

# Description of *Chytridiopsis trichopterae* N. Sp. (Microspora, Chytridiopsidae), a Microsporidian Parasite of the Caddis Fly *Polycentropus flavomaculatus* (Trichoptera, Polycentropodidae), with Comments on Relationships Between the Families Chytridiopsidae and Metchnikovellidae

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**ABSTRACT.** The microsporidium *Chytridiopsis trichopterae* n. sp., a parasite of the midgut epithelium of larvae of the caddis fly *Polycentropus flavomaculatus* found in southern Sweden, is described based on light microscopic and ultrastructural characteristics. All life cycle stages have isolated nuclei. Merogonial reproduction was not observed. The sporogony comprises two sequences: one with free spores in parasitophorous vacuoles, the other in spherical, 5.6–6.8 µm wide, sporophorous vesicles which lie in the cytoplasm. The free sporogony yields more than 20 spores per sporont. The vesicle-bound sporogony produces 8, 12 or 16 spores. The envelope of the sporophorous vesicle is about 82 nm thick and layered. The internal layer is the plasma membrane of the sporont; the surface layer is electron dense with regularly arranged translucent components. Both spore types are spherical. They have an ~35-nm thick spore wall, with a plasma membrane, an electron-lucent endospore, and an ~14-nm thick electron-dense exospore. The polar sac is cup-like and lacks a layered anchoring disc. The polar filament is arranged in two to three isofilar coils in the half of the spore opposite the nucleus. The coupling between the polar sac and the polar filament is characteristic. The surface of the polar filament is covered with regularly arranged membranous chambers resembling a honeycomb. There is no polaroplast of traditional type. The cytoplasm lacks polyribosomes. The nucleus has a prominent, wide nucleolus. The two spore types have identical construction, but differ in dimensions and electron density. Free living spores are about 3.2 µm wide, the diameter of the polar filament proper is 102–187 nm, the chambers of the honeycomb are 70–85 nm high, and the polar sac is up to 425 nm wide. Living spores in the vesicle-bound sporogony are about 2.1 µm wide, the polar filament measures 69–102 nm, the chambers of the honeycomb are about 45 nm high, and these spores are more electron dense. Comparisons of cytology (especially the construction of the spore wall and the polar filament and associated structures) and life cycles reveal prominent differences among the *Chytridiopsis*-like microsporidia, and close relationships between the families Chytridiopsidae and Metchnikovellidae.

**Supplementary key words.** Classification of microsporidia, production of micro- and macrospores, ultrastructure.

A small number of microsporidia, mostly with spherical spores, exhibit a spore cytology that differs from that of the group norm, and they are usually recognized as the most simple or primitive representatives of the phylum. Five families have been established for these species: Metchnikovellidae Caullery & Mesnil, 1914, Chytridiopsidae Sprague, Ormières & Manier, 1972, Hesseidae Ormières & Sprague, 1973, Burkeidae Sprague, 1977, and Buxtehudeidae Larsson, 1980. The two recent classifications of the microsporidia, by Sprague [31] and by Weiser [38], divided the phylum Microspora into two classes, one with rather few, aberrant species, and the other with the majority of species, mostly "typical" microsporidians. However, the two classifications delimited the classes differently. Weiser united Metchnikovellidae, Chytridiopsidae and Hesseidae in the class Metchnikovellidea, leaving the class Microsporididea to microsporidia with traditional cytology [38]. Sprague restricted the class Rudimicrosporea to the family Metchnikovellidae, and combined the other families of aberrant microsporidia with the "normal" microsporidia in the class Microsporea, which was divided into two orders: Chytridiopsida, with the families Chytridiopsidae, Hesseidae and Burkeidae, and Microsporida, containing all the other microsporidia [31]. Burkeidae was established by Sprague in the monograph where the new classification was published [31], and Buxtehudeidae was not created at the time these two classifications appeared [12]. In 1982 when Sprague revised his classification, the order Chytridiopsida changed name to Minisporida, and the family Buxtehudeidae was included [32]. About 25 named species seem to belong to the order Minisporida, if Sprague's classification is preferred, and they use a wide spectrum of invertebrate hosts, including Oligochaeta, Myriapoda, Insecta and Mollusca.

Without exception, the approximately 11 species of the genus *Chytridiopsis* Schneider, 1884 inhabit the gut epithelium of their hosts. The type species, *C. socius* Schneider, 1884, is a parasite of adult beetles of the genus *Blaps* [30]. The other *Chytridiopsis* species live in various Coleoptera, Myriapoda and Oligochaeta.

Except for two doubtful *Chytridiopsis* species of Oligochaeta and *C. aquaticus* Léger & Duboscq, 1909, a parasite of larvae of the beetle *Helodes minuta*, which use hosts living in fresh water, all other *Chytridiopsis* species are found in a terrestrial environment. Only two species, both parasites of terrestrial beetles, have been investigated using electron microscopy: *C. socius* Schneider, 1884 [21, 33] and *C. typographi* (Weiser, 1954) [24, 25]. These are also the only *Chytridiopsis* species that have been discussed in publications other than the description.

A microsporidium of the *Chytridiopsis* type was found in a sample of larvae of the caddis fly *Polycentropus flavomaculatus* collected in July 1987 in southern Sweden. The species is new to science, and it does undoubtedly belong to *Chytridiopsis*, even though it is not typical for these species to use freshwater hosts for their development. Herein we briefly describe the light and electron microscopic cytology and discuss some aspects of the cytology and identification of the species. Similarities in the ultrastructure and life cycle between *Chytridiopsis*-like microsporidia and Metchnikovellidae suggest a closer relationship than that expressed in the classification adopted by the Society of Protozoologists [19].

## MATERIALS AND METHODS

Caddis fly larvae of the species *P. flavomaculatus* (Pictet, 1834) (Trichoptera, Polycentropodidae) were collected in a small pond at Gårdstänga, in Scania, southern Sweden, on July 7, 1987. Fresh squash preparations were made using the agar method of Hostounský & Žížka (Hostounský, Z. & Žížka, Z. 1979. A modification of the "agar cushion method" for observation and photographic recording microsporidian spores. *J. Protozool.*, 26(suppl.):41A–42A; abs. no. 117) and examined using phase contrast microscopy and dark field illumination. Permanent squash preparations were air dried lightly and fixed in Bouin-Duboscq-Brasil (BDB) solution overnight. For paraffin sectioning, infected animals were immersed in BDB or in 4% formaldehyde overnight or longer. After washing and dehy-

drating in a graded series of ethanols, specimens were cleared in butanol and embedded in Paraplast (Lancer, St. Louis, MO). Sections were cut longitudinally at 10  $\mu\text{m}$ . Squash preparations and sections were stained using Giemsa solution or Heidenhain's iron haematoxylin. For details on the general histological techniques used, see the manual by Romeis [29]. All permanent preparations were mounted in D.P.X. (BDH Chemicals Ltd., Poole, England). Measurements were made with an eye-piece micrometer at  $\times 1,000$ .

For transmission electron microscopy, individual segments were excised and fixed in 2.5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4°C for 48 h. After washing in cacodylate buffer and postfixing in 2% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4°C, the tissue samples were washed and dehydrated in an ascending series of buffer-acetone solutions to absolute acetone and embedded in Epon. Sections were stained using uranyl acetate and lead citrate [27].

For scanning electron microscopy, infected tissue was smeared on circular cover glasses, air dried lightly, and fixed in 2.5% glutaraldehyde in cacodylate buffer at 4°C for 48 h. After washing in buffer and critical point drying, smears were covered with gold and palladium.

## RESULTS

**Prevalence, pathology and life cycle.** The sample of caddis fly larvae collected on July 7, 1987, was not intended for the study of microsporidia. Fresh smears were made directly, and spherical bodies of different types and sizes were noticed and recorded, but they were not recognized as microsporidia. Their microsporidian nature was not obvious until the first ultrathin sections were made in 1991. The sample contained six specimens of *P. flavomaculatus*, and all of them were infected with the *Chytridiopsis* species. Another sample, collected on September 23, 1987, was devoid of the parasite. Specimens of the same and related hosts, collected at the same locality in earlier years, were free of the microsporidium, and so were the larvae of *P. flavomaculatus* collected in July 1991. Another microsporidium, *Vavraia holocentropi*, is common in the population, and it occurred together with the *Chytridiopsis* species in four of the six specimens collected in July 1987. This species utilizes various tissues, but avoids the gut epithelium and fat cells [17].

