Available online at www.sciencedirect.com
ScienceDirect
European Journal of Protistology 45 (2009) 13–20
European Journal of PROTISTOLOGY
www.elsevier.de/ejop

Pankovaia semitubulata gen. et sp. n. (Microsporidia: Tuzetiidae) from nymphs of mayflies *Cloeon dipterum* L. (Insecta: Ephemeroptera) in Western Siberia

Anastasia V. Simakova[a], Yuri S. Tokarev[b,*], Irma V. Issi[b]

[a]*Research Institute of Biochemistry and Biophysics, Tomsk State University, pr. Lenina 36, Tomsk 634050, Russia*
[b]*All-Russian Institute for Plant Protection, Russian Academy of Agricultural Sciences, sh. Podbelskogo 3, St-Petersburg, Pushkin 196608, Russia*

Received 16 September 2007; received in revised form 24 April 2008; accepted 30 April 2008

Abstract

The ultrastructure of a new microsporidian, *Pankovaia semitubulata* gen. et sp. n. (Microsporidia: Tuzetiidae), from the fat body of *Cloeon dipterum* (L.) (Ephemeroptera: Baetidae) is described. The species is monokaryotic throughout the life cycle, developing in direct contact with the host cell cytoplasm. Sporogonial plasmodium divides into 2–8 sporoblasts. Each sporoblast, then spore, is enclosed in an individual sporophorous vesicle. Fixed and stained spores of the type species *P. semitubulata* are $3.4 \times 1.9\,\mu m$ in size. The polaroplast is bipartite (lamellar and vesicular). The polar filament is isofilar, possessing 6 coils in one row. The following features distinguish the genus *Pankovaia* from other monokaryotic genera of Tuzetiidae: (a) exospore is composed of multiple irregularly laid tubules with a lengthwise opening, referred to as "semitubules"; (b) episporontal space of sporophorous vesicle (SPV) is devoid of secretory formations; (c) SPV envelope is represented by a thin fragile membrane.

Keywords: *Cloeon dipterum*; *Pankovaia semitubulata* gen. et sp. n.; Microsporidia; Ephemeroptera; Ultrastructure

Introduction

Microsporidia of mayflies of Europe and North America have been extensively studied. Ten species of these parasites were listed by Kudo (1924) in his monograph summarizing results of research on microsporidia from all groups of animal hosts. Three of these 10 species were attributed to the genus *Nosema* according to the taxonomic criteria of that time. The others were diagnosed as *Thelohania* (two species), *Plistophora* (= *Pleistophora*), *Gurleya*, *Stempellia, Telomyxa* and one undefined species. Thirty seven years later, Weiser (1961) extended this list with two species of *Nosema,* two species of *Plistophora* (= *Pleistophora*), three species of *Thelohania* and a species of a new genus *Trichoduboscqia*.

Based upon ultrastructural features of sporogonial stages, three *Nosema* species from mayflies were then transferred to the new genus *Tuzetia* (Maurand et al. 1971; Maurand 1973). In a review that followed, Larsson (1983) included 6 microsporidian species parasitizing Ephemeroptera in the genus *Tuzetia*. The main taxonomic features of the *Tuzetia* genus proposed by the author were: (a) monokaryotic nuclear apparatus in all

*Corresponding author. Tel.: +78 12 377 2923;
fax: +78 12 470 5110.
E-mail address: jumacro@yahoo.com (Y.S. Tokarev).

0932-4739/$ - see front matter
doi:10.1016/j.ejop.2008.04.003

stages of the life cycle; (b) small number of sporoblasts (up to 8), resulting from rosette-like sporont fission; (c) individual sporophorous vesicle (SPV) around each spore. Therefore, at the beginning of our studies there were as many as 20 species of microsporidia in mayflies, belonging to 7 genera. However, microsporidia from the mayflies (Insecta: Ephemeroptera) of Russian fauna were not studied before.

The goal of our study is to describe the life cycle and morphology of a microsporidian parasite from nymphs of *Cloeon dipterum* in Russia, and to determine its taxonomic position using morphological and ultrastructural criteria.

Materials and methods

Nymphs of the mayfly *Cloeon dipterum* were collected in flood-plain ponds around the town of Tomsk in July 1996. For light microscopy (LM), smears of infected insect tissues were fixed with methanol for 5 min, stained with Giemsa for 24 h (in a modification proposed by Weiser, 1961) and subsequently washed with deionized water. Digital images were acquired using Carl Zeiss Axio 10 Imager M1 with an attached digital camera. Measurements of spores were performed with Carl Zeiss Axiovision software version 4.4.6.

