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Ultrastructural study of *Agglomerata connexa* sp. nov. (Microspora, Duboscqiidae), a new microsporidian parasite of *Daphnia longispina* (Cladocera, Daphniidae)

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Abstract. The new microsporidium, *Agglomerata connexa* sp. nov., is described, based primarily on ultrastructural characteristics. The parasite infects the adipose tissue of *Daphnia longispina* O.F. Müller, 1785. All life cycle stages have isolated nuclei. Sporogonial reproduction is by rosette- or finger-like budding. Sporophorous vesicles contain not more than 4 mature spores, most vesicles are monosporous, and usually devoid of inclusions. Rare aggregates of tubules are visible inside multisporous and outside of individual sporophorous

vesicles. Often two or more individual vesicles form a chain-like structure. Unfixed spores are pyriform, measuring $4.08 \pm 0.27 \times 2.72 \pm 0.24 \mu\text{m}$. The exospore is layered, approximately 30 nm thick. The polar filament is isofilar, 140 nm wide, making 5–7 coils in the posterior half of the spore. The polaroplast has three subdivisions: wide lamellae, narrow lamellae and tubules. Discrimination from other microsporidian species is discussed.

Key words: *Agglomerata connexa* sp. nov., Microspora, ultrastructure, *Daphnia longispina*, Cladocera

Introduction

Jírovec (1937) published a short description of *Plistophora obtusa* a microsporidium, infecting *Daphnia pulex*, *D. magna* and *D. longispina*. Another microsporidium, mentioned by Jírovec (1937), *Glugea* sp., was transferred by Vávra to the genus *Norlevinea* (Vávra, 1984). A third species, *Gurleya vavrai*, was briefly described by Green (1974) without mentioning the tissue specificity of this microsporidium. On page 19, of "Structure of the Microsporidia", Vávra and Larsson (1999) showed part of the lobate plasmodia of *Microsporidium* sp. from *D. longispina* without any taxonomic comment.

Daphnia longispina, the host of the microsporidium infecting the adipose tissue, has been found in a small pond near Kosewo Górne, north-east Poland. The ultrastructural features of this microsporidium resemble representatives of the genera *Agglomerata* Larsson et Yan, 1988, and *Larssonia* Vidtmann et Sokolova, 1994. The new microsporidium is

described with emphasis on the ultrastructural characteristics, and its taxonomic position is discussed.

Materials and methods

Infected hosts were collected in a small pond located on the territory of Kosewo Górne Biological Station of the Institute of Parasitology, Polish Academy of Sciences (north-east region of Poland), in the second half of the years 1997 and 1999 (July to October). Freshly collected daphnias were dissected and examined under a light microscope. Infected tissues were smeared and dry smears were stained using Giemsa solution. Squash preparations were made by the usual techniques (Vávra and Maddox 1976).

For transmission electron microscopy small pieces of infected tissues or whole microcrustaceans were fixed in 2.5% (w/v) glutaraldehyde solution in 0.05 M sodium cacodylate buffer (pH 7.4) containing 0.12 M sucrose and 5 mM CaCl₂ at room temperature. They were then washed in cacodylate

