

# Light and Electron Microscope Studies on *Jirovecia involuta* sp. nov. (Microspora, Bacillidiidae), a New Microsporidian Parasite of Oligochaetes in Sweden

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## SUMMARY

The new microsporidium *Jirovecia involuta* is described, based on light and electron microscopy preparations. The host was the oligochaete *Limnodrilus hoffmeisteri*, collected from a stream in southern Sweden. All developmental stages are diplokaryotic. Mature spores are cylindrical, with a short tail-like projection of exospore material. The spore wall has a uniform exospore. The polaroplast has two regions, anteriorly with closely packed lamellae, posteriorly with tubules. The polar filament has a wide, straight anterior portion, and an approximately equally long, narrow posterior section, forming one coil. Each spore is enclosed in a double-layered sac-like structure, formed by the sporoblast from exospore material. The identity of the species and some traits on the ultrastructural cytology are discussed.

## Introduction

The microsporidia of the family Bacillidiidae Larsson, 1986, with cylindrical spores, are primarily parasites of aquatic oligochaetes. They are usually easily identified from the great size of the spores, the distinct, elongate diplokaryon, and from the presence of a "manubrium", a stiff, straight and wide anterior portion of the polar filament. These characters are obvious both in fresh and stained smears. Until the present time 9 species have been described from oligochaetes, and one species each from copepods, midge larvae and fish. The family is divided into two genera, which are distinguished by the shape of the spore. *Bacillidium* species have spores with blunt ends, or with a somewhat pointed posterior pole. In species of *Jirovecia* the terminal part of the spore is prolonged to a thin, tail-like structure, in some species longer than the spore itself. Few publications have dealt with the supraspecific taxonomy of these microsporidia [6, 8, 10, 18, 25].

There are few ultrastructural investigations on the cytology and development of *Bacillidium-Jirovecia* species. There are five reports on *Bacillidium* species [10, 12–13, 21, 24] and five on *Jirovecia* [2, 5, 15–16, 24]. The most thorough investigation treated *Jirovecia* (*Mrazekia*)

*brevicauda*, a parasite of midge larvae, with focus on the morphogenesis and morphology of the polar filament, illustrated by a series of excellent electron micrographs [2].

*Jirovecia* species are now and then observed in oligochaetes in southern Sweden. In the summer of 1987 two specimens in a sample of *Limnodrilus hoffmeisteri* were infected with a *Jirovecia* species, morphologically similar to *J. brevicauda* at the light microscopic level. The different size of the spores and systematically distant hosts indicated that the species were different, and further differences appeared during the ultrastructural study. The Swedish species is new to science. It is named *Jirovecia involuta* and described here. Some traits on the ultrastructural cytology and the taxonomy of the species are discussed.

## Material and Methods

The microsporidium was present in two specimens of *Limnodrilus hoffmeisteri* Claparède, 1862 (Oligochaeta, Tubificidae) (slide series no. 870713-L2 and L4 RL), collected in the small stream Höje å, close to the village of Esarp, in Scania, Sweden.

Fresh squash preparations were prepared by the agar method of Hostounský and Žižka, and studied using phase contrast micro-

copy and dark field illumination [3]. A number of spores ejected their polar filament spontaneously by the squashing. Permanent squash preparations were lightly air-dried and fixed in Bouin-Duboscq-Brasil solution for at least one hour. For paraffin sectioning infected segments were fixed in the same fixative overnight, washed and dehydrated in an ascending series of ethanol, cleared in butanol, and embedded in paraplast. Sections were cut longitudinally at 10 µm. Smears and sections were stained using Giemsa solution and Heidenhain's iron haematoxylin. For details on the histological techniques used see Romeis [17]. Permanent preparations were mounted in DePeX. Measurements were made with an eye-piece micrometer at  $\times 1000$ .

For transmission electron microscopy infected 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 45 hours. After washing in cacodylate buffer and post fixation in 2% (w/v) osmium tetroxide in cacodylate buffer for one hour at 4°C, the pieces were washed and dehydrated in an ascending series of buffer-acetone solutions to absolute acetone, and embedded in epon. Sections were stained with uranyl acetate and lead citrate.

## Abbreviations

A	= anchoring apparatus
AC	= collar-like part of anchoring apparatus
AP	= pad-like part of anchoring apparatus
C	= chromatin
CP	= centriolar plaque
D	= diplokaryon
EN	= endospore
EX	= exospore
FA	= anterior part of polar filament
FP	= posterior part of polar filament
G	= Golgi apparatus
H	= holotype
M	= mitochondrion
N	= nucleus
P	= plasma membrane
PL	= lamellar part of polaroplast
PS	= polar sac
PT	= tubular part of polaroplast
R	= ribosomes
S	= sac-like spore envelope
T	= mitotic spindle tubule
V	= posterior vacuole

## Results

### Pathology

The microsporidium developed in the chloragogen tissue, the cellular cover on the coelomic side of the digestive tract (Fig. 1). Microsporidia-filled cells were greatly hypertrophic and cyst-like (Figs. 1–2), with microvillus-like surface projections (Fig. 4). They were pluri-nucleate with up to 8 nuclei visible in the same sectioned cell. The nuclei were compressed, and like the mitochondria, dislocated to the peripheral zone of the host cell (Figs. 3–4). Presporal stages of the microsporidium were accumulated at the periphery, while the centre was filled with densely packed and fairly regularly arranged mature spores (Fig. 4).