For transmission electron microscopy (TEM), tissue pieces were fixed with 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) with 4% sucrose for 1–2 h and postfixed with 1% cacodylate-buffered osmium tetroxide for 1 h. The tissues were dehydrated in ascending ethanol series and absolute acetone and embedded in epon-araldite resin. Ultrathin sections were stained with 2% uranyl acetate in 50% ethanol and lead citrate for 10–20 min. The material was examined in a JEM-100 CX II electron microscope at an accelerating voltage of 80 kV.

Repeated screening for microsporidian infection of mayfly populations around Tomsk in 2003–2004, failed to yield new material, and diagnosis using molecular techniques was not available, restricting our research to light and electron microscope analysis.

Results

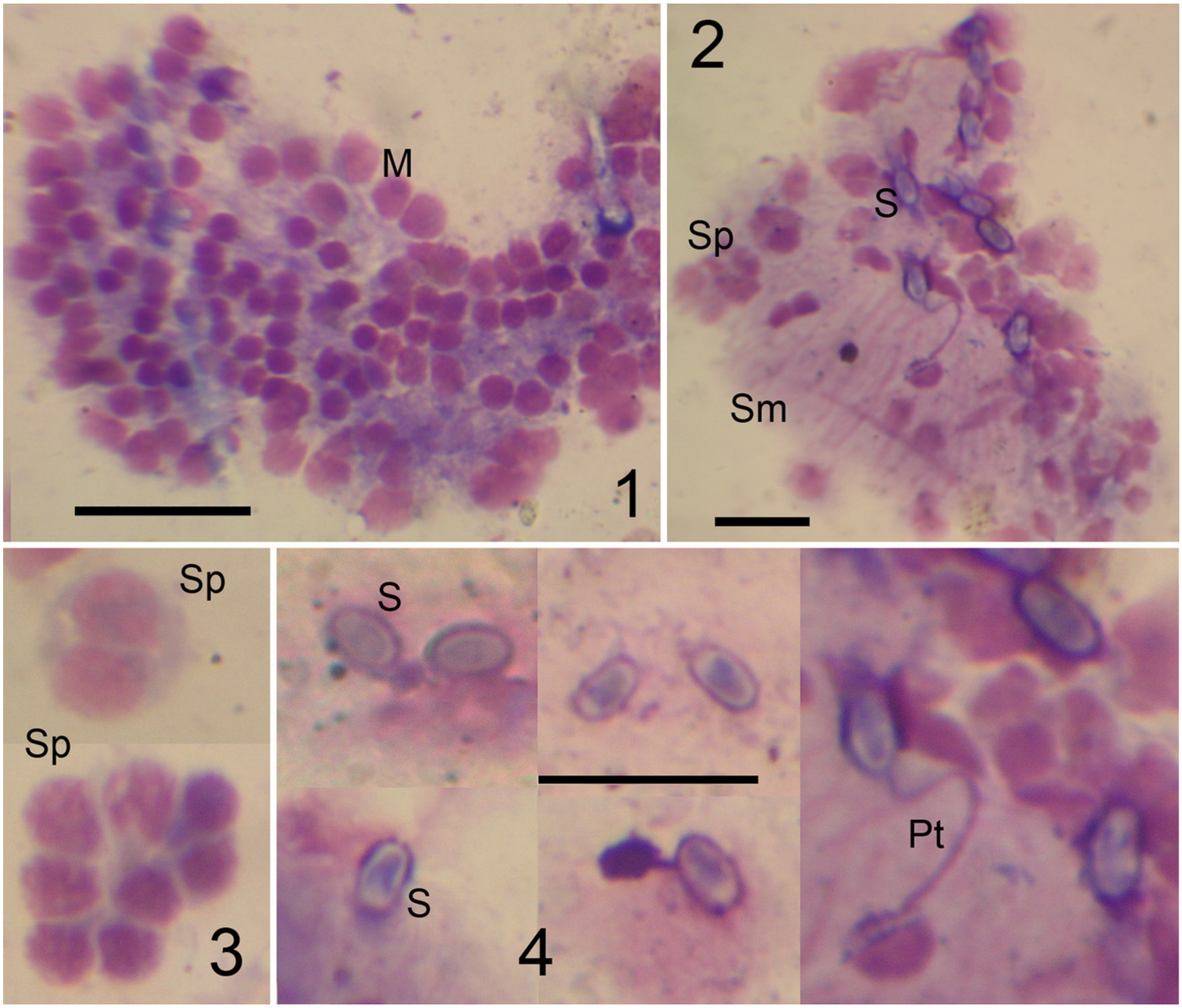
Studies by light microscopy (LM) of the life cycle stages of *Pankovaia semitubulata* gen. et sp. n. found in adipose tissue of *Cleon dipterum* revealed that all stages of the life cycle are monokaryotic and develop in direct contact with host cell cytoplasm (Figs 1 and 2). Some of the developmental stages of microsporidia are found accumulated at the border of the striated muscle cells (Figs 2 and 4). A rosette-like merogonial plasmodium produces 2–4 transitional meront-sporont stages, which give rise to rosette-like sporogonial plasmodia, each dividing into 4–8 sporoblasts, then spores (Figs 3 and 4). Spore development is monomorphic, the stained spores being 3.4 μm × 1.9 μm (2.6–3.9 × 1.9–2.2 μm) in size and oval or oval-cylindrical. On Giemsa-stained slides the exospore is visible as a broad blue rim around the spores, suggesting that the uneven surface of the spores facilitates deposition of the dye. Spores with an extruded polar tube may be observed on the slides (Fig. 4). Discharged spores with ejected invasion tubes are observed regularly on fixed smears, suggesting the possibility of parasite's dissemination due to autoinvasion within the host organism.

