J. Appl. Ent. 126, 148–154 (2002)

ISSN 0931-2048

Unikaryon polygraphi sp.n. (Protista, Microspora): a new pathogen of the four-eyed spruce bark beetle *Polygraphus poligraphus* (Col., Scolytidae)

J. Weiser[1], U. Händel[2], R. Wegensteiner[2] and Z. Zizka[3]
[1]Insect Pathology, Institute of Entomology, Academy of Sciences of the Czech Republic, Ceské Budejovice, Czech Republic; [2]Institute of Forest Entomology, Forest Pathology and Forest Protection, University-BOKU-Vienna, Wien, Austria; [3]Department of Electron Microscopy, Institute of Microbiology, Academy of Sciences of the Czech Republic, Praha, Czech Republic

Ms. received: May 17, 2000; accepted: January 25, 2001

Abstract: The microsporidium *Unikaryon polygraphi* sp.n., a pathogen of *Polygraphus poligraphus* in Austria is described based on light microscopic and ultrastructural characteristics. All life stages have isolated nuclei. Sporogony ends with uninucleate single sporoblasts and spores. Mature oval spores measure 2.5–3.0 μm × 1.0–1.5 μm. The larger spores (3 × 1.5 μm) belong to the 'early spore type' with a polar filament coiled in five turns and the smaller spores (2.5 × 1 μm) with polar filament coiled in 6/7 turns belong to the 'environmental spore type'. Columnar cells of the midgut, longitudinal and circular muscles and the secretory part of Malpighian tubules of adult beetles are infected. Mature spores are excreted together with the faeces.

1 Introduction

The four-eyed spruce bark beetle *Polygraphus poligraphus* (L.) is a secondary pest of Norway spruce (*Picea abies* (L.) Karst.) during innocuous phase, but after large snow-breaks or storm events this species may develop into outbreak phase. In such cases, *P. poligraphus* is able to attack old vigorous trees together with *Ips typographus* (L.), causing considerable economical damage (Postner, 1974). Its flight activity is from late April to early May and in Central Europe it develops usually two generations per year. After laying one complement of eggs, the parental beetles emerge and attack uninfested parts of the affected tree and establish a second brood ('sister generation') which hibernates. They usually accompany the *Ips* bark beetles and often their second galleries overlap the border galleries of *I. typographus*.

In different bark beetles microsporidia cause only occasional damage and limitation of field populations. Their distribution was studied recently by Weiser et al. (1995, 1998). The pathogens belong to the genera *Nosema*, *Pleistophora*, *Chytridiopsis* and *Unikaryon*. *Chytridiopsis* is typical and common for many bark beetles, infecting cells of the midgut epithelium and forming thick-walled cysts which are unique among microsporidia. Among microsporidia that are more specialized for a single host are species belonging to the Unikaryonidae, with two known species, *Unikaryon minutum* in *Dendroctonus frontalis* (Knell and Allen, 1978) and *Unikaryon montanum* in *I. typographus* (Weiser et al., 1998). The closely related *Canningia spinidentis* infects *Pityokteines spinidens* (Weiser et al., 1995). These species primarily infect the midgut; for some it is the principal organ infected whereas in others the midgut infection is healed and the microsporidian proceeds within the host body to the skeletal muscles, the gonads and the fat body. The type species of Unikaryonidae was described from Trematode larvae (Canning et al., 1974) and species from Trematoda are slightly different from species infecting beetles.

Investigations of larvae, pupae and adult bark beetles have brought evidence that diseases appear mainly in adult beetles, especially in black or dark-stained ones. These beetles have had time to move around in free galleries with remains of faeces of beetles of the former generation and this is the presumed way of horizontal transmission (Wegensteiner and Weiser, l996).

2 Materials and methods

Log segments of Norway spruce were cut from a trap tree infested with *P. poligraphus* and *I. typographus* in a managed forest (Kreisbach, Lower Austria, 500 m a.s.l.). The segments were incubated in a rearing chamber in an insectary at 20 ± 2°C and long day conditions (16 h light : 8 h dark). Emerging bark beetles were removed daily and stored in an incubator at 15°C until inspection (at max. 1 week).

