FOLIA PARASITOLOGICA 42: 1–10, 1995

Canningia spinidentis gen. et sp. n. (Protista: Microspora), a new pathogen of the fir bark beetle *Pityokteines spinidens*

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Key words: Microspora, *Canningia*, bark beetle, *Pityokteines*, Austria, *Unikaryon*

Abstract. *Canningia spinidentis* gen. et sp. n. infects the fir bark beetle *Pityokteines spinidens* Rtt. in Austria. The pathogen attacks mainly the fat body, Malpighian tubules, the muscles and the connective tissue of larvae and adults, and the gonads of adults. The development is haplokaryotic, with single spores. Spores are short tubular, uninucleate, with globular anchoring disc inserted subapically, laterally, in a depression of the endospore wall. Polar filament is isofilar, with 5/6 coils. Polaroplast is composed of two lamellar parts of different density. A new genus *Canningia* gen. n. is proposed based on differences in ultrastructures of spores from *Unikaryon* Canning, Barker, Hammond et Nicholas, 1974.

More than eleven microsporidia have been described from different bark beetles, older descriptions are based on studies in the optical microscope and only recently some materials were studied in ultrathin sections. Detailed classification of material described long before electron microscopy was accessible for this study on old type slides. Its ultrastructural study and revision should be performed with newly collected material. In adult beetles, the microsporidia infect different tissues: the midgut, the Malpighian tubules, the tracheal matrix or the gonads. In some infections, just one tissue is infected; in others they cause a general infection (see Table 1). Several species have a rather broad host range and appear in different zoogeographic regions under identical ecological conditions. They are transmited in old galleries in infected trees mainly during the maturation of newly hatched beetles, under crowded conditions where old and new systems of galleries are interconnected. Another opportunity for transmission is the period of flights and aggregation of animals on damaged trees. Different species of bark beetles inhabit different parts of the same tree and there is a possibility of transmission of infections from one host species to another. This report describes a new microsporidian infecting the fir tree bark beetle *Pityokteines spinidens* in Austria.

MATERIALS AND METHODS

Adult *Pityokteines spinidens* Rtt. were collected from fir trees (*Abies alba*) in the locality Flatz, near Neunkirchen, South of Vienna, Austria, in different collection periods during the year. Living beetles were brought to the laboratory and were maintained on chips of fresh bark in vials till examination. Other material was maintained on cut trunks in screened boxes in the insectary. Beetles were dissected and all tissues of the beetle were inspected in water-mount under the optical microscope to ascertain the distribution of the infection in different organs of the host. The material from water-mounts was used for preparation of dry smears stained with Giemsa. Fresh dissected material was fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH = 7.2) at 4°C over night. After washing in cacodylate buffer and post-fixation in 2% OsO_4 in cacodylate buffer for 4 h at 4°C, material was washed and dehydrated in ascending series of ethanol to acetone and embedded in Vestopal W. Sections were stained in uranyl acetate and lead citrate. Material for histology was fixed in Duboscq-Bresil fixative and after washing and dehydration was embedded in Technovit 7100. Serial sections 4 μm thick were stained in Toluidin Blue and embedded in Eukit for light microscopy.

RESULTS

Infected adults were present in the locality Flatz during the whole cycle of activity of *P. spinidens*, with a prevalence of 3 to 8 %. In insectary reared colonies, infection levels reached 20–28 % in subsequent populations. The infected beetles showed no external signs of infection in growth, motility or behaviour. Spores were present in fecal pellets in late stages of the infection.