Studies of life cycle stages by TEM revealed more details. The meronts are the largest developmental stages (Figs 5–7). Division of merogonial plasmodium produces several, usually 4, spherical meronts, 2.6–3.5 μm in size in late stages, with nuclei up to 1.8 μm in diameter occupying much of the cell (Fig. 7). These developmental stages possess nucleoplasm and cytoplasm of low electron density. Nucleoli were not seen, but the presence of centriolar plaques indicates mitotic activity.

During sporogony each sporont forms a sporogonial plasmodium with 4 to 8 nuclei. Sporont nuclei are 1.6–1.8 μm in diameter. The sporogonial plasmodium divides by rosette-like budding followed by lengthening of each "rose-petal". Electron-dense granular material is accumulated in a spotty pattern on the surface of the sporogonial plasmodium, making the cell surface to look like a leopard's skin. These spots are up to 180 nm in height and 100–200 nm in diameter (Fig. 8). Division of a sporogonial plasmodium produces up to 8 sporoblasts (Fig. 9). Later sporoblasts differ from the early ones by higher amounts of electron-dense granules on the surface, denser cytoplasm and the presence of nucleoli (Fig. 10). During their development, sporoblasts change their shape from broad-oval to oval and decrease in size from 4.9 × 2.6 to 3.0 × 1.3 μm.

The next transitional stage from late sporoblast to young spore is a long-oval cell with smaller size and denser cytoplasm as compared to the early sporoblast (Fig. 11). The cytoplasm of the young spore contains primordia of the polar filament and anchoring disc. This cell is covered with a thick continuous layer of secretory material, undulating due to its varying thickness, ranging from 10 to 130 nm (Fig. 11). Cross sections of fine tubular structures can be seen in the core of the secretory material.

The mature spores are oval-cylindrical, with a size (when fixed and stained) of 2.5–3.2 × 1.0–1.3 μm (Figs 4,5,15). The anchoring disc is a flattened structure under the polar sac, the latter covering the polaroplast (Figs 15 and 16). The polaroplast is bipartite; the anterior