Only living beetles were dissected according to the method of Wegensteiner et al., 1996. The gut was removed together with parts of the adhering fat body and the gonads and was

 0931–2048/2002/2602–0148 $ 15.00/0

inspected under a light microscope. In case of infection the distribution of the spores in different organs was determined and dry smears were prepared from crushed organs. They were fixed with methanol and stained with Giemsa. Spores were measured with an ocular micrometer in water mounts and after Giemsa staining (magnification 1000×). Microsporidia-infected tissues were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.2) at 4°C for 12 h, washed in buffer and post-fixed in 1% osmic acid at 4°C for 1 h. After dehydration in a descendent series of ethanol and acetone the material was embedded in Vestopal W (Chemische Werke Huls Co., Switzerland). Ultrathin sections were contrasted in uranyl acetate and lead citrate and inspected in a Philips EM300 TEM (Philips Co., the Netherlands).

3 Results

3.1 Prevalence and pathogenicity

The microsporidian was found only in adults with hardened callow cuticle. All inspected larvae, pupae and callow adults were without evidence of infection. The prevalence in the dissected group of adult beetles ($n = 316$) was 0.6%. Only one type of microsporidian was present, infecting the columnar cells of the midgut, the circular and longitudinal muscles of the midgut and the Malpighian tubules. The infected zones in the midgut are the area of crypts (Plate 1, fig. 4) in the medial part and the posterior part of the midgut. The microsporidia are concentrated in the area of the basal membrane and form individual local groups there. The coeca (Plate 1, fig. 5) are infected only in sporadic stages, without massive development in their depth. The posterior part of the midgut and the hindgut (Plate 1, fig. 1) have the spores arranged in irregular circular rows with all spores arranged in one direction. In many situations groups of haemocytes were found adhering to the basal membrane of the gut and eventually absorb the extruded filaments and released germs. Masses of spores are located in the muscularis of the gut (Plate 1, fig. 4), forming long columns of densely aggregated spores in the space of the muscle fibres, which were dissolved during the invasion of the microsporidian. The Malpighian tubules were infected in the massive cells of the secretory part (Plate 1, fig. 2), the thin walls of the absorbent basal part were not infected. Nevertheless, the spores were mixed with crystals of secretions in the lumen of this part and transported with the secretion to the hindgut, leaving the gut with the faeces. The haemocytes associated with development of the microsporidian, which were injected from the gut, transport the spores around the host body cavity. They infect the Malpighian tubules from the haemocoel. With absence of motility in all stages of microsporidia, the infection of the Malpighian tubules from the midgut, against the direction of motion of the secretions, seems to be difficult. The spores produced are stored in the tissues to which they have been brought during the primary infection and there is a steady release of viable spores in the faeces of the infected animal. The presumed pathogenic effect is a decreasing motility of the midgut and a reduced secretory activity of the Malpighian tubules. There was no direct evidence of any other symptom indicating the infection.

3.2 Pre-sporal stages

The development of the microsporidian as seen using the light microscope appears to be rather simple; in native smears the stages of merogony are quite rare. They are minute uninucleate oval to spherical bodies, 2–3 μm in diameter, without any present formation of plurinucleate stages. Sporoblasts are elongated bodies measuring 2 μm × 3 μm. Spores (Plate 1, fig. 3) are single, free, short tubular to oval bodies, 2.5–3 μm long and 1–1.5 μm wide (in water mount). On Giemsa-stained smears, the measurements of length were reduced to 2–2.5 μm. In ultrathin sections we found in our material meronts (M; Plate 2, fig. 8) with a badly fixed interior and a thin plasmalemma forming the wall of the stage. The sporonts (Sn; Plate 2, fig. 7) have their cell wall thickened with electron-dense deposit, closed in a fine vesicle (arrowheads in Plate 2, fig. 7). In the material prefixed under field conditions, we found multiple oval coils of membranes with some plasmatic interspace, which have the size and shape of spores (M, Plate 2, figs 7, 8.T, Plate 2, figs 6, 9). They probably were the remains of autolysed young spores.