The *Chytridiopsis* species was confined to the midgut epithelium, and the infection caused no externally visible signs (Fig. 1). Infection was fairly weak, involving less than half of the midgut cells. It increased in posterior direction in the midgut, visible both as an increased number of infected cells and as a greater number of microsporidia per cell. Infected cells were rarely hypertrophic, and most cells contained only one or two groups of sporulating microsporidia in the superior region (Fig. 2). The developing microsporidia were closely associated with the nucleus and mitochondria of the host cell (Fig. 2-4). The close association with the nucleus, where groups of microsporidia were located into characteristic invaginations of the nuclear membrane (Fig. 2), was also apparent using light microscopy. Sporulated microsporidia were released into the gut lumen (Fig. 1).

Vegetative reproduction (merogony) was not observed and is presumed to be absent. Two types of sporogonies producing spores of two sizes were recognized (Fig. 2, 5). The largest spores, measuring approximately 3.2  $\mu\text{m}$  unfixed, 2.0–2.7  $\mu\text{m}$  when fixed and stained, were produced in greater numbers. Sectioned groups contained up to 20 spores in membrane-lined cavities, which apparently were parasitophorous vacuoles generated by the host cell (Fig. 3). The associated ribosomes on the cytoplasmic side of the membrane spoke for that, and so did the fact that sporoblasts and spores sometimes occurred together in

different parts of the same vacuole. Occasionally no vacuoles were formed and the spores were free in the cytoplasm (Fig. 4). The other and more commonly observed sporogony yielded spherical, thick-walled sporophorous vesicles measuring 5.6–8.8  $\mu\text{m}$  unfixed or 5.2–7.0  $\mu\text{m}$  after fixation and staining (Fig. 2, 5). They contained eight, 12 or 16 spores, eight being the most common number. These spores measured approximately 2.1  $\mu\text{m}$  unfixed, 1.2–1.6  $\mu\text{m}$  when fixed and stained. Vesicles were fairly resistant, but they opened under moderate pressure between slide and cover glass, releasing the spores (Fig. 5). Sporophorous vesicles were in intimate contact with the host cytoplasm, and no parasitophorous vacuoles were formed (Fig. 4).

Spores of both types were present in the lumen of the midgut (Fig. 1, 5). The largest spores occurred free or were temporarily enclosed in vacuole membranes. The small spores remained enclosed in sporophorous vesicles. Spores in sporophorous vesicles were also observed in the lumen of the foregut, which indicated that they must have been ingested. The distribution of the spore types suggests a life cycle where spores in thick sporophorous vesicles are released from the host, and, when ingested, infect new host specimens. The free sporogony in parasitophorous vacuoles is probably the initial phase of reproduction in the new host, and the free spores disperse the parasite inside the host and probably initiate the vesicle-bound sporogony. It is unknown if more than one cycle of free sporogonies precedes the production of vesicle-bound, durable spores.

**The free sporogony.** The earliest life cycle stages recognized as such were plasmodia with a small number of nuclei (Fig. 6). Their plasma membrane was about 8 nm thick, and the cytoplasm was uniformly granular with numerous free ribosomes and vacuoles filled with electron-dense material (Fig. 7). Sectioned nuclei measured up to 1.4  $\mu\text{m}$  in diameter. The nucleoplasm was moderately dense and there was a distinct area of nucleolus-like, denser material (Fig. 7). The envelope of the nucleus was of the traditional type, composed of two unit membranes with pores and a perinuclear cisterna. The plasmodia divided by plasmotomy into cells with smaller numbers of nuclei and finally into uninucleate sporoblasts (Fig. 8). Depressions of the nuclear envelope and intranuclear tubules were observed, both in undivided and dividing plasmodia (Fig. 6, 7, 9). There were no distinct, layered centriolar plaques. Remainders of plasmoidal material persisted between newly released daughter cells and in small amounts between fully mature spores (Fig. 9).

The youngest plasmodia were embedded in the cytoplasm of the epithelium cell (Fig. 6), but a parasitophorous vacuole developed progressively with the plasmodium preparing for plasmotomy, and the vacuole was usually complete before the sporoblasts were released (Fig. 8, 9).

The first traces of the exospore layer appeared as electron-dense spots on the plasma membrane at the time the plasmodia began dividing (Fig. 10). Simultaneously, the cytoplasm was rearranged and cisternae of rough endoplasmic reticulum became visible. The polar filament and the polar sac were initiated before the plasmotomy was complete, and nearly mature filaments were present in plasmodia with a small number of nuclei (Fig. 10). The filament originated from a Golgi apparatus of aggregated membrane-lined sacs (Fig. 11); initially it appeared as an electron-dense substance with radiating protrusions. The youngest polar sacs seen were unit membrane lined, like the filament primordia, and appeared as crescent-shaped bodies filled with electron-dense material. The differentiation of the filament proceeded rapidly, and mature filaments were present in still irregularly shaped sporoblasts (Fig. 12). The peripheral zone of the maturing filament, internally to the unit membrane, progressively became vacuolated (Fig. 13) and subsequently the vacuoles were arranged in regular order. Occasionally two polar

