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Microsporidia in a Woodland Pool I. *Lanatospora costata* sp. n. (Opisthosporidia, Microsporidia), Parasite of *Megacyclops viridis* (Crustacea, Copepoda): Fine Structure and Molecular Phylogeny

Jiří VÁVRA[1,2], Miroslav HYLIŠ[3], Ivan FIALA[1,2], Dominik REFARDT[4], J. I. Ronny LARSSON[5]

[1] Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic; [2] Department of Parasitology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic; [3] Laboratory of Electron Microscopy, Faculty of Science, Charles University in Prague, Czech Republic; [4] Institute of Natural Resource Sciences, Zurich University of Applied Sciences, 8820 Wädenswil, Switzerland; [5] Department of Biology, Lund University, Lund, Sweden

Abstract. *Lanatospora costata* sp. n., a new microsporidian parasite of the adipose and connective tissue cells of *Megacyclops viridis* (Jurine, 1820) (Copepoda: Cyclopidae) is described. It was isolated from a woodland pool with a rich and diverse fauna of microsporidia-infected small crustaceans and insect larvae. The parasite is structurally similar to the genus *Lanatospora* Voronin, 1989, characterized by a complex decorated exospore. In the described species the exospore is armoured by a conspicuous layer of interwoven ribs forming labyrinth on the spore surface. SSU rRNA phylogeny places the organism in the "aquatic outgroup" of microsporidia (Vossbrinck *et al*. 2004), which includes a number of microsporidia from aquatic microcrustacea of the classes Copepoda and Cladocera. The increasing number of available SSU rRNA gene sequences of these microsporidia allows a more detailed interpretation of their developmental histories.

Key word: Lower fungi, phylogeny, utrastructure, spore, exospore.

INTRODUCTION

Microsporidia are widespread protist parasites of animals, infecting a wide range of hosts from protists to man with more than 200 genera and 1500–2000 species described so far (Vávra and Lukeš 2013, Becnel *et al*. 2014). Microsporidia have been presently ranked into the superphylum Opisthosporidia, a group of parasitic organisms representing a sister clade to Fungi. The Opisthosporidia (containing besides microsporidia the algal parasites "aphelids" and water mold parasites "rozellids") (Karpov *et al*. 2014), have special invasive mechanism in which an infective cell is injected into the host cell by an invasion tube. This mechanism is perfected in microsporidia, in which a preformed tube, present in the spore, everts explosively during spore germination and the spore contents pass through the tube and are injected into cells of the host (Cali and Takvorian 2014).

The evolutionary relationship of microsporidia to other representatives of the Opisthosporidia suggests that microsporidia originated in an aquatic environment and spread from there to terrestrial hosts. This assumption, corroborated by the recent discovery of a basal

Address for correspondence: Jiří Vávra, Charles University, Fac. Sci., Dept. Parasitol., Viničná 7, 128 44 Prague 2, Czech Republic; E-mail: vavrajir@natur.cuni.cz

microsporidian in *Daphnia magna* (Haag *et al.* 2014), substantiates the search for ancestral microsporidia in the rich microsporidian fauna of aquatic crustaceans, namely copepods and daphnids. These are known as frequent hosts of microsporidia from which a number of microsporidian genera was described (Becnel *et al.* 2014; Voronin 1986, 1999). The present paper describes a new species of a microsporidium infecting copepods, completes the recent review of microsporidia from the Arthropoda subphylum Crustacea (Stentiford *et al.* 2013), and allows a more detailed view of their evolutionary history.

MATERIALS AND METHODS

Host and habitat. Microscopy methods

The described microsporidian infects the copepod *Megacyclops viridis* (Jurine, 1820). Infected hosts were found in a small, permanent woodland pool located in the vicinity of the town Přerov nad Labem, Central Bohemia Region, Czech Republic (50°167′N, 14°810′E) during three consecutive years (2007–2009) from mid-April to May, always as rare individuals among solitary, non-infected *M. viridis* copepods. The pool (about 30–40 m^2, 30–50 cm deep, rich in decaying leaves) (Fig. 19) hosted a rich fauna of small crustaceans (copepods spp. and daphnids) and mosquito larvae, some of them infected with their specific microsporidia (undescribed). Infected specimens of *Megacyclops viridis* were conspicuously white in appearance which allowed fishing them out of the water with a sieve. Routine methods of light microscopy (LM) were used for examination: spore measurement of immobilized spores using QuickPHOTO MICRO 3.0 (Promicra) software, staining of smears by Giemsa and Giemsa after hydrolysis (Robinow method), staining of non-fixed developmental stages by 1% aqueous solution of Gurr's Azidine Scarlet Red (AZSR), negative-contrast staining of spores by the bacteriological Burri-Ink (Becnel 2012, Vávra and Larsson 2014, Vávra and Maddox 1976). For transmission electron microscopy (TEM), the methods described in Refardt *et al.* 2008, were used. Scanning electron microscopy (SEM) observations were performed on glutaraldehyde fixed spores, washed in water, dehydrated by ascending ethanol series to 100% ethanol, dried on stubs, coated by gold in an ion sputter and observed with a JEOL 6380 LV microscope.