Sporoblasts (Sb, Plate 2, fig. 9) are rare, they keep the thickened, slightly undulated outer wall of the exospore (e) and a thin area of deposition of the electron-lucent endospore. In mature spores the regular thin layer of the exospore (e) is closed in the same vesicle (arrowhead, Plate 3, fig. 13), which was evident in the sporont, and the internal layer of the endospore is of equal thickness with an attenuated part between the area of the anchoring disc and the spore wall. The electron-lucent endospore (E, Plate 3, figs 10–13) adheres tightly to the exospore, sometimes the interface is not apparent (Plate 3, figs. 10, 12), whereas in other spores the exospore is a very distinct layer. In most spores the sporoplasm is condensed as a result of fixation and between the spore wall and the plasm is a rather large empty zone (x, Plate 3, figs 11–13). The polar filament is fixed to the anchoring disc with a broadened dense area, which fills the arch of the anchoring disc. From there the filament crosses the polaroplast and is coiled in the posterior part of the spore in an irregular coil with 5 or 7 turns with a distinct tilt. The short filament is isofilar and is connected with the products of the Golgi system in the posterior vacuole ('posterosome' in Weiser and Zizka, 1975). The posterior vacuole usually has an empty zone (fixation artefact) with remains of the tubules of the Golgi system. The polaroplast in the apical end is a binary structure, with a dense lamellar outer cover zone and a tubular interior mass. In Plate 3, figs 12 and 13 (P) the outer zone is activated during fixation, the lamellae are melted and the material forms clusters of irregular masses of electron-dense material in the former zone of the lamellae. This arrangement is typical in spores prepared to eject the filament. The circular part of the anchoring disc, the umbrella, is