### Presporal stages

The most immature life-cycle stages observed were plasmodia with diplokaryotic nuclei (Fig. 5). Their nucleoplasm and cytoplasm were fairly electron-dense, and there were numerous free ribosomes in the cytoplasm. The plasmodia were limited by a ca 8 nm thick unit membrane. In a number of plasmodia dividing nuclei were seen. They had bundles of ca 18 nm wide microtubules in a plane parallel to the contract area between the nuclei (Fig. 5). This stage was interpreted as the merogonial plasmodium. The bouts of merogony and the number of merozoites produced by each plasmodium is unknown. Sectioned plasmodia with more than two diplokarya were not seen.

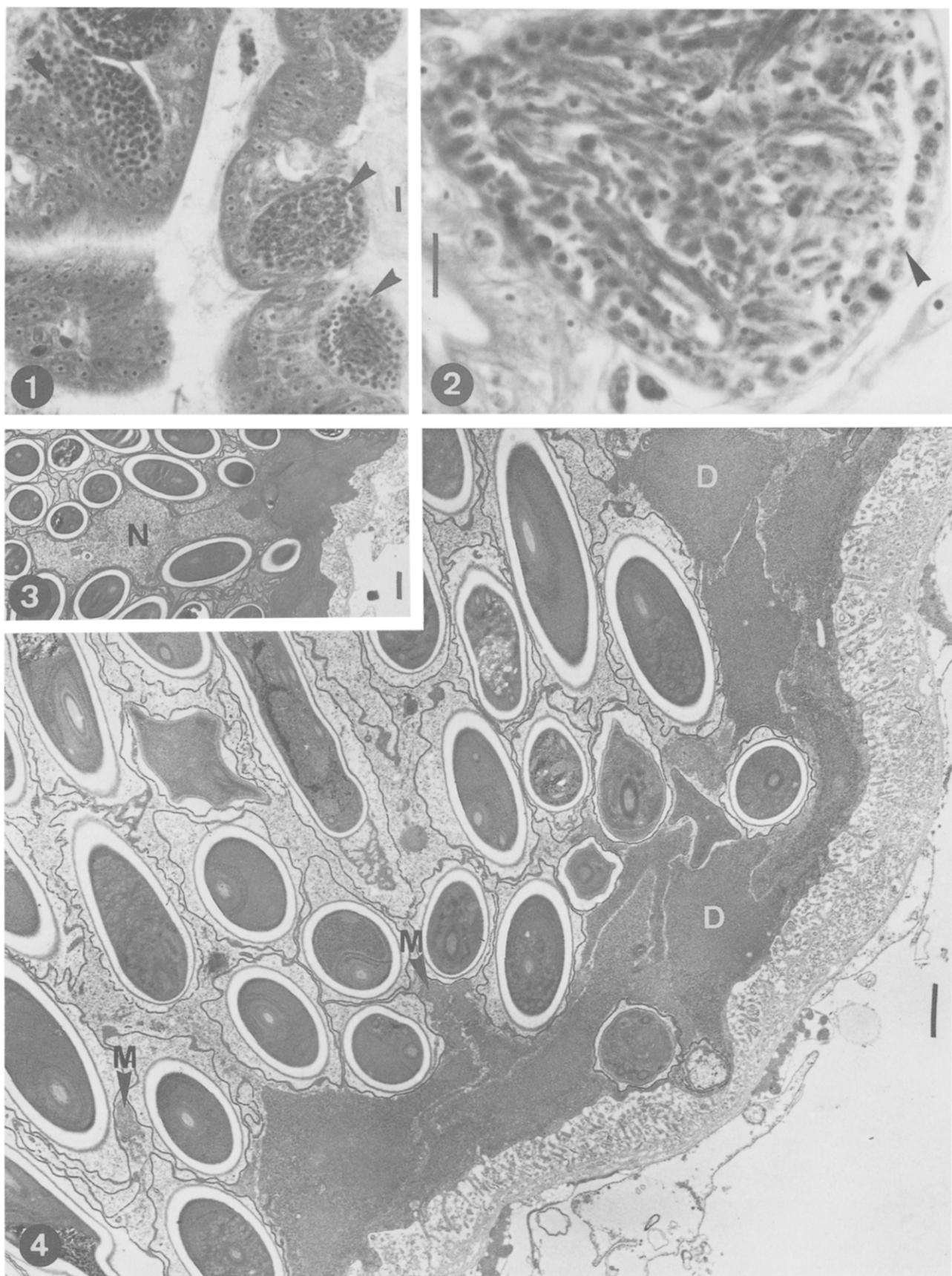
The daughter-cells, more or less rounded, 5–7 µm wide, merozoites, were grouped (Fig. 7). They had a normal ca 8 nm thick plasma membrane. The cytoplasm was less dense than in the plasmodial stage, and the ribosomes were to a great extent associated with membranes, which enveloped the nuclei. There were also rounded aggregates of vesicles of the type interpreted as Golgi apparatuses (Fig. 7). More than one apparatus was never seen in the same section.