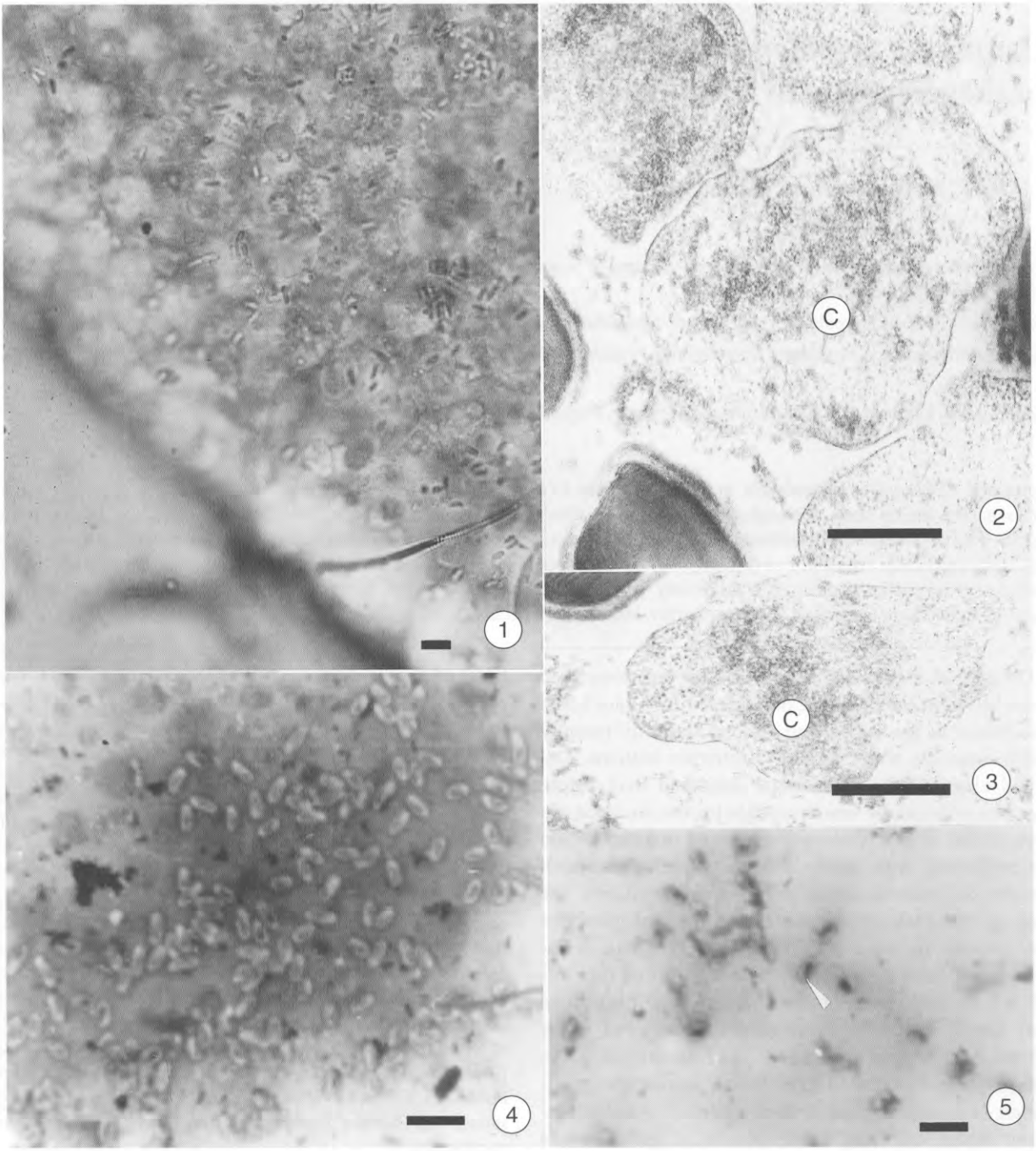
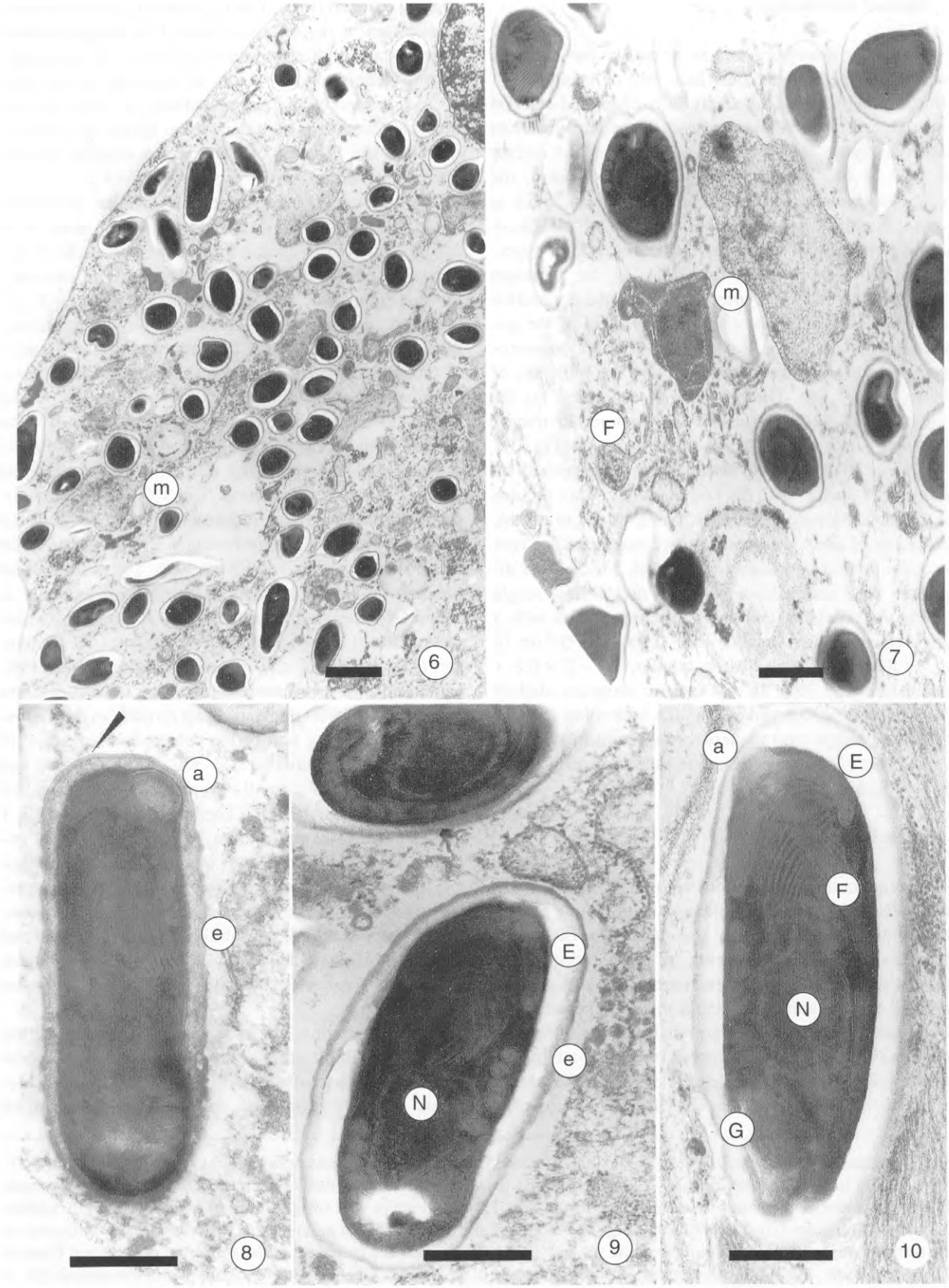