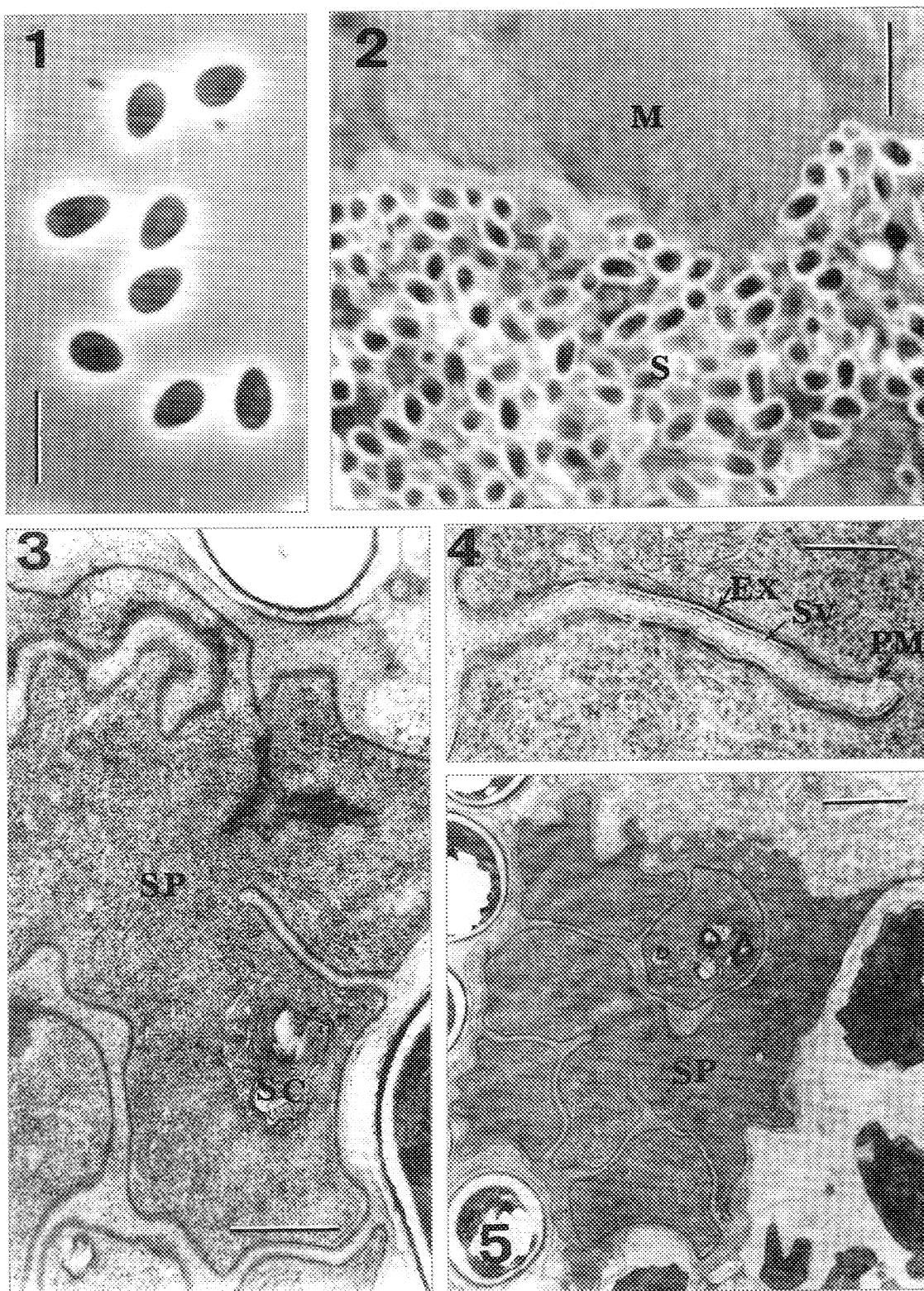


Fig. 1. Living spores under the phase contrast microscope. Scale bar = 10 µm. **Fig. 2.** Semithin section of infected adipose tissue filled with spores (S). Muscle tissue (M) is not infected. Scale bar = 10 µm. **Fig. 3.** The rosette-like dividing sporont (SP), with the scindosome-shaped structure (SC). Scale bar = 0.8 µm. **Fig. 4.** Initiation of the spore wall primordium during the rosette-like budding of the sporont. Plasmalemma (PM), exospore primordium (EX), and thin membrane of sporophorous vesicle (SV) are visible. Scale bar = 0.2 µm. **Fig. 5.** Early sporogonial plasmodium (SP) delimited by a plasma membrane. Scale bar = 1.1 µm

buffer and post-fixed in 2.0% (w/v) osmium tetroxide in the same buffer for 1 h at 4°C. After washing in buffer, the pieces were dehydrated in an ascending series of ethanol, transferred to absolute acetone and embedded in Epon resin. The semithin 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 microscopes at 80 kV accelerating voltage.

For scanning electron microscopy a spore suspension was placed on a cover slip, 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. Part of the fixed smears, after dehydrating in ethanol to absolute acetone, was covered with carbon.

Results

Light microscopic data

The primary site of infection was adipose tissue. In heavily infected daphnias, nearly all tissues, except muscles and the digestive tube, were full of spores (Fig. 2). Infected cladocerans were colored milky white. The earliest developmental stages observed in Giemsa stained smears were rounded, uni- to tetranucleate. No diplokarya were noticed. Rounded sporogonial plasmodia measured approximately 9.0–9.5 µm in diameter. The early sporoblasts were rounded, about 5.0 µm in diameter with the nuclei measuring 1.5–2.0 µm. The living spores in squash preparations were pyriform, measuring 4.08 ± 0.27 × 2.72 ± 0.24 µm (n = 50). The spores were weakly aggregated and they were easily separated by squashing (Fig. 1). Most of the spores in the squash preparations were