The nuclei were coupled as a central great diplokaryon. The widest diameter of sectioned diplokarya, measured perpendicularly to the plane of apposition, was 4 µm. The nucleoplasm was mottled from material of different electron densities. The translucent areas were sectioned, ca 18 nm wide, mitotic spindle tubules, associated with dark isles of chromatin (Fig. 6). The envelope of the nuclei was a double unit membrane with pores. Centriolar plaques appeared as electron-dense shallow depressions (Fig. 7). The widest sectioned plaque measured 140 nm. There were no signs of meiotic division.

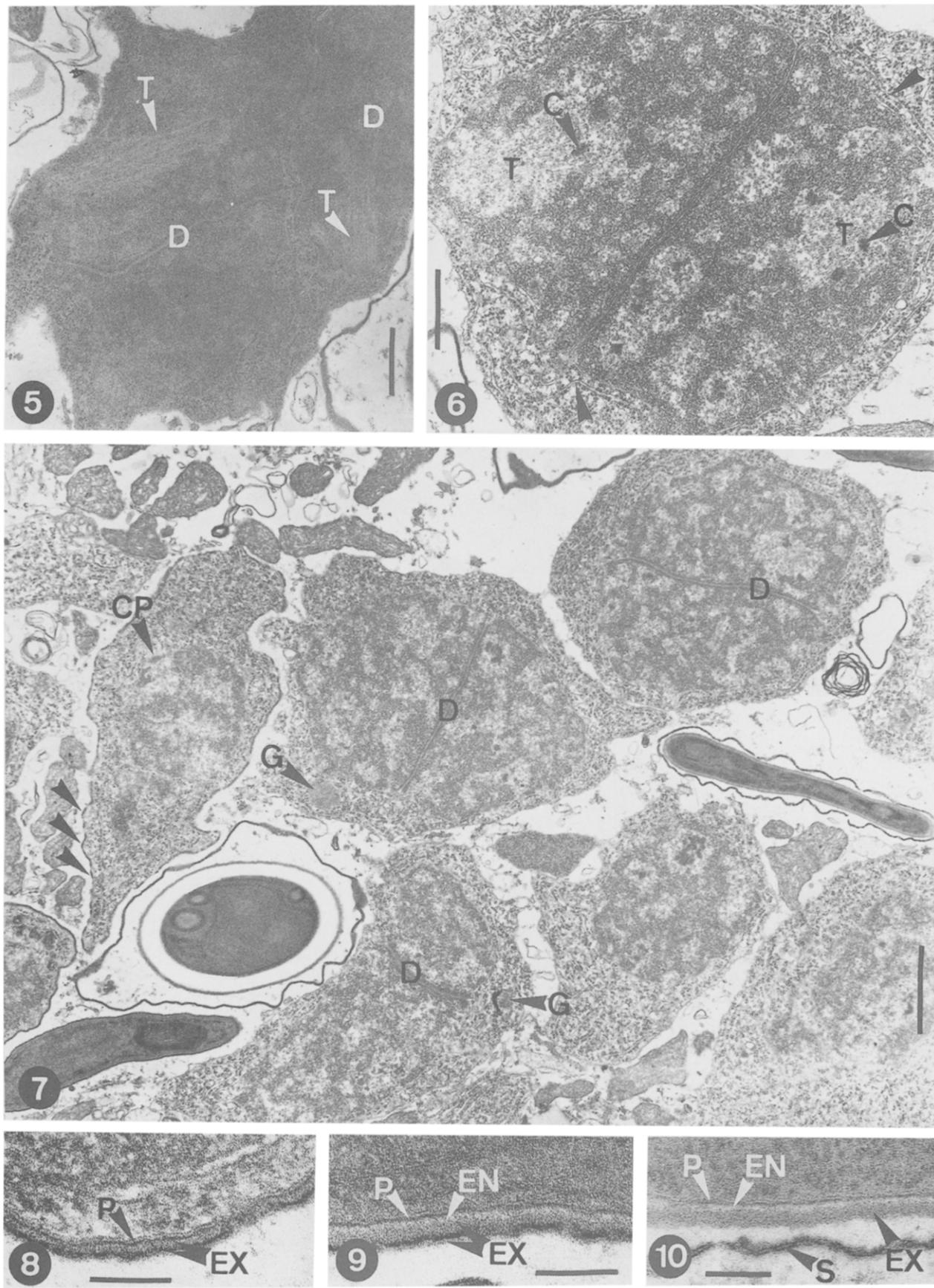
The merozoites matured to sporonts, and in the cells with dividing nuclei, the plasma membrane was covered with ca 10 nm thick patches of electron-dense material (Fig. 7). The release of sporoblasts was not observed, but as sporoblasts and spores were frequently seen in pair-wise associations, the sporogony was apparently disporoblastic, like the normal for Bacillidiidae.