Fig. 1. *Canningia spinidentis*, spores in the eggs of *Pityokteines spinidens*. Bar = 5 μm. **Fig. 2.** Thin-walled meronts of *C. spinidentis*, c - chromosomes in nuclei. Bar = 0.5 μm. **Fig. 3.** Late meront with chromososes in equatorial plate. Bar = 0. 5μm. **Fig. 4.** Spores of *C. spinidentis* on Giemsa-stained smear. Bar = 5 μm. **Fig. 5.** Spores stained with Giemsa showing subapical insertion of the polar filament (arrowhead). Bar = 5 μm. **Fig. 6.** Spores and vegetative stages of *Canningia spinidentis* in the cytoplasm of a host cell. Spores thick-walled, with irregular empty zones of host cytoplasm. Meronts (m) single. Bar = 2 μm. **Fig. 7.** Meront (right) and early sporoblast (left-m) in host cell. F - germ with extruded polar filament and sections of extruded filaments. Empty zones around mature spores. Bar = 1 μm. **Fig. 8.** Young tubular spore of *C. spinidentis*: Foamy exospore (e) surface with a double membrane (arrowhead). Secretion of the endospore at its beginning. Polar filament with globular anchoring disc (a) inserted in a subapical pit of the spore wall. The binary polaroplast with an anterior system of fine lamellae and the posterior part with loose meshes. Bar = 0.5 μm. **Fig. 9.** Maturing spore of *C. spinidentis* with 5/6 coils of the isofilar filament. Single nucleus in membrane (N). Exospore (e) reduced to a thin layer covering the endospore (E) replacing its foamy part. Bar = 0.5 μm. **Fig. 10.** Mature spore with typical subapical insertion of the anchoring disc (a) in the attenuated part of the endospore (E). Polar filament crossing the polaroplast (F) and coiled on the spore wall. Lamellae of both parts of the polaroplast are visible. Nucleus (N) in the central part. Coil of tubules of the Golgi (G) in the posterosome. Bar = 0.5 μm.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Optical microscopy

The microsporidium infects primarily the fat body and the Malpighian tubules; later foci of infection appear in other tissues, especially in tracheal end cells on the midgut and the tracheal matrix. The microsporidium infects circular and longitudinal muscles of the midgut as well as the segmental muscle strands. There, the pathogen invades the sarcoplasmic reticulum which is replaced with elongated masses of stages of the microsporidium. Infected tissues are infiltrated without hypertrophy or other immune reactions as the infection spreads in tissues. With infected cells of the connective tissue the pathogen enters the surface sheet of the gonads and in maturating ovaries of the females the microsporidium invades the nurse cells and the follicules of the maturing eggs. The infection is transmited via the egg and infects the tissues of the embryo. In mature eggs are mainly mature spores of the pathogen (Fig.1).

