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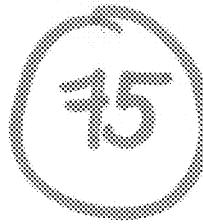
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***Helmichia anomala* sp. nov. (Microspora, Striatosporidae) a new microsporidian parasite of *Microtendipes pedellus* (Diptera, Chironomidae) in Poland**

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Abstract. The new microsporidium, *Helmichia anomala* sp. nov., a parasite of the adipose tissue of the midge larva *Microtendipes pedellus* (Diptera, Chironomidae) is described, based primarily on ultrastructural characteristics. Each diplokaryotic sporont yields eight monokaryotic sporoblasts in a thin-walled sporophorous vesicle containing tubular and granular inclusions. Sporogonial reproduction is by rosette-like budding. The live spores are rod-shaped measuring 1.3 ± 0.2 (0.9–1.7) \times 2.9 ± 0.4 (2.3–4.2) μm . The spore wall consists

of a plasmalemma, a thin endospore, and a two layered exospore 30–34 nm wide. The polaroplast has two subdivisions: tightly packed lamellae and a posterior, probably, spongy zone. The uncoiled isofilar polar filament measured 104–109 nm in diameter. A small number of single diplokaryotic spores and anomalous spores are recorded. Discrimination from other microsporidian species and its systematic position are discussed.