The newly formed sporoblasts were irregular cells, which successively reached a more elongate shape, and at the same time, the cytoplasm became more electron-dense (Fig. 19). The organelles of the spore were initiated in the normal way for microsporidia.

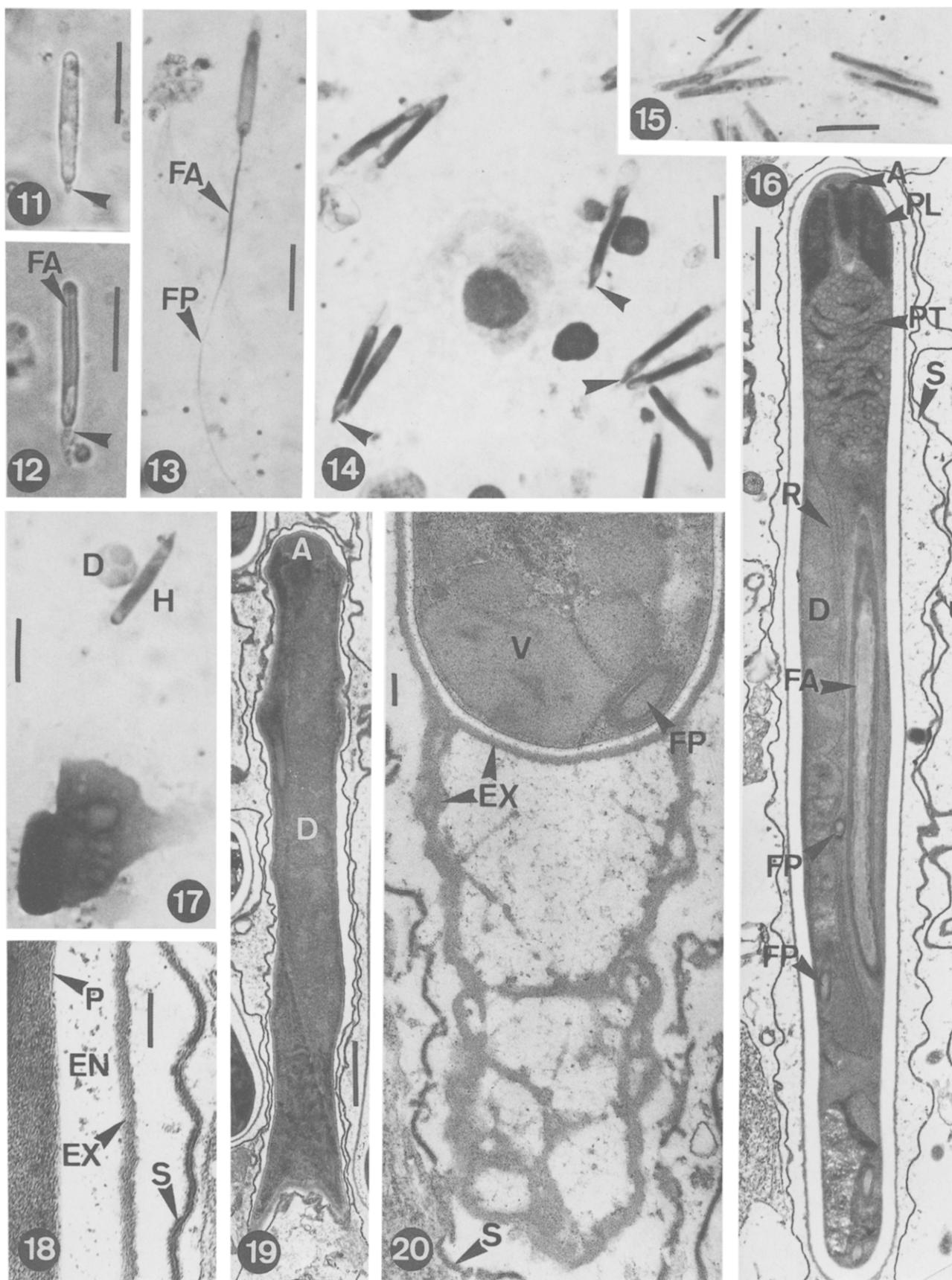
The envelope of the newly formed sporoblast was a plasma membrane covered with a ca 10 nm thick, uniform, electron-dense layer. Later on a moderate electron-dense substance was laid down between the plasma membrane and the dense cover, forming a ca 17 nm thick sporoblast wall (Fig. 8). In the next phase translucent material was added, the primordium of the endospore, which separated a two-layered exospore from the plasma membrane (Fig. 9). At this stage the sporoblast wall varied somewhat in thickness, measuring up to 41 nm. Still at the unpolarized sporoblast stage the electron-dense cover lost contact with the internal layer of the exospore, forming a ca 10 nm thick sac (Fig. 10). The surface material was uniformly electron-dense when initiated, but was successively re-



Figs. 1–4. Pathology of *Jirovecia involuta*. – Fig. 1. Chloragogen cell (arrow-heads) filled with microsporidia. – Fig. 2. Hypertrophic chloragogen cell with immature microsporidia at the periphery (arrow-head), mature spores in the centre. – Fig. 3. Ultrathin section close to the periphery of a chloragogen cell, showing one compressed host nucleus. – Fig. 4. The periphery of an infected cell, showing the stratification of immature and mature microsporidia, the microvillus-like surface projections, and the accumulation of host cell mitochondria at the periphery. Figs. 1–2. Haematoxylin staining. Scale bars: Figs. 1–3 = 10 µm; Fig. 4 = 1 µm.