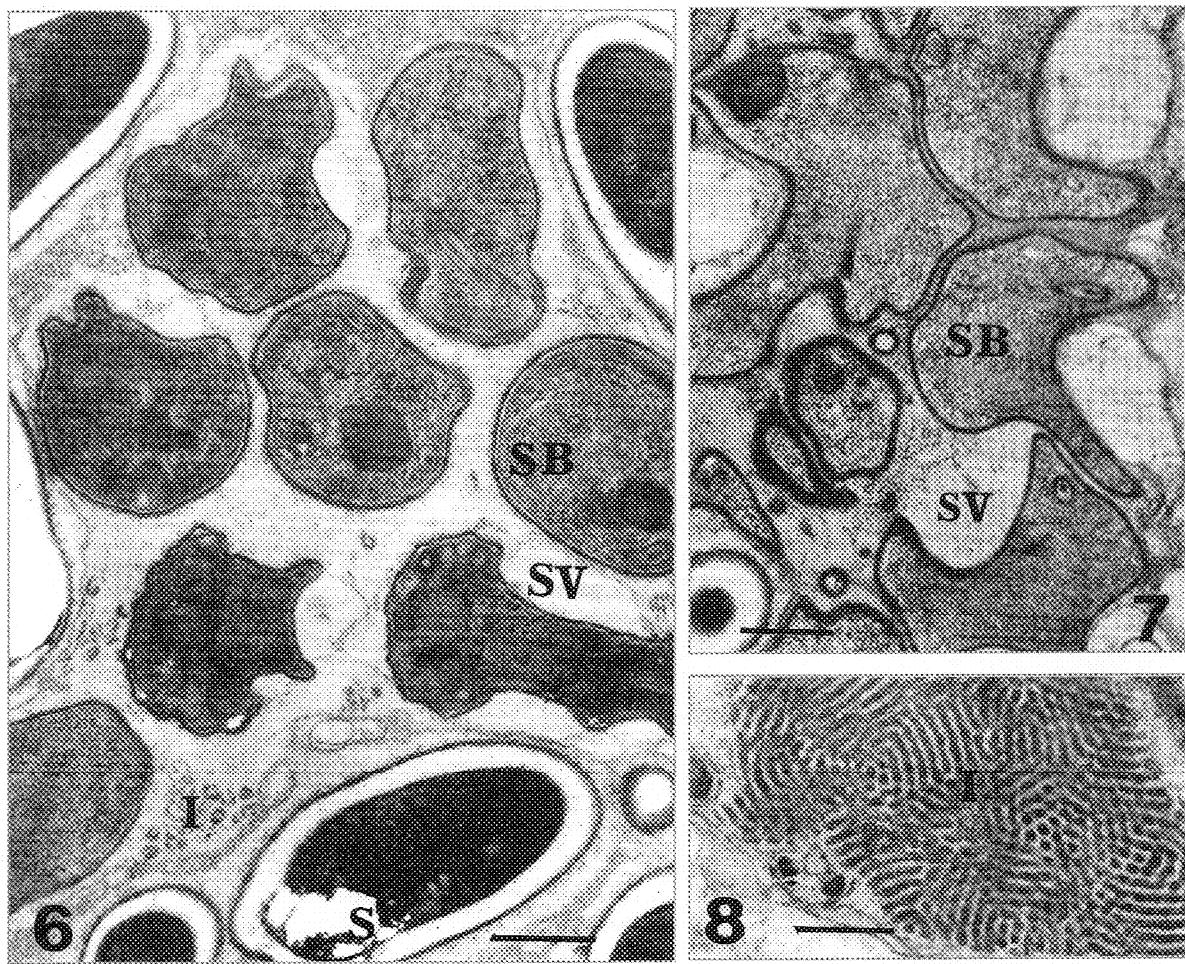


Fig. 6. Late sporogony. Multisporoblastic sporophorous vesicle, containing sporoblasts (SB) and inclusions (I) gives rise to individual sporophorous vesicles (SV). Scale bar = 1.0 µm. **Fig. 7.** Late sporogonial stage. The electron-lucent space of individual sporophorous vesicles (SV) adjoining the sporoblasts (SB) are visible. Scale bar = 1.0 µm. **Fig. 8.** Structure of aggregated episporontal inclusions. Scale bar = 0.2 µm.