Key words: *Helmichia anomala* sp. nov., Microspora, taxonomy, ultrastructure

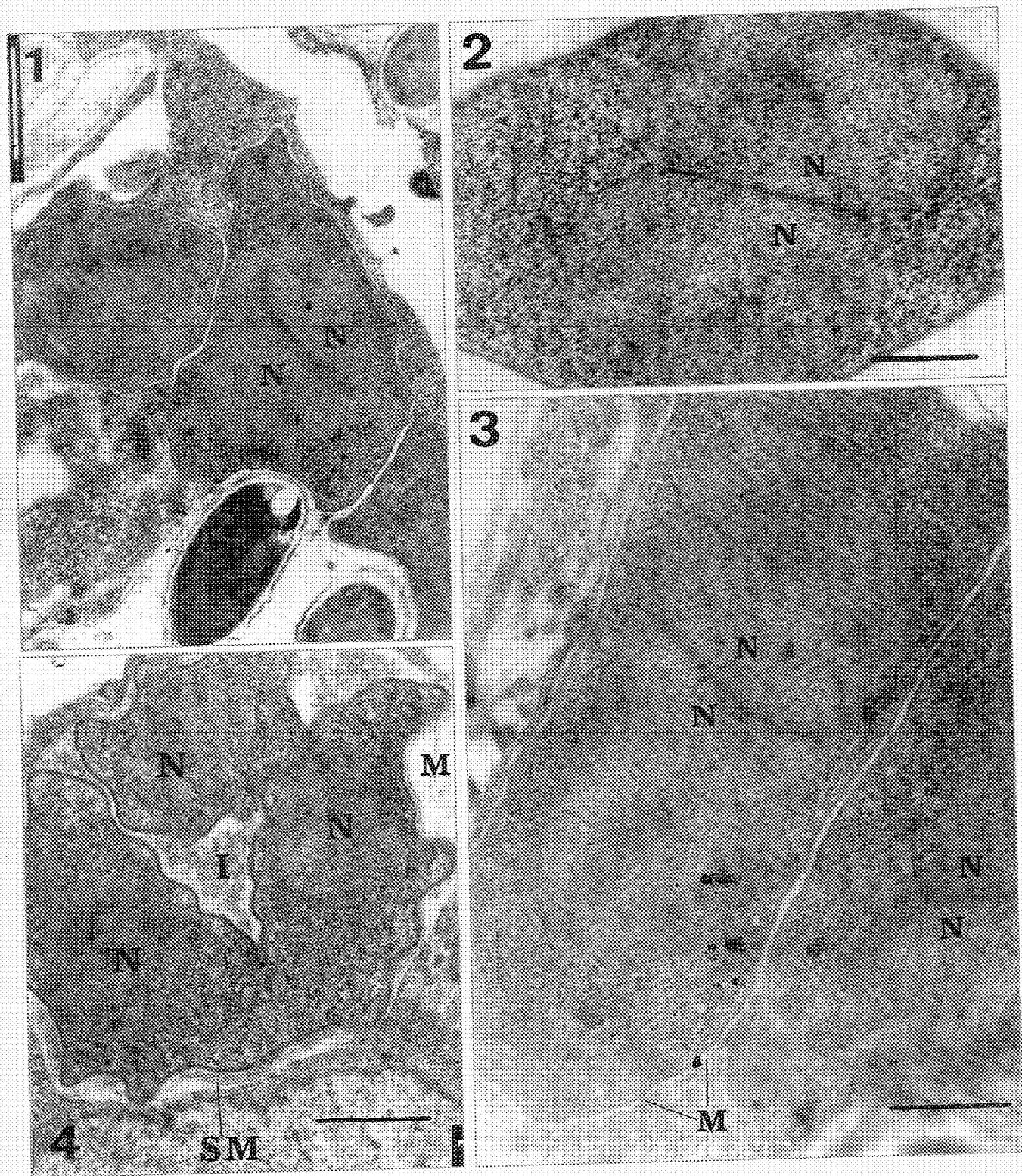
Introduction

In the summer, 1995, a microsporidium with ovocylindrical octospores was found in a midge in a highly polluted pond in the Mazurian region of Poland. The adipose tissue of the midge larva was filled by parasites. The spore organization was briefly described, but the systematic position of the microsporidium was not determined (Ovcharenko *et al.* 1998). Later the microsporidium was studied in detail. The presence of diplokaryotic stages, the octosporoblastic sporogony, the short cylindrical spores having a thin, uncoiled polar