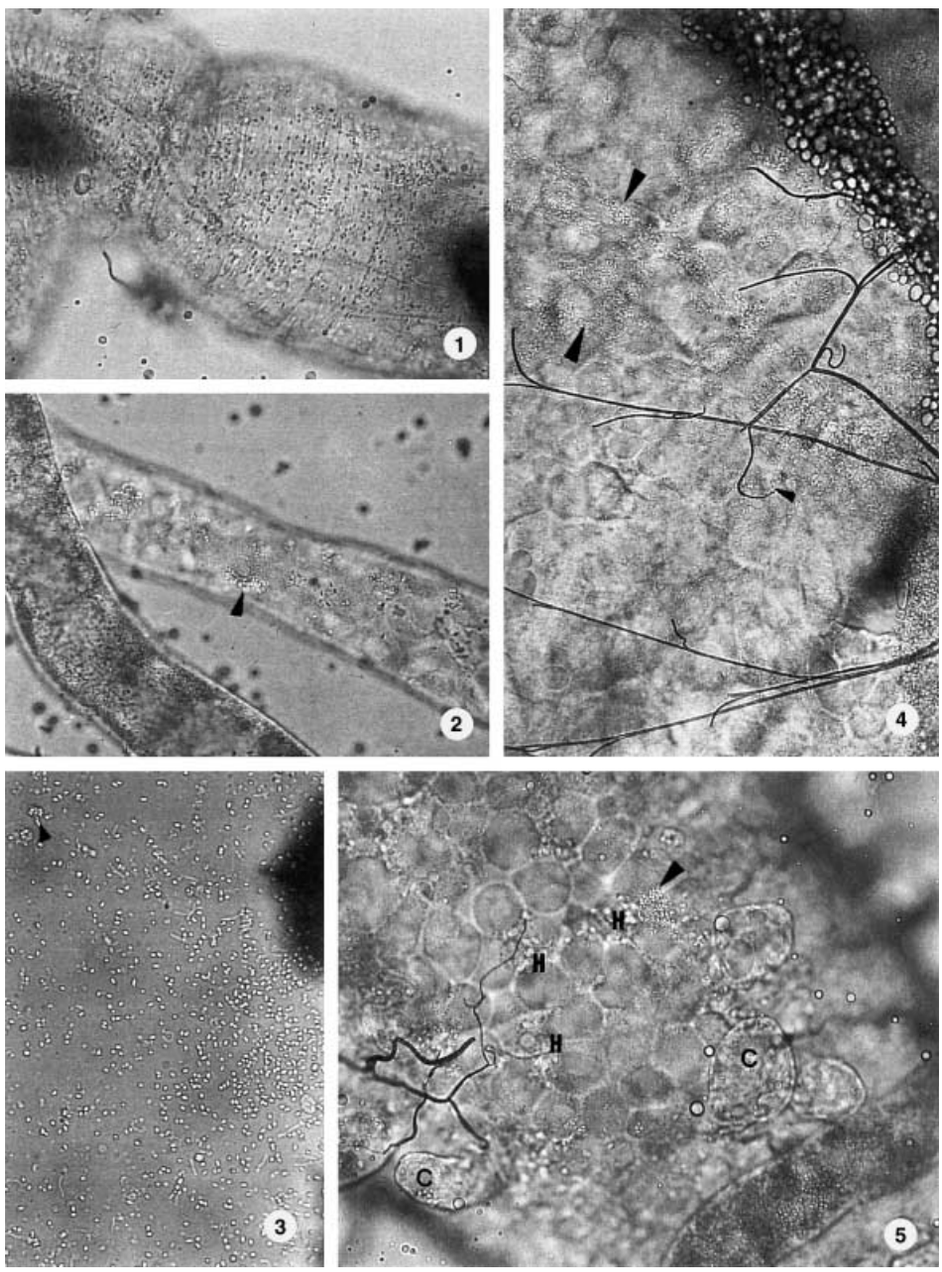

Plate 1. fig. 1. Unikaryon polygraphi *spores in the wall of the posterior part of the gut of* Polygraphus poligraphus. *Spores are arranged in regular lines due to the peristaltics of the gut wall. Magnification: 400×;* ***fig. 2.*** *Malpighian tubule with spores in individual cells of the secretory part of the tubule. Arrowhead indicating the empty space of the nucleus in the infected cell. The absorbing basal part with thin wall is without infection. Magnification: 400×;* ***fig. 3.*** *Compressed central region of the midgut of* P. poligraphus *with Unikaryon polygraphi in the muscularis. Individual muscle fibres are filled with spores in rows (arrowheads), irregular groupings of spores are in columnar cells. The end cells of tracheoles (T) are not infected. Magnification: 400×;* ***fig. 4.*** *Spores of* U. polygraphi *released from infected midgut cells. Spores are single except cases (arrowhead) phagocytized in a blood cell. Magnification: 400×;* ***fig. 5.*** *Part of the midgut of* P. poligraphus *with groups of spores of* U. polygraphi *in epithelial cells and coeca (C). Numerous haemocytes (H) adhere to the surface of the gut. Magnification: 400×*

indistinct in most sections of the spores. The single nucleus adheres to the polaroplast complex and ribosome strands are indistinct. In the ultrathin sections the two types of spores, those with 6/7 and those with 5 turns of the filament, do not differ from each other in size and construction of the interior. In sections of the midgut we found groups of empty spores among normal ones and part of the empty spores was filled with coiled filaments.

4 Discussion

4.1 Pathology and distribution

The infection is located in the midgut and is present also in the Malpighian tubules. The spores in the columnar cells are localized in depth, at the basal membrane surface. This location is perfect for contamination of the muscularis of the gut with germs from extruded polar filaments. The orientation of the