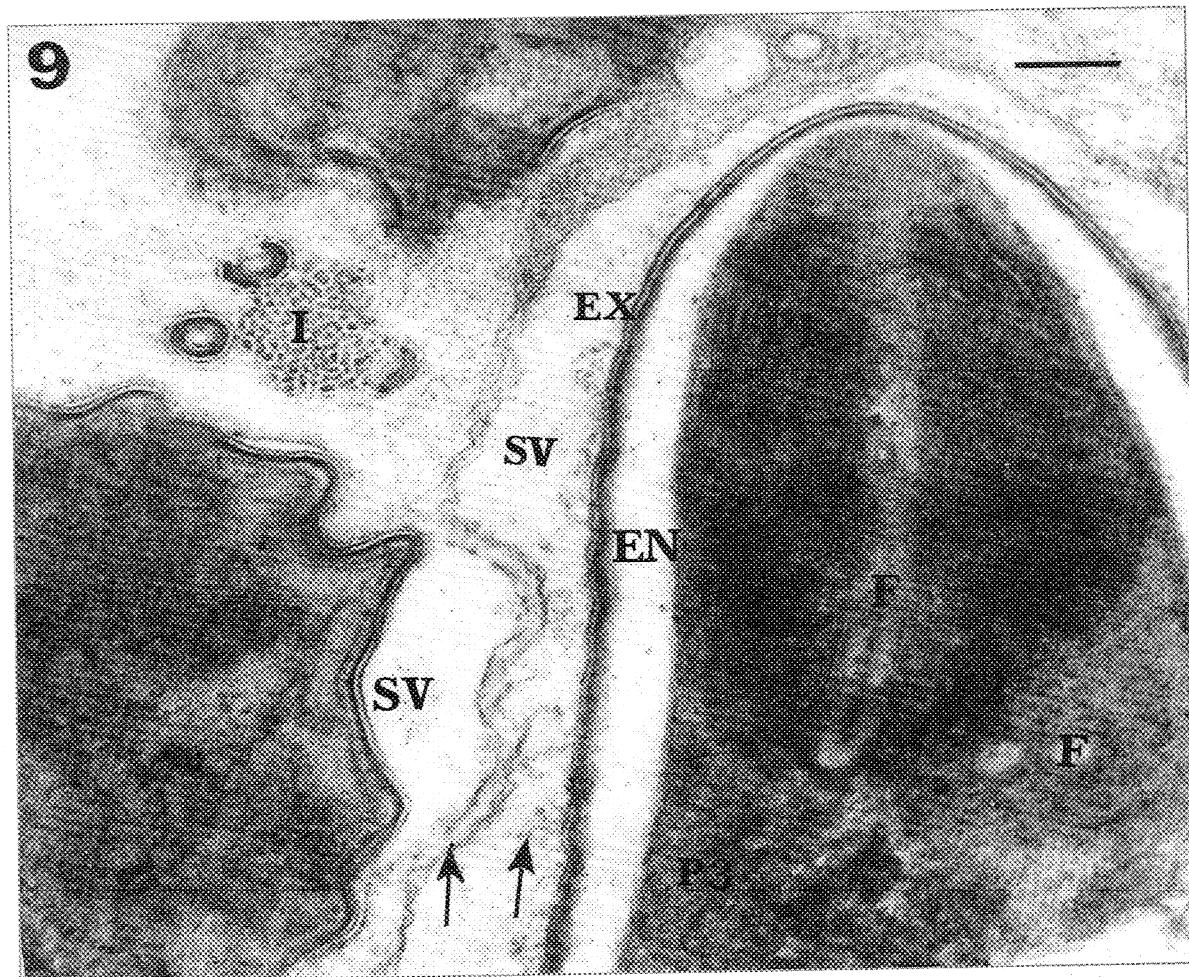


Fig. 9. Parts of longitudinal section of the mature spore and late sporoblast. The structure of the envelopes enclosing the mature spore and late sporoblast is identical with the thin granulated material of the exospore coat protuberances (arrowed). Tripartite polaroplast (P₁, P₂, P₃), polar filament (F), endospore (EN), and layered exospore (EX), are visible. Inclusions (I) are situated outside of the individual sporophorous vesicles (SV). Scale bar = 0.2 µm

free or weakly aggregated in doublets or triplets, which were connected in chains. The size of the Giemsa stained spores was $3.37 \pm 0.34 \times 1.97 \pm 0.63$ µm ($n = 50$).