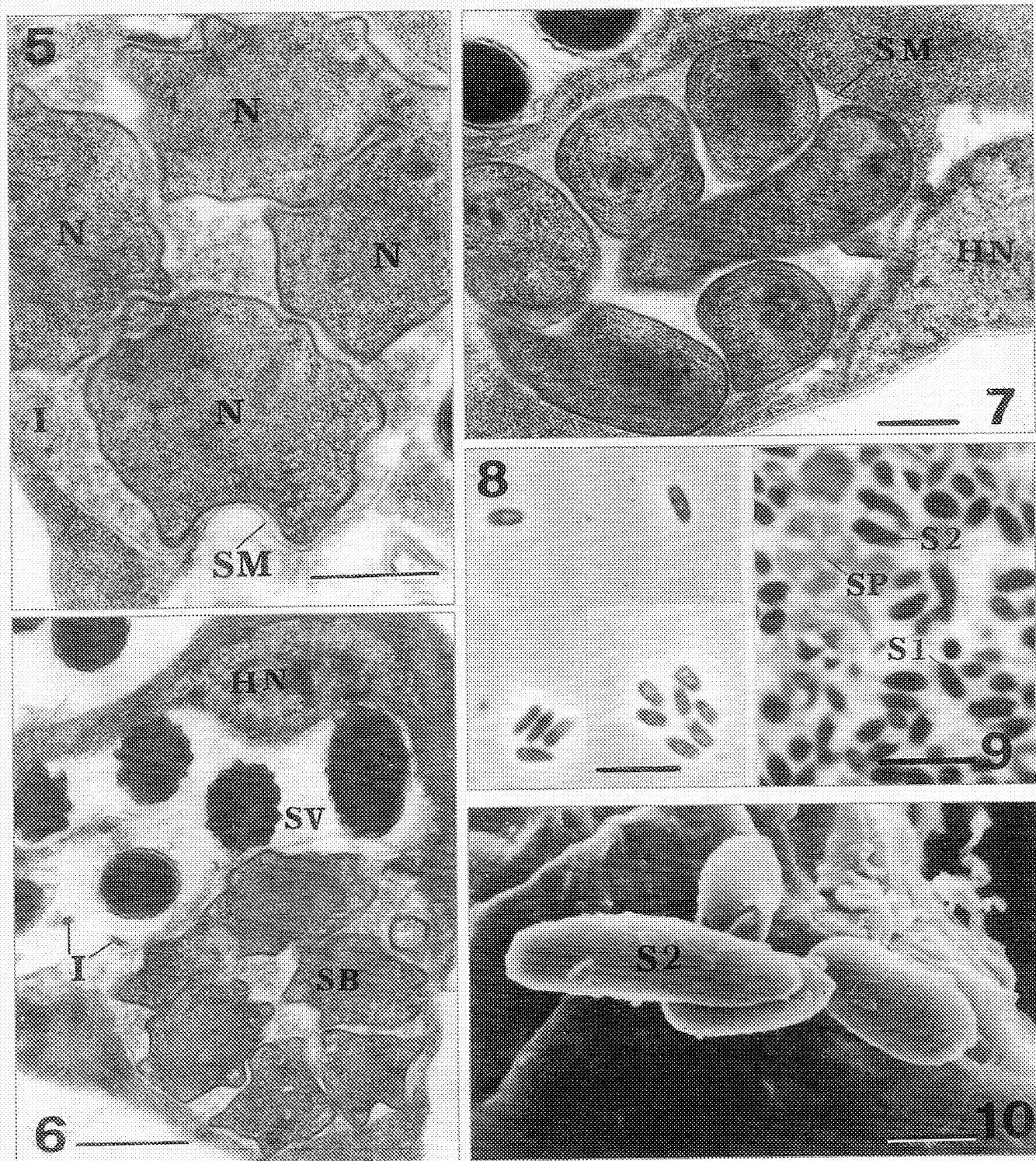
filament are structures suggesting a ranking in the family Striatosporidae Issi et Voronin, 1986. The organization of the spore organelles and the development within a host cytoplasm derived vacuole make it possible to consider this microsporidium as a new species belonging to the *Helmichia* Larsson, 1982.

The new microsporidium is described with emphasis on the ultrastructural characteristics. It is compared to octosporoblastic microsporidia from midge larvae having an uncoiled polar filament. The taxonomic considerations are discussed with special attention to ultrastructural features.

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Figs. 1-4. *Helmichia anomala* sp. nov.: 1. Three early sporonts with diplokaryotic nuclei (N). The narrow electron-lucent zone surrounding the parasites is interpreted as parasitophorous vacuole. Scale bar = 1.0 μm . 2. Oval-shaped early sporont with nuclei (N) arranged as a diplokaryon. The cytoplasm is filled with numerous free ribosomes. Scale bar = 0.6 μm . 3. Elongate sporonts with nuclei (N) arranged as diplokarya. The thin uniform membrane of the parasitophorous vacuole (M) is visible. Scale bar = 0.5 μm . 4. Dividing sporont with separate diplokarya (N). Episporontal space contains granular inclusions (I). The sporont is surrounded by thin envelope of the sporophorous vesicle (SM) and the parasitophorous vacuole (M). Scale bar = 0.5 μm . Figs. 1-4: TEM micrographs