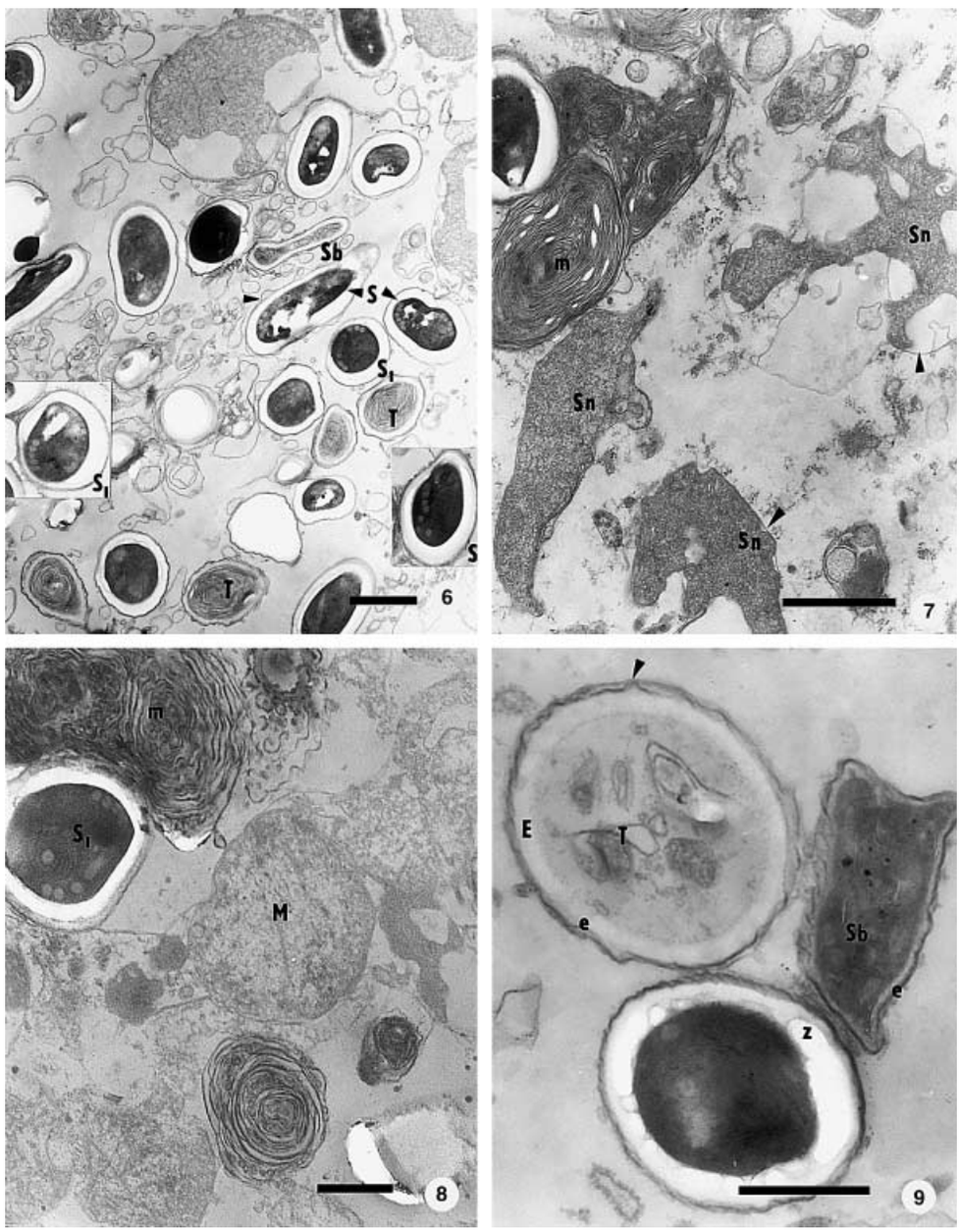

Plate 2. fig. 6. *Group of spores of* U. polygraphi *in the infected midgut cell. Young spores (Sb), teratospores (T) and mature spores of two types: S persistent type with 7 turns of the polar filament and Sl 'early' (primary) type spores with 5 turns of the filament. Bar = 1 μm;* ***fig. 7.*** *Late merogonial stages (early sporonts) (Sn) of* U. polygraphi *closed in a plasma membrane (arrowhead). Complicated membranous wicks (m) in the depleted host cell. Bar = 1 μm;* ***fig. 8.*** *Meronts of* U. polygraphi *(M) and mature early spore (Sl), one sporont (Sn) and a membranous wick in the depleted midgut cell. Bar = 500 nm;* ***fig. 9.*** *Sporoblast (Sb) with electron-dense deposit in the wall (e), teratological spore (T) with membranous structures in the interior, regular endospore (E), exospore (e) and exospore derived membrane (arrowhead). Bar = 500 nm*

spores is in some areas arranged by peristaltic motion of the midgut and the active contractions of muscles give the spores a chance to reach the muscularis muscle fibres with extruded polar filaments. During the development in muscles large masses of spores are produced, filling the fibres. (Plate 1, fig. 4, arrowheads). At the same time, groups of haemocytes (H, Plate 1, fig. 5) are infected in the manner that was demonstrated in infections with microsporidia (David and Weiser, 1994) and the generation developing into spores inside the haemocytes spreads germs in tissues to which they are fixed during their further circulation. In the cases studied it was only in the system of midgut and Malpighian tubules where injected germs were able to develop. There was no infection in the end cells (astrocytes) of tracheoles on the surface of the midgut, which are the target of primary development of microsporidia infecting the fat body (Weiser, 1957; in *Nosema lymantriae*). The spores which are present in the hindgut faecal pellets are spores released from the infected cells of the midgut and the spores coming with urate secretions from infected Malpighian tubules.