Electron microscopy and life cycle

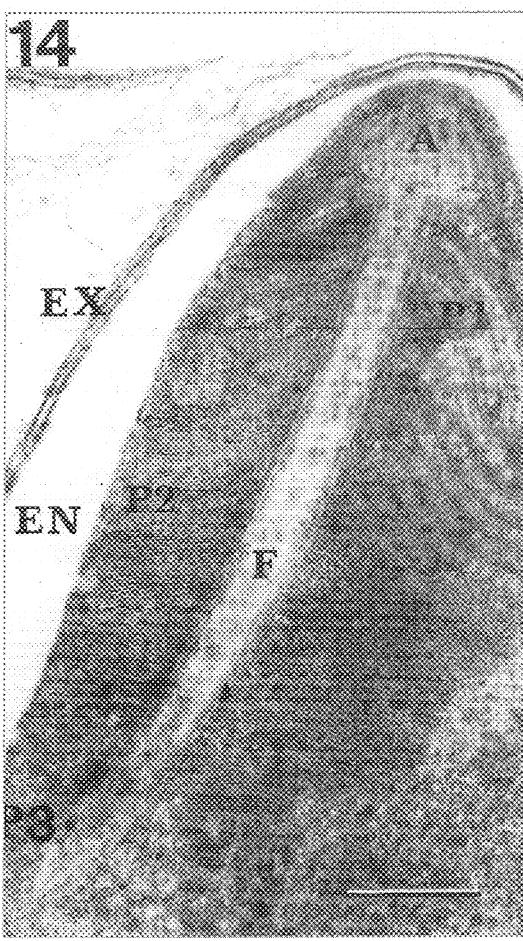
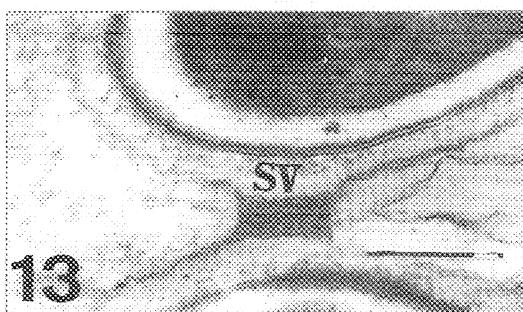
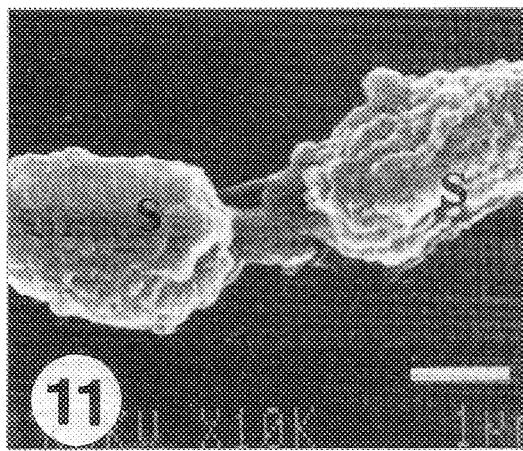
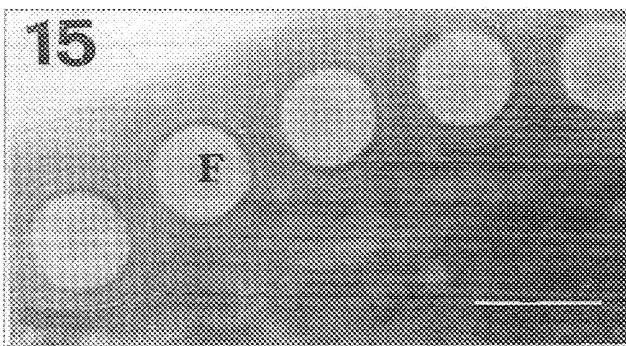
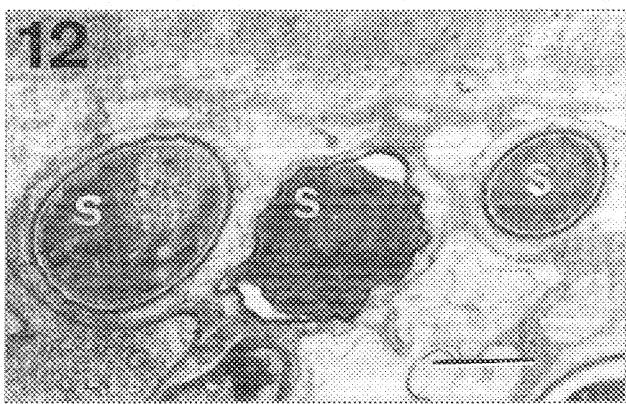
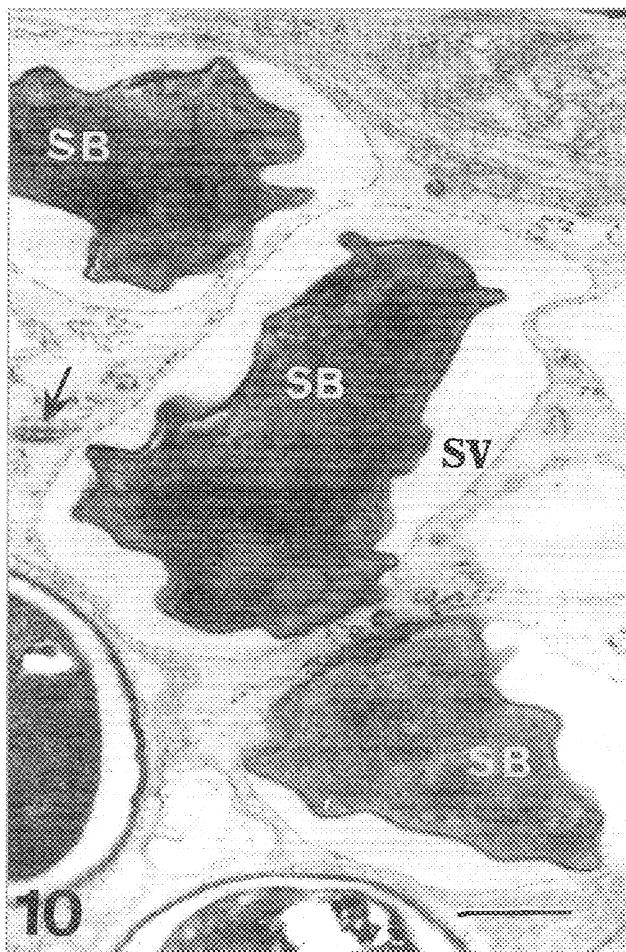
All stages of the life cycle had isolated nuclei. The earliest documented stages were pluri-nucleate sporogonial plasmodia delimited by a plasma membrane (Fig. 5). Sporoblasts were formed by rosette- or finger-like budding (Figs. 3 and 5). Meiosis was not observed.