DNA extraction, amplification, and sequencing. Phylogeny analysis

DNA was extracted from infected animals and the 16S ribosomal RNA (rRNA) gene, internal transcribed spacer (ITS), and partial 23S rRNA were amplified using microsporidia-specific primers as described previously (Refardt *et al.* 2008). Amplified fragments were purified (QIAquick PCR purification kit, Qiagen) and cloned (TOPO TA cloning kit, Invitrogen). Colonies were selected by blue/white-screening, plasmids were purified (QIAprep spin miniprep kit, Qiagen), and digested with EcoRI to check whether they contained the desired insert. Sequencing was done on both strands using T3 and T7 priming sites (Microsynth, Balgach, Switzerland). The obtained microsporidian SSU rDNA sequence was aligned with sequences from closely related microsporidian species available in GenBank. *Vavraia culicis* was set as outgroup. Sequences were aligned using MAFFT v6.626b (Katoh *et al.* 2005) with the E-INS-i multiple alignment method, a gap opening penalty of 1.0, and gap extension penalty of 0.0. The alignment was checked using SEAVIEW v3.2 (Galtier *et al.* 1996) and both ends were trimmed to a final length of 1328 bp. Phylogenetic trees were constructed using maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). ML analysis was performed in RAxML v7.2.8 (Stamatakis 2006) under a GTR + Γ model. MP was done in PAUP* v4.0b10 (Swofford 2001) with a heuristic search, random addition of taxa and Ts:Tv = 1:2. Bootstrap support was calculated from 1000 replicates in both ML and MP analysis. BI was done using MrBayes v3.0 (Ronquist and Huelsenbeck 2003) with the GTR + Γ model of evolution (6 rates of substitution; gamma rate variation across sites; 4 categories used to approximate gamma distribution). MrBayes was run to estimate posterior probabilities over 1 million generations via 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Tracer v1.4.1 (Rambaut and Drummond 2007) was used to ascertain a sufficient length of the burn-in period.

RESULTS

Light microscopy (LM): infected tissues, spores and developmental stages

The infected animals were conspicuously white in appearance, none of them had ovisacs (Fig. 1). Cross-section through the mid-part of the body of infected animals showed that practically all tissues, except muscles, digestive tube and nervous tissue, were full of spores and earlier developmental stages of the parasite, revealed as a single confluent mass (Fig. 2). This made it impossible to determine, which tissue was the primary target of infection, but the general spread of infection indicated that it might be the connective and adipose tissue.

Fresh spores liberated from the animal were egg-shaped, 4,7 (4,4–4,8) × 2,7 (2,5–2,8) µm (n = 10) with a small posterior vacuole (Fig. 3). The shape of spores was well preserved in dry smears and the spores negatively stained by Burri Bacteriology Ink measured 4,4 (4,3–4,7) × 2.5 (2,3–2,6) µm (Fig. 3b). Spores had a single, relatively small nucleus, seen in smears stained by the Robinow method (Fig. 3c). The following developmental stages were identified in Giemsa stained smears: large cells with single nucleus and plasmodia with two to four nuclei (Figs 4a–4c). *In vivo* staining of developmental stages by AZSR (a stain which specifically stains sporophorous vesicle – SPOV-materials) showed

that the plasmodial stages mentioned above actually belonged to the sporogonial sequence of the development, which proceeded as "rosette-like" budding (Figs 5a–f). Four-member rosettes giving rise to four spores were typical (Fig. 5d), but rosettes with 8 daughter cells also existed (Fig. 5e). Individual spores were the final stage of sporogony (Fig. 5f). The AZSR staining demonstrated that extensive SPOV material existed on the surface of young spores (Fig. 5f). This material disappeared as the spore matured and mature spores were AZSR negative.

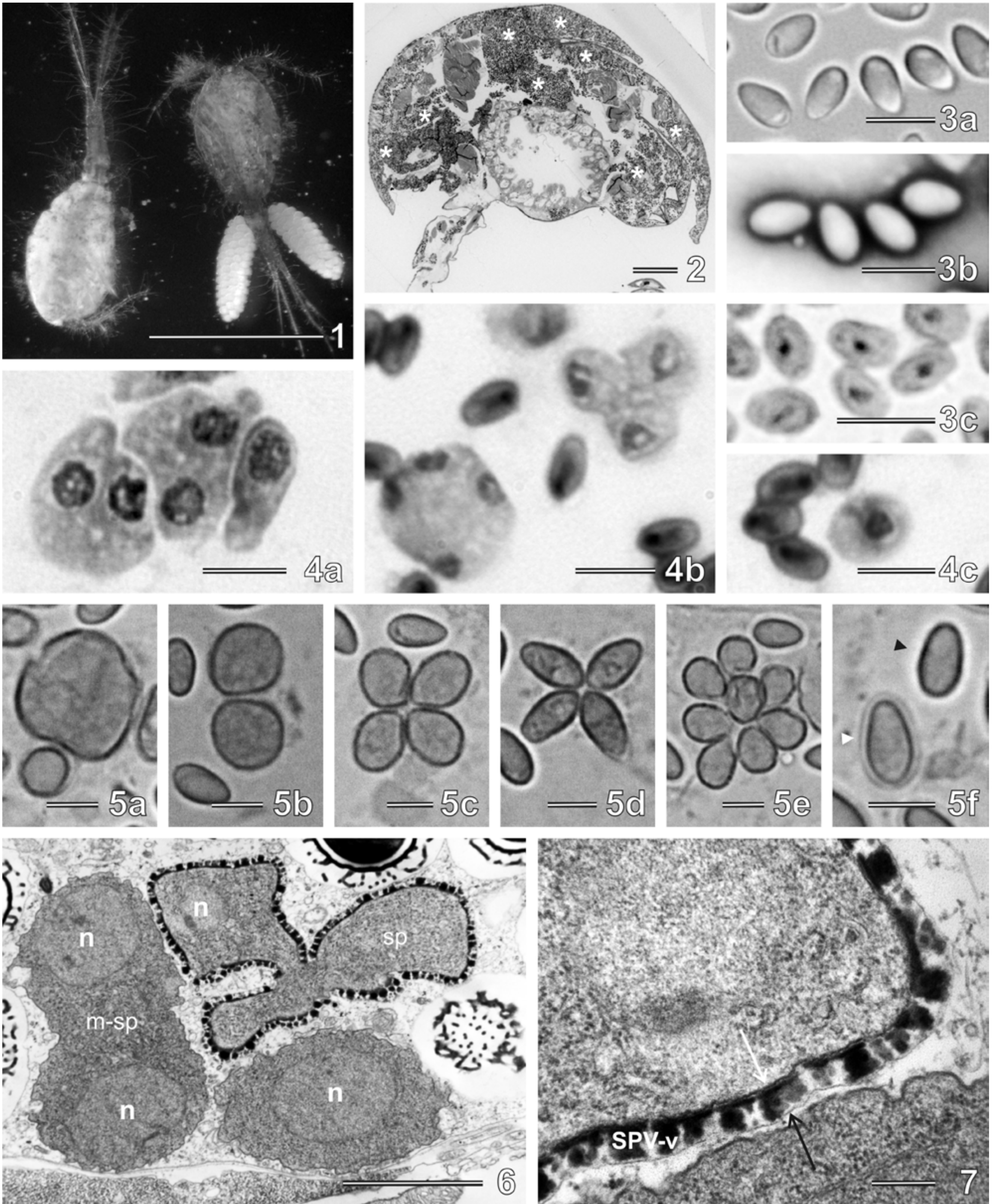
Electron microscopy, transmission (TEM) and scanning (SEM)