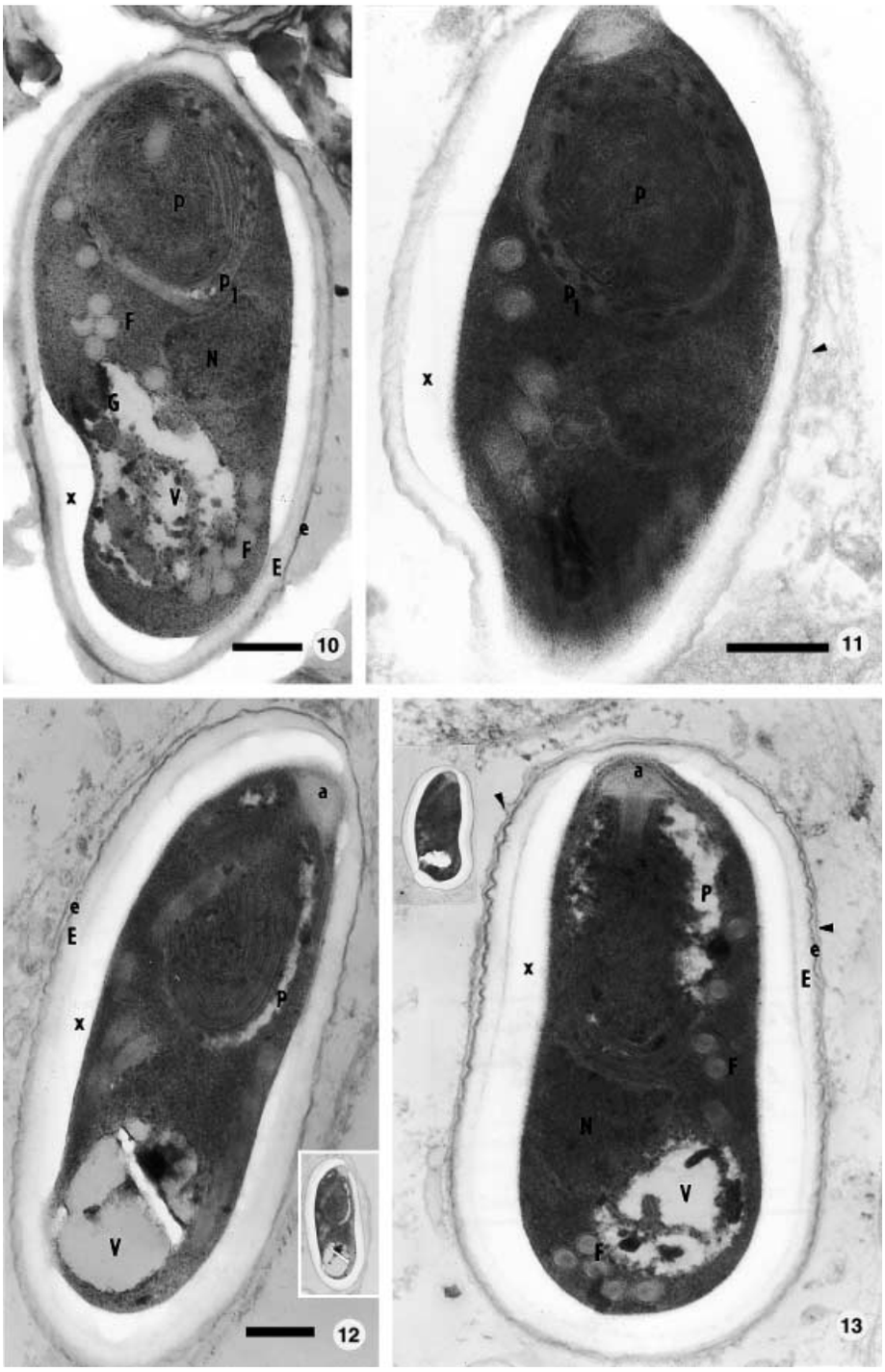

Plate 3. fig. 10. *Immature persistent spore of* U. polygraphi. *Endospore (E) closed in a soft exospore (e). The sporoplasm is constricted in a circular empty space (x). The outer polaroblast (Pl) is a continuous layer of dense lamellae enclosing the internal, tubular or broad lamellar part (P). Nucleus (N) is located on the side of the posterior vacuole (V) with products of the Golgi system (G). Isofilar polar filament is coiled in 6/7 turns. Bar = 200 nm;* ***fig. 11.*** *Immature early spore with plastic wall, circular empty space (x), visible polaroplast (P, Pl) and polar filament in 5 coils. On outer surface partly developed exospore derived membrane (arrowhead). Bar = 200 nm;* ***fig. 12.*** *Spore of* U. polygraphi *in lateral view with anchoring disc (a) with subapical location. Polar filament isofilar with 5 coils and posterior vacuole (V) with minor deposit. Spore wall with plastic endospore (E) and wrinkled exospore (e) without distinct derived membrane. Bar = 200 nm;* ***fig. 13.*** *Mature spore in saggital section with regular, anteriorly attenuated endospore (E) and exospore with finely wrinkled surface (e) and distinct electron dense surface membrane (arrowhead). The empty space around the sporoplasm (x) is regular, the exospore directly adheres to the anchoring disc (a) only at the anterior end. Outer layer of the polaroplast is vacuolized and the polar filament (F) is isofilar and coiled in 5 turns. Single nucleus (N) and posterior vacuole (V) with Golgi products are in the posterior half of the spore. Bar = 200 nm*

Environmental spores in faeces cause infections to adult beetles that inhabit the galleries of the colony during the time of hardening of their chitinous cuticle. In the larval stages, pupae and callow adults the infection was not present.