Figs. 5–10. *Helmichia anomala* sp. nov.: 5. Dividing tetranucleate sporont with separate nuclei (N). The sporophorous vesicle with granular inclusions (I) has a thin, fragile envelope (SM). Scale bar = 0.6 μm . 6. Infected host cell. The separate sporoblasts (SB) develop in a sporophorous vesicle with granular inclusions. The sporophorous vesicle (SV) contains mature spores and tubules (I). The host nucleus (HN) is displaced to the periphery of the cell. Scale bar = 1.2 μm . 7. Late sporogonial stage. The elongate sporoblasts are covered by the envelope of the sporophorous vesicle (SM). The displaced host cell nucleus (HN) is visible. Scale bar = 1.0 μm . 8. Three phase contrast images of live spores. Scale bar = 10.0 μm . Separate spores, tetraspores and octospores are visible. Scale bar = 8.0 μm . 9. Semithin section images of live spores. Scale bar = 10.0 μm . Separate spores, tetraspores and octospores are visible. Scale bar = 8.0 μm . 10. Critical point of the infected fat body illustrating the rosette-like dividing sporont (SP) and spores (S1, S2). Scale bar = 1.3 μm . Figs. 5–7: TEM micrographs; Figs. 8 and 9: Light micrographs; Fig. 10: SEM micrograph.

Materials and methods

An infected larva of *Microtendipes pedellus* (De Geer, 1773) with hypertrophied white lobes of the fat body was recorded in August 1995 in a heavily polluted pond at a deer-farm near Kosewo Górne, northeast Poland.

Squash preparations were made by the standard techniques (Vávra and Maddox 1976). Permanent squash preparations were air-dried, fixed in absolute methanol and stained using Giemsa solution.

For transmission electron microscopy (TEM), pieces of the infected segments were excised and fixed in a 2.5% (v/v) glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.2) for 1–3 days. After washing in cacodylate buffer and post-fixation in 2.0% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4°C, the pieces were washed and dehydrated in an ascending series of ethanol, transferred to absolute acetone and embedded in Epon-Araldite. The semithin plastic sections stained with toluidine blue were observed under the light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined in a JEM 100B and JEOL-JEM 1200 transmission electron microscope at 80 kV accelerating voltage. For scanning electron microscopy (SEM) a spore suspension was placed on a cover glass, air-dried and fixed in 2.5% glutaraldehyde in cacodylate buffer. After washing in buffer, the smears were critical point dried using CO₂ (Schwartz *et al.* 1994). After coating with carbon, the preparations were observed in a JEOL-JEM 1200 scanning electron microscope.

Results

Pathogenicity and prevalence

One in a sample of eight larvae was infected. The hypertrophied white coloured lobes of the infected fat body were evident through the hypodermis. No syncytium was formed. The nuclei of infected cells were not hypertrophied. The displacement of the host nucleus to the periphery of the infected cell, caused by the spores and developmental stages of the parasites, was recorded (Figs. 6 and 7).

Sporogony

The earliest stages observed were young diplokaryotic sporonts, inside of a thin-walled vacuole of host cell origin (Figs. 1–3). The cytoplasm of the sporonts had numerous ribosomes, and a weakly developed endoplasmic reticulum. Fixed and stained sporonts were oval (Fig. 2) or elongate oval (Fig. 3) measuring approximately 2.5 × 4.3 µm. The cytological features of the earliest sporonts were similar to the late meronts, but the thickening of the cell border indicated that they were sporogonal stages. During sporogony the two components of the diplokaryon separate, and divide, probably reductionally. The rosette-like budding initially yields lobed

plasmodia with four isolated nuclei (Fig. 5), and after mitosis eight nuclei. A delicate envelope of a sporophorous vesicle was present around the lobate plasmodia (Figs. 4, 5 and 7). Usually eight uninucleate sporoblasts were observed during sporogony. The rounded octosporoblastic sporophorous vesicles were approximately 6.5 µm wide (Figs. 6 and 7). Rarely separate sporogonal stages containing two nuclei were occasionally registered (Fig. 14). When the plasmodium has reached the lobed stage, fine granular inclusions appear inside the sporophorous vesicle (Figs. 4 and 5). During spore maturation the episporontal inclusions transform into tubules (Fig. 6). The elongate sporoblasts matured into spores. Maturation was accompanied by the differentiation of spore organelles. The shape of the future spore became more regular, and the cytoplasm condensed to a more electron-dense condition (Fig. 7).