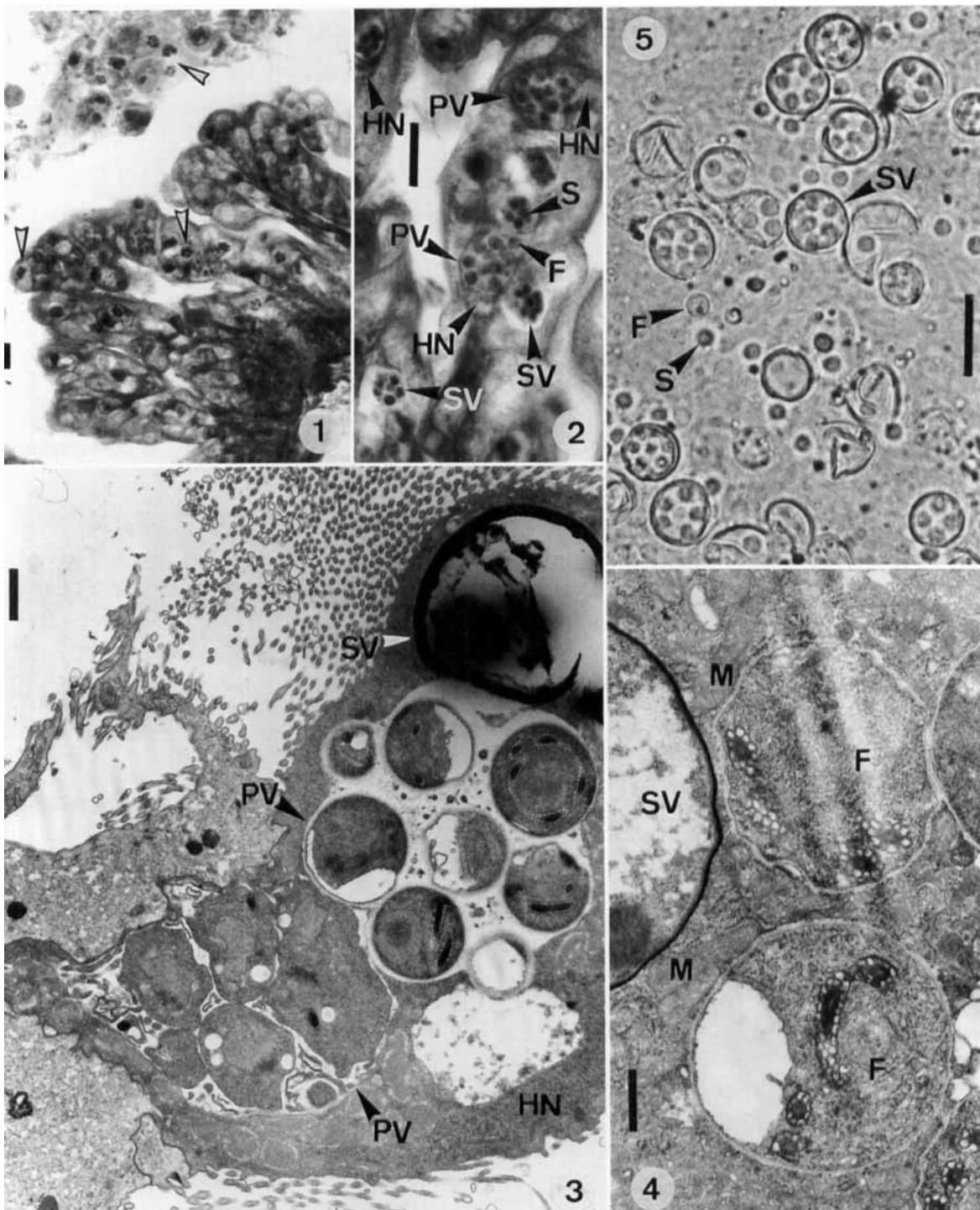
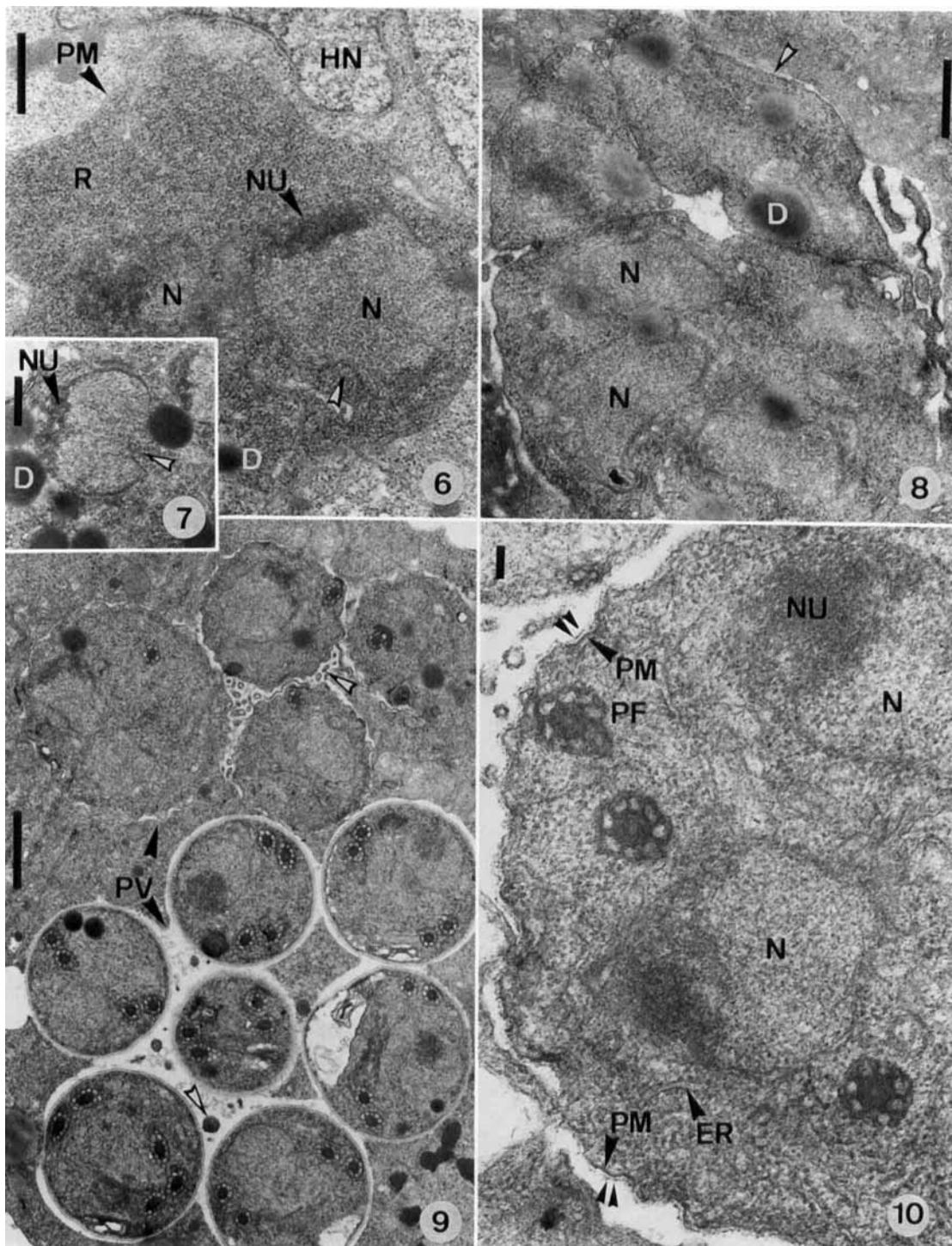
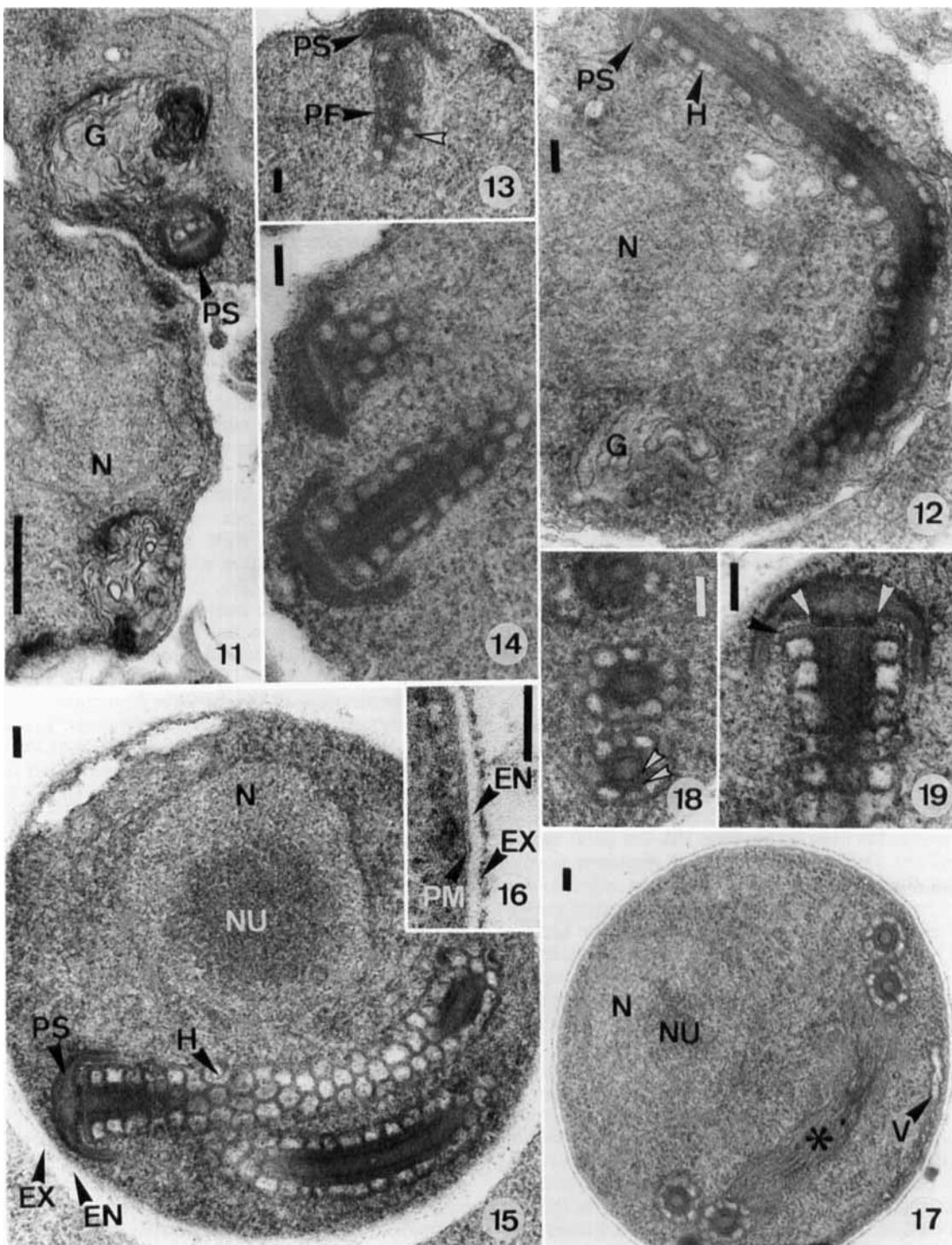


Fig. 1–5. Pathology and gross morphology of *C. trichopterae* n. sp. 1. Midgut epithelium and gut lumen of *Polycentropus flavomaculatus* with sporulated microsporidia (arrowheads). 2. Detail of the midgut epithelium with free spores in parasitophorous vacuoles and smaller spores in sporophorous vesicles. 3. The superior region of the midgut epithelium with dividing sporogonial plasmodia and free spores in parasitophorous vacuoles, and one sporophorous vesicle. 4. Detail of an epithelium cell exhibiting one sporophorous vesicle and four free spores; no parasitophorous vacuoles are formed. 5. Living spores of both sporogonies; sporophorous vesicles open under pressure. F, free spore; HN, host nucleus; M, host mitochondrion; PV, parasitophorous vacuole; S, vesicle-bound spore; SV, sporophorous vesicle. Fig. 1, 2, haematoxylin staining; Fig. 3, 4, ultrathin sections; Fig. 5, phase contrast. Bars = 10 µm (1, 2, 5), 1 µm (3), 0.5 µm (4).