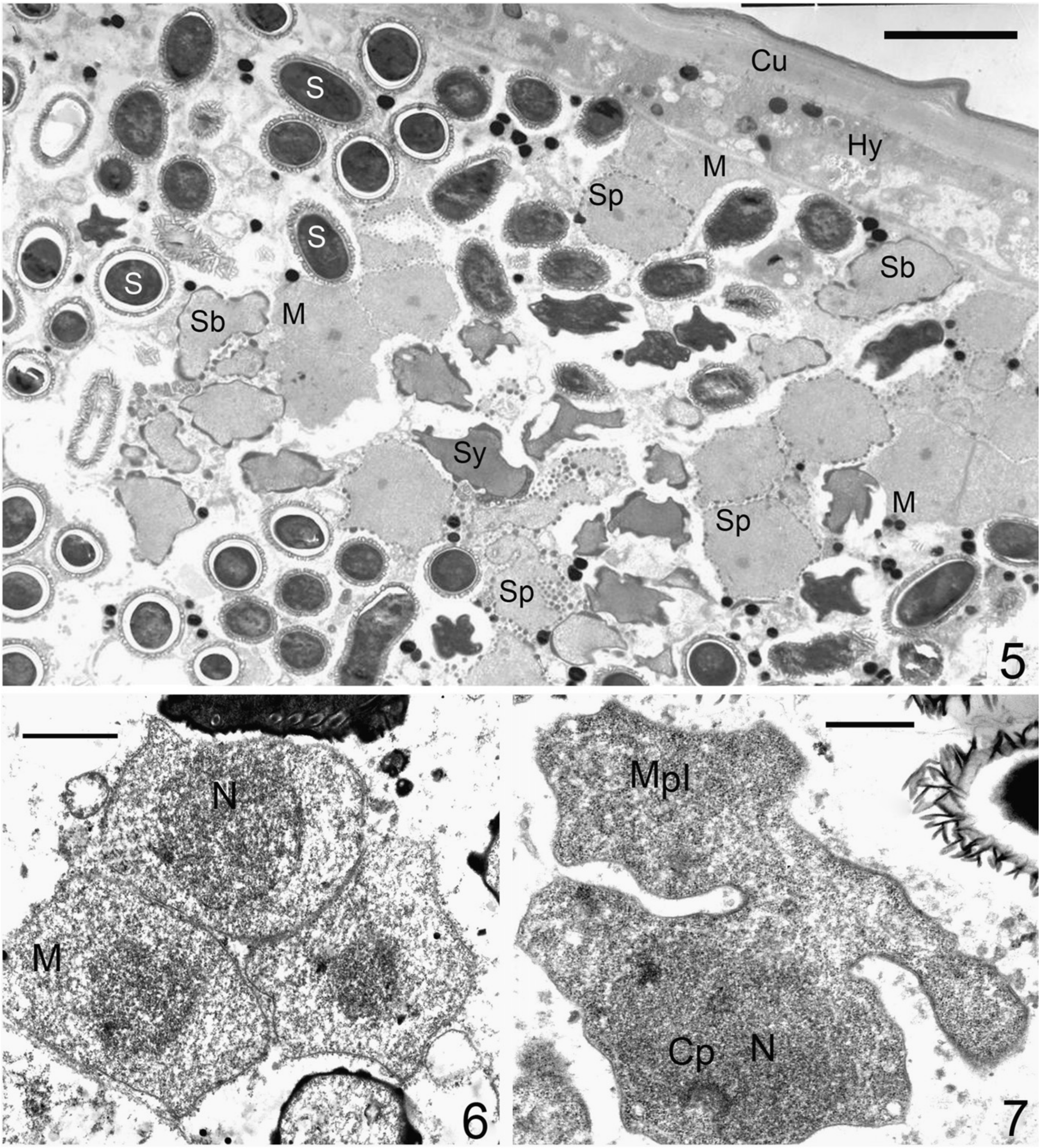


Figs 1–4. *Pankovaia semitubulata* sp. n. light microscopy of the Giemsa-stained slides. **(1)** Adipose tissue cells of the mayfly, filled with meronts with large nuclei. **(2)** A region of infected tissue at the border of the striated muscle. (**3**) A binucleate and an octonucleate sporont. **(4)** Mature spores with intensively stained outer layer of the spore wall. M – meront, Pt – polar tube, S – spore, Sp – sporont, Sm – striated muscle. Scale bars: 10 μm.

horseshoe-shaped (close to circular) region of several tightly packed lamellae surrounds a region of small vesicles filled with inclusions of varied electron density (Fig. 16). The isofilar polar filament is 150–130 nm in diameter and has 6–6.5 coils in one row (Fig. 15). No posterior vacuole was found on a series of sections of mature spores.