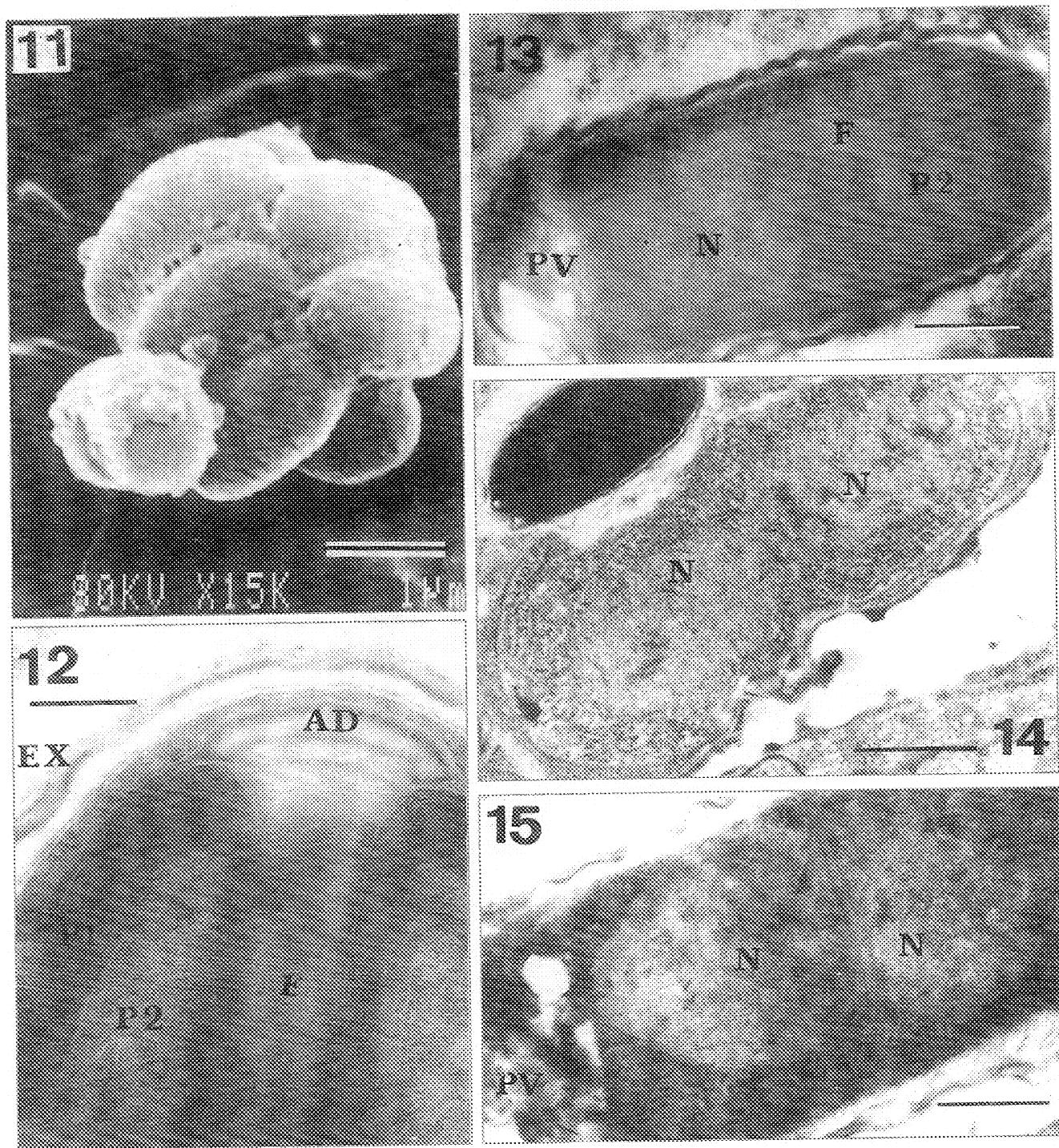
The mature spore

The fresh uninucleate spores in the squash preparations were rod-shaped, measuring 1.3 ± 0.2 (0.9–1.7) × 2.9 ± 0.4 (2.3–4.2) µm (Fig. 8). Spores fixed in glutaraldehyde were ovocylindrical, measuring 0.8–1.2 × 1.9–3.8 µm (Figs. 9–11). Majority of the spores measured 2.7–3.2 µm long. Spores with diplokaryotic nuclei were occasionally observed during ultrastructural analysis (Fig. 15). Usually they were not enveloped in a sporophorous vesicle, and they had a typical for the other spores structure and size. A single teratospore with a double set of the anchoring disc-polaroplast complex was reported previously (Ovcharenko *et al.* 1998). The spore wall has three layers: an approximately 5 nm thick plasma membrane, a lucid 40–45 nm thick endospore, and outermost a 30–34 nm thick exospore. The exospore was constructed as an electron-dense basal layer and an approximately 20 nm thick surface layer (Fig. 12).

The uncoiled polar filament measured 104–109 nm in diameter. The anterior part of the polar filament was attached to a typical anchoring apparatus. The anchoring disc was composed of layers of different electron-density with a central invagination for the pad-like attachment of the polar filament (Fig. 12). The polar filament passed obliquely through the polaroplast towards the spore wall, and ended at the posterior part of the spore near the posterior vacuole (Fig. 13).

The polaroplast occupied approximately the anterior third of the spore. It was composed of tightly packed lamellae anteriorly and a posterior zone (Figs. 12 and 13). The anterior region of the polaroplast was narrow and umbrella-shaped. The ultrastructural construction of the posterior part of polaroplast was not determined exactly, but may have been constructed of interlacing narrow tubules or arranged as spongy structures. The posterior end of the polaroplast was straight, nearly perpendicular to the long axis of the spore (Fig. 13).