**Fig. 6-10.** Free sporogony. **6.** Early sporont with two nuclei visible. There is no parasitophorous vacuole, and the cell has only a plasma membrane. White arrowheads indicate depressions of the nuclear envelope (in Fig. 6, 7). **7.** Detail of a nucleus and electron-dense vacuoles. **8.** Sporogonial plasmodium dividing by plasmotomy; electron-dense material is present on the plasma membrane (arrowhead). A parasitophorous vacuole is being formed, but there is no distinct vacuole membrane. **9.** Sporoblasts (above) and mature spores in parasitophorous vacuoles; arrowheads indicate remaining plasmodium material. **10.** Detail of a sporogonial plasmodium older than that seen in Fig. 8. Two nuclei are visible together with an almost mature polar filament. Electron-dense material (arrowheads) is being deposited on the plasma membrane. D, vacuole with dense material; ER, endoplasmic reticulum; HN, host nucleus; N, microsporidian nucleus; NU, nucleolus; PF, polar filament; PM plasma membrane; PV, parasitophorous vacuole; R, ribosomes. Bars = 0.5  $\mu\text{m}$  (6-8), 1  $\mu\text{m}$  (9), 100 nm (10).



**Fig. 11–19.** The free sporogony. 11. Two irregularly shaped sporoblasts; the polar sac and the polar filament are generated by the Golgi apparatus. 12. The polar filament is almost complete before the spore has reached the spherical shape. 13. Polar sac and polar filament of a sporoblast. Vacuoles (arrowhead) are being formed at the periphery of the filament; they are still not arranged into regular orders. 14. Sporoblast with two polar sacs on adjacent filament coils. 15. Mature spore. There is no angle between the anterior part of the polar filament and the coils. 16. Detail of the spore wall exhibiting the layers. 17. Mature spore with transversely sectioned filament coils; \* indicates continuous membranes between the honeycomb at opposite poles of the coil (probably tangential sections through the surface of the honeycomb). 18. Transversely sectioned polar filament coils, exhibiting the regularly arranged chambers of the honeycomb and the simple stratification of the filament (arrowheads indicate the two electron-dense layers of the filament). 19. The polar sac and the connection to the polar filament. There is no stratified anchoring disc; the posterior surface of the polar sac is shaped like a socket (white arrowheads). The anterior surface of the polar filament, including the honeycomb, is covered by a lamellar disc (black arrowhead). The chambers of the honeycomb are arranged in regular layers. EN, endospore; EX, exospore; G, Golgi apparatus; H, honeycomb-like layer of the polar filament; N, nucleus; NU, nucleolus; PF, polar filament; PM, plasma membrane; PS, polar sac; V, posterior vacuole. Bars = 0.5  $\mu$ m (11), 100 nm (12–19).

sacs developed on the same filament, probably at opposite ends (Fig. 14). When sporoblasts matured to spores their shape changed to spherical (Fig. 3, 15). The last sporal structure to develop was the endospore layer (Fig. 12, 15).

Mature spores had a three-layered spore wall, measuring 28–35 nm, with an internal, ~8 nm thick plasma membrane, a median, electron-lucent endospore, and a uniform, electron-dense, 11–14 nm wide exospore with a rough surface (Fig. 15, 16). The cytoplasm was uniformly granular with free ribosomes but devoid of polyribosomes (Fig. 15). The nucleus, which measured ~0.9  $\mu\text{m}$  in diameter in living spores, had a uniform, granular nucleoplasm and a distinct, rounded, more electron-dense nucleolus measuring about half the diameter. At the pole opposite to the nucleus was a clear, membrane-lined space, obviously corresponding to a posterior vacuole (Fig. 17).

The polar filament was arranged in two to three isofilar coils in the half of the spore opposite the nucleus (Fig. 15, 17). An anterior straight part, forming an angle to the coils, was lacking. The filament was 102–187 nm wide and stratified in a rather simple way by material of two electron densities. The external layer was ~10 nm and electron dense, and, moving inward, was followed by a moderately dense and slightly wider layer, an ~10-nm wide dense layer, and a moderately dense center, measuring about half the diameter of the filament (Fig. 15, 18).

The surface of the filament was covered by 70–85 nm high, hexagonal chambers, delimited by 7-nm thick unit membranes (Fig. 12, 15). They were arranged like a honeycomb, in regular layers consisting of 8–9 chambers (Fig. 18). There was no traditional polaroplast.

The anterior end of the filament was connected to a cap-like polar sac, measuring up to 425 nm in diameter (Fig. 15). Duplicated polar sacs, like those seen in some sporoblasts (Fig. 14), were not observed in mature spores. The anterior surface of the polar sac was bent semi-circularly. The posterior surface had a straight central part and almost perpendicular, posteriorly directed sides, which approximately covered the two anterior orders of chambers in the honeycomb (Fig. 19). The polar sac was filled with uniform, electron-dense material. An internal, stratified anchoring disc of the type normally seen in microsporidia was lacking. The posterior, central region of the polar sac had a socket-like extension of the same diameter as the filament proper (Fig. 19). The proximal end of the filament was anteriorly covered with a disc-like structure as wide as the filament together with the honeycomb. The disc had an external unit membrane and linearly arranged electron-lucent globular or fibrous structures in the center.

**Sporogony in sporophorous vesicles.** Mature sporophorous vesicles were with difficulty permeable to solutions used for fixation and embedding, and only undivided plasmodia were well preserved. This sporogony proceeded in intimate contact with the cytoplasm of the epithelium cell, without provoking production of parasitophorous vacuoles (Fig. 3). Each cell normally hosted only one or two sporophorous vesicles (Fig. 2), which indicated that the invading cells sporulated without previous vegetative reproduction.

The earliest stage observed were lightly irregular cells with one nucleus per sectioned cell (Fig. 20). The largest sectioned cell measured 3.8  $\mu\text{m}$  in diameter and the nucleus was 1.1  $\mu\text{m}$  wide. The plasma membrane was already covered with a continuous, up to 45-nm thick, electron-dense envelope (Fig. 20) (the primordium of the sporophorous vesicle). The nucleus was identical to nuclei of the corresponding stage in the free sporogony sequence, having a denser, nucleolus-like structure at the periphery (Fig. 20). The cytoplasm had strands of endoplasmic reticulum. There were also up to 650-nm wide vacuoles filled with electron-dense material. Membrane whorls and elec-

tron-lucent vacuoles were present at the periphery. Simultaneously with increasing numbers of nuclei the vacuolation increased to all parts of the cell (Fig. 21), and finally the vacuolation split the plasmodium into uninucleate sporoblasts (Fig. 22). Each sporoblast generated a polar filament and a polar sac of identical construction to corresponding structures of sporoblasts in the free sporogony (Fig. 23, 24).

Sporulating sporophorous vesicles were frequently associated with up to 10 more-or-less circular structures, arranged in one row in the host cytoplasm, close to the vesicle (Fig. 20). They were not connected with the sporophorous vesicle. In a few sectioned vesicles the plane of sectioning passed tangentially through this region, revealing that the structures were tubules (Fig. 25). Occasionally the sporophorous vesicles were associated with two rows of transversely sectioned tubules at opposite poles (Fig. 26), suggesting that one long tubule might be coiled several times around the vesicle. The tubules measured 106–124 nm in diameter, and the wall was up to 34-nm thick. They were not built of vesicle material, but layered with 3–5 narrow, electron-dense layers alternating with a less dense substance (Fig. 27).