4.2 Two spore types

The ultrathin sections reveal two types of spores which are not distinguishable in water mounts of fresh material; namely spores with 6/7 turns of the coiled polar filament and spores with 5 turns. Lack of other differences in ultrastructures of both types of spores support the idea of Larsson (Vavra and Larsson, 1999) that 'early spores' which extrude their filaments in the host and use this process for propagation of the microsporidian inside the host, are in fact young spores of the 'persistent' type which later, when not emptied, develop to spores that resist environmental conditions and serve the spreading and survival of the pathogen in the environment. In other similar cases, such as in *Unikaryon montanum* Weiser et al. (1998) or *Unikaryon ixodis* Weiser et al. (1999), spores which are identified as 'early' spores have a minor number of turns in the coil of the polar filament and are shorter than spores indicated as 'persistent' or 'environmental'. This is analogous to the structures of spores in our material. The large empty space inside the maturing spore, dividing the sporoplasm on most of its surface from the spore wall is a fixation artefact, but it indicates the autonomous 'management' of the sporoplasm inside the final rigid wall of the spore, with one exception: the anchoring disc is fixed firmly to the apical pole of the spore and communicates with outer conditions.

4.3 Vegetative stages

The vegetative stages, schizonts and sporonts are rather rare in smears (Plate 1, fig. 3) and in ultrathin sections. One characteristic feature in this microsporidian is the outer thin electron-dense sporophorous vesicle which is formed at the end of merogony and is evident also around spores after maturation (Plate 3, fig. 13). It is not present on each stage in sporogony and spore formation, but it is present in well-fixed mature spores. In available materials it is evident that the thickening of the wall of the formed sporont appears rather late, at a moment when the electron-lucent endospore is also formed. The multiplication of stages in merogony is rather slow and multinucleate stages are not seen in our material. There is no evidence that in sporogony, the sporont undergoes several divisions.

4.4 Taxonomic classification

Single uninucleate minute spores are typical for Unikaryonidae. All Microsporidia of the genus *Unikaryon* have similar spore wall structures as summarized in Weiser et al. (1995). The thin exposure is lining the electron-lucent layer without interspace and most probably is acting as a cortical impregnation. They have the binary polaroplast with a dense lamellar outer layer and tubular inner coil. The outer layer undergoes different changes in connection with the type of fixation or the stage of activation. Among the Unikaryonidae Sprague, 1977 with genera *Unikaryon*, *Oligosporidium* and *Orthosomella* (Sprague et al., 1992) and *Canningia* (Weiser et al., 1995) are several species in the genus Unikaryon, described from helminths (including the type species) as differing in spore shape and host parazitation from species that attack beetles. The pathogens of Trematodes have more oval spores whereas the pathogens of beetles have short tubular spores with rounded ends. In both categories there is some tendency to move the fixing point of the anchoring disc to a subapical position and this is used as a specific marker in the genus *Canningia* (Weiser et al., 1995). The individual Unikaryonidae of beetles, especially bark beetles, differ mainly in specificity for host insects, in their location in the insect body and in some ultrastructures in their spores (table 1).

Evidently there are only minute differences in spore size, but important differences in the number of turns of the coiled filament, different hosts and different locations of the infection in these hosts. The polaroplast is binary, but the structure of both its regions is different. The differences between *U. montanum* in *I. typographus* and our *Unikaryon* in *P. poligraphus* are important. Both organisms differ in spore size and in the number of cross-sections in the coil of the polar filament. They also differ in details of organization of the spore. They eventually live close to each other in

Table 1. *Some differences among* Unikaryon *in bark beetles and ticks*

			Tissue						
Microsporidian	Host	Spore size (in μm)	Turns Pf	Gut	Malp.	Mus.	Gon.	Fb	Egg
Canningia spinidentis	*Pityokteines spinidens*	1.9–2 × 0.8-1	5/6	–	+	+	+	+	+
Unikaryon minutum	*Dendroctonus frontalis*	2–2.5 × 0.8-1	6/6	+	+	–	–	+	–
Unikaryon montanum	*Ips typographus*	2 × 1	7/8	–	+	+	+	+	+
		1.5 × 1	5/6						
Unikaryon ixodis	*Ixodes ricinus*	2.5 × 1.5	6	+	+	+	+	+	+
		1.6–2 × 1.4	3/4						
Unikaryon polygraphi	*Polygraphus poligraphus*	2.8 × 1	6/7	+	+	+	–	–	–
		2.5–2 × 1	5						

Gut = midgut, Malp. = Malpighian tubules; Mus. = muscles (segmental muscles where gut is negative, and midgut muscularis layer in where gut is +); Gon. = ovary or testes; Fb = fat body; Egg = egg follicles in ovary; Pf = polar filament.

colonies in the same tree, but during our studies pathogens occurring in associated bark beetles (Haidler et al., 1998), both microsporidia did not appear in adjacent colonies of beetles. We therefore consider these two microsporidia to be different and propose *Unikaryon* in *Polygraphus poligraphus* in Austria as a new species, *Unikaryon poligraphi* sp.n.