The uninucleate spore had a round nucleus which, like the fusiform nuclei of the diplokaryotic spore, was located in the posterior half of the spore (Figs. 13 and 15).



Figs. 11–15. *Helmichia anomala* sp. nov.: 11. Critical point dried octospores within the sporophorous vesicle, a separate spore is visible. Scale bar = 1.0 µm. 12. The longitudinally sectioned anterior part of a mature spore with layered exospore (EX). The polar filament (F) is attached to a typically constructed anchoring apparatus. The anchoring disc (AD) is composed of layers of different electron-density. There is a central area for the pad-like attachment of the polar filament. The bipartite polaroplast is composed of tightly compressed lamellae (P1), and a posterior zone (P2). Scale bar = 0.1 µm. 13. Longitudinal section of a mature spore. The uncoiled polar filament (F) ends at the posterior part of the spore. The posterior border of the polaroplast (P2) is nearly perpendicular to the long axis of the spore. The rounded nucleus (N) is located in the posterior half of the spore. Posterior vacuole (PV) occupies about one fifth of the spore length. Scale bar = 0.6 µm. 14. Longitudinally sectioned sporogonial stage with two nuclei (N). Scale bar = 0.7 µm. 15. The longitudinally sectioned posterior part of a diplokaryotic spore. The nuclei (N) of the fusiform diplokaryon are located in the posterior half of the spore near the posterior vacuole (PV). Scale bar = 0.5 µm. Fig. 11: SEM micrograph; Figs. 12–15: TEM micrographs

The posterior vacuole occupied about one fifth of the spore, and contained electron-dense inclusions (Figs. 13 and 15).

Mature spores, as well as other stages of sporogony, were enclosed both by a thin unit membrane of the host cell origin (parasitophorous vacuole) and the thin envelope of the sporophorous vesicle. The episporontal space contains few tubules (Fig. 6).