The earliest developmental stages seen by TEM were meronts in the form of large plasmodia, with dense cytoplasm, single nucleus (or nuclei), surrounded by wrinkled cytoplasmic membrane (Fig. 6). Slight thickening of the membrane at some place was the first marker of starting sporogony (not shown). Sporonts were seen as cells surrounded partly or completely by thick, uniform layer of material showing patches of dense inclusions deposited at intervals filled with less dense material in the form of irregular patches and globules (Fig. 6). A thin membrane delimited the outer rim of the material deposited at the sporont surface. The inner rim of the material at the sporont surface was the cytoplasmic membrane of the sporont in early sporonts, later a clear zone (trace of the future endospore) separated the cytoplasmic membrane from progressively thickening inner rim of the deposited material, now representing future exospore (Fig. 7). We interpret the outer membrane delimiting the deposited material on the sporont surface as the SPOV membrane. The "thickness" of the deposited material is, in fact, the "volume" (the episporontal space) of the SPOV, and the deposited dense material corresponds to the "secretory material" ("metabolic granules") occurring within SPOVs of many microsporidia (Fig. 7).

Sporonts covered by SPOV divided in finger-like fashion with the SPOV and its material remaining attached to the cell surface (Fig. 6). At the same time a uniform layer of dense material started to form outside the cell membrane of sporonts and thickened progressively. This is the future exospore. Sporonts divided into daughter cells with single nucleus each, in which spore components started to form, defining the "early spores". Young spores had a dense, thick limiting outer wall (on former cell membrane), had the polar filament formed, but were surrounded by the thick layer of granular material inside the SPOV (Fig. 8). As the early spores matured, transparent endospore was formed (Fig. 9). The SPOV membrane detached from the dense material inside and the dense material, formerly located in the SPOV, changed into a system of dense rib-like extension from the exospore, forming in some places completely, and in other places incompletely enclosed system of thick-walled lacunae (Fig. 10). Spores proved to be difficult to fix and preserve for TEM, but their organization was typical for most microsporidia (Fig. 9–14). Each spore was enclosed in its individual SPOV, delimited by a semipersistent membrane, which loosely ensheathed the spore. Spore had a complex exospore (see below), a thick transparent endospore (70–80 nm) (Figs 11 and 13), single nucleus (Fig. 9), lamellar polaroplast of closely parallel membranes, occupying about 1/3 of the spore length (Figs 14 and 16) and isofilar polar filament with 8–9 isofilar coils in a single row (Fig. 17), ending at the spore apex in a complex anchoring disc of several layers of material. The exospore had a uniquely built structure composed of a dark layer of 50 nm, with three sublayers at the limit of resolution (from center to the outside: less dark and diffuse layer, solid dark line, less dense slightly granular layer). A complex system of branched ribs stretched out of the outermost layer of the exospore, extending to a distance of 160–200 nm from the spore surface (Figs 10–14). Each rib was constructed from material of medium electron density with internal, dense axis, this central element being particularly well visible on tangential sections thru the exospore (Fig. 12). The branched ribs formed a kind of basket-like armour at the spore surface. The complex pattern of the exospore structure is best shown at tangential sections of the spore surface. SEM images reveal convincingly that the spore surface is constructed as a complex, irregular and thick mesh-like armour (Fig. 15).

Molecular phylogeny

The described microsporidium falls into the large clade of aquatic microsporidia ("aquatic outgroup" of Vossbrinck *et al.* 2004) (Fig. 18 B–F). Its closest relative, in the SSU rRNA tree, is an uncultured microsporidium UBN_Seattle 5, the DNA of which was isolated from soil (NCBI: KC111804). These two microsporidia are sister related to the group of three species including two undescribed microsporidia (UBN_Seattle 6, with DNA isolated from soil, NCBI: KC111798, and BLAT9 with DNA isolated from a gammarid crustacean in lake Baikal, Russia, NCBI: FJ756058) and *Trichotuzetia*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guttata, parasite of the copepod *Cyclops vicinus*. A sister clade to the above mentioned microsporidia clades is a clade containing the mosquito parasite *Hazardia* (Fig. 18 E). Although these relationships were stable in all performed analyses, the nodal supports were low by most of the nodes.