Figs. 5–10. The presporal development. – Fig. 5. Merogonial plasmodium, showing diplokarya with mitotic spindle tubules. – Fig. 6. Diplokaryotic merozoite with intranuclear spindle tubules associated with areas of chromatin. Arrow-heads indicate the arrangement of the membrane-associated ribosomes – Fig. 7. Group of merozoites maturing to sporonts. Arrow-heads indicate accumulation of electron-dense material on the sporont wall. – Figs. 8–10. The successive development of the layers of the spore wall and the initiation of the sac-like envelope of sporoblasts and spores. Scale bars: Figs. 5–6 = 0.5  $\mu\text{m}$ ; Fig. 7 = 1  $\mu\text{m}$ ; Figs. 8–10 = 100 nm.



arranged to a two-layered structure similar to a ca 10 nm thick unit membrane (Figs. 7–10). After the delamination of the surface layer the sporoblast wall measured ca 40 nm.

#### The mature spore

The cylindrical mature spores had blunt ends, but carried a narrow posterior projection (Figs. 11–12). Unfixed spores measured  $2.1\text{--}2.5 \times 16.5\text{--}18.2 \mu\text{m}$ , except for the projection, which measured  $0.9\text{--}1.0 \times 1.4\text{--}2.1 \mu\text{m}$ . The dimensions of fixed and stained spores and spore projections were  $1.2\text{--}2.1 \times 15.0\text{--}17.3 \mu\text{m}$ , and ca  $0.6 \times 1.2\text{--}1.7 \mu\text{m}$ .

The spore wall was 119–179 nm thick. The thickness was fairly constant in each spore, although reduced to approximately half the size at the front end. The spore wall exhibited the normal three layers: an internal ca 8 nm thick plasma membrane, a translucent endospore layer of variable size, and a uniform, moderately electron-dense, 28–34 nm thick exospore (Fig. 18). Each spore was enclosed in a ca 10 nm thick, double-layered envelope, produced from the surface layer of the exospore by delamination (Figs. 10, 16). The relations between the posterior projection and the spore wall were not visible using light microscopy. Ultrathin sections revealed that the projection was a sac-like structure of fairly irregular shape. It was formed from exospore material of approximately the same dimensions as the exospore, and it was traversed by exospore material (Fig. 20).

The polar filament had an anterior, 302–371 nm wide, straight part, about 3/4 of the spore length, a short median section with successively reduced diameter, and a narrow, ca. 160 nm wide, final portion, which touched the posterior pole, turned around, and continued in anterior direction approximately to the middle of the spore (Fig. 16). The diameter of the wide, straight filament widened slightly in posterior direction (Fig. 13). In the ejected polar filament the wide and narrow parts were approximately equally long (Fig. 13). The longest ejected, stained filament measured ca 36  $\mu\text{m}$ . The filament passed through the centre of the spore to the level of the nuclei, and then followed the spore wall to the posterior pole (Figs. 16, 23, 24).

The polar filament exhibited a series of cylindrical layers of varying thickness and electron density (Figs. 21, 27). In the wide, straight part the three external layers had approximately constant thickness: an external ca 5 nm thick unit membrane, a ca 14 nm wide, fairly electron-dense layer, and a ca 6 nm thick translucent zone (Fig. 27).

The next layer, the fourth, was the widest, 28–64 nm. It had a characteristic mottled appearance. The electron density increased towards the periphery, sometimes giving the impression of a distinct dark, peripheral band. In the 159–194 nm wide centre lucent and dense material were mixed. This region had a basic structure of concentrical layers of material with small differences in electron density. In addition distinct dense material was present in the very centre and close to the periphery (Fig. 27). In longitudinal sections the external dense material looked like an irregular, perforated cylinder (Figs. 16, 21). In transverse sections it appeared either as a continuous ring-like structure, or it was broken into symmetrically arranged spots (Figs. 23–25, 27). The different configurations in transverse sections were not characteristic for specific levels in the spore. Close to the anchoring apparatus the material of the external translucent layer united with the internal translucent zone (Fig. 21).