According to our observations, the formation of the sporophorous vesicle and the spore wall primordium occurs simultaneously with the beginning of the rosette-like budding (Figs. 3 and 4). The electron-dense material accumulated externally to the plasma membrane splits into electron-lucent endospore and dense exospore primordia. The thin uniform

layer of sporophorous vesicle wall appears between the lobes externally to the future exospore (Fig. 4).

The lobate plasmodium sometimes exhibits scindosome-shaped structures (Fig. 3). Finally, the lobes of the sporogonial plasmodium were released as individual cells – the sporoblasts (Figs. 6 and 7). Sporoblasts matured to spores without further division.

The sporophorous vesicle divided together with the budding of pluri-nucleate sporogonial plasmodia and usually only one sporoblast surrounded by the individual sporophorous vesicle (Fig. 10), was left. Multisporous sporophorous vesicles containing two or more separate sporoblasts and spores were also visible (Figs. 6 and 12). Initially, the narrow zone of episporal space lacked inclusions. The episporal space of individual sporophorous vesicles containing mature spores, usually had no inclusions, too (Figs. 6, 7 and 10). The fine-granular material surrounding the spores is most proba-



bly connected to the exospore coat (Figs. 9, 12 and 13). The episporontal space between the sporoblasts of multisporous sporophorous vesicles, as well as the space outside of individual sporophorous vesicles contains prominent inclusions, which transform during the sporogony into tubules (Figs. 6, 8 and 9). Probably, they take part in the formation of the exospore (Fig. 9). Sometimes, two or more spores, enclosed within the sporophorous vesicle, were noticed. Often a few individual sporophorous vesicles form a chain (Figs. 11, 12 and 13).

Spores fixed in 2.5% glutaraldehyde were typically pyriform (Figs. 2 and 11). The wall of the mature spore measured 150–160 nm, except at the anterior pole, where it was considerably thinner. The layered exospore was composed of a dense internal layer and the double membrane-like layers, separated by an intermediate translucent zone (Figs. 9 and 14). The surface of the exospore was covered with a narrow coat of granular material which formed thin protuberances (Figs. 9 and 13). The polar filament was isofilar, 140 nm wide, making 6–7 coils in the posterior half of the spore (Figs. 9 and 15). The angle of tilt of the anterior coil to the long axis of the spore was about 30°. Three distinctly separated regions of the polaroplast could be discriminated: wide lamellae occupied one third of the polaroplast length anteriorly, followed by closely packed narrow lamellae and posterior tubules (Figs. 9 and 14). The posterior vacuole was poorly defined.

Discussion

Sporophorous vesicle

The microsporidium described herein produces uninucleate spores, usually surrounded by the individual sporophorous vesicles, containing no tubules. However, multisporous sporophorous vesicles with tubular inclusions were also observed.

Individual sporophorous vesicles are characteristic of the genera *Tuzetia*, *Janacekia*, *Alfvenia*, *Nelliemelba*, *Lanatospora*, *Berwaldia* and *Agglomerata* (Maurand *et al.* 1971; Larsson 1981, 1983; Sprague *et al.* 1992, Bronnvall and Larsson 1995; Larsson and Voronin 2000).

The young vesicles surrounding sporoblasts of *Janacekia udinarum* Larsson, 1983 are two-layered. The internal layer is reduced during maturation of the spores (Larsson 1983).

Contrary to *Janacekia*, the sporophorous vesicles surrounding the late sporoblasts and mature spores of microsporidium studied herein were identically constructed (Fig. 9).

The sporoblastogenetic vesicles of *Alfvenia ceriodaphniae* Vidtmann et Sokolova, 1994 develop by a separation of the episporontal coat from the late sporont. The thin uniform vesicles contain no inclusions (Vidtmann and Sokolova 1994). We consider that the episporontal granular coat of the microsporidium we found is the source of the developing vesicle wall. The observation that the material of the exospore coat is structurally identical to the sporophorous vesicle material (Fig. 9) supports this idea.

The fragile vesicle of *Agglomerata volgensae* Larsson et Voronin, 2000 either collects all daughter cells of the sporont, or the vesicle divides together with the plasmodium to enclose spores in individual vesicles. The mode of development of the sporophorous vesicles of microsporidium treated herein is similar to *A. volgensae*, but we have never seen multisporous vesicles, containing more than 4 mature spores, and nearly all of the spores were enclosed in individual vesicles. Often, neighbouring vesicles were connected into short chains (Fig. 12).

Taxonomy

More than 40 species have been recorded in daphniid hosts (Weiser 1945, Bradbury 1994, Larsson *et al.* 1998). Only a few of them have been studied at the ultrastructural level (Loubes and Akbarieh 1977, 1978; Vávra 1984; Voronin 1986, 1988; Larsson and Yan 1988; Vávra and Larsson 1994; Vidtmann and Sokolova 1994, 1995; Friedrich *et al.* 1996; Larsson *et al.* 1996, 1997; Larsson and Voronin 2000). Considering that many species were described more than fifty years ago, the definition of their contemporary systematic position is fairly complicated.