Acknowledgements

This research was conducted as part of the Special Research Program Forest Ecosystem Restoration (SFB008). The authors gratefully acknowledge the funding by the Austrian Science Foundation and the Ministry of Agriculture and Forestry.

References

Canning, E. U.; Lai, P. F.; Lie, K. J., 1974: Microsporidian parasites of trematode larvae from aquatic snails in West Malaysia. J. Protozool. **21,** 19–25.

David, L.; Weiser, J., 1994: Role of hemocytes in the propagation of a microsporidian infection in larvae of *Galleria mellonella*. J. Invertebr. Pathol. **63,** 212–213.

Haidler, B.; Wegensteiner, R.; Weiser, J., 1998: Pathogen occurrence in associated bark beetles (Coleoptera, Scolytidae). In: Book of Abstracts, 6th Eur. Congr. Entomol. Brunnhofer, V.; Soldan, T. (eds.) Ceske Budejovice, Czech Republic, August 23–29, p. 668.

Knell, J. D.; Allen, G. E., 1978: Morphology and ultrastructure of *Unikaryon minutum* sp. n. (Microsporida, Protozoa), a parasite of the southern pine beetle *Dendroctonus frontalis*. Acta Protozool. **17,** 271–278.

Postner, M., 1974: Scolytidae (= Ipidae), Borkenkäfer. In: Die Forstschädlinge Europas, Bd. 2, Ed. by Schwenke, W. Hamburg, Berlin: Parey, 334–482.

Sprague, V.; Becnel, J. J.; Hazard, E. I., 1992: Taxonomy of Phylum Microspora. Crit Rev. Microbiol. **18,** 285–395.

Vavra, J.; Larsson, R., 1999: Structure of the Microsporidia. In: The Microsporidia and Microsporidiosis. Ed. by Wittner, M.; Weiss, L. M. Washington, DC, USA: Am. Soc. Microb, 7–84.

Wegensteiner, R.; Weiser, J., 1996: Occurrence of *Chytridiopsis typographi* (Microspora: Chytridiopsida) in *Ips typographus* L. (Coleoptera, Scolytidae) field populations and in a laboratory stock. J. Appl. Ent **120,** 595–602.

Wegensteiner, R.; Weiser, J.; Führer, E., 1996: Observations on the occurrence of pathogens in the bark beetle *Ips typographus* L. (Coleoptera, Scolytidae). J. Appl. Ent. **120,** 199–204.

Weiser, J., 1957: Mikrosporidien des Schwammspinners und der Goldafter. Zschr. Angew. Entomol. **40,** 509–527.

Weiser, J.; Zizka, Z., 1975: Stages in sporogony of *Plistiphora debaisieuxi* Jirovec (Microsporidia). Acta Protozool. **14,** 185–194.

Weiser, J.; Rehacek, J.; Zizka, Z.; Ciampor, F.; Kocianova, E., 1999: *Nosema slovaca* Weiser et Rehacek, 1975 and *Unikaryon ixodis* (Weiser, 1957) comb.n. in ixodid ticks. Acta Parasitol. **44,** 99–107.

Weiser, J.; Wegensteiner, R.; Zizka, Z., 1995: *Canningia spinidentis* General et sp. n. (Protista: Microspora), a new pathogen of the fir bark beetle *Pityokteines spinidens*. Folia Parasitol. **42,** 1–10.

Weiser, J.; Wegensteiner, R.; Zizka, Z., 1998: *Unikaryon montanum* sp.n. (Protista: Microspora), a new pathogen of the spruce bark beetle, *Ips typographus* (Coleoptera: Scolytidae). Folia Parasitol. **45,** 191–195.

Authors' address: Prof. Dr R. Wegensteiner, Institute of Forest Entomology, Forest Pathology and Forest Protection, University-BOKU-Vienna, Hasenauerstrasse 38, 1190 Wien, Austria