Living (Fig. 28) and carefully fixed (Fig. 29) sporophorous vesicles were spherical, but vesicles in routinely made permanent preparations for light microscopy were more-or-less shrunken (Fig. 30). Mature spores were spherical or with one pole lightly compressed (Fig. 28). The spore wall measured ~35 nm and had a plasma membrane, a median electron-lucent endospore, and an ~14 nm wide, uniformly dense exospore (Fig. 31). The cytoplasm was rather electron dense, with free ribosomes but lacking polyribosomes (Fig. 32). The polar filament was arranged in two or three horizontal coils in the half of the spore opposite the nucleus. The filament proper measured 69–102 nm in diameter, and the chambers of the honeycomb were ~45 nm high. The transversely sectioned filament had identical layering to the filament of free spores (Fig. 33). In addition, the honeycomb was identical, but the chambers were less distinct here as they were filled with a substance of approximately the same density as the cytoplasm. These spores also lacked a traditional polaroplast. The greatest polar sac observed measured 266 nm in diameter. The construction was identical to polar sacs of free spores and also the coupling to the polar filament was identical. The nucleus had a wide, dense nucleolus, like that found in free spores. A distinct posterior vacuole was not seen in the enveloped spores.

When the progressive vacuolation generated the sporoblasts, the envelope of the sporophorous vesicle, including the original plasma membrane, lost contact with included cells. The membrane of the vacuoles became the plasma membrane of the sporoblasts. The envelope of mature sporophorous vesicles measured ~82 nm and exhibited a distinct stratification (Fig. 34). The internal layer was the original ~8 nm wide plasma membrane, often with remainders of cytoplasm attached, and, moving outward, was followed by a strand of moderately dense material of the same thickness, and a thick, dense surface coat. The surface layer had a distinct structure. When sectioned longitudinally it appeared layered, with more lucent material in regular order in the external zone (Fig. 34). Tangential sections revealed that the surface was chambered, with ~9 nm wide, rounded, electron-lucent components (Fig. 35).

## DISCUSSION

The four families of *Chytridiopsis*-like microsporidia contain 20 species, eight of which have been investigated using electron microscopy: *Chytridiopsis socius* [21, 33, 37], *C. typographi* [24, 25], *Steinhausia brachynema* [28], *Nolleria pulicis* [2], *Burkea gatesi* [26], *B. eisenia* [4], *Buxtehudea scaniae* [12], and *Hessea*

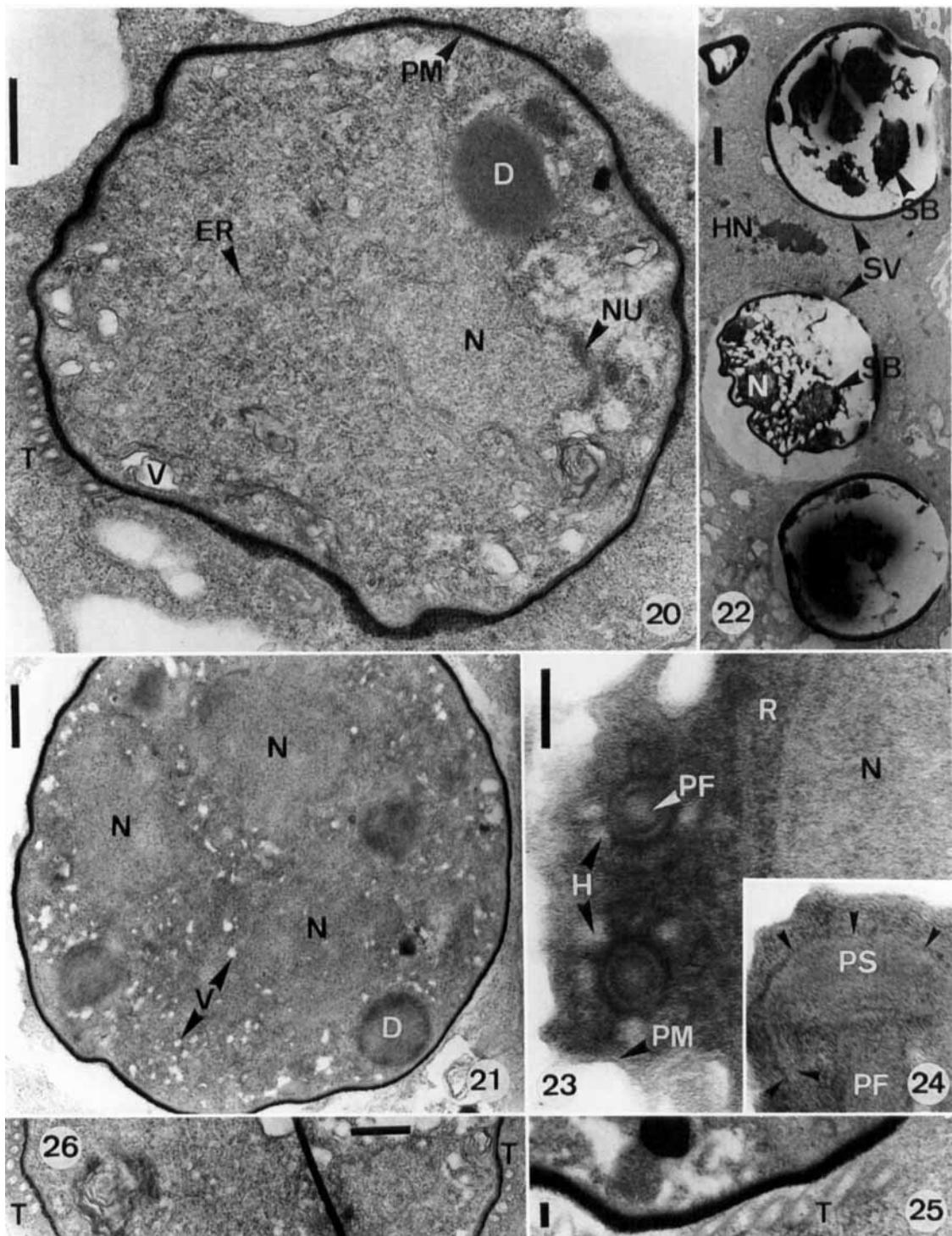


Fig. 20–26. The vesicle-bound sporogony. 20. Sporont with one nucleus visible. The plasma membrane is covered with electron-dense material; a row of tubules is present close to the envelope. Beginning vacuolation at the periphery of the sporont. 21. Older sporogonial plasmodium; vacuoles are dispersed in the cytoplasm. 22. Sporophorous vesicles closely associated with the host nucleus; sporoblasts are being formed by progressive vacuolation. 23. Periphery of a sporoblast; transversely sectioned polar filament coils with honeycomb layer are visible. 24. The polar sac and the anterior part of the polar filament of a sporoblast. Arrowheads indicate the semicircular anterior surface and the narrow posterior projection of the polar sac. 25. Periphery of a sporophorous vesicle exhibiting longitudinally sectioned cytoplasmic tubules. 26. Transverse section of a sporogonial plasmodium revealing tubules at opposite poles. D, vacuole with electron-dense material; ER, endoplasmic reticulum; H, honeycomb-like layer of the polar filament; HN, host nucleus; N, nucleus of the microsporidium; NU, nucleolus; PF, polar filament; PM, plasma membrane; PS, polar sac; R, ribosomes; SV, sporophorous vesicle; T, tubule; V, vacuole. Bars = 0.5 µm (20, 21, 26), 1 µm (22), 100 nm (23, 24 [with common bar on 23], 25).