The schizogonial development is characterized by uninucleate stages, with the exception of cells in process of division. Meronts are single, not arranged in chains. In smears of adult beetles vegetative stages are not very frequent. Merozoites are round or oval, 3–3.5 μm in diameter. Oval uninucleate sporonts divide in a single sporogonial division and form oval sporoblasts with a single round central nucleus, 4.5 ± 0.5 × 2.5 ± 0.3 μm (n = 30). Spores are long oval to tubular, 1.9 – 2 × 0.8–1 μm in size (n = 100). In dry smears, some are slightly curved or kidney-shaped. With HCl hydrolysis (Weiser 1976) one single oval nucleus was demonstrated in the central part of the spore. On smears the spores were all single (Figs. 4–5).

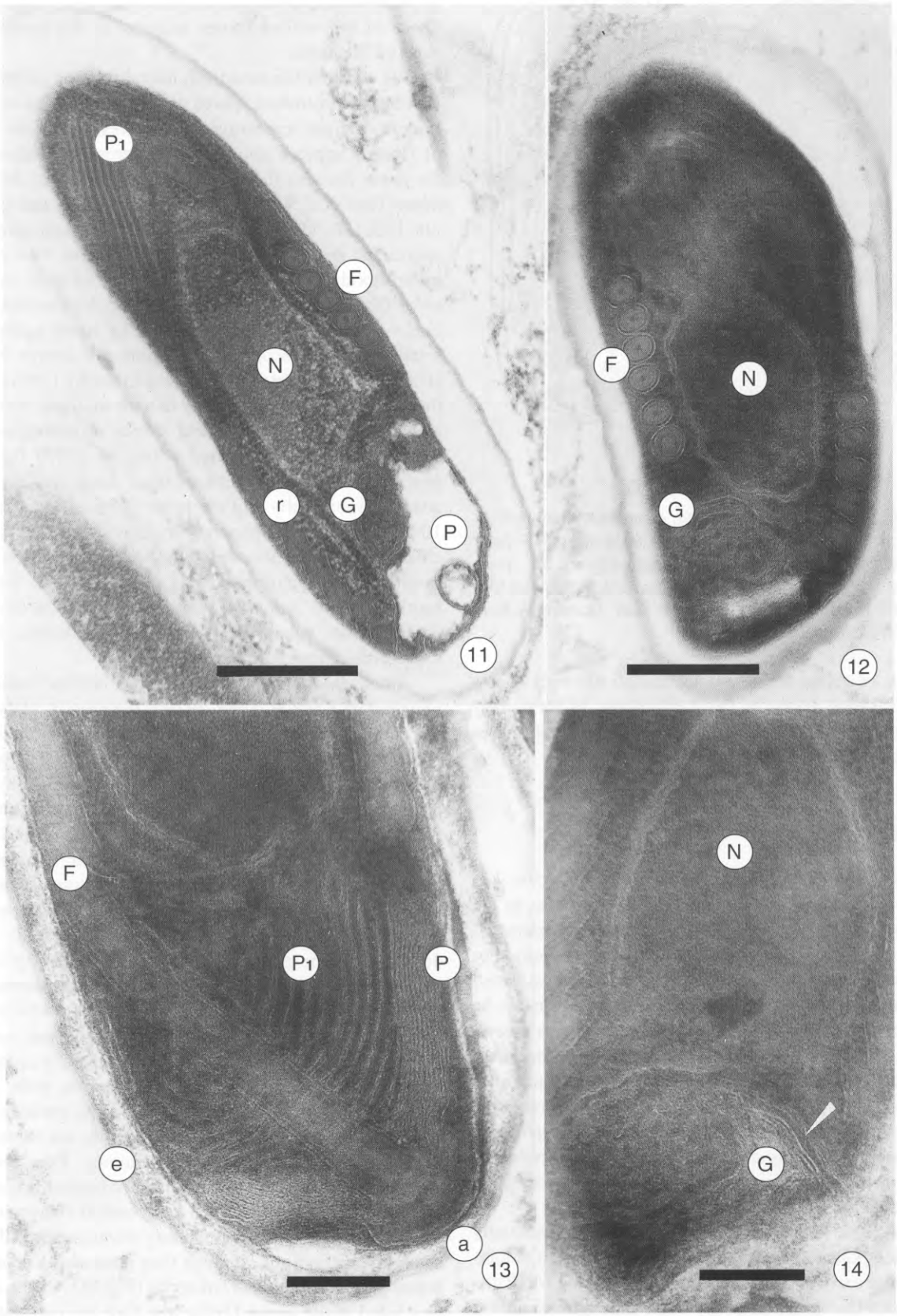
Ultrastructure

Meronts in ultrathin sections are uninucleate, broadly oval cells with a thin plasmalemma, without any prominent endoplasmic reticulum and masses of ribosomes (Figs. 2–3). In sections meronts are in small foci, without any delimitation by a membrane or vacuole and are in close contact with the cytoplasm of the host cell. In some cases chromosomes were visible in nuclei of late meronts (Figs. 6–7).The sporont is limited by a thin unit membrane without surface deposit or plaque-like thickening of the membrane. The size of the sporoblasts is slightly reduced (Fig. 7) and a system of polyribosomes is disposed in dense layers around its single nucleus. Sporonts are in direct contact with host cell cytoplasm. During maturation of spores, an irregular empty zone appears around each spore, eventually as result of fixation, but this area is confluent when spores are close together in the infected cell forming a vacuole without distinct membrane.

Immature spores are long oval to tubular, with broad round poles (Fig. 8). The exospore, 100 nm thick, is of foamy consistence, enclosed from the outside by a system of membranes 30 nm thick (Fig. 8). The exospore, of equal thickness, is attenuated and protruding in the region of fixation of the spherical globular anchoring disc of the polar filament. The lateral subapical fixation of the polar filament is a typical feature of this microsporidian. During the maturation of the spore, the foamy electron-dense exospore is replaced by the electron-transparent layer of the endospore, and the depression with the anchoring disc of