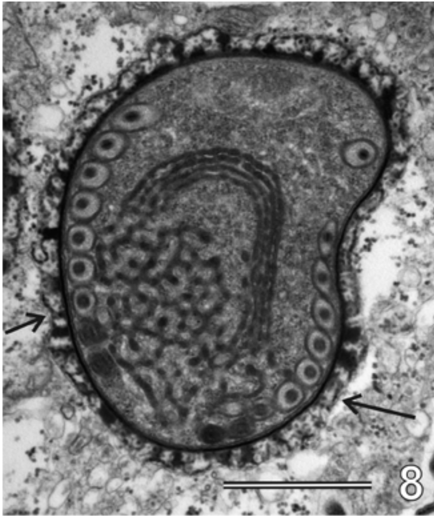
DISCUSSION

Copepod microsporidia and *Megacyclops viridis* as microsporidia host

Copepoda (Crustacea, Maxillipoda) are frequent hosts of microsporidia, documented by the fact that 20 microsporidian genera have a copepod as (sometimes unique) host and 11 genera have copepods as their type hosts (Bronnvall and Larsson 1995, Becnel *et al*. 2014). This encompasses about 20% of the genera from the subphylum Crustacea of the class Arthropoda. The number of microsporidia species described from copepods is estimated to be around 50 species (Bronnvall and Larsson 1995, 2002). The available evidence suggests that many microsporidia are host specific organisms (Vávra and Lukeš 2013), this being characteristic for some aquatic microsporidia (Voronin 1995). The following discussion is therefore focused on microsporidia found in *Megacyclops* sp.

Four microsporidia species were described from *Megacyclops viridis*, the host of the herein described species (Weiser 1945). Hesse (1935) described two species of the genus *Cougourdella* from *M. viridis*, yet the shape of their spore is strikingly different from the species described herein ("squash-like" against "egg-shaped"). The third species, *Tuzetia cyclopis* (Kudo, 1921) Larsson, 1983, (originally described as *Nosema cyclopis* and transferred into the genus *Microsporidium* by Sprague in 1976) has spores of the same shape and nearly the same size (4.2–4.7 × 2.7–3 µm) as the species described herein, but its original host was *Cyclops fuscus*. Its description is documented with a spore drawing only. A microsporidium of the same name, with spores of similar shape and size as described by Kudo, was seen by Jírovec (1936) and Weiser (1945) "in the body" of *Megacyclops viridis* in several habitats (pools and ponds) in what is now Czech Republic. Jírovec reported that living spores in his material were 5 x 2.5 µm in size, and that the fixed spores were of the same size as indicated by Kudo for *Nosema cyclopis* in 1921. Only spore drawings and data on spore size and shape (4.5–4.7 × 2.7–3 µm) were given by Weiser (1945). Jírovec's and Weiser's findings suggest that a microsporidium possibly identical with that one described here occurs frequently in *Megacyclops viridis*. This seemed confirmed by Loubès (1979), who found in the fat body of *M. viridis* a microsporidium, identified by him as Kudo's *Nosema cyclopis* (referred to as *Tuzetia cyclopis* by Larsson in 1983). Spores of his material had similar size and shape as the species described here and those found by Jírovec (1936) and Weiser (1945). However, on Loubès's TEM micrographs the spores have 13 coils of the polar filament and the exospore is relatively thin and smooth. This clearly shows that several (at least two) microsporidia with pyriform spores of nearly the same size parasitize *Megacyclops viridis* and that without TEM data these microsporidia cannot be properly separated. In this situation we feel that the most conservative approach is to retain the name *Tuzetia cyclopis* for the microsporidium studied using TEM by Loubès 1979, and to describe the parasite treated here as a new species. The true identity of *Megacyclops* parasites seen by Jirovec (1936) and Weiser (1945) remains "in limbo".