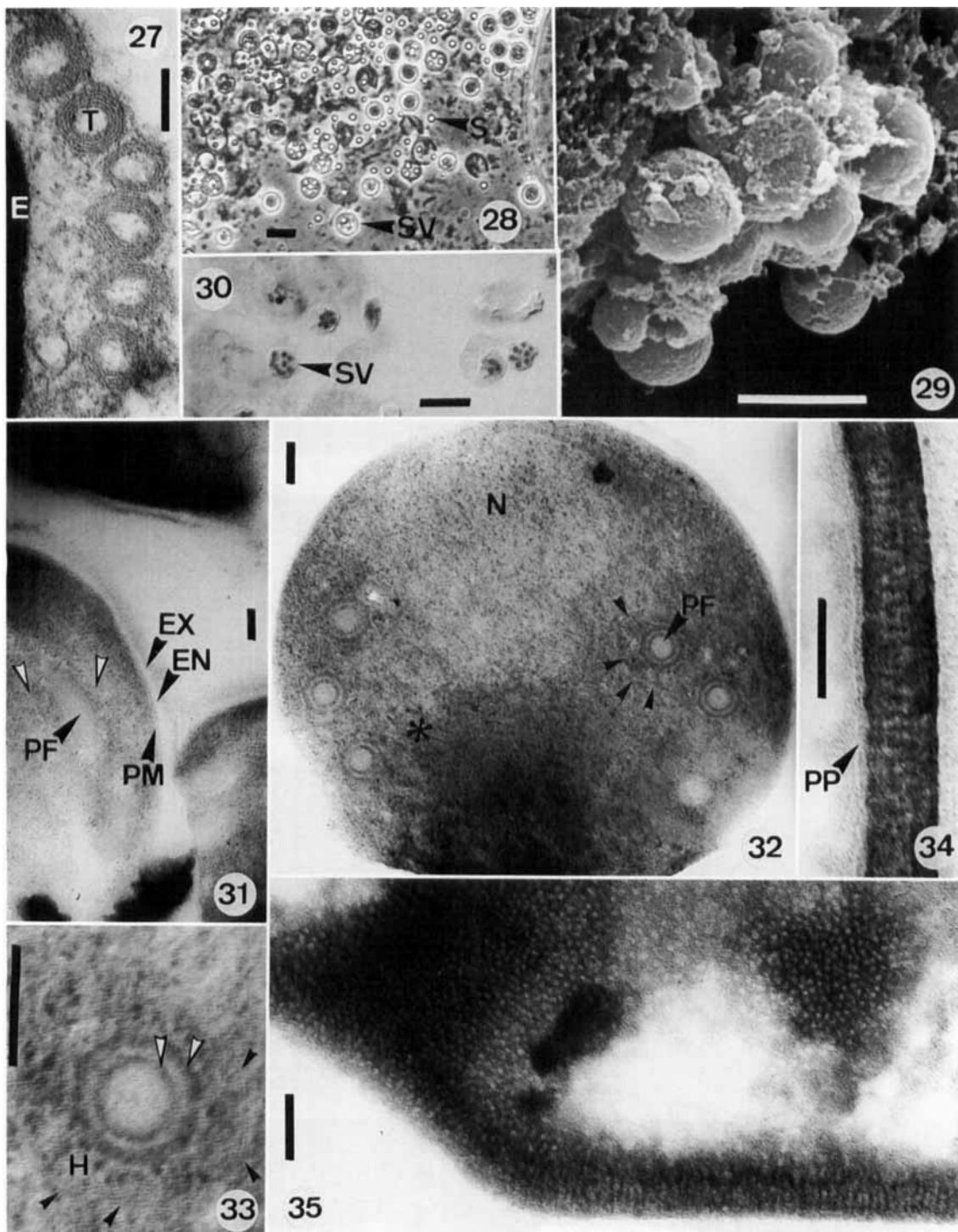


Fig. 27-35. The vesicle-bound sporogony. 27. Transversely sectioned cytoplasmic tubules in the proximity of a sporophorous vesicle. 28. Living sporophorous vesicles and vesicle-bound spores. 29. Carefully fixed sporophorous vesicles. 30. Routine fixations for light microscopy cause vesicles to shrink. 31. Parts of three mature spores, exhibiting three layers of the spore wall. The polar filament is visible and the arrowheads indicate the border of the honeycomb. 32. Mature vesicle-bound spore; arrowheads indicate the border of the honeycomb, \* continuous membranes in the honeycomb region. 33. Transversely sectioned filament coil; white arrowheads indicate the electron-dense layers of the filament; black arrowheads the border to the honeycomb. 34. Longitudinally sectioned envelope of a sporophorous vesicle. 35. Tangentially sectioned envelope; regularly arranged translucent components are present in the surface layer. E, envelope of the sporophorous vesicle; EN, endospore; EX, exospore; H, honeycomb-like layer of the polar filament; N, nucleus; PF, polar filament; PM, plasma membrane; PP, plasma membrane of the sporogonial plasmodium; S, vesicle-bound spore; SV, sporophorous vesicle; T, tubule. Fig. 28, phase contrast; Fig. 29, SEM; Fig. 30, Heidenhain's haematoxylin. Bars = 100 nm (27, 31, 35), 10  $\mu$ m (28, 30), 5  $\mu$ m (29).

*squamosa* [23]. Many of these investigations suffered from problems with fixation and infiltration, and the interpretations were clearly influenced by the technical problems. Little is known of the mature spores in particular, and all aspects of the cytology cannot be compared. No study has given a complete description of the ultrastructure of spores in thick-walled sporophorous vesicles. In addition four species of the family Metchnikovellidae have been studied at the ultrastructural level: *Metchnikovella hovassei* [35], *M. wohlfarthi* [9], *Amphiamblrys laubieri* [8], and *A. bhatiellae* [22]. These species must also be included in the discussion.

**Cytology and life cycle.** The spores of the *Chytridiopsis* species described herein have a cytology that differs from the usual condition of microsporidia, for example, as exhibited by the spherical octospores of *Pilosporaella chapmani* [3]. However, it corresponds well with the organization of other microsporidia of *Chytridiopsis* and related genera. Details of the cytological specializations, e.g. the nature of the honeycomb-like layer, have been discussed in previous studies, and there is no need to repeat them here. However, the uniquely organized connection between the polar sac and the polar filament has not previously received attention.

In a typical microsporidian spore the anterior half is occupied by the voluminous infection apparatus consisting of the straight, anterior part of the polar filament, the bell-shaped polar sac and the lamellar or sac-like polaroplast. The filament enters the polar sac and attaches to a wide, layered, biconvex anchoring disc in the center of the polar sac. The unit membrane of the polar sac is continuous with the polaroplast. The construction is clearly visible as seen in, for example, the cytological studies of *Janacekia debaisieuxi* (called *Pleistophora debaisieuxi*) [20] and *Napamicium dispersus* (called *Chapmanium dispersus*) [14]. The *Chytridiopsis* species treated herein has a rather tiny and compact polar sac filled with uniform electron-dense material (Fig. 19). There is no anchoring disc and the posterior, central area of the polar sac is shaped like a socket to which the anterior, disc-like part of the polar filament attaches. Only the center of the polar filament enters the polar sac. This is probably a widespread type of anchoring apparatus among the *Chytridiopsis*-like microsporidia, even if previous investigations, with the exception of the studies of *S. brachynema* [28] and *N. pulicis* [2], have failed to reveal it. However, this type of polar sac and attachment between sac and filament is also present in the Metchnikovellidae, clearly visible in *M. hovassei* (Fig. 24 in [35]), and also apparent in *M. wohlfarthi* [9], *A. laubieri* [8], and *A. bhatiellae* [22].

One of the sporogonies of the *Chytridiopsis* species yields spores enclosed in thick envelopes generated by the sporont. The envelope of the species described herein is a complex, layered structure (Fig. 34). The envelopes of *C. socius* [33] and *C. typographi* [25] have similar dimensions, and probably similar layers, but details are not visible in the published micrographs. *Hessea squamosa* is another species with thick-walled spore-containing sacs. The surface layer is characteristic, composed of plaque-like structures [23], which might be compared to the compartmentalized surface layer of the present species (Fig. 35).

Also the microsporidia of the Metchnikovellidae sporulate in thick-walled sacs and, for example, the envelope of *M. hovassei* modifies from uniformly electron-dense when initiated to layered with a translucent, almost spongyous surface when mature (Fig. 33 in [35]). These envelopes are similar to the envelopes of the *Chytridiopsis*-like microsporidia. A comparison between the envelopes of *A. laubieri* (Fig. 24 and 28 in [8]) and of the present species (Fig. 34, 35) reveals striking similarities.

Previous investigations have applied names like cysts [23] and pansporoblasts [24] to the spore-containing sacs of the *Chytridiopsis*

-like microsporidia. In modern microsporidiology a sac-like structure, normally produced by the sporont, that collects the spores is called a sporophorous vesicle [5]. This is normally a thin and uniform structure, but there are examples of more complex vesicles, like the layered vesicle of *Telomyxa glugeiformis* [13]. Merontogenetic sporophorous vesicles produced by *Pleistophora typicalis* [6], *Vavraia culicis* [5], and *Vavraia holocentropi* [17] are thick, and for each genus with characteristic construction. Merontogenetic sporophorous vesicles might look similar to the envelopes produced by the *Chytridiopsis* and *Hessea* species, but there is one important difference. A typical sporophorous vesicle, both of the sporontogenetic and the merontogenetic type, is a secretory product generated at the surface of the plasma membrane. The envelope of the *Chytridiopsis*-like microsporidia incorporates the plasma membrane of the sporont. Consequently it is not a typical sporophorous vesicle, and it might deserve a name of its own. Cyst and pansporoblast are used for other structures and should be avoided. However, Beard and colleagues accepted the term sporophorous vesicle (even if slightly modified to polysporophorous vesicle) for the thin envelope of *N. pulicis*, even if the plasma membrane was one component of the envelope [2], and therefore the term sporophorous vesicle has also been used in this description. The Metchnikovellidae have sporophorous vesicles of identical construction to the *Chytridiopsis*-like microsporidia, i.e. the plasma membrane is incorporated into the envelope.