the polar filament remains in its lateral position. The 100 nm thick endospore is in the depression reduced to 50 nm, but does not protrude (Fig. 10). The anchoring disc remains globular 100–150 nm in diameter, 80 nm high. The isofilar polar filament descends obliquely to the central axis and is coiled 5/6 times in the posterior part. A bipartite polaroplast encloses the apical end of the filament. Its anterior part is formed of usually 10 tightly adhering fine lamellae closed in a membrane and forming a mass 600 nm broad and 100–150 thick adhering directly to the endospore. The posterior part of the polaroplast is formed of broader loops of lamellae 200–300 nm in diameter and 300–350 nm thick, usually formed of 5–6 individual lamellae (Figs. 8, 10, 13). The single nucleus, 0.5 × 1 μm, is situated in the middle of the spore and is encircled by multiple layers of polyribosomes (Figs. 10–12). At the posterior end of the spore the posterosome, visible in fresh spores as a distinct vacuole, contains an oval coil of Golgi tubules (Figs. 10, 12, 14) and remains of electron-dense products of the Golgi system disposed in a vacuole with some membranous structures (Figs. 9– 10).

In some sections of infected host tissues there are areas with multiple cross sections of tubules which most probably are partially dissolved extruded polar filaments (Fig. 7). Some of them are connected with

Fig. 11. Mature spore with thick endospore and thin exospore. Posterior part of the polaroplast (p) and longitudial layers of polyribosomes (r) surround the nucleus (N). Posterior end with posterosome including the Golgi coil (G) and the posterior vacuole (P) with its empty membranous structure. Bar = 0.5 μm. **Fig. 12.** Mature spore with cross sections of polar filament (F), nucleus (N) and Golgi tubules (G). Bar = 0.5 μm. **Fig. 13.** Details of the anterior pole of an immature spore with foamy exospore (e) and detailed view of the structure of the dense (p) and lose (p_l) parts. Detailed structures of the anchoring disc (a) and filament (F). Bar = 0.2 μm. **Fig. 14.** Detailed structure of the Golgi coil of tubules (G) adhering to the posterior part of the nucleus (N). A lateral tubule connects the Golgi with the space of the polar filament (arrowhead). Bar = 0.2 μm.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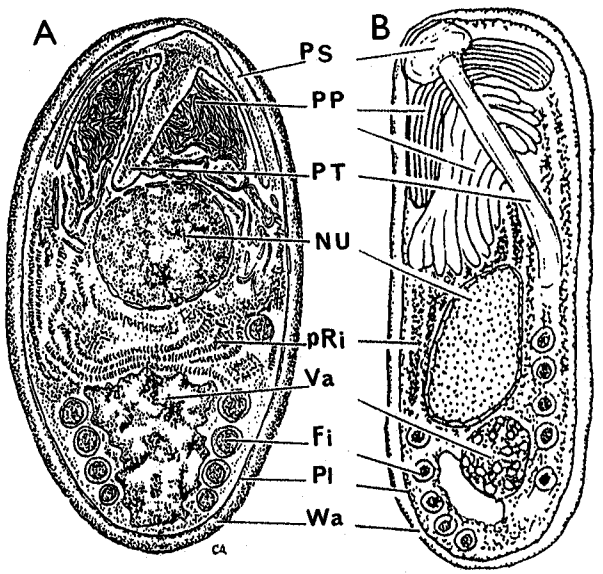