Discussion

A few microsporidian genera have a straight or bent uncoiled polar filament. Some genera like *Bacillidium*, *Jirovecia*, *Mrazekia*, and *Ormieresia* have a manubroid polar filament with a small coiled region in representatives of the genera *Mrazekia* and *Ormieresia* (Larsson 1980, 1984, 1986; Götz 1981; Knell 1981). *Coccospora micrococcus* (Léger et Hesse, 1921) produces spherical spores with an uncoiled, slightly bent polar filament (Bylén and Larsson 1994a). *Baculea daphniae* Loubès et Akbarieh, 1978 has rod-shaped spores with a polar filament of manubroid region only, not extending to the posterior end of the spore (Loubès and Akbarieh 1978). The microsporidium described herein differs distinctly from these genera by using a host belonging to a different order, by the polar filament and/or polaroplast construction, and in the mode of sporogony.

More than 45 species of microsporidia are found in midges. Twenty eight of them have been studied on the basis of ultrastructural data (Loubès and Maurand 1975; Coste-Mathiez and Tuzet 1977; Loubès 1979; Codreanu-Balcescu and Codreanu 1980; Götz 1981; Larsson 1982, 1984, 1985, 1986; Issi *et al.* 1985; Issi 1986; Bylén and Larsson 1991, 1994 b, c, 1996; Voronin 1991, 1993, 1998; Wülker and Weiser 1991; Kilochitskiy and Cholan 1993; Ovcharenko and Wita 1995; Ovcharenko *et al.* 1998). If we disregard the species whose sporogony and a spore organization are clearly different from the new species, seven named species remain. These microsporidia produce bacilliform, short-cylindrical and rod-shaped spores with uncoiled polar filaments.

Scipionospora tetraspora Bylén et Larsson, 1996, *Striatospora chironomi* Voronin, 1986 and *Pernicivesicula gracilis* Bylén et Larsson, 1994 have bacilliform spores. The diplokaryotic sporont of *S. tetraspora* divides in a rosette-like manner, producing four diplokaryotic sporoblasts (Bylén and Larsson 1996) while the new microsporidium is octosporoblastic and produces mostly uninucleate sporoblasts. The tightly packed lamellae of the anterior polaroplast of both microsporidia were similar, but the posterior parts differed distinctly. The exospore of *Striatospora chironomi* was ornamented with longitudinal rows of electron-dense material (Issi 1986) and its polar filament was distinctly shorter in comparison with the new *Helmichia*. In contrast to this microsporidium, *Pernicivesicula gracilis* has multisporoblastic sporogony and a polaroplast with lamellar and chambered parts (Bylén and Larsson 1994b).

Cylindrospora chironomi Issi et Voronin, 1986 has short-cylindrical spores. Like in the new microsporidium its octo-

sporoblastic sporogony occurs inside of parasitophorous vacuole, but the spores of *C. chironomi* have a funnel-shaped, short polar filament and spongy polaroplast without lamellae (Issi 1986).

Helmichia spp. have rod-shaped spores with uncoiled polar filament. This genus was established by Larsson for a microsporidium infecting midge larvae of *Endochironomus* sp., collected in the south of Sweden (Larsson 1982). Diplokaryotic sporont, octosporoblastic sporogony within sporophorous vesicles and parasitophorous vacuoles, and uninucleate rod-shaped spores with isofilar uncoiled polar filament are characteristic features of this genus. All of them are also characteristic for the species described herein. Four species belonging to the genus *Helmichia* have been described: *H. aggregata* Larsson, 1982, *H. glandulicola* Wülker et Weiser, 1991, *H. tetrasticta* Kilochitskiy et Cholan, 1993, and *H. lacustris* Voronin, 1998.

The stained spores of *H. aggregata* were rod-shaped, lightly bent measuring $1.0-1.2 \times 3.1-7.0 \mu\text{m}$ (Larsson 1982). Like the microsporidium described herein, it has a layered exospore, bipartite polaroplast with a narrow tightly lamellar anterior part and a clear-cut, posterior border. However, two important differences exist: the spores of the new microsporidium studied herein were distinctly shorter, and their host belongs to a different genus.