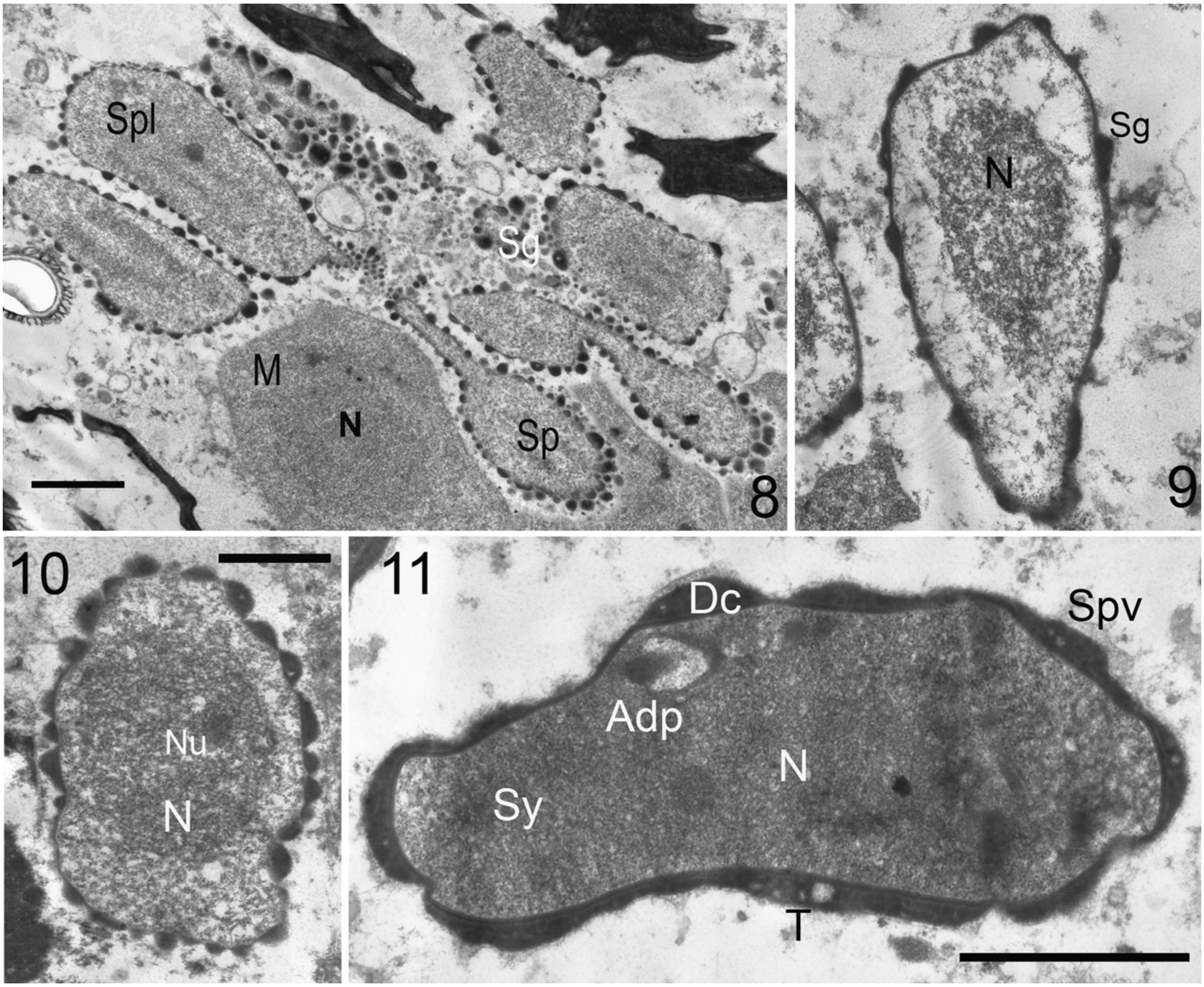
The exospore is a complex structure, composed of numerous "gutter-shaped" elements. In cross sections, they possess the shape of a Latin letter "C", while in tangential sections they look like multiple tubules with a lengthwise opening, irregularly laid on the spore surface, with their openings outwards (Figs 12–17). Due to these features, we refer these formations as "semitubules". They are up to 130 nm high and 60–100 nm wide; the length of some of the semitubules reaches 500 nm (Fig. 17). Due to the presence of these structures on the spore surface, the thickness of the exospore reaches 130 nm, similar to the thickness of the endospore.

The SPV envelope in this species is formed only at the final phase of sporogony. An additional thin layer of moderate electron density appears on small regions of the electron-dense layer surrounding the young spore (Fig. 11). At the phase of spore maturation a single-layered envelope can be seen tightly bound to the complex protrusions of the exospore (Figs 11,12,15). Some regions of this envelope bear tubular structures or form long protrusions into the host cell cytoplasm



Figs 5–7. Merogonial stages of *Pankovaia semitubulata* sp. n. (TEM) **(5)** Section of adipose tissue of mayflies with different developmental stages of microsporidia. **(6)** Late meronts with large nuclei. **(7)** Rosette-like merogonial plasmodium with dividing nuclei. Cp – centriolar plaque, Cu – cuticle; Hy – hypoderm, M – meront, Mpl – merogonial plasmodium, N – nucleus, S – spore, Sb – sporoblast, Sp – sporont. Scale bars: Fig. 5–5 μm; Figs. 6, 7–1 μm.

(Fig. 12). The envelope is gradually detached from the spore, forming an electron-translucent SPV cavity (Figs 13 and 14). The vesicle envelope is ephemeral and hardly visible around the mature spore. Tubular structures or filaments connecting the exospore with the SPV envelope, as well as the reticulate structure of the



Figs 8–11. Sporogonial stages of *Pankovaia semitubulata* sp. n. (TEM) **(8)** Rosette-like sporogonial plasmodium with abundant electron-dense granules on the surface. **(9–10)** Sporoblasts with secretion material accumulated in a spotty pattern. **(11)** Transitional stage late sporoblast to young spore. Adp – primordium of anchoring disc. Dc – dense surface coat. M – meront. N – nucleus. Nu – nucleolus. Sg – secretion granules. Spl – sporogonial plasmodium. Spv – sporophorous vesicle. Sy – young spore. Scale bar: Figs. 8–11–1 μm.

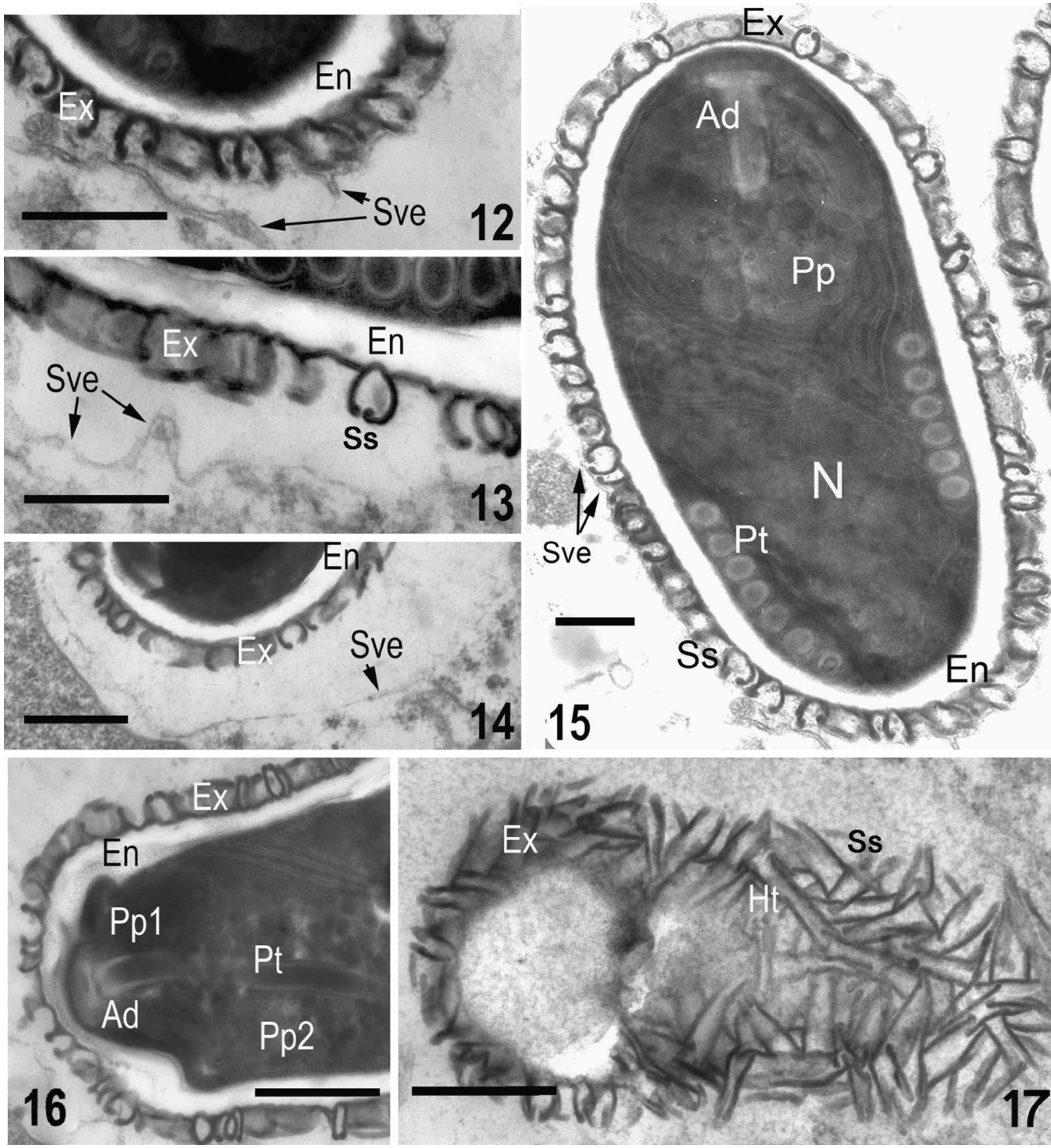
SPV wall itself, typical of *Tuzetia* species, are absent from this microsporidian.

Discussion

This is the first description of a microsporidian parasite infecting a mayfly from Russia. This microsporidium belongs to a new genus and species, which we have named *Pankovaia semitubulata*.

Pankovaia semitubulata has an individual SPV sheath around each spore and therefore should be attributed to the family Tuzetiidae (see Larsson 1983 for the family diagnosis). This family at present incorporates seven genera, namely *Tuzetia*, *Alfvenia*, *Janacekia*, *Nelliemelba*, *Lanatospora*, *Trichotuzetia* and *Paratuzetia* (Larsson 1983; Poddubnaya et al. 2006; Vavra et al. 1997; Voronin 1989, 2001). Only three of these genera are monokaryotic during late merogony and sporogony, as well as the newly established genus *Pankovaia*. Therefore these genera, *Tuzetia, Lanatospora* and *Trichotuzetia*, need to be compared with the type species of the genus described in the present work.

These three genera share following similar features in cell morphology of the sporogonial stage: (a) rosette-like division of sporonts with simultaneous production of sporoblasts enclosed within an individual SPV sheath; (b) accumulation of discrete electron-dense material on the sporoblast surface; (c) epispore structures (tubules,



Figs 12–17. Sporophorous vesicles and spores of *Pankovaia semitubulata* sp. n. (TEM) **(12–14)** Formation of episporontal space of SPV. **(15)** Mature spore with cross-section of "semitubular" structures of exospore. **(16)** Anterior part of the spore with flattened anchoring disc and bipartite polaroplast, in which the anterior horseshoe-like lamellar part surrounds the posterior vesicular one. **(17)** Longitudinal section of spore wall showing semitubular structure of the exospore. Ad – anchoring disc. En – endospore. Ex – exospore. Ht – half-tube sectioned longitudinally. N – nucleus. Pp1, Pp2 – anterior and posterior parts of polaroplast. Pt – polar filament. Ss – semitubular structures. Sve – membrane of SPV. Scale bar: Figs. 12–17–0.5 μm.

fibrils or fleecy layer) filling the episporontal space of the SPV.

Distinctions between these genera are as follows: in *Tuzetia,* the sporont gives rise to 2–8 sporoblasts, the exospore is thin, the SPV cavity is partially filled with secretory tubules, the polar filament is isofilar and the SPV sheath is reticular; in *Trichotuzetia,* the sporont gives rise to 20–30 sporoblasts, the exospore is 4-layered, the SPV cavity is completely filled with fibrils, and the polar filament is isofilar; in *Lanatospora*, 6–16 sporoblasts are formed, the exospore is 2-layered, covered by blister-like structures filled with a fleecy material, a typical SPV is absent, and the polar filament is heterofilar.

The type species of the microsporidian described in the present work is distinguished from these genera by the following features: (a) a continuous layer of amorphous secreted material on the surface of late sporoblasts and young spores; (b) the texture of the exospore, consisting of semitubular structures (see above); (c) the absence of secretory formations in the episporontal space of the SPV. An exospore with such a complicated structure is not known for other genera of the family Tuzetiidae, or for other microsporidia. For these reasons, we propose to establish the new genus *Pankovaia* with the type species of *Pankovaia semitubulata,* described in the present work.

The finding of a new microsporidian species parasitizing *Cloeon dipterum* L. in Western Siberia is interesting for two reasons. First, this is the first record of microsporidia from mayflies in Russia. Second, it is an addition to the list of parasites found in this widely distributed palearctic insect. Two microsporidia have been described from *Cloeon dipterum* in Europe. A discovery of new microsporidian parasitizing the same mayfly is an indication of the originality of parasitic fauna in the Asian part of Russia.

It is noteworthy that the presence of more than one microsporidian species is often revealed in intensively studied insect and crustacean hosts. For example, there are ten species of microsporidia in *Chironomus plumosus* (Chironomidae), eight and six species in the cladoceran crustaceans *Daphnia pulex* and *D. magna*, respectively. It is probable that the mayfly *Cloeon dipterum* possesses various sets of parasites in different parts of its vast distribution area.

A certain level of similarity is found between *Pankovaia semitubulata* and *Tuzetia weidneri* (Canning et al. 2002). The spore wall of the latter is decorated with ridges and its attribution to the genus *Tuzetia* is supplied with the following stipulation: ‘‘We have tentatively assigned the species to the genus *Tuzetia* in spite of the difference which exists between the episporontal secretions and although the prominent system of ridges on the spore wall is a new feature for the genus’’ (Canning et al. 2002, p. 73).

T. weidneri and *P. semitubulata* possess the following similar features: (a) complicated composition of the spore wall; (b) absence of any secretory material in the episporontal space of the SPV; (c) typical membranous structure of the SPV sheath. However, meronts in *T. weidneri* are ribbon-like in contrast to those of *P. semitubulata*. Confirmation of a close relationship between these two species of microsporidia will be possible only after comparison of their respective rDNA nucleotide sequences.

A few words about the composition of the Tuzetiidae are necessary. In our opinion, there are currently four genera that undoubtedly belong to this family: *Tuzetia* (Larsson 1983), *Lanatospora* (Voronin 1989), *Trichotuzetia* (Vavra et al. 1997) and the newly established genus *Pankovaia*. They are characterized by development in direct contact with the host cell cytoplasm, monokaryotic developmental stages, rosette-like fission of meronts and sporonts (as a rule; less frequently, ribbon-like or chain fission is observed), spores of the same type, formed within individual SPV, and isofilar polar filaments. The position of the other four genera (*Janacekia*, *Alfvenia*, *Nelliemelba* and *Paratuzetia*) forming diplokaryotic developmental stages and attributed by Larsson (1983), Poddubnaya et al. (2006) to the Tuzetiidae family, is uncertain. The former author himself regarded their position as temporary: ‘‘In the future it will probably be necessary to split the genera of the Tuzetiidae into more than one family’’ (Larsson 1983, p. 354). The same should be applied to the genus *Paratuzetia* as well.

Diagnosis of *Pankovaia* gen. n.

Monomorphic and monokaryotic throughout the life cycle. All stages develop in direct contact with host-cell cytoplasm. Merogonial and sporogonial plasmodia fission is performed by a rosette-like budding with production of 2–4 late meronts and 4–8 sporoblasts, respectively. During sporogony, amorphous electron-dense material is arranged in a spotty pattern on the surface of rosette-like sporonts, transforming into a continuous layer on the surface of sporoblasts and young spores. Spores possess a bipartite polaroplast and isofilar polar filament. The exospore is single-layered, with a complicated texture. The SPV envelope is represented by a thin fragile membrane. The SPV episporontal space is devoid of secretory inclusions. Parasites of Ephemeroptera. Type species: *Pankovaia semitubulata*.

Etymology. Named in honour of Tamara F. Pankova, an associate professor of Tomsk State University, who initiated research of insect microsporidia in Western Siberia.

Diagnosis of Pankovaia semitubulata sp. n.

Type host: mayfly *Cloeon dipterum* (L.) (Ephemeroptera, Baetidae).
Localization: adipose tissue.
Type locality: vicinity of Tomsk, Western Siberia.

Developmental stages: Merogony and sporogony with characteristics of the genus. Spores are oval-cylindrical, $3.4 \times 1.9\,\mu m$ in size after fixation. The exospore is composed of multiple irregularly laid tubules with a lengthwise opening, referred to as “semitubules”. The polaroplast is bipartite (lamellar and vesicular). The isofilar filament possesses 6–6.5 coils. The SPV envelope is ephemeral, virtually disappearing in mature spores.

Etymology

The species name reflects the shape of complicated structures (semitubules), composing the exospore in mature spores.

Acknowledgements

The research is supported by the Russian Foundation for Basic Research, Grant nos. 07-04-00269 and 07-04-00468, and a grant from the President of Russian Federation no. MK-653.2007.4.

References

Canning, E.U., Curry, A., Overstreet, R.M., 2002. Ultrastructure of *Tuzetia weidneri* sp n. (Microsporidia: Tuzetiidae) in skeletal muscle of *Litopenaeus setiferus* and *Farfantepenaeus aztecus* (Crustacea: Decapoda) and new data on *Perezia nelsoni* (Microsporidia: Pereziidae) in *L. setiferus*. Acta Protozool. 41, 63–77.

Kudo, R., 1924. A biologic and taxonomic study of the Microsporidia. Illin. Biol. Monogr. 9, 1–268.

Larsson, R., 1983. A revisionary study of the taxon *Tuzetia* Maurand, Fize, Fenwick and Michel, 1971, and related forms (Microspora, Tuzetiidae). Protistologica 19, 323–355.

Maurand, J., 1973. Recherches biologiques sur les Microsporidies des larves des Simulies. These. Universite des Sciences et Techniques du Languedoc, Academie de Montpellier, France, pp. 1–199.

Maurand, J., Fize, A., Fenwick, B., Michel, R., 1971. Etude au microscope electronique de *Nosema infirmum* Kudo 1921. Microsporidie parasite d’un Copepode Cyclopoide, creation du genre nouveau *Tuzetia* a propos de cette espece. Protistologica 7, 221–225.

Poddubnaya, L.G., Tokarev, Y.S., Issi, I.V., 2006. A new microsporidium *Paratuzetia kupermani* gen et sp. n. (Microsporidia), a hyperparasite of the procercoid of the cestode *Khawia armeniaca* Chol. 1915 (Cestoda, Caryophyllidea). Protistology 4, 269–277.

Vavra, J., Larsson, R., Baker, M., 1997. Light and electron microscopic cytology of *Trichotuzetia guttata* gen et sp. n. (Microspora, Tuzetiidae), a microsporidian parasite of *Cyclops vicinus* Uljanin, 1875 (Crustacea, Decapoda). Arch. Protistenkd. 147, 293–306.

Voronin, V.N., 1989. The ultrastructure of *Lanatospora macrocyclopis* (Protozoa: Microsporidia) from the cyclope *Macrocyclops albidus* (Jur) (Crustacea, Copepoda). Arch. Protistenkd. 137, 356–357.

Voronin, V.N., 2001. Macrotaxonomy of the phylum Microsporidia. Parazitologya 35, 35–44.

Weiser, J., 1961. Die mikrosporidien als parasiten der insekten. Monogr. Angew. Entomol. 17, 1–149.