Fig. 15. *Unikaryon* and *Canningia*, comparative morphology of the spore: PS - anchoring disc, PP - polaroplast, PT - polar filament, Nu - nucleus, pRi - polyribosome layers, Va - posterosome with Golghi tubules, Fi - cross sections of polar filament, Pl - plasmalemma, Wa - spore wall. Drawing A from Azavedo and Canning (1987).

remains of germs. There are no remains of empty spores or thin-walled spores in these groupings. In some meronts in the same group are evident chromosomal figures in slightly hypertrophic nuclei.

DISCUSSION

The microsporidium of *Pityokteines spinidens* belongs to infections transmited via infected feces in the galleries and on food of the bark beetles, or transovarially via infected eggs from adults to their progeny. For microsporidia infecting bark beetles *Nosema calcarati* (Purrini and Halperin 1982) is the only suspected to be transovarially transmited. In other cases, there is no evidence of direct transovarial transmission. In cases where tissues are infected without direct communication with the intestine, such as infections of the fat body, the transmission is mediated by predators and saprophagous organisms destroying dead bodies of infected beetles. In cases of eventual transovarial infection the microsporidium appears automatically in larvae.

The cross sections of tubular structures of eventually extruded polar filaments indicate evidence of an autoinfection in early cycles of the pathogen serving for distribution of the infection in the host. This type of spread of the infection was described by Iwano and Ishihara (1991) in infections with *Nosema bombycis*, mediated by thin-walled spores. In our material there is no evidence of thin-walled spores adjacent to the cross sections of filaments.

We consider the subapical, lateral fixation of the polar filament in mature spores as a typical feature of our microsporidium, appearing in all spores. The same type of fixation appears also in other Microspora such as in the genus *Baculea* (Loubes and Akbarieh 1978), *Microfilum* (Faye et al. 1991) and *Desportesia* (Issi and Voronin 1986). In all of these cases all other structures in spores and stages in sporogony are different. Two other microsporidia with eventual subapical fixation of the polar filament are *Endoreticulatus* and *Nosemoides*. In *Endoreticulatus* (Brooks et al. 1988) some subapical fixation can be recognized in figures of spores of *E. schubergi* as presented by Cali and El Garhy (1991), but the development in sporogony is with multiple division and groups of sporoblasts and spores in sporophorous vesicles. *Nosemoides vivieri* (Vinckier 1975) has an oval spore where the hemispherical anchoring disc is attached subapically and the polar filament is coiled in 10 posterior coils. The sporogony again is with multinucleate sporogonial plasmodia and sporoblasts formed in rosettes. This type of sporogony differs from the microsporidium in *P. spinidens* and also different is the anchoring disc which is flat and not inserted in a depression of the spore wall.

Among Microspora infecting bark beetles *Unikaryon minutum* Knell et Allen, 1978 is the closest related to the pathogen in *P. spinidens*. It has oval to cylindrical spores 0.9 × 2.3 μm and its development in *Dendroctonus frontalis* in Mississippi, USA is very similar to our pathogen. It differs in that it was not reported from the infected gonads and that the spore has a rather thick endospore. The type of fixation of its polar filament is identical with that in *P. spinidens*. Questionable is the insertion of *U. minutum* into the genus *Unikaryon* if published data on ultrastructures are compared. The definition of the genus *Unikaryon* was given by Canning et al. (1974) on the basis of a limited material in optical microscopy of spores on smears and on the basis of a single species. It defines the genus as "a microsporidium with nuclei unpaired throughout the life cycle, in schizonts, sporonts, sporoblasts and spores, with sporonts dividing into 2 sporoblast each. The parasite develops diffusely in the cytoplasm and does not stimulate production of a cell hypertrophy tumor. Parasites in Platyhelmiths". On the basis of further studies Azevedo and Canning (1987) redefined the essential characters of the genus, but did not include any ultrastructure of the spore in the diagnosis although they presented a detailed drawing of the spore of *Unikaryon* (Fig.15). Of the species listed in the genus *Unikaryon,* four are parasites of Trematoda, one infects Crustacea (Table 2) and four are

Table 1. Microsporidia reported from bark beetles.

Pathogen	Host	Tissue	Spore (µm)	Author
Nosema typographi	*Ips typographus*	fat body	3.6-5.3 × 2-3.5	Weiser 1955
Nosema curvidentis	*Pityokteines curvidens*	fat body	2.5-3.6 × 1.5-2.0	Weiser 1961
Chytridiopsis typographi	*Ips typographus*	midgut	1-1.5 × 1.5	Weiser 1955
Pleistophora scolyti	*Scolytus scolytus*	midgut	3.0 × 2.0	Weiser 1968
Stempellia scolyti	*S. scolytus* *S. ensifer* *S. pygmaeus* *S. multistriatus*	midgut	2.6-3.6 × 1.0-2.0	Lipa 1968
Nosema scolyti	*Scolytus scolytus* *S. multistriatus*	Malpighian tubuli, midgut	4.0-5.0 × 2.0-3.3	Lipa 1968
Nosema dendroctoni	*Dendroctonus pseudotsugae*	Malpighian tubuli, fat body	2.6-3.0 × 2.0-1.0	Weiser 1970
Unikaryon minutum	*Dendroctonus frontalis*	muscle, fat body, midgut, Malpighian tubuli	2.0-2.5 × 0.8-1.0	Knell, Allen 1978
Nosema dryocoeti	*Dryocoetes autographus*	fat body, lymphocytes	2.5-4.0 × 1.2-1.5	Purrini, Ormieres 1981
Pleistophora xyloteri	*Xyloterus domesticus*	midgut, oenocytes	2.5-3.0 × 1.2-1.5	Purrini, Ormieres 1981
Nosema calcarati	*Pityogenes calcaratus*	male, female gonads	3.5-5 × 2.5-3.0	Purrini, Halperin 1982
Canningia spinidentis	*Pityokteines spinidens*	fat body, muscle, gonads, Malpighian tub.	1.9-2 × 0.8-1.0	this paper

parasites of beetles. More detailed comparison of spore structures splits the group into two distinct morphological formations: the first includes mainly the species infecting trematode larvae, the second the parasites of the bark beetles. The species in Trematoda have all a flat anchoring disc and a rather thick endospore, whereas parasites of bark beetles have elongate to tubular spores with hemispherical anchoring discs of their filament fixed subapically in the endospore. Microsporidia of chrysomelid beetles remain with the pathogens of flukes as they have also flat anchoring discs, but their spores are broad oval to spherical. There are further details to be elucidated such as the sporophorous vesicle appearing during sporogony in *U. legeri* which does not appear in other species in trematodes or in beetles. The rule of a short filament with 6/7 coils is not found in both species with piriform spores. In *U. piriformis* the extruded polar filament is 130 µm long, corresponding with at least 15 to 18 coils. In *U. slaptonleyi* where the filament is 145 µm long, the filament is coiled in 17–21 coils. The genus *Unikaryon* seems to be a heterogenous group and we propose to split it into two types, one for pathogens of trematodes and the other for pathogens of bark beetles. The pathogens of chrysomelid beetles and Crustacea remain provisionally with *Unikaryon*. We propose the following definitions of both genera:

Genus *Unikaryon* Canning, Lai et Lie, 1974. Nuclei unpaired, single in all stages of schizogony, binary fission. Sporogony with one single binary fission, electron dense surface coat of sporonts. Spores oval to pyriform, polar filament isofilar, with plate-like flat anchoring disc fixed apically, centrally. Bipartite polaroplast, lamellar, anterior part composed of broad flat sacs adhering to the anchoring disc. Posterior part made of losely packed shorter sacs. Sometime thickened endospore. Parasites of trematodes or chrysomelids.

Type species: *U. piriformis* Canning, Lai et Lie, 1974.

Comment: Emendatio based on discussion and figures in Canning et al. (1974), Canning and Madhavi (1977) and Azevedo and Canning (1987) (Fig. 15A).