Helmichia glandulicola was described from the salivary glands of *Chironomus anthracinus* larvae (Wülker and Weiser 1991). The rod-shaped spores measured $1.2-1.5 \times 3.0-4.0 \mu\text{m}$ in stained condition. As in the new species the polaroplast of *H. glandulicola* was bipartite with narrow lamellae anteriorly and an almost perpendicular posterior border. However, *H. glandulicola* has a different site of infection, different host genus and slightly longer and more narrow spores.

The stained spores of *H. tetrasticta* were oval or kidney shaped $2.4 \pm 0.19 \times 1.19 \pm 0.01 \mu\text{m}$ (Kilochitskiy and Cholan 1993). The polaroplast was composed of narrow lamellar and spongy parts. This microsporidium was found in Ukraine within the fat body of the larva of *Ablabesmyia tetrasticta* Kieffer, 1936. The stained spores of the microsporidium described herein were smaller than the spores of *H. tetrasticta* and their shape was ovocylindrical.

Helmichia lacustris produces rod-shaped spores $1.0-1.3 \times 3.4$ ($3.0-3.7 \mu\text{m}$) in size with a polar filament, partly coiled and slightly narrowed at the posterior end of the spore. Polaroplast was composed of rare thin lamellae anteriorly, and filled with granular secrets (Voronin 1998). Like the new microsporidium, the episporontal space of *H. lacustris* contains thin granules transforming into tubules. Spores of *H. lacustris* isolated from the adipose tissue of larvae of *Chironomus plumosus* in Russia have approximately the same size as the present microsporidium, but slightly difference proportions, and no binucleate sporoblasts and diplokaryotic spores were observed. Contrary to *H. lacustris*, the polar filament of the present microsporidium is straight isofilar, and the polaroplast is constructed of anterior part with tightly packed lamellae and distinctly separated posterior region.

We consider that the distinctive and original characters of the microsporidium described herein justify establishing of a new species. Basic characters, together with the additional morphological information presented, are evidence that this new species can be included in the genus *Helmichia*. The presence of rare diplokaryotic spores, the spore size and the host affinity clearly separate this new species from other species within the genus. We suppose that the presence of rare binucleate sporogonial stages and isolated diplokaryon-containing spores may represent another type of sporogony, previously unknown in this genus.

Helmichia anomala sp. nov.

Synonym: *Microsporidium* sp. Ovcharenko, Molloy et Wita, 1998

Type host: *Microtendipes pedellus* (De Geer, 1773), larva.

Site of infection: Fat body cells.

Sporogony: As for the genus. Each sporont produces mostly eight sporoblasts with a single nucleus. Episporontal space around sporogonic stages contains granular inclusions. The space between the mature spores contains tubules.

Spore: Uninucleate, rod-shaped measuring 1.3 ± 0.2 (0.9–1.7) $\times 2.9 \pm 0.4$ (2.3–4.2) μm . Spores fixed in glutaraldehyde were ovocylindrical measuring $0.8\text{--}1.2 \times 1.9\text{--}4.3 \mu\text{m}$. More than half of the spores were $2.7\text{--}3.2 \mu\text{m}$ long. Exospore layered, 30–34 nm wide. Polar filament straight uncoiled, isofilar, 104–109 nm wide, reaching the posterior pole of the spore. Polaroplast occupied about one-third of the spore length. Its anterior region was narrow, with tightly packed thin lamellae, the ultrastructural organization of the posterior part was not precisely determined. The single nucleus was rounded. Nuclei of diplokaryotic spores were fusiform.

Locality: A polluted pond located near the village of Kosewo Górne, northeast Poland.

Types: Hapantotypes on slides Nos. D011237–D011241.

Deposition of type slides: International Protozoan Type Slide Collection, Smithsonian Institution, Washington, U.S.A. Slides Nos. D011237–D011241 and TEM-blocks in the protozoological collection of the Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Warszawa, Poland.

Etymology: After latin *anomala* = anomalous, alluding to the rare diplokaryotic spores which are atypical for the genus *Helmichia*.

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