Figs 1–7. *Lanatospora costata* n. sp., parasite of *Megacyclops viridis*. **1** – Uninfected (with ovisacs) and infected host animals. Note the whithis appearance of the infected individual. Scale bar: 2 mm. **2** – Cross sesction throughout infected *M. viridis*. Infected tissue is shown at asterisks. Semi-thin section, Toluidine blue. Scale bar: 0.1 mm. **3** – Spores of the parasite (3a – fresh; 3b – dry smear stained by Burri Ink; 3c – nuclei revealed by Robinow method). Scale bar: 5 µm. **4** – Giemsa stained developmental stages (4a – binucleate and uninucleate meronts characterized by large nuclei; 4b – four-nucleate and respective non-divided and dividing sporont. Note the smaller size of nuclei in relation to stages on Fig. 4a; 4c – one presporal cell and three young spores. Scale bar: 5 µm. **5** – Developmental stages and spores stained *in vivo* by Azidine Scarlet Red (AZSR) to reveal presence of materials of future sporophorous vesicle and spore coat (5a–5e – multinucleate sporont and cells originated by its rosette-like division; 5f – young spores are nearly AZSR negative, but are covered by a layer of a fuzzy material – arrowheads). Scale bar: 5 µm. **6** – Meront to sporont (m-sp) transitional stages with relatively large nuclei (n) and dividing sporont (sp). Scale bar: 2 µm. **7 –** Construction of the sporont cell wall. From the interior outwards the cell is covered by cell membrane (white arrow), followed by dark, patchy deposits of the material of future spore exospore and its ornamentation. This last, thick layer is externally limited by sporophorous vesicle membrane (dark arrow). Scale bar: 200 nm.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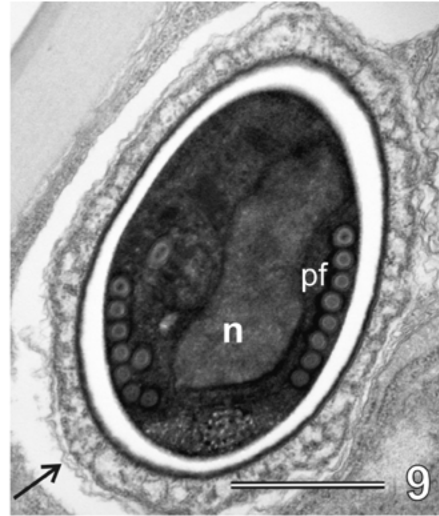
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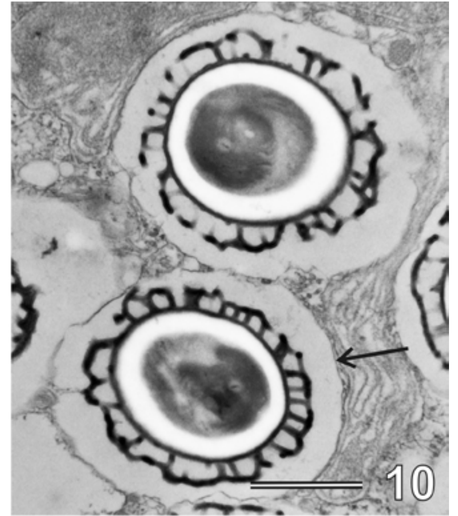
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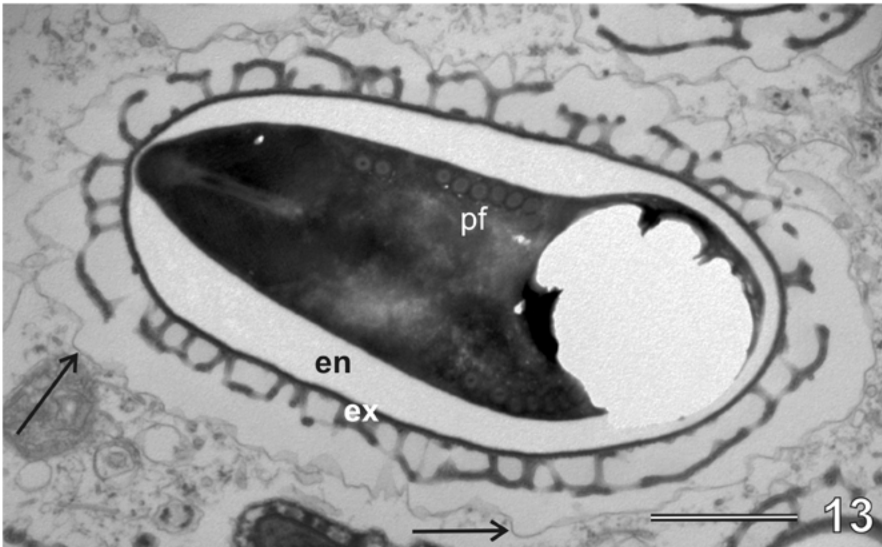
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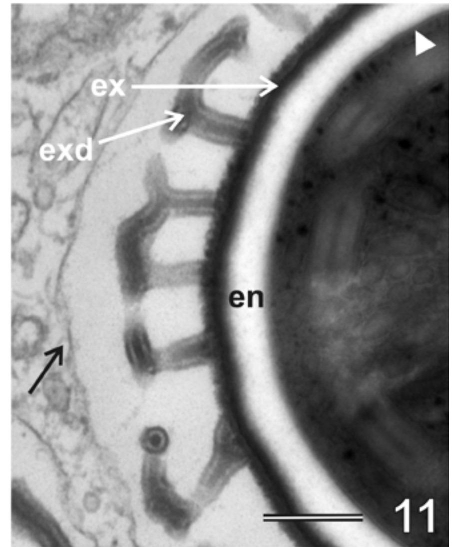
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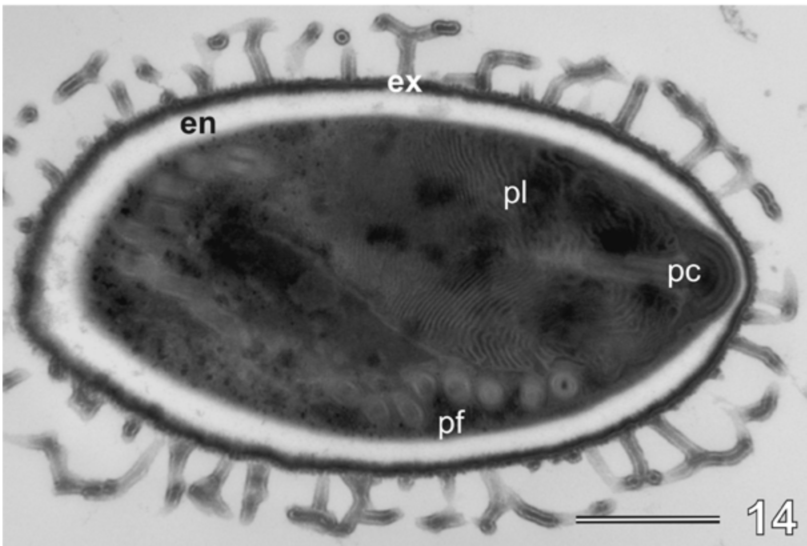
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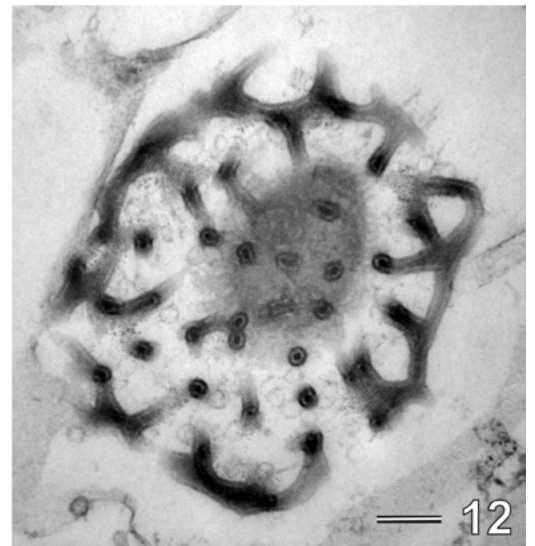
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Which genus and family should be chosen for the microsporidium described herein?

The organism described bears a general structural similarity to microsporidia of the genus *Lanatospora.* So far two described species of the genus *Lanatospora*, are parasites of copepods: *L. macrocyclopis* Voronin, 1989 from *Macrocyclops albidus*, type species of *Lanatospora* and *L. tubulifera* Bronnvall and Larsson, 1995 from *Acanthocyclops vernalis*. The third species of the genus *Lanatospora, L. bosminae* Voronin, 1986 is a sparsely documented species described from connective tissue surrounding the ovaries of the cladoceran *Bosmina* (Voronin 1986). This last species evidently does not belong to the genus *Lanatospora* (see discussion in Bronnvall and Larsson 1995) and two figures showing fine structure of its spores (Voronin 1990) imply resemblance of its spores to *Agglomerata*-like microsporidia from cladocerans (see e.g. Larsson and Voronin 2000).

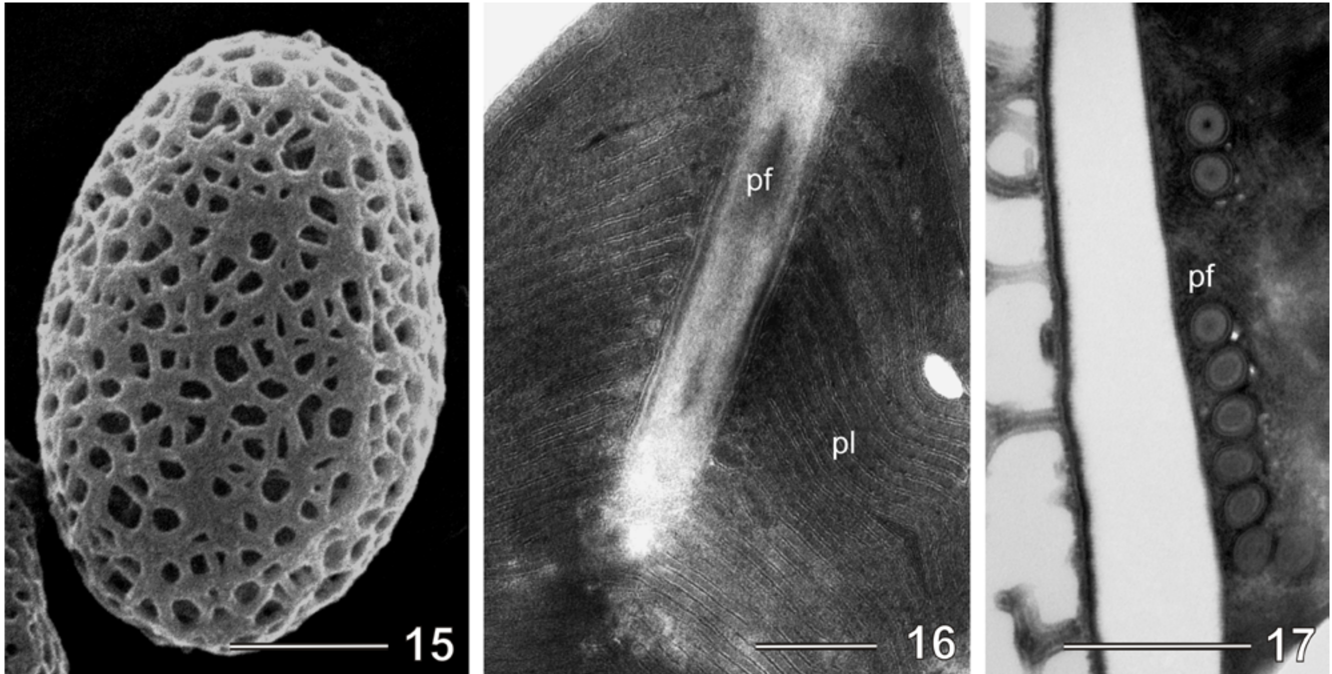
In both *Lanatospora* spp. from copepods, the exospores are decorated by electron-dense extensions filling the SPOV volume between the exospore and the SPOV membrane: "woolskin-like coat", i.e. a granulofibrillar fleece, distributed in an undulatory pattern on the surface of spores in *L. macrocyclopis* and, "numerous tubuli" connecting the exospore to the SPOV in *L. tubulifera*. The branching ribs of *L. costata* sp. n., ornamenting the exospore, seem to have the same structural relationship to the exospore as the exospore ornamentation in both species cited above, however, the ornamentation in *L. costata* sp. n. is much more developed and complex, forming in fact an armour on the spore surface as seen in SEM photomicrographs. In contrast to *L. macrocyclopis* and to *L. tubulifera* the ribs in the species described do not fill completely the SPOV. In favour of the *Lanatospora* identity of our organism is the fact that both so far described species of the genus *Lanatospora* have the same tissue specificity (fat body and connective tissue) as the species under description.

The ranging of our parasite into the genus *Lanatospora* seems to be the most conservative approach, respecting the contemporary microsporidia taxonomy based mainly on minute structural characters. This ranking may be tentative, however, awaiting that the type organism of the genus *Lanatospora* is sequenced and its relationship to *L. costata* sp. n. is confirmed. Concerning the assignment of the family, Sprague *et al*. 1992 ranked the genus *Lanatospora* into the family Tuzetiidae, which disagrees with the oppinion of Voronin (1989), who believed that *Lanatospora* belongs to Tetramicridae Ralphs et Matthews, 1986. We believe that the type of development, in which rosette-like division of sporonts leads to spores enclosed in individual SPOVs, indicates that the microsporidium described here belongs to the family Tuzetiidae Maurand, Fize, Michel and Fenwick, 1971, as emended by Larsson, 1983. A strong argument supporting this ranking is the presence of extrasporal coats in several microsporidia of Tuzetiidae (Larsson 1983), evidently an adaptation to life in water dwelling hosts. It should be noted here, that the above ranking is rather conservative, respecting the conventional microsporidia classification. The ongoing accumulation of molecular phylogeny data is expected to provide means to test the valididy of this, and many others microsporidia taxons.

Spore ornamentations in aquatic microsporidia

The exospore ornamentation in the described species is a conspicuous example of spore surface decoration, frequently occurring in microsporidia infecting aquatic hosts. In some aquatic microsporidia the exospore is grossly modified and forms long tail and

Figs 8–14. *Lanatospora costata*, parasite of *Megacyclops viridis*, structure of spores as seen in TEM. **8** – A young sporoblast stage with polar filament coils being formed, several cisternae of the endoplasmic reticulum and a hypertrophied Golgi zone. The cell is covered by layers of materials corresponding to those in the sporont. Scale bar: 1 µm. **9** – Young spore with its nucleus (n), polar filament coils (pf), transparent endospore, single layer exospore surrounded by fibrillo-granular material of future exospore ornamentation. The outermost layer around the spore is the sporophorous vesicle (SPOV) membrane (arrow). Scale bar: 1 µm. **10** – Cross section through the fully formed spores, showing the exospore connection to the labyrinth of exospore ribs and lacunae and that each spore is surrounded by its own SPOV vesicle (arrow). Scale bar: 1 µm. **11** – Detailed view of the spore envelopes with chitinous endospore (en), single layer exospore of medium thickness (ex, arrow), to which exospore ribs are connected. The rib has a complex substructure represented by "stalks" of material of medium electron density with a central, dense element (exd, white arrow). SPOV membrane is at dark arrow. Scale bar: 200 nm. **12** – Tangential section through spore ornamentation showing the substructure of the ribs described in Fig. 11. Scale bar: 200 nm. **13, 14** – Longitudinal section of spore, showing its organelles and elements (pc – polar cap, pf – polar filament, pl – polaroplast, en – endospore, ex – exospore, arrow – SPOV membrane) and the system of spore ribs, forming irregular, open to semi-open or closed lacunae. Scale bar: 1 µm (Fig. 13), 0.5 µm (Fig. 14).



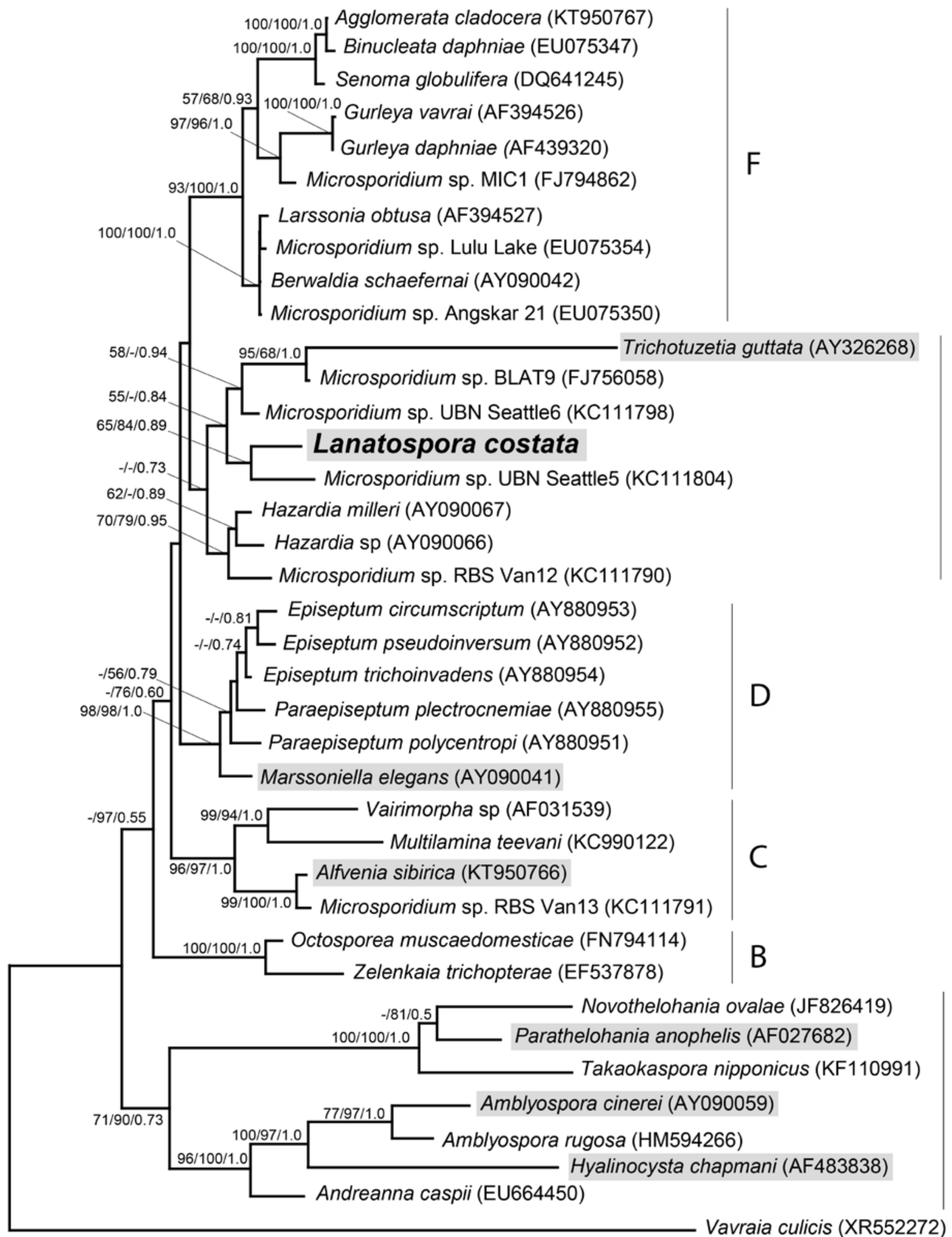
Figs 15–17. *Lanatospora costata*, parasite of *Megacyclops viridis*, structure of spores as seen in SEM and TEM. **15** – Spore surface ornamentation as seen by SEM. Note that the exospore ribs form a complex armour on the spore surface. Scale bar: 1 µm. **16** – Detail of the polaroplast lamellae (pl) in the apical part of the spore, pf – polar filament. Scale bar: 200 nm. **17** – Details of the polar filament coils (pf) in cross section. Scale bar: 500 nm.

wing-like appendages, in other species the exospore is decorated by fine bristles or fibers, imitating the "mucous envelopes" (Lom and Vávra 1963, Vávra *et al*. 2005, Vávra and Larsson 2014). It is believed that spore ornamentations aid buoyancy to spores or modify their rheological properties, however, no data exist to support this assumption (Vávra and Lukeš 2013). It is also possible that spore ornamentations play role in food particle selection by respective microsporidia host, or that the ornamentations play role in adherence of spores to intestinal epithelium of their hosts during peroral infection process. The number of so far isolated and characterized exospore proteins in spores of some terrestrial microsporidia, some of them modifying infectivity of spores to their hosts (Yang *et al*. 2015), indicates that possibly spore ornamentations play a similar role in microsporidia from aquatic hosts. Enormous variability in structural appearance of exospore ornamentations suggest, that characterization of exospore proteins in aquatic microsporidia may represent a fertile research area.

The *Lanatospora* phylogeny

Lanatospora costata n. sp. is presently the first representative of the genus *Lanatospora* with a known molecular phylogeny. It belongs to microsporidia assembled into clade I ("class Aquasporidia") of the molecular and ecological distribution pattern of microsporidia of Vossbrinck and Debrunner-Vossbrinck 2005, and specifically to the part comprising organisms

Fig. 18. Maximum likelihood tree based on microsporidian SSU rDNA sequences containing *Lanatospora costata* sp. n. Support values are given for each node, the first two numbers are bootstrap values based on the maximum likelihood and maximum parsimony analyses (1000 replicates, less than 50% not shown), the third values representing the posterior probabilities of Bayesian inference. Scale bar is given under the tree. Microsporidia from copepods are in grey boxes, clades A–F represent well defined phylogenetic groups of organisms. Clades B–F belong to the "aquatic outgroup" of Vossbrinck *et al*. 2004. The sister clade (A) to the aquatic outgroup clade is the *Amblyospora-Parathelohania-Hyalinocysta* clade in which some species have a copepod host.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Gurleya daphniae (AF439320)
Microsporidium sp. MIC1 (FJ794862)
Larssonia obtusa (AF394527)
Microsporidium sp. Lulu Lake (EU075354)
Berwaldia schaefernai (AY090042)
Microsporidium sp. Angskar 21 (EU075350)
Trichotuzetia guttata (AY326268)
Microsporidium sp. BLAT9 (FJ756058)
Microsporidium sp. UBN Seattle6 (KC111798)
Lanatospora costata
Microsporidium sp. UBN Seattle5 (KC111804)
Hazardia milleri (AY090067)
Hazardia sp (AY090066)
Microsporidium sp. RBS Van12 (KC111790)
Episeptum circumscriptum (AY880953)
Episeptum pseudoinversum (AY880952)
Episeptum trichoinvadens (AY880954)
Paraepiseptum plectrