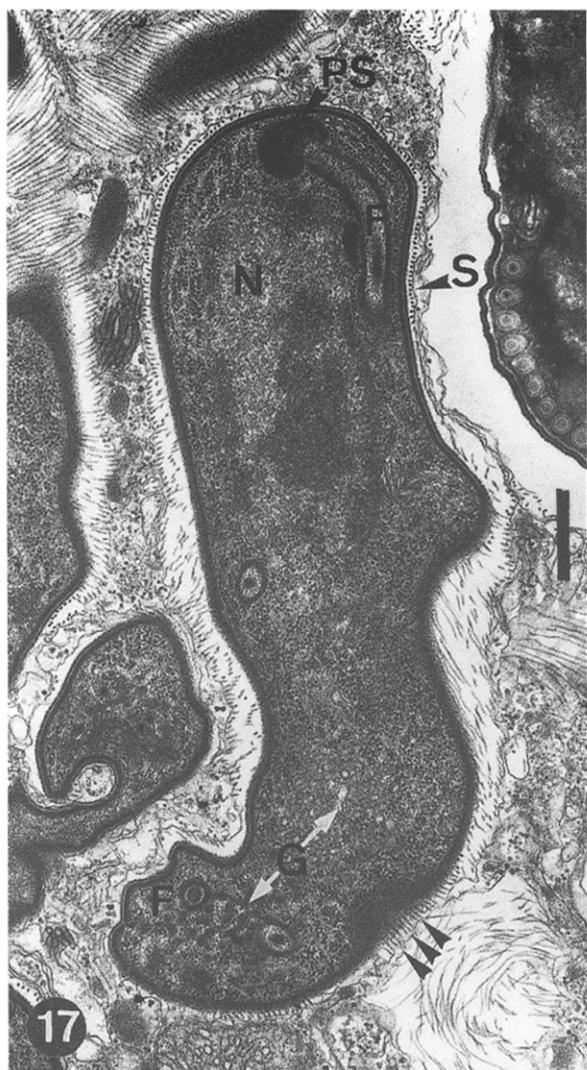
### Presporal stages and life cycle

All life cycle stages from the merogony to mature spores had isolated nuclei. Stages belonging to the merogonic part of the reproduction were rarely found. The earliest stages seen were more or less oval merogonial plasmodia, measuring approximately 15×10 µm, with several (usually 8–10, sometimes more) nuclei (Fig. 2), belonging to the last, or possibly only, sequence of merogony. Their cytoplasm was rich in free

**Figs. 1–12.** Pathology and early development of *Trichotuzetia guttata* n. sp. **1.** Semithin section of an adult specimen of *Cyclops vicinus* showing that all tissues except for the musculature are filled with spores (arrowheads). **2–3.** Light and electron microscopic aspects of a dividing merogonial plasmodium. **4.** The cell wall of the merogonial plasmodium is a plasma membrane. **5–6.** Multinucleate plasmodia early in the sporogonial sequence. **7–10.** Later the plasmodium splits into a rosette-like formation with uninucleate lobes. The earliest lobed plasmodia have a moderate number of wide lobes with large nuclei (7, 8, 9). A second division yields an increased number of more narrow lobes, with reduced nuclear dimensions (8, 10). **11.** Nearly mature rosette. **12.** Mature rosette releasing sporoblasts. Fig. 1. Toluidine and methylene blue. Figs. 2, 5–12. Giemsa staining. Scale bars: 1 = 50 µm, 2, 5–12 (with common bar on 10) = 10 µm, 3 = 1 µm, 4 = 50 nm.







ribosomes, but there were practically no traces of an endoplasmic reticulum (Fig. 3). Nuclei were prominently granular. The widest sectioned nuclei measured 2.8 µm in diameter. The merogonial plasmodia were delimited by an approximately 8 nm thick plasma membrane, a unit membrane lacking external reinforcements (Fig. 4). The plasmodia divided by plasmotomy (Figs. 2–3), finally yielding uninucleate merozoites, which matured to sporonts.

In light microscopic preparations the earliest sporogonial stages observed were plasmodia with a small number of nuclei (Figs. 5–6). Cytoplasmic fission followed the nuclear division, yielding rosette-like lobed plasmodia where the nucleus of each lobe was localized to the periphery (Figs. 7–11). Meiosis was not observed in the sporogony. Several rounds of divisions occurred, which were evident from the decreasing size of the nuclei and the increasing number of lobes in the formations (Figs. 8, 10–12). Thus 17–20 µm large rosettes with 20–30 lobes were formed (Fig. 11). The lobes became progressively longer, and finally they were released as individual cells, the sporoblasts (Fig. 12). The nucleus, in the anterior part of the cell, condensed and a clear zone appeared, separating the nucleus from the posterior part of the cell in which the Golgi apparatus is located (see below) (Fig. 12).

In ultrathin sections sporogonial stages were discriminated by the membrane-bound ribosomes, organized as a prominent rough endoplasmic reticulum in concentric layers around the nuclei (Fig. 13). Sectioned nuclei measured up to 1.4 µm in diameter. The nucleoplasm was granular and identical to nuclei of the merogonial reproduction.

While the plasmodium still had a small number of nuclei, moderately electron-dense material appeared spot-wise on the plasma membrane. The newly formed layer was about 4 nm thick. With increasing production of material the layer lost contact with the plasma membrane, forming wide protrusions, which were the pri-

mordia of the sporophorous vesicle (Figs. 13–14). Their cavity, the episporontal space, was initially filled with a spongy material (Fig. 13), which later broke up into c. 15 nm wide, fibrous components, arranged in a regular labyrinth-like way (Fig. 14). Inside the vesicle primordia new secretions of electron-dense material accumulated on the plasma membrane, generating the primordium of the exospore (Fig. 14). Initially the material had the same fibrous character as the material of the episporontal space, but it was more electron-dense (Fig. 14). It is remarkable that even deeply lobed plasmodia had surface areas where the exospore layer was not continuous and complete (Fig. 15). In the lobed plasmodium the cytoplasm was slightly modified. It was still granular with rough endoplasmic reticulum, but the regular arrangement of the components of the reticulum disappeared (Fig. 15). An aggregation of vesicles of the type interpreted as a Golgi apparatus was present in each lobe close to the nucleus, in the region of the lobe directed towards the centre of the rosette (Fig. 15).

The fibril-like primordial exospore developed into a distinctly layered structure, 42–47 nm thick (Fig. 16). Externally to the plasma membrane were two lucent, approximately 10 nm wide layers, separated by a layer of approximately the same thickness resembling a double membrane. The surface was covered by a slightly more narrow layer of electron-dense material, which was continuous with the fibrous material of the episporontal space.

The transition to spores seems to be extremely rapid as no stages between the sporoblasts in star-like formations (Fig. 12) and mature spores were observed using light microscopy. Ultrathin sections revealed that the sporophorous vesicle divided together with the plasmodium enclosing each sporoblast lobe separately (Fig. 15), and when the daughter cells were released, each sporoblast (Fig. 16) occupied a vesicle of its own. A dense layer of regularly arranged fibrils, about 15 nm

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**Figs. 13–16.** Sporogony of *T. guttata*. **13.** Sporogonial plasmodium with initiation of the sporophorous vesicle. **14.** Detail of the plasmodium wall with one vesicle primordium with regularly arranged fibrous material in the episporontal space and with initiation of the surface layer of the exospore inside the primordium. **15.** Rosette-like lobed sporogonial plasmodium with almost complete exospore-layer; arrowheads indicate positions where the exospore still is lacking. **16.** Detail of the plasmodium wall with completely developed exospore layer. Arrowheads indicate the layer resembling a double-membrane. Scale bars: 13 = 0.5 µm, 14 = 100 nm, 15 = 1 µm, 16 = 50 nm.

**Figs. 17–20.** Sporoblasts and spores of *T. guttata*. **17.** Sporoblast or immature spore showing the initiation of the polar filament and the regularly arranged fibrous material of the episporontal space (arrowheads). The Golgi apparatus consists of two structurally different areas. **18.** The posterior end of an immature spore exhibiting the two regions of the Golgi apparatus, a scindosome communicating with the cell wall, and the regularly arranged fibrils of the episporontal space, continuous between the exospore and the envelope of the sporophorous vesicle (arrowheads). **19.** Fibrils of the episporontal space at greater magnification. Arrowheads indicate a regular organization of the substructure. **20.** The fibrous material (arrowhead) is reduced at the maturation of the spores. Scale bars: 17–18, 20 = 0.5 µm, 19 = 100 nm.

apart, connected the exospore of the sporoblast and immature spore stages with the wall of the sporophorous vesicle (Figs. 17–18). The approximately 10 nm thick fibrils appeared to be divided into compartments by more electron-dense transverse strands, 8–10 nm apart (Fig. 19). When the sporoblasts matured to spores the episporontal space widened, and the traversing fibrils lost their regular arrangement and successively disappeared (Fig. 20).

The morphogenesis of the spore equalled the normal for microsporidia. The nucleus was initially close to the anterior pole (Fig. 17). At the opposite pole the polar filament was generated by the Golgi apparatus (Fig. 17–18), and the developing filament was pushed in anterior direction when the length increased. Simultaneously a polar sac originated at the anterior pole of the filament, and when the filament reached the anterior end of the spore, it carried a polar sac filled with electron-dense material (Fig. 17). The Golgi apparatus of sporoblasts and immature spores had two distinct regions, an anterior region of the same vesicular structure as the primordial Golgi apparatus, which was initiated close to the nucleus of each sporoblast lobe (Figs. 15, 17–18), and a posterior spongious region with more electron-dense structure from which the polar filament was generated (Figs. 17–18). Sporoblasts often exhibited scindosome-like structures in the Golgi region (Fig. 18). The cell wall remained unchanged from the time the layered exospore was complete in the sporogonial plasmodium (Figs. 15–16) to the point of maturation when the polar filament of the immature spore had reached the anterior pole (Fig. 17).

### The mature spores

Usually the visibly infected copepods contained only spores. The average spore load was 6–9×10<sup>5</sup> spores per copepod. Living spores were shortly pyriform with

pointed anterior pole and with a small, eccentrically located posterior vacuole (Fig. 21). Fixed and stained spores appeared more blunt (Fig. 22). Spores fixed for SEM retained the shape of living spores (Fig. 23). The average dimensions of unfixed spores was 5.0×2.9 μm (4.6–5.7×2.7–3.3 μm; n = 50). Fixed and stained spores measured 4.2×2.2 μm (3.7–5.0×2.0–2.8 μm; n = 40). Mature spores, when stained following acid hydrolysis, exhibited one small (1.3×0.7 μm) nucleus (Fig. 22). No mucous layer was observed around spores mixed with India ink.

The wall of the mature spore measured 170–195 nm, except anteriorly where it was considerably thinner, down to 70 nm (Figs. 20, 24). It had the three subdivisions characteristic for the microsporidian spore wall, with a lucent endospore layer of variable thickness inserted between the plasma membrane and the exospore (Fig. 25). The exospore measured about 30 nm. Its three external components (the dense surface layer, a translucent zone, and the double membrane-like layer) remained unchanged from the primordial state, but the internal less dense layer was reduced to a narrow granular zone between the double-layer and the endospore (Figs. 16, 25).

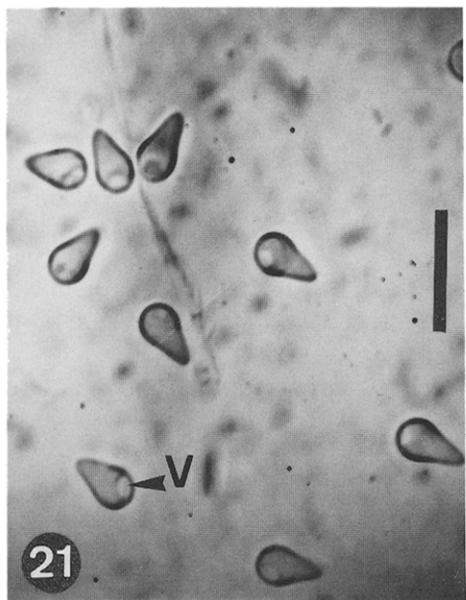
The polar filament was anteriorly connected to a biconvex anchoring disc of stratified more or less electron-dense material (Fig. 24). The widest sectioned disc measured 298 nm in diameter. The uncoiled part of the filament proceeded straight backwards in the centre of the spore for about 1/5 of the spore length, then turned to the side, and the posterior part was arranged as 9–10 coils, in a single layer of coils close to the spore wall, in the posterior half of the spore. The last coil touched the posterior pole. The angle of tilt of the most anterior coil to the long axis of the spore was 60–65°. The filament coils were approximately uniformly thick (isofilar filament), measuring 102–131 nm diameter.

The filament was composed of layered material, and 7 subdivisions could be discriminated, in direction

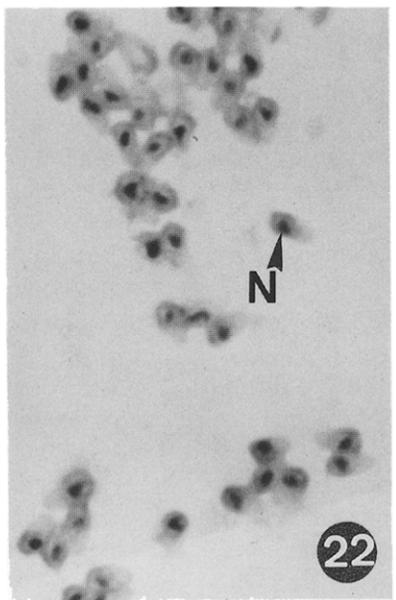
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**Figs. 21–25.** The mature spore of *T. guttata*. **21.** Fresh, unstained mature spores. **22.** Mature spores stained using Giemsa solution. **23.** SEM picture of a mature spore; no traces of fibrils are visible. **24.** Longitudinal ultrathin section of a mature spore revealing the characteristic organelles; the episporontal space is devoid of fibrils. Arrowhead indicates closely packed lamellae, arrows anastomoses. **25.** Detail of the spore wall of a mature spore; arrowhead indicates the double-layer of the exospore. Scale bars: 21–22 (with common bar on 21) = 10 μm, 23–24 = 0.5 μm, 25 = 100 nm.

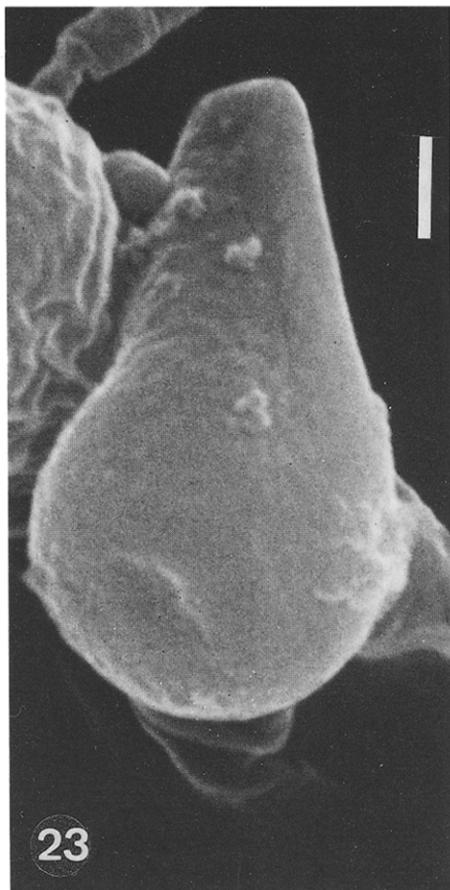
**Figs. 26–30.** Ultrastructural details of mature spores. **26.** Anterior end exhibiting the anterior polaroplast region and the anchoring apparatus. Black arrowheads indicate the regularly spaced transverse fibrous components of the polar filament, white arrowhead an anastomosis between lamellae of the polaroplast, arrows the double-membrane-like layer of the exospore. **27.** Posterior region of the polaroplast; arrowheads indicate the closely packed lamellae. **28.** The layers (numbered 1–7) of the transversely sectioned polar filament; black arrowheads indicate differences in the central part; white arrowheads the fibres of layer 6. **29.** Intranuclear isometric virus-like particles (arrow). **30.** Teratological macrospore. Scale bars: 26–27, 29 = 100 nm, 28 = 50 nm, 30 = 1 μm.



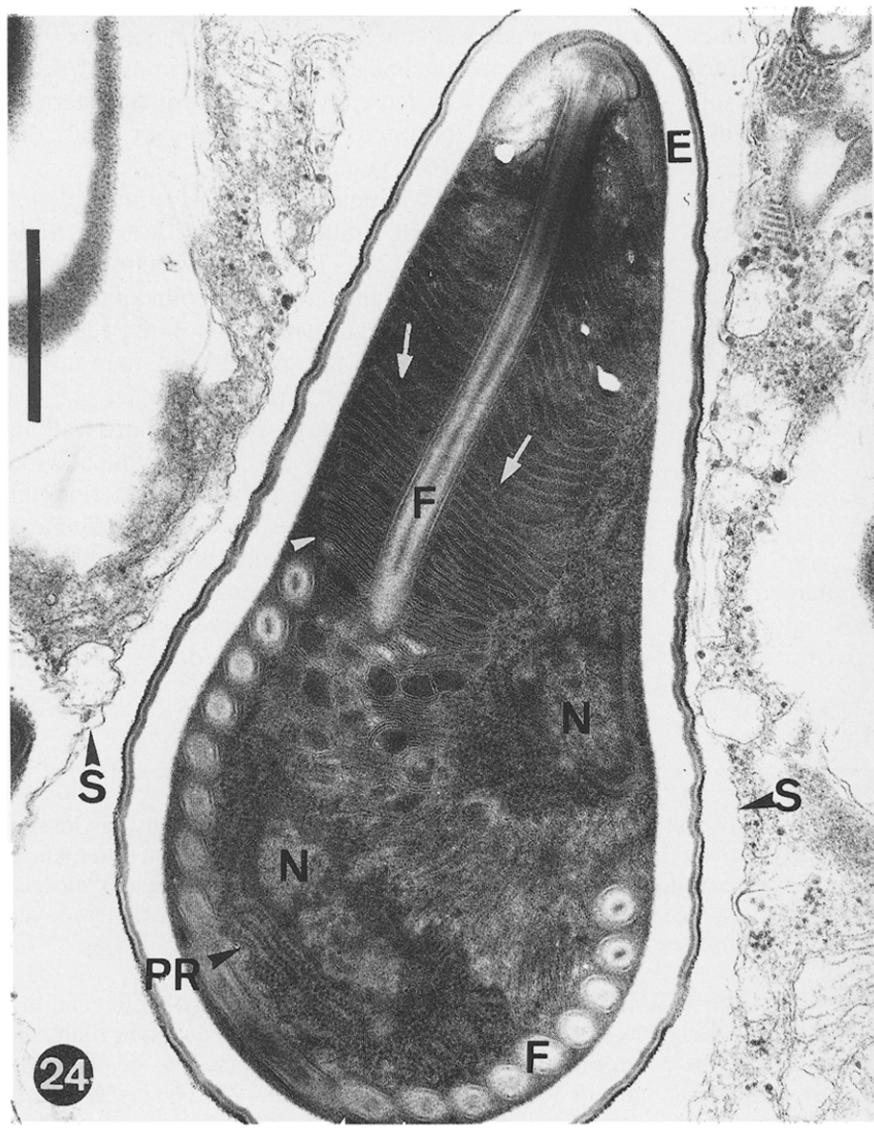
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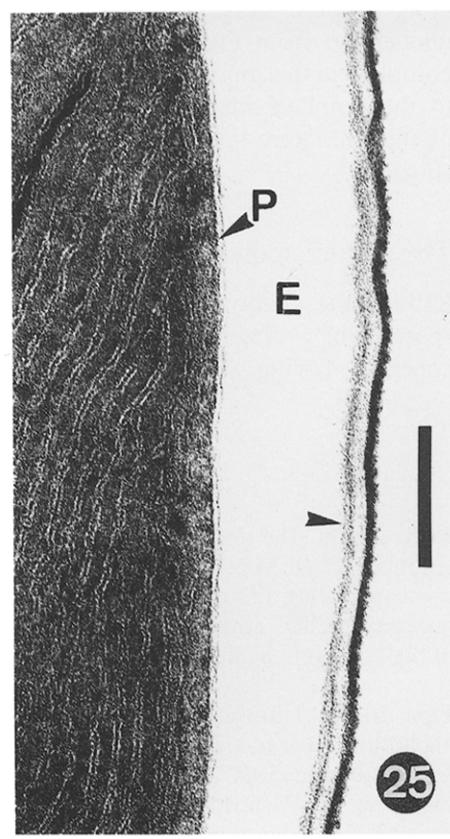
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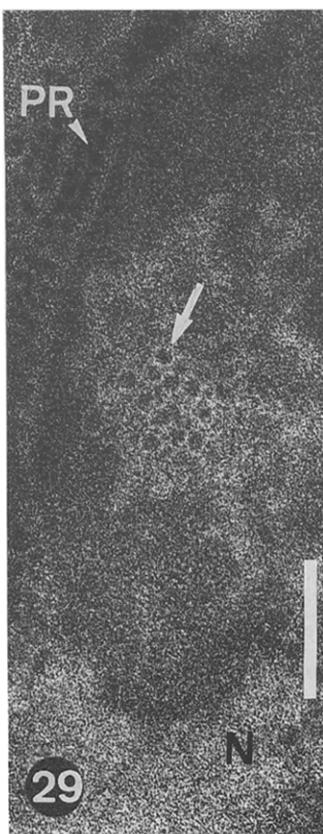
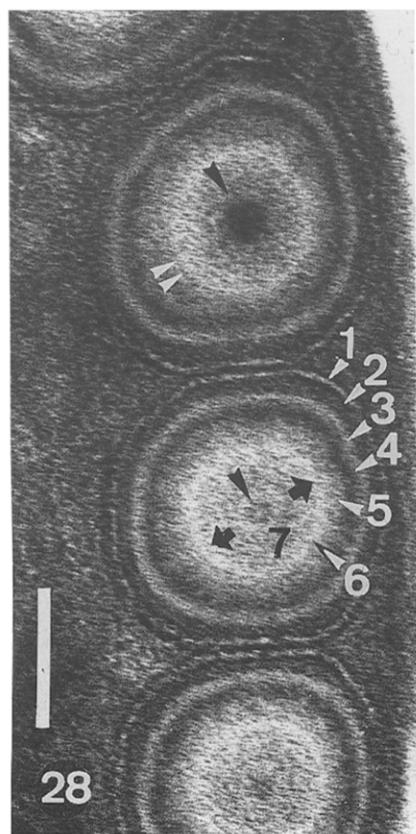
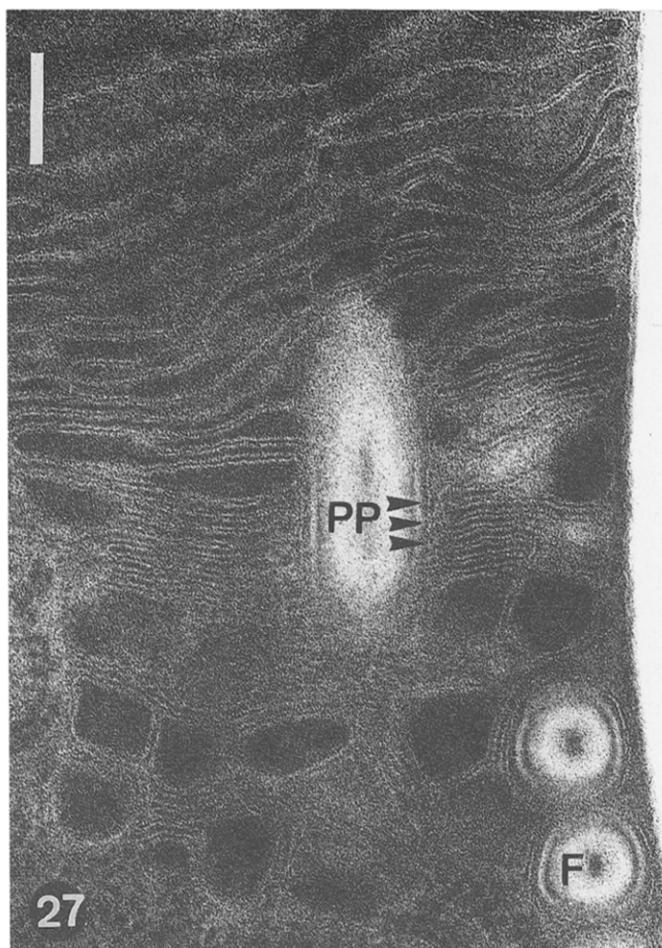
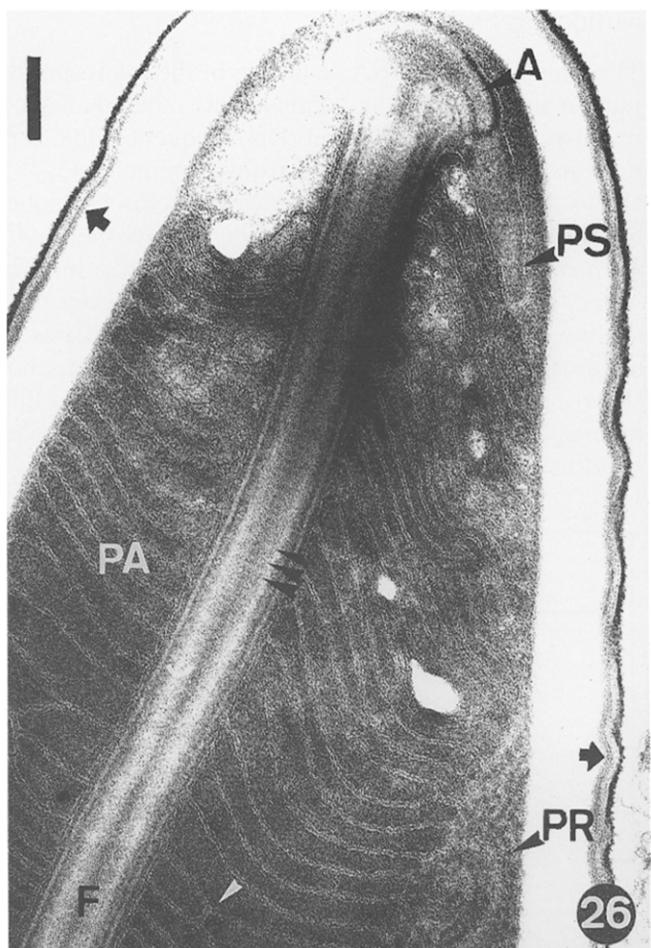
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inwards (Figs. 24, 26, 28): an approximately 5 nm thick unit membrane layer (1) was followed by three approximately equally wide layers (5–6 nm) that were moderately dense (2), fairly lucent (3), and moderately dense (4); the following c. 9 nm wide layer was moderately dense (5); next layer (6) was the most lucent and gave the impression of being fibrillar (approximately 24 components could be discriminated in the sections). The central part (7) had indistinct layering, and the very centre of the uncoiled part and the two anterior coils differed from the centre of posterior coils. The distinct strand of electron-dense material in the centre disappeared after the second coil (Figs. 24, 28). In the following 1–3 coils the transversely sectioned centre appeared ring-like. Longitudinal sections close to the centre in the uncoiled part of the filament exhibited at transverse striation probably caused by the fibrous layer (Fig. 26). The polaroplast was composed of lamellae in layers around the uncoiled part of the polar filament. Two indistinctly separated regions could be discriminated. The anterior lamellae, around the unbent part of the filament, were fairly regularly arranged, 17–26 nm wide (Figs. 24, 26). At the point where the filament bent to the side the lamellae widened up to 56 nm (Figs. 24, 26–27). Anastomoses between the unit membranes indicated that all lamellae were not completely continuous around the filament (Figs. 24, 26). In the posterior part of the polaroplast, close to the coils, the lamellae were compressed to about 10 nm thickness close to the filament, but they were wider (up to 70 nm) in their peripheral parts and more sac-like (Fig. 27).

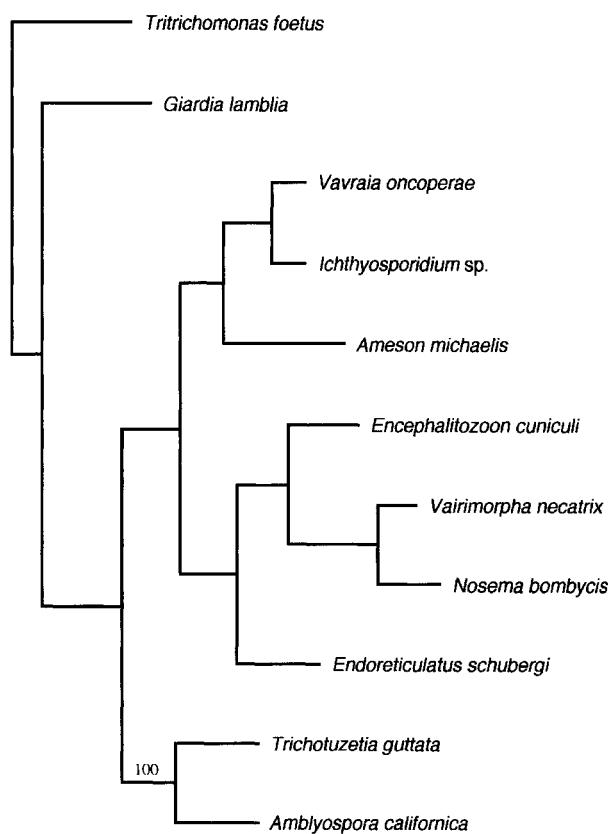
The polar sac was an almost cup-shaped structure, enclosing the anchoring apparatus and extending backwards for approximately the same distance as the diameter of the anchoring disc, enclosing only a short part of the anterior polaroplast lamellae (Fig. 26). The polar sac, the polaroplast compartments and the surface layer of the polar filament shared a continuous system of about 5 nm thick unit membranes.

The nucleus was localized to the posterior half of the spore, inside the coils (Fig. 24). The cytoplasm was dense, with strands of polyribosomes around the nucleus and the polaroplast (Figs. 24, 26). In an oblique position at the posterior pole of the mature spore was a unit membrane-lined posterior vacuole, measuring up to  $\frac{1}{4}$  of the spore length (Fig. 20), clearly visible also in living spores (Fig. 21).

A small number of anomalous macrospores were present in the ultrathin sections (Fig. 30). The spore wall was of the normal construction but wider, caused by increased dimensions of the endospore layer, and measured up to 280 nm. There was also an increased number of polar filament coils. The layering of the coils was disturbed. The polaroplast was an irregular mass of lamellar or sac-like components.

## Molecular biology

The small subunit rDNA sequence of the microsporidium treated herein was compared to other published microsporidian small subunit rDNA sequences to determine its phylogenetic position using parsimony. Fig. 31 shows that this microsporidium is most closely related to a group of microsporidia including *Amblyospora californica* (see BAKER 1995). Support for this relationship is very high with 100% of 100 bootstrap replicates possessing this clade. Furthermore, preliminary analysis of additional small subunit rRNA sequences (results not shown) suggests a very close relationship between this microsporidium and *Hazardia* sp. from *Anopheles quadrimaculatus*.



**Fig. 31.** Most parsimonious tree (1331 steps) found using *Giardia lamblia* and *Tritrichomonas foetus* as outgroups; *Amblyospora/Trichotuzetia guttata* clade supported by 100% of 100 bootstrap replicates.

## Intranuclear virus-like particles

In nuclei of mature spores from one sample (collected on July 11, 1994 at the type locality), aggregations of isometric 21–25 nm wide particles with a lucent periphery and an electron-dense centre were present (Fig. 29). The particles resembled virus-like particles of the type previously reported from microsporidia

## Infection experiments

All attempts to infect the original host with spores failed even when the host copepods were kept in water with spores for several weeks.

## Discussion

### Cytology

The cytology conforms with the normal for microsporidia and only a few observations need comments.

Normally the Golgi apparatus of the sporoblast and immature spore is a uniform spongius structure [cf. Fig. 7 B in LARSSON (1986)]. In the microsporidium two distinct regions of the Golgi apparatus are visible (Figs. 17–18). However, this is not unique, and a differentiated Golgi region was also observed in *Bacillidium criodrili* [cf. Fig. 16 in LARSSON (1994)].

The 10 nm fibrils appearing within the sporophorous vesicle at a certain stage of spore maturation are probably homologous to the intermediate cytoskeletal filaments present at the same site in several *Thelohania* species from Crustacea (WEIDNER et al. 1990). The role of such fibrils or filaments is not known, but apparently they are a particular case of the so-called "secretions", occurring in various forms (granules, tubules, fibrils etc.) in the episporontal space of many microsporidia (VÁVRA 1976).

Virus-like particles have repeatedly been observed in parasitic protozoa, reviewed by MILES (1988), WANG & WANG (1991) and LIPSCOMB & RIORDAN (1995). Those of proven virus nature are all RNA viruses (WANG & WANG 1991). From microsporidia two reports exist. In the earliest, by LIU (1984), virus-like particles in the cytoplasm of lysed spores of the honey bee parasite *Nosema apis* were described. These particles resembled bee viruses. The second report described virus-like particles from two unidentified microsporidia with rod-shaped spores, belonging in the family Thelohaniidae (LARSSON 1988). These particles were intranuclear, spherical and 20–24 nm wide. The virus-like particles reported herein are similar concerning shape, size and location in the nucleus. The role of viruses in the life of protozoa is unknown.

### Taxonomy

About 40 named species microsporidia have been described from copepods (SPRAGUE 1977; VORONIN 1986). Most of the descriptions are superficial, and few of the species have been studied at the ultrastructural level. Detailed comparison is not always possible. Many of the species differ from the microsporidium

treated herein by obvious and easily compared characters, e.g. the shape and size of the spores, and they need no comments here. Two species have been reported from the same host, *Cyclops vicinus*, and the same country, the Czech Republic: *Gurleya elegans* (LEMMERMANN, 1900) and *Thelohania cyclopis* WEISER, 1945. As these microsporidia produce distinct sporophorous vesicles with regular numbers of spores the differences to the species treated herein are obvious (KOMÁREK & VÁVRA 1968; WEISER 1945). It is clear that the microsporidium treated by us differs from all other microsporidian species as they are recognized at present time.

Isolated nuclei in all stages of the life cycle and polysporoblastic sporogony by rosette-like budding, yielding spores in individual sporophorous vesicles, are characteristics of the family Tuzetiidae SPRAGUE, TUZET & MAURAND, 1977 since ISSI and colleagues created the family Janacekiidae and thereby removed the diplokaryotic microsporidia (ISSI et al. 1991). None of the three genera *Tuzetia* MAURAND, FIZE, FENWICK and MICHEL, 1971, *Nelliemelba* LARSSON, 1984 and *Lanatospora* VORONIN, 1986 can accommodate the present microsporidium. The exospores, polaroplasts and inclusions of the episporontal space are different (LARSSON 1984; MILNER & MAYER 1982; BRONNVALL & LARSSON 1995). The primordia of the sporophorous vesicle are electron-dense pad-like structures on the surface of the sporont of the *Tuzetia* and *Lanatospora* species, not labyrinth-like as seen herein (Fig. 13). Further *Nelliemelba boeckella* differs by having an anisofilar polar filament.

There are two reasons to presume that the microsporidium described herein might have a more complex life cycle, involving a second host. One reason is the unsuccessful infection experiments, which indicate that the spores might not be infective to the host from which they have been harvested. The second reason is the molecular similarity between this microsporidian parasite of a crustacean and microsporidia of *Amblyospora* and related genera, especially the mosquito parasite *Hazardia* sp. (Fig. 31).

The microsporidium treated by us might represent an undescribed morph (in the intermediate host) of a polymorphic microsporidium. In that case a dipteran is most likely to be the definite host. It has been proven that a number of microsporidia from mosquitoes (the genera *Amblyospora*, *Culicosporella*, *Edhazardia*, *Hazardia*, *Parathelohania*) have complex life cycles, involving diploid and haploid generations, and that their sporogony is polymorphic with production of different kinds of spores in the different hosts. In the life cycles so far proven, where the two hosts are two not closely related organisms, not two generations of the same species, the alternating hosts are copepods and mosquitoes. For

microsporidia of the genera *Amblyospora* and *Parathelohania* copepod-mosquito life cycles have been established (ANDREAS 1985; AVERY & UNDEEN 1990). *Hazardia*, *Culicosporella* and *Edhazardia* are horizontally and vertically transmitted in the mosquito, and the life cycles so far examined need no alternate host (HAZARD & FUKUDA 1974; HAZARD et al. 1984; BECNEL & JOHNSON 1993). If the copepod microsporidium treated herein is the haplophase of a polymorphic species, the probable candidate to be the definite host is a bottom-dwelling midge larva. The fact that the spores released from dying copepods accumulate at the bottom of the pond (see below) suggests that. However, it is remarkable that the microsporidium described herein is cytologically different from the copepod morphs so far proven to be involved in polymorphic life cycles, both concerning the spore shape, and the construction of the exospore, polaroplast and sporophorous vesicle (ANDREADIS 1985; SWEENEY et al. 1988).