Three species of microsporidia have been reported from the same host, *Daphnia longispina*. Microsporidia *Norlevinea daphniae* and *Gurleya vavrai* produce distinct sporophorous vesicles with a regular number of spores (Green 1974, Vávra 1984). The third species, *Pleistophora obtusa* is similar to the microsporidium we found.

Pleistophora obtusa (Moniez, 1887) (= *Microsporidium obtusa*, *Plistophora obtusa*, *Microsporidium obtusum*) was reported from five cladoceran species (Moniez 1887, Jírovec

Fig. 10. Three late sporoblasts (SB) enclosed in individual sporophorous vesicles (SV). Rare tubular inclusions (arrowed) are visible inside of the multisporous vesicle between the vesicles containing single spores. Scale bar = 1.1 µm. **Fig. 11.** Two spores (S) fixed in glutaraldehyde and coated with carbon. The spores are interconnected with the envelope of sporophorous vesicle. **Fig. 12.** Transversal section of three immature spores (S) in a chain-like disposition. Scale bar = 1.3 µm. **Fig. 13.** Ultrastructure of the sporophorous vesicles connection. Dense material, connecting the neighbouring vesicles is similar to the material of the sporophorous vesicle envelopes. Sporophorous vesicle (SV) is devoid of inclusions. The surface of the exospore is covered with a thin coat of granular material forming thin protuberances. Scale bar = 0.8 µm. **Fig. 14.** Longitudinal sectioned anterior part of the mature spore. The layered exospore (EX), electron-lucent endospore (EN), lamellar (P1, P2) and tubular (P3) parts of polaroplast, anchoring apparatus (A), and uncoiled part of polar filament (F) are visible. Scale bar = 1.3 µm. **Fig. 15.** Part of the transversal sectioned polar filament coils. Scale bar = 0.1 µm.

1937, Sprague 1977). In *Simocephalus vetulus* and *Daphnia reticulata* this microsporidium produces obtuse spores up to $4.0 \times 2.5 \mu\text{m}$, inflated posteriorly (Moniez 1887), while in *Daphnia magna*, *D. pulex* and *D. longispina* the spores were pyriform, $3.0-4.0 \times 1.5-2.0 \mu\text{m}$ in size (Jírovec 1937). Later, the species infecting *D. magna* was transferred to the genus *Larssonia* (Vidtmann and Sokolova 1994). The authors did not mention a sporophorous vesicle (pansporoblastic membrane) of *P. obtusa*. Sprague has written that this species seems not to belong to any established genus, and has placed it in the collective group *Microsporidium* (Sprague 1977). There are two similarities between *Plistophora obtusa* described by Jírovec and the microsporidium described herein, according to Jírovec (1937) and Weiser (1945) the sporogonial plasmodium of *P. obtusa* produces aggregates of spores which are loosely stuck together and soon become free, similar to the microsporidium described herein; another similarity is the shape and size of the spores. However, the microsporidium described by Jírovec was recovered from the hemolymph, while the species we found occupied primarily adipose tissue.

In cytology and development the microsporidium treated by us resembles the genera *Agglomerata* and *Larssonia*. The microsporidium has the layered exospore of *Agglomerata*-type. The polaroplast has three subdivisions: wide lamellae, narrow lamellae and tubules, like *Agglomerata* and *Larssonia*. The polar filament is isofilar, similar to both genera. The tubular inclusions of the episporontal space resemble tubules of *Agglomerata*, but differ slightly from the tubular and lamellar inclusions of *Larssonia*.

The genus *Larssonia* was established by Vidtmann and Sokolova (1994), for the species known as *Plistophora obtusa* (Moniez, 1887). This genus differs from *Agglomerata* by the presence of diplokarya at the end of merogony, which was not illustrated ultrastructurally by authors (Vidtmann and Sokolova 1994).

The genus *Agglomerata* was established in 1988 based on an ultrastructural study of the microsporidium belonging to *Duboscqia sidae* Jírovec, 1942 (Larsson and Yan 1988). Five species infecting cladocerans were included in this genus. Four of them were originally described in other gen-

era. *Agglomerata (Duboscqia) sidae* infects cladoceran *Holopedium gibberum* in Canada. The spores measuring $1.5-2.0 \times 2.5-3.5 \mu\text{m}$ were firmly aggregated and their number per vesicles varied from 8 to 32 (Larsson and Yan 1988). *Agglomerata (Glugea) cladocera* (Pfeiffer, 1895) infects the hypoderm of *Daphnia magna* in West Europe and produces spores $3.0-3.5 \times 1.5-1.8 \mu\text{m}$ in size (Pfeiffer 1895, Larsson et al. 1997). *Agglomerata (Lanatospora) bosminaiae* (Voronin, 1986) infects the ovary of *Bosmina obtusirostris*, *B. longirostris* and *B. coregoni* (Larsson and Voronin 2000). The spores measured $1.7 \times 2.9 \mu\text{m}$ in *B. obtusirostris* and $2.0 \times 3.6 \mu\text{m}$ in *B. longirostris* (Voronin 1986). Ovoid spores measuring $5.0 (4.7-5.5) \times 2.8 (2.5-3.4) \mu\text{m}$ produces *Agglomerata (Thelohania) simocephali* (Voronin 1986). The last mentioned two species were described in Russia.

The microsporidium described herein differs from the mentioned above four microsporidia in spore size and host affinity. The species conforms to *Agglomerata* in nuclear characteristics, mode of sporogonial divisions and the shape and structure of the spore, but the late sporogony is different. Closest to the microsporidium found by us is *A. volgensae* Larsson et Voronin, 2000. The microsporidium infects hypoderm and adipose tissue of *Daphnia magna* in Kostroma district, Russia (Larsson and Voronin 2000). Some important differences between *A. volgensae* and the microsporidium we found are marked in Table I.

The tubular and the fibrillar inclusions of *A. volgensae* were situated inside the individual and multisporous vesicles, while individual sporophorous vesicles of the species described herein contain no inclusions. The polaroplast of *A. volgensae* was constructed of two lamellar parts of *Agglomerata*-type, while the polaroplast of the microsporidium we found exhibits three typical regions (wide lamellae, narrow lamellae, tubules). In addition, the spores of *A. volgensae* were distinctly smaller, had a pyriform shape with a pointed pole and their polar filament was thinner and shorter than in the species, found in Poland.

The microsporidium of *Daphnia longispina* described in this paper is clearly new and can be placed in the genus *Agglomerata* based on nuclear characteristic, mode of sporogonial divisions, and the shape and structure of the spores. The

Table I. Comparative data of *Agglomerata volgensae* and *Agglomerata connexa* sp. nov.

	<i>Agglomerata volgensae</i>	<i>Agglomerata connexa</i> sp. nov.
Host	<i>Daphnia magna</i>	<i>Daphnia longispina</i>
Spore shape	pyriform with pointed anterior pole	pyriform
Spore size (μm):		
fresh	$3.2-3.7 \times 1.7-2.0$	$4.08 \pm 0.27 \times 2.72 \pm 0.24$
stained	$3.0-3.4 \times 1.9-2.2$	$3.37 \pm 0.34 \times 1.97 \pm 0.63$
Polaroplast	inverted lamellar	inverted lamellar + tubular
Polar filament, coils	lightly anisofilar, 4–6	isofilar, 5–7
Tubular inclusions in episporontal space of sporophorous vesicles (SV)	present	absent in individual SV, rare in multisporous SV, present outside of individual SV

late sporogony of microsporidium is similar to *A. volgensae*, which was included into genus *Agglomerata* only temporally, however (Larsson and Voronin 2000). The distinctive and original development of the sporophorous vesicles of both microsporidia is a deviation from the typical *Agglomerata* cytology.

Description

Agglomerata connexa sp. nov.

Synonym: *Pleistophora obtusa* (Moniez, 1887) partim

Development: All stages with isolated nuclei. Sporogony polysporoblastic, accompanied by rosette- or finger-like budding of pluri-nucleate plasmodium and production of uninucleate sporoblasts. The sporophorous vesicle divides together with the budding of sporogonial plasmodia, resulting usually in single spore sporophorous vesicles. The episporontal space of individual sporophorous vesicles contains no inclusions. Multisporous sporophorous vesicles containing up to 4 spores as well as the space outside of individual vesicles contain prominent inclusions, transformed during sporogony into tubules. Often, neighbouring vesicles are interconnected into short chains.

Spore: Uninucleate, pyriform, $4.08 \pm 0.27 \times 2.72 \pm 0.24 \mu\text{m}$ (fresh), $3.37 \pm 0.34 \times 1.97 \pm 0.63 \mu\text{m}$ (stained), ovoid (critical point dried). The spore wall is 150–160 nm thick, with a 40 nm thick, layered exospore. The surface of the exospore is covered by narrow zone of granular material which forms sparse protuberances up to 60 nm high. The polar filament 140–165 nm wide is arranged in 5–7 coils in a single row close to the spore wall. The angle of tilt is about 30°. The polaroplast has three subdivisions: wide lamellae, narrow lamellae and tubules. The posterior vacuole is poorly defined.

Host tissue involved: Adipose cells.

Type host: *Daphnia longispina* O.F. Müller, 1785.

Type locality: A small pond located near the village of Kosewo Górne, north-east Poland.

Types: Hapantotypes on slides No. D011235–D011243.

Deposition of type slides: In the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, USA. Slide No. D011235–D011242 and TEM-blocks in the protozoological collection of the Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Warszawa, Poland.

Etymology: Alluding to the mode of the spore disposition.

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