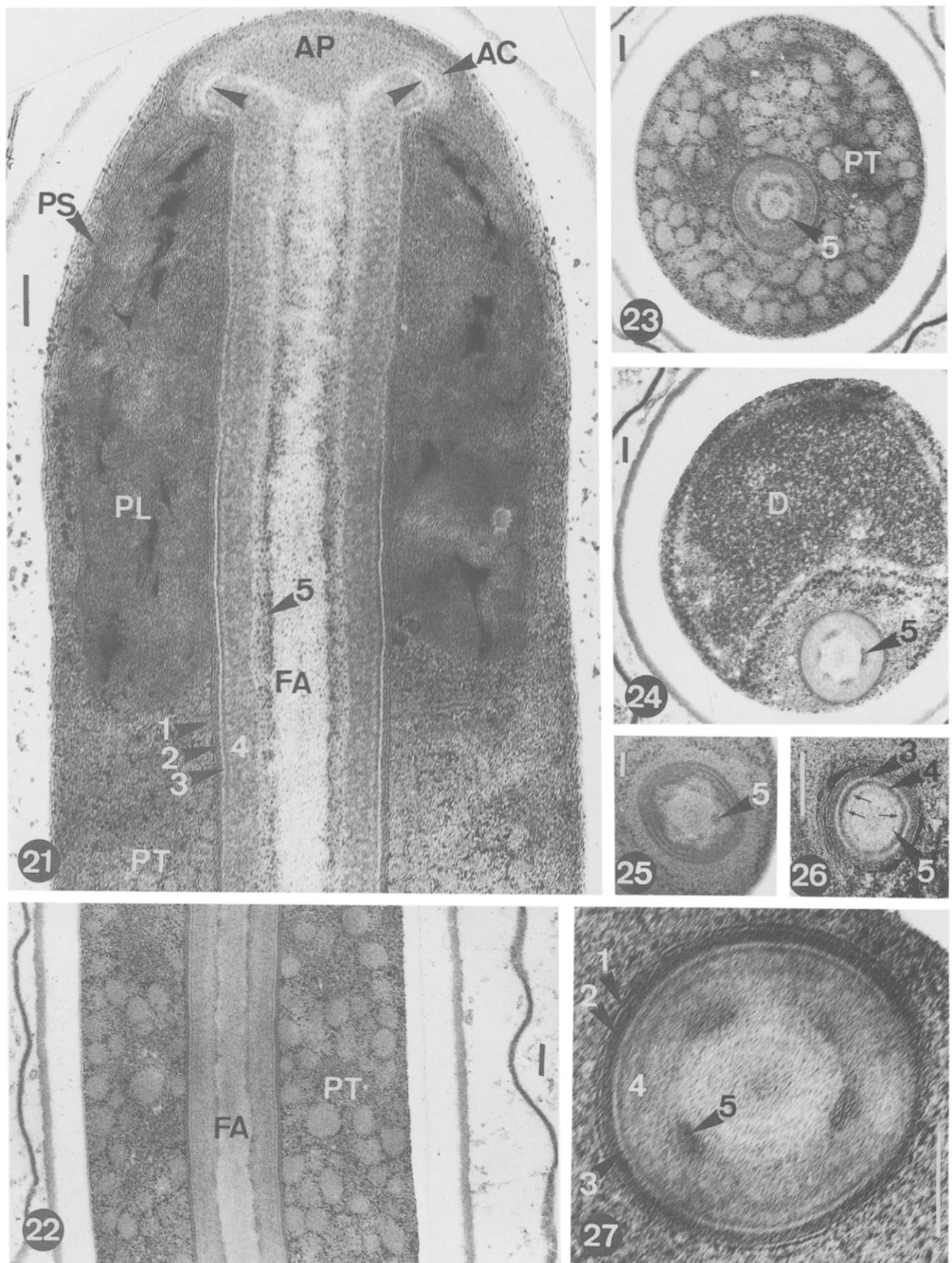
The internal organization of the narrow filament differed in some respects. The mottled layer was reduced to a narrow, uniformly dense zone, and the dense material at the periphery of the centre was reduced to a narrow band (Fig. 26). The lucent material enclosed between these bands appeared as sectioned longitudinal fibrils.

The anchoring apparatus had two components: a central, moderately electron-dense, up to 540 nm wide pad, and a collar-like, up to 562 nm wide, convex, peripheral part, with a lamellar structure (Fig. 21). The translucent centre of the filament widened anteriorly and attached to the pad, while the mottled layer was connected to the collar, although with a zone of translucent material in between (Fig. 21).

The two regions of the polaroplast had compartments limited by a ca 5 nm thick unit membrane (Fig. 21). The anterior region was lamellar, with so closely packed lamellae that the compartments lacked a lumen. This part was about 1/10 of the spore length. The second region had tubular, 43–106 nm wide, compartments (Fig. 22). It was about 1/3 of the spore length, and followed closely the anterior polaroplast. The posterior end tapered. The polar sac was limited by a ca 5 nm thick unit membrane, identical to the membrane of the polar filament and the polaroplast. It was filled with a dense, granular substance. The umbrella-like peripheral part extended backwards to the middle of the anterior polaroplast (Fig. 21).