Genus *Canningia* gen. n.

Schizogony uninucleate, binucleate during division, meronts single, in direct contact with host cytoplasm, without sporophorous vesicle. Electron-dense cover on sporonts thin. Spores long oval to tubular, polar filament isofilar, with 5 – 7 coils, fixed subapically, laterally. Anchoring disc globular. Polaroplast binary,

Table 2. Species in the genera *Unikaryon* and *Canningia*.

Pathogen	Host	Author	Spore size (µm)	Shape	Anchor. disc	Coils
Unikaryon piriforme	*Trematoda*	Canning, Lie, Lai 1974	3.8 × 2.7	pyriform	flat	18/21
Unikaryon legeri	*trematoda*	Canning, Nicholas 1974	2.8 × 1.6	oval	flat	6/7
Unikaryon allocreadii	*Trematoda*	Canning, Madhavi 1977	3.5 × 2.7	oval	flat	
Unikaryon slaptonleyi	*Trematoda*	Canning, Barker, Mammond, Nicholas 1983	5 × 2.8	pyriform	flat	17/21
Unikaryon bouixi	*Coleoptera Chrysomel.*	Toguebaye, Marchand 1983	1.6-2.5× 1.4-1.6	broad oval	flat	3/4
Unikatyon matteii	*Coleoptera Chrysomel.*	Toguebaye, Marchand 1984	3.7 × 1.9	oval	flat	5/12
Unikaryon euzetii	*Coleoptera Chrysomel.*	Toguebaye, Marchand 1988	2.2 × 1.4	oval	flat	7/9
Unikaryon mytilicola	*Crustacea*	Durfort, Vallmitiana, Vivares 1980		oval		
Canningia spinidentis	*Coleoptera Scolytidae*	this paper	1.9-2 × 0.8-1.0	tubular	bulbose	5/6
Canningia minutum	*Coleoptera Scolytidae*	Knell, Allen 1978	2.0-2.5 × 0.8-1.0	tubular	bulbose	6/6

lamellar, anterior part composed of thin layers of flat sacs, adhering to the anchoring discs. Posterior part with shorter broader lamellae. Endospore wall attenuated at the anchoring pit. Parasites of bark beetles.

Type species: *Canningia spinidentis* sp. n. (Fig. 15B). The name proposed is in honour of the distinguished protozoologist and specialist in research of microsporidia, Prof. Elizabeth U. Canning, Imperial College, London, UK. Both genera remain in the family Unikaryonidae, Sprague 1977.

Canningia spinidentis sp. n.

Type host and site of location: *Pityokteines spinidens* (Coleoptera: Scolytidae), fat body, Malpighian tubules, muscles, tracheal matrix, connective tissue and ovaries. In larvae, pupae, adults and eggs.

Type locality: Flatz, near Neunkirchen, South of Vienna, Austria, on fir trees, *Abies alba*.

Transmission: Contamination of food and galleries with infected feces; transmission via the egg.

Interface: Vegetative stages in direct contact with cytoplasm of host cells till spore maturation. Irregular empty zone around each spore during maturation. No immune reaction in tissues.

Development: Uninucleate schizonts (3 × 4 µm) divide immediately into merozoites (3–3.5 µm). Sporonts without thickenings of their plasmalemma, undergoing one division. Sporoblasts single, uninucleate, thin-walled, with dense ribosomes, with a single central nucleus. Spores long oval to tubular, (1.9–2 × 0.8–1 µm) with broad rounded ends. Polar filament attached subapically, laterally, with a globular anchoring disc adhering to a depression of the endospore. Spore wall 100–150 nm thick. Polar filament isofilar, short, with 5/6 coils in a single row. Polaroplast bipartite, anterior with long fine lamellae closed in a membrane, posterior with shorter lamellae with broader meshes. Posterosome with persistent oval coil of Golgi tubules and a posterior vacuole (visible in fresh spores). In late schizonts nuclei with sections of chromosomes. Autoinfection with spores with normal wall.

Canningia minuta (Knell et Allen, 1978) differs in spore size and thickness of spore wall, in details of structures of the polaroplast and the posterosome (see Fig. 15). It infects the midgut and does not infect the ovaries. Details must be reconfirmed on fresh material.

Acknowledgements. The authors wish to thank Prof. J. Vávra, Prague and Prof. R. Larsson, Lund for critical reading of the manuscript and helpful suggestions and comments. This report is part of a project supported by the Federal Ministry of Science and Research of Austria.

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Received 29 August 1994

Accepted 9 February 1995