Beard and colleagues redefined the family Chytridiopsidae, using the spore-containing structures as a part of the definition: "Polysporophorous vesicles present, two types (one a thick cell wall and the other a fragile membrane) in some species, only one type (the fragile membrane) in others" [2]. This means that all spore-containing sacs of microsporidia of the Chytridiopsidae, in their opinion, are of parasite origin, like the sporophorous vesicles of *N. pulicis* [2]. Purrini and Weiser initially interpreted the envelopes of *C. typographi* in the same way [24], but in the second contribution on the species the thin envelopes are described as vacuole membranes generated by the host [25]. Everything speaks for the unit membrane-lined cavities collecting the larger spores of the species treated herein as being parasitophorous vacuoles of host cell origin, not parasite-derived sporophorous vesicles. The associated ribosomes on the cytoplasmic side of the membrane is one argument, another is the fact that sporoblasts and spores occasionally occur together in different parts of the same vacuole. The same situation is clearly visible in the micrograph of *C. socius*, the type species of *Chytridiopsis*, used by Weiser in the second edition of the "Atlas of Insect Diseases" (Fig. 256 in [37]). If these cavities were sporophorous vesicles produced by the sporont, the mixed stages would indicate that the daughter cells of the sporont develop unsynchronously. That interpretation seems less probable than that two sporonts occasionally are caught in the same parasitophorous vacuole.

Léger and Duboscq noticed that *C. schneideri* produced spores of two size classes, and they were of the opinion that it would be necessary to create a new genus for this species [18]. However, micro- and macrospores were produced by the *Chytridiopsis* species described herein (Fig. 3), and a close examination of illustrations of related species reveals that this apparently is typical for the genus *Chytridiopsis*. The drawing Léger and Duboscq used for *C. socius*, the type species, shows that (Fig. 1 in [18]) as do the micrographs of *C. typographi* used in Weiser's Atlas (e.g. Fig. 333 in [36]); and this was further confirmed by Purrini and Weiser [25].

Manier and Ormières discussed three hypotheses about the role of the free and enveloped spores in the life cycle of *C. socius* [21]. One possibility was that the free spores functioned inside

Table 1. Comparison of *Chytridiopsis*-like microsporidia from which the ultrastructural cytology is known.<sup>a</sup>

	Exospore thin	Exospore thick	Endospore thin	Endospore thick	Polar filament short	Polar filament long	Polar sac on coil	Polar sac anteriorly, filament with angle to coils	Honeycomb-like layer	Merocony observed	Diplokarya present	Polyribosomes present	Spores of two sizes	Sporophorous vesicle thick	Sporophorous vesicle thin	Parasitophorous vacuole	Association with nucleus of the host cell	References
<i>C. socius</i>	+	?	+	?	+	+	+	+	+			+	+	+	+	+	18, 21, 33, 37	
<i>C. typographi</i>	+	+	+	+	+	+	+	+	+			+	+	+	+	+	24, 25, 36	
<i>C. trichopterae</i> n. sp.	+		+		+	+	+	+	+			+	+				This study	
<i>Steinhausia brachynema</i>	+			+	+	+	+	+	?								28	
<i>Nolleria pulicis</i>	+		1		+	+	+	+	+								2	
<i>Burkea eisenia</i>	+	?	?	?	+	?	?	?	?				?	?	?		4	
<i>B. gatesi</i>		+		?		+	+	+								+	26	
<i>Buxtehudea scaniae</i>	+			+		+	+	+	2							+	12	
<i>Hessea squamosa</i>	+		+	+		+	+	+	+				+				23	

<sup>a</sup> +, Character present; 1, endospore lacking; 2, tubules substitute honeycomb.

the host, in the dispersal between cells, and that the enveloped spores spread the parasite to new host specimens, like the life cycle of the *Chytridiopsis* species treated herein has been interpreted. It has been noticed in related microsporidia, like *S. brachynema* (Fig. 22 in [28]) and *B. scaniae* (Larsson, unpubl. observ.), that spores are capable of ejecting their polar filament in the host where the spores originated. This life cycle can be compared with the life cycle of the coccidian *Cryptosporidium parvum*, inhabiting the superficial zone of the gut epithelium of mice [7]. A minor part of the sporozoites, which only are enclosed by a thin membrane, are released already inside the host, ready to infect new epithelium cells, while most sporozoites, protected by thick envelopes, leave the host. A similar, although considerably more complex, life cycle characterizes the microsporidium *Amblyospora connecticus*, where the free spores inject their sporoplasms into the oocytes of the female hosts where the spores originated, while the spores in sporophorous vesicles are released to infect the intermediate host [1]. The life cycle of *M. hovassei* expresses free and vesicle-bound sporogony of identical type to the *Chytridiopsis* species [35].

**Taxonomy.** The spherical spores and the absence of a traditional polaroplast unite the *Chytridiopsis*-like microsporidia. The Metchnikovellidae also lack a polaroplast, but their spores are either spherical or rod shaped.

The cytological variation between the *Chytridiopsis*-like microsporidia is most distinct in the spore wall and the polar filament. Most commonly the exospore is unspecialized, thin and uniform, like the exospore of the *Chytridiopsis* species treated herein (Fig. 16). This type of exospore is shared by at least five species belonging in four genera (Table 1). *Chytridiopsis socius*, the type species of the genus, probably has the same type of exospore, but no study of this species reveals the construction of the spore wall of mature spores distinctly. *Hessea squamosa* and *B. gatesi* have wide, prominent exospores. The exospore of *B. eisenia* can only be traced in the published micrographs (Fig. 3 in [4], but it seems to be of the narrow type).

The endospore is distinct but narrow in the species treated herein; the same is true for the spores of *C. typographi* (Fig. 6, 7 in [24], Fig. 14 in [25]). The ultrastructural treatises on *C. typographi* describe the spore wall to have a rigid outer membrane, the exospore, and an electron-dark endospore, but Fig. 7 in [24] and Fig. 18 in [25] reveal an electron-dense plasma

membrane, a narrow, lucent endospore, and a narrow, granular exospore. *Buxtehudea scaniae*, *S. brachynema* and *H. squamosa* have wide, lucent endospores of the dimensions normally seen in microsporidia [15, 34]. *Burkea gatesi* probably has a similar endospore, unless the electron-lucent space between the exospore and the plasma membrane, visible in published micrographs, is an artifact (Fig. 9, 11 in [26]). The endospore is not always mentioned in studies of *Chytridiopsis*-like microsporidia, and for example not reported for *B. eisenia* [4]. The endospore layer is the last layer of the spore wall to develop, and consequently it is necessary to be convinced that mature spores have been studied before it can be concluded that the endospore is missing (cf. Fig. 12, 15). Micrographs of what appears to be immature spores of *B. eisenia* have a narrow, less electron-dense zone between the plasma membrane and the exospore (Fig. 3 in [4]). The first sign of the developing endospore normally looks like this. Mature spores of *B. eisenia* can be expected to have a distinct wide or narrow endospore. The only convincingly proven case of a missing endospore seems to be in *N. pulicis* (Fig. 23 in [2]).

Microsporidia of Metchnikovellidae have spore walls of the *Chytridiopsis* type, with narrow exo- and endospore layers, as can be seen in micrographs of *M. hovassei* (Fig. 29 in [35]), *M. wohlfaerti* (Fig. 11 in [9]), and *A. bhatiellae* (Fig. 14 in [22]).

A typical *Chytridiopsis*-like microsporidium has a short polar filament with two to three coils. The polar sac is directly attached to the anterior coil and no part of the filament forms an angle to the coils. The surface is covered with unit membrane-lined chambers in a honeycomb-like arrangement. The *Chytridiopsis* species, *S. brachynema* and *N. pulicis* exhibit this construction (Table 1). The micrographs of spores of *B. eisenia* do not clearly reveal filament details, but it cannot be excluded that the polar filament is of this type. The veil-like structure of Fig. 2 in [4] might be a honeycomb-like layer. Spores of *B. scaniae* and *B. gatesi* have long coiled filaments, where the proximal part forms an angle to the tightly arranged coils. The filament of *B. scaniae* has projecting tubules instead of a honeycomb. It is unclear if the filament of *B. gatesi* has a complex surface cover. Complexes of membrane-lined compressed sacs of suspected polaroplast nature have been observed in spores of *B. scaniae* (Fig. 16 in [12]) and *B. gatesi* (Fig. 11 in [26]). The polar filament of *H. squamosa* has few coils, but the anterior portion forms a distinct

angle to the coils and there is no evidence for a honeycomb or other complex surface structure.