The eccentric diplokaryon was long and narrow, with an external flat side, separated from the spore wall by a narrow strand of cytoplasm, and a concave internal side (Figs. 16, 24). The diplokaryon of the immature spore was almost as long as the spore (Fig. 19). In mature spores the

◀ Figs. 11–20. Gross morphology of the spore. Figs. 11–14, 16–20. *J. involuta*; Fig. 15. *Bacillidium criodrili*. – Figs. 11–12. Unfixed mature spores. Arrow-heads indicate the posterior projection. – Fig. 13. Mature spore with ejected polar filament. – Fig. 14. Squash preparation showing coupled spores. Arrow-heads indicate the posterior projection. – Fig. 15. Mature spores of *B. criodrili*. – Fig. 16. Longitudinally sectioned mature spore. – Fig. 17. Localization of the holotype, slide no. 870713-L2-2 RL. – Fig. 18. The spore wall and sac-like envelope of a mature spore. – Fig. 19. Longitudinally sectioned immature spore. – Fig. 20. The posterior end of a mature spore. The posterior projection is formed by exospore material. Figs. 13–15. Haematoxylin staining. Scale bars: Figs. 11–15, 17 = 10  $\mu\text{m}$ ; Figs. 16, 19 = 1  $\mu\text{m}$ ; Figs. 18, 20 = 100 nm.



diplokaryon was about half as long as the spore, and it was localized to the middle of the spore (Fig. 16). The fixed and stained diplokaryon of mature spores measured 7.6–9.2  $\mu\text{m}$  long.

The cytoplasm was electron-dense, with prominent strands of membrane-associated ribosomes, surrounding the nuclei, the polaroplast and the polar filament (Fig. 16). The posterior region of the spore was usually not correctly fixed and appeared spongy (Fig. 16). However, there were posterior membrane-lined compartments, filled with granular, dense material (Fig. 20). It was probably a system of small posterior vacuoles, not a single, lobed vacuole.

## Discussion

### *On the Identity of the Species*

Of the six species of *Jirovecia*, all but *J. brevicauda* have spore projections of the size of the spore or longer. The spores of *J. brevicauda* measured 1.40–1.50  $\times$  20–22  $\mu\text{m}$  in the material used for the description, and they had a 3.5  $\mu\text{m}$  long tail [11]. In the material studied by Götz, the spores were more variable in size, 1.6–3.2  $\times$  16–25.6  $\mu\text{m}$ , and they had considerably longer tails, 4.0–9.6  $\mu\text{m}$  [2]. The spores and spore projections of *J. brevicauda* are greater than the spores and spore projections of the Swedish species, which speaks for the species being different. As *J. brevicauda* is a parasite of midge larvae, the distant hosts indicate further differences.

The species treated herein is superficially similar to *Bacillidium criodrili*, the type species of *Bacillidium* [4]. The spores of both species are widest at the level of the diplokaryon in the middle of the spore (Figs. 14–15). *B. criodrili* (Fig. 15) differs from the other *Bacillidium* species by having spores with pointed posterior end. However, the spores lack a tail-like posterior projection. The spores of *B. criodrili* measured 1.6  $\times$  20–22  $\mu\text{m}$  according to the description [4]. Jírovec investigated the species in greater details, using material provided by Janda. He found spores of three size classes: 1.2–1.4  $\times$  15.5–17  $\mu\text{m}$ , 1.4–1.5  $\times$  18–20  $\mu\text{m}$  (dominant), and ca 1.6  $\times$  24–25  $\mu\text{m}$  [6]. Whether the dimensions relate to fresh or fixed spores is unclear. The Swedish microsporidium has spores of similar length to the smaller size class, but are distinctly smaller than the dominant size class. They are also smaller than the spore size mentioned in the description [4]. The small but distinct difference in shape, the fairly clear difference in size, and a probable difference in the polar filament indicate that the species are different. Jírovec

found that spores of *B. criodrili* only ejected the straight, wide manubrium-like part of the polar filament [6]. The explanation is probably that *B. criodrili* either has a very short narrow posterior portion of the filament, or lacks a narrow filament completely. The species described here has a fairly long narrow part, which in stained smears has similar length to the wide, anterior part (Fig. 13).

### Cytology

A series of publications on the ultrastructure of *Bacillidium*-*Jirovecia* species appeared about 25 years ago [12, 13, 15, 16, 21, 24]. They all focused on the polar filament, the most resistant organelle of the spore, which survives in a fairly good state of preservation, even under conditions where other organelles of the spore normally are destroyed. None of the papers described the polaroplast, and Puytorac, who commented on the absence of a polaroplast, suggested that the polar filament had an analogous role [16]. Only two publications have given a complete picture of the organization of the mature spore of the genera *Bacillidium* and *Jirovecia* [2, 10].

The polar filament of a microsporidium has a basic organization of electron-dense and -translucent layers [9, 23]. The filament of *Bacillidium* and *Jirovecia* species follows the scheme with some modifications. The wide, mottled layer (Figs. 21, 27:4) seems to be unique to these microsporidia, and the variable arrangement of the dense material close to the centre (Figs. 23–25, 27:5) appears to be characteristic, too. It seems further to be characteristic that a zone with successively reduced diameter separates the wide and narrow parts of the filament. A normal anisofilar polar filament is abruptly constricted.

Two previous publications [2, 10] and the present investigation reveal that *Bacillidium* and *Jirovecia* species have a polaroplast divided into two sections (Figs. 21–22). In all three species the lamellae of the anterior part are closely associated to a compact structure, similar to the arrangement described for *Ameson michaelis* (*Nosema* sp.) by Sprague and collaborators [19]. In the posterior part the compartments are either lamellar [10], or like the situation here, tubular (Figs. 21–22).

The exospore of the mature spores of *Bacillidium* and *Jirovecia* is a uniform, electron-dense layer (Fig. 18) [2, 10, 16, 21]. During maturation of the sporoblast, electron-dense material loses contact with the basal layer of the exospore, and it is organized to tubular projections [2, 10], or, as is the case here, forms a complete sac (Figs. 8–10, 16, 19). A number of microsporidia have spores enclosed in individual sac-like structures. In the family Tuzetiidae the sac is a normal sporophorous vesicle, produced by the

◀ Figs. 21–27. The ultrastructure of the polar filament and the polaroplast. – Fig. 21. Longitudinal section through the anterior end of a mature spore. The layers of the polar filament are numbered 1–5 from the surface. Arrow-heads indicate the connection between the translucent layers. – Fig. 22. Longitudinal section through the tubular part of the polaroplast. – Figs. 23–25. Transverse sections through the wide part of the polar filament at different levels. The variable nature of the layer 5 is apparent. – Fig. 26. Transverse section through the narrow part of the filament. Arrows indicate the fibril-looking layer. – Fig. 27. Transverse section through the wide part of the filament at higher magnification. Scale bars = 100 nm.

sporont, and dividing together with the sporoblasts, enclosing each sporoblast in a sporophorous vesicle of its own [7]. In three genera of the *Tuzetia*-like microsporidia, in addition, exospore material is given off from the sporoblast, forming a persistent or transient coat between the sporophorous vesicle and the spore wall [7]. That envelope is obviously an identical structure to the spore-containing sac of the present species. It is further known that the trimorphic species of *Amblyospora* produce a spore morph in copepods, which is enclosed in a sac of uniform electron-dense material [20]. It appears to be a sac, produced by delamination of exospore material from the sporoblast, not a sporophorous vesicle produced by the sporont.

In the present species the tail-like prolongation of the spore is formed by exospore material alone (Fig. 20). A similar condition was observed in a *Jirovecia* species with long-tailed spores (Larsson, unpublished). This is a difference to *Jirovecia (Mrazekia) brevicauda*, where all three layers of the spore wall (plasma membrane, endospore, exospore) are present in the posterior projection [2]. With increased knowledge of the microsporidia of Bacillidiidae, it will probably be necessary to move *J. brevicauda* to another genus. Spore projections are characteristic for a number of microsporidia, and, for example, *Caudospora simulii* [22], *Indosporus spraguei* [14] and *Hirsutosporos austrosimulii* [1] have spore projections formed by exospore material alone, similarly to the microsporidium described here.

## Description

### *Jirovecia involuta* sp. nov.

**Presporal stages:** Plasmodia with diplokaryotic nuclei divide into diplokaryotic merozoites. The number of merozoites and the cycles of merogony are unknown. The regular occurrence of coupled sporoblasts and spores in squash preparations suggests disporoblastic sporogony. Meiotic division was not observed.

**Spores:** Cylindrical with blunt ends. Dimensions: unfixed  $2.1\text{--}2.5 \times 16.5\text{--}18.2 \mu\text{m}$ , fixed and stained  $1.2\text{--}2.1 \times 15.0\text{--}17.3 \mu\text{m}$ . Posterior end with a narrow tail-like projection:  $0.9\text{--}1.0 \times 1.4\text{--}2.1 \mu\text{m}$  (unfixed), ca  $0.6 \times 1.2\text{--}1.7 \mu\text{m}$  (fixed and stained). Spore wall 119–179 nm thick, exospore uniform, 28–34 nm. Polar filament with a 302–371 nm wide, straight part (3/4 of the spore length), and a posterior, ca 160 nm wide, coiled section, reaching the middle of the spore. Polaroplast with two parts: anterior region about 1/10 of the spore length, with closely packed lamellae, posterior section, about 1/3 of the spore length, with 43–106 nm wide, tubular compartments. Polar sac encloses anterior half of anterior polaroplast region. Diplokaryon long and narrow,  $7.6\text{--}9.2 \mu\text{m}$  (fixed and stained), in the middle of the spore. Posterior end with a system of small posterior vacuoles (or, less probably, a great lobed vacuole).

**Sporophorous vesicle:** Absent. Each spore enclosed in a ca 10 nm thick double-layered, persistent envelope, produced at the sporoblast stage by exospore material.

**Type host:** *Limnodrilus hoffmeisteri* Claparède, 1862 (Oligochaeta, Tubificidae).

**Host tissues affected:** Chloragogen tissues, causes hypertrophy.

**Type locality:** Höje å, close to Esarp, Scania, Sweden.

**Type material:** Holotype (Fig. 17) on slide no. 870713-L2-2 RL, paratypes on slides no. 870713-L2-(1–6) RL.

**Deposition of types:** The slide with the holotype in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, D.C., USA. Paratypes in the collections of Dr. J. Weiser, Prague, Czechoslovakia, and in the collection of the author.

**Etymology:** The name alludes to the enveloped spores.

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