An alternative hypothesis would be that the microsporidium is monomorphic, using the copepod as the single host. The spores released from dead copepods accumulate at the bottom of the pond. They are later brought into circulation by the water currents and thereby made available for new generations of copepods. When the infection culminates considerable numbers of spores are released into the habitat. With an observed load of about  $6\text{--}9 \times 10^5$  spores per copepod, the density of the infected copepod population studied would yield about  $2.7 \times 10^9$  spores to be released over each  $\text{m}^2$  of the bottom of the pond. This means that every  $\text{mm}^2$  receives at least 2–3 spores (VÁVRA & LARSSON 1994). The seasonal dynamics of the infection indicates that the material infective for copepods might be dependent on what is occurring at the bottom of the pond. The seasonal peak of infection takes place about 5–6 weeks after the spring mixing of the water column of the pond, when the bottom material is brought up to the water surface. An explanation for the unsuccessful infection experiments could be that spores resting on the bottom of the pond need special conditions for maturation – conditions not fulfilled in the experiments. Such requirements have not been registered in the microsporidia so far, which makes this hypothesis less probable.

None of the genera of Tuzetiidae can accomodate the microsporidium, and the same is true for the *Amblyospora*-like microsporidia. The copepod morphs of *Amblyospora* and *Parathelohania* are cytologically different (ANDREADIS 1985; SWEENEY et al. 1988). Even if the spore shape resembles one of the spore morphs of *Hazardia milleri* produced in the mosquito [cf. Figs. 2, 22 and 24 with Figs. 6 and 15 in HAZARD & FUKUDA (1974)] (ultrastructural details cannot be compared), there are prominent differences between *Hazardia* and

this microsporidium. *Hazardia* species sporulate in the absence of sporophorous vesicles and the merogonic reproduction, and the sporonts (or "sporont mother cells"), are diplokaryotic (WEISER 1977; SPRAGUE et al. 1992).

As no genus can accomodate the new microsporidium treated herein our choice is either to establish a new genus, which can be characterized clearly, or to put the species in the genus *Microsporidium*, an unclassified taxonomic monster without diagnostic characters. Both solutions might be temporary. To choose *Microsporidium* is actually to hide the species away from comparison. To establish a new species and a new genus is to take the risk of creating synonyms, for it cannot be excluded that the species some time in the future will be proven to belong to the life cycle of a (probably undescribed) *Hazardia* species or another polymorphic microsporidium of a genus where the polymorphic nature is unknown today. However, we cannot deal with presumed new microsporidia in a useful way without naming them, so we consequently decide to describe a new species and a new genus.

## Description

### *Trichotuzetia* gen. nov.

**Diagnosis:** All life cycle stages with isolated nuclei. Merogonial reproduction by plasmotomy. Sporogony by rosette-like division, yielding a variable number of sporoblasts in individual sporophorous vesicles generated by the sporont. Spores pyriform with pointed anterior pole. Exospore four-layered, with one layer appearing like a double-membrane. Polar filament isofilar. Polaroplast with two lamellar regions, where the anterior lamellae are less densely packed than the posterior ones. Sporogony associated with production of fibrillar material that connects the exospore of sporoblasts and immature spores to the envelope of the sporophorous vesicle.

**Etymology:** Alluding to the hair-like cover of the immature sporal stages.

### *Trichotuzetia guttata* sp. nov.

**Merogony:** As for the genus. Plasmodial nuclei (sectioned)  $2.8 \mu\text{m}$  wide. The number of merogonial divisions unknown.

**Sporogony:** As for the genus. Up to 30 elongated sporoblasts are formed.

**Spores:** Unfixed spores measure  $5.0 \times 2.9$  ( $4.6\text{--}5.7 \times 2.7\text{--}3.3$ )  $\mu\text{m}$ . The spore wall is 170–195 nm thick, with a 30 nm thick, layered exospore. The 102–131 nm wide polar filament is arranged in 9–10 coils in a single row of coils close to the spore wall in the posterior half of the spore. The angle of tilt is

60–65°. The polaroplast surrounds the uncoiled part of the filament. The lamellae of the posterior short polaroplast region (close to the anterior filament coil) are tightly compressed close to the filament, but the peripheral parts are dilated. A single nucleus, measuring about 1.7×0.7 µm (stained) in the posterior half of the spore.

**Sporophorous vesicle:** Approximately of the same shape as the spore. Episporontal space of vesicles containing sporoblasts and immature spores traversed by about 10 nm wide fibrils, which are successively reduced and practically totally absent from vesicles with mature spores.

**Host tissues involved:** Initially in gonads of both sexes. In advanced cases systemic infection, muscles and gut epithelium excepted.

**Type host:** *Cyclops vicinus* ULJANIN, 1875 (Crustacea, Copepoda, Cyclopidae).

**Type locality:** A small pond in the village of Biskoupky, near Zbiroh, County Rokycany, Czech Republic.

**Types:** Hapantotypes on slides No. 0952–0959.

**Deposition of types:** Slides No. 0953 and 0957 in the International Protozoan Type Slide Collection at Smithsonian Institution, Washington, DC; slides No. 0954 and 0958 in the collection of Dr. J. WEISER, Prague; slides No. 0952, 0955, 0956 and 0959 in the collection of J. VÁVRA., Department of Parasitology, Charles University, Prague, Czech Republic.

**Etymology:** Alluding to the drop-shaped mature spore.

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