The polar filament of the Metchnikovellidae is straight and stout and no complex surface structures have been described. However, there is a slight similarity between the developing honeycomb of the *Chytridiopsis* species treated herein (Fig. 15) and the filament of *A. bhatiellae* (Fig. 12, 15 in [22]). The terminal part of the filament of Metchnikovellidae is usually swollen and it has been called a gland. Sections through the gland of *M. hovassei* (Fig. 25 in [35]) and *A. laubieri* (Fig. 34, 37 in [8]) show an enlarged portion of a filament, but nothing except for traditional polar filament structures. The terminal part of the polar filament has never received attention, and we know practically nothing of how it is constructed in "normal" microsporidia. The polar filament of *Cylindrospora fasciculata* ends with a slightly swollen portion, where the internal organization deviates from the rest of the filament (Fig. 4A in [16]). The gland of the Metchnikovellidae seems not to be a structure unique enough to merit an isolated taxonomic position for Metchnikovellidae.

Summarizing the cytological characteristics, the *Chytridiopsis* species, *S. brachynema*, *N. pulicis*, and probably *B. eisenia*, constitute a rather homogeneous group (Table 1). *Burkea gatesi* and *B. scaniae* combine *Chytridiopsis*-like characteristics with traditional microsporidian cytology. The last species, *H. squamosa*, diverges from the other species by having merogonial reproduction, nuclei coupled as diplokarya, and by having spores with polyribosomes. It seems to be more closely related with microsporidia of the order Microsporida, containing the "normal" microsporidia, than with Minisporida.

A closer comparison between the *Chytridiopsis* species and, for example, *M. hovassei* [35] reveals striking similarities: two sporogonies with free and enveloped spores, sporophorous vesicles of similar type, with multilayered walls where the plasma membrane of the sporont is incorporated, identical spore walls, identical polar sacs, identical, and as far as we know, unique coupling between polar sac and polar filament, spores devoid of polyribosomes, and no polaroplast of traditional type. Undoubtedly Chytridiopsidae and Metchnikovellidae are related. In the revised classification of the protozoa by the Committee on Systematics and Evolution of the Society of Protozoologists [19], the updated classification by Sprague [32] was used for the microsporidia, which means that Metchnikovellidae and Chytridiopsidae are separated into different classes. However, a two-class system can still be defended, but the classes must be delimited differently. Metchnikovellidae, Chytridiopsidae, Burkeidae and Buxtehudeidae (if Burkeidae and Buxtehudeidae are distinct families) appear to be a natural group, and they might be joined in one class, while Hesseidae could go together with the "normal" microsporidia in the second class. The International Code of Zoological Nomenclature provides no rules for nomenclature above the level of the superfamily [10], so the taxonomist is free to decide if Metchnikovellidea, Rudimicrosporea or a new name should be used. However, the restriction of the class as outlined above corresponds fairly well with Weiser's conception of Metchnikovellidea [38].

**Identity of the species.** About 15 species of microsporidia have been reported from caddis fly larvae; none has spherical spores and none uses the gut epithelium for development. The species described herein is clearly different from them. The distinction from the *Chytridiopsis*-like microsporidia is not that simple. Cytological characteristics are usually not sufficient for their discrimination and their spores are of approximately the same size. Their status as new species has principally been based upon postulated host specificity. Four species of *Chytridiopsis*-like microsporidia use freshwater hosts: *C. hahni* Jírovec, 1940,

*C. limnodrili* Jírovec, 1940, *C. aquaticus* Léger & Duboscq, 1909, and *S. brachynema* (Richards & Sheffield, 1971). *Steinhausia* is a clearly defined genus and the distinction to *S. brachynema* needs no further comment. Thick-walled sporophorous vesicles are not mentioned in the descriptions of *C. hahni* and *C. limnodrili* [11], and Sprague doubted that they belong in the genus *Chytridiopsis* [31]. When preparing the description of *B. scaniae*, the type slide of *C. limnodrili* was studied. The absence of thick-walled vesicles was verified, and no clear association between the parasite and the nucleus of the host cell was found. This is clearly not a *Chytridiopsis* species and, lacking another possible genus affiliation, the new genus *Jiroveciana* was created for *C. limnodrili* [12]. Undoubtedly these two species are different from the species treated herein. The last species, *C. aquaticus*, lives in the gut epithelium of the aquatic larva of a beetle. The three-line description devoid of illustrations tells us that spores are produced in thick-walled vesicles [18]. Léger's types have been destroyed (Degrange, pers. commun.), so we have lost the opportunity to extract more information from them. It cannot be excluded that there is an overlooked free sporogony in the life cycle. There might also be a close association between the parasite and the host nucleus, and it might be a good *Chytridiopsis* species. The thick-walled sporophorous vesicles are 11–18 µm wide, which is twice the size of the vesicles of the species from caddis fly larvae, but the spores are of similar size, 1–2 µm in diameter. The discrepancies between the vesicle sizes are probably great enough to indicate that the species are different, and we do not need to rely on the different orders of hosts to distinguish them.

#### Description

##### *Chytridiopsis trichopterae* n. sp.

**Merogony.** Not observed.

**Sporogony I.** Multinucleate sporogonial plasmodia divide by plasmotomy into uninucleate sporoblasts. The sporont produces at least 20 daughter cells.

**Spore I.** Spherical. Living spores measure approximately 3.2 µm in diameter, fixed and stained spores 2.0–2.7 µm. The spore wall is up to 35 nm thick, with plasma membrane, narrow, electron-lucent endospore, and up to 14 nm wide, electron-dense exospore. Nucleus with large nucleolus. Cytoplasm devoid of polyribosomes. Their polar filament is 102–187 nm wide, arranged in 2–3 isofilar coils in the half of the spore opposite to the nucleus. The surface of the filament is covered with regularly arranged, membrane-lined chambers in honeycomb-like arrangement. The height of the chambers is 70–85 nm. No traditional polaroplast. The polar sac is cap-like, up to 425 nm in diameter, and covers the anterior two orders of chambers in the honeycomb. A layered anchoring disc is absent. A membrane-lined vacuole at the pole opposite to the nucleus.

**Sporogony II.** Multinucleate sporogonial plasmodia divide by progressive vacuolation into uninucleate sporoblasts in sporophorous vesicles. Each sporont generates 8, 12 or 16 daughter cells; 8 is the most common number.

**Spore II.** Spherical. Living spores measure approximately 2.1 µm in diameter, fixed and stained spores 1.2–1.6 µm. The spore wall is about 35 nm thick and identical to the wall of spore I. Nucleus with large nucleolus; cytoplasm devoid of polyribosomes. Polar filament with 2–3 coils arranged and constructed identically to the filament of spore I. The filament is 69–102 nm wide; the chambers of the honeycomb are ~45 nm high. Polar sac identical to the sac of spore I, up to 266 nm wide.

**Sporophorous vesicle.** Spherical. Living vesicles are 5.6–8.8 µm wide, 5.2–7.0 µm wide fixed and stained. Envelope persistent, ~82 nm thick, and layered: the plasma membrane of the sporont, a moderately dense layer, and an electron-dense surface layer, with about 9 nm wide, translucent components in the superior region.

**Host tissues involved.** Midgut epithelium, causes no or slight hypertrophy. Sporogony I in parasitophorous vacuole or occasionally free in the cytoplasm; sporophorous vesicles of sporogony II in direct contact

with the cytoplasm. Sporophorous vesicles associated with 106–124 nm wide tubular structures with layered walls.

**Type host.** *Polycentropus flavomaculatus* (Pictet, 1834) (Trichoptera, Polycentropodidae), larvae.

**Type locality.** A small pond, communicating with the river Kävlingeån, at Gårdstånga, Scania, in the south of Sweden.

**Types.** Syntypes on slides No. 870707-F-(5-9) RL.

**Deposition of types.** In the International Protozoan Type Slide Collection at Smithsonian Institution, Washington, DC (USNM #43105 and #43106), in the collection of Dr. J. Weiser, Charles University, Prague, Czechoslovakia, and in the collection of the author.

**Etymology.** As this is the first microsporidium of this type from caddis flies, the species name was derived from the name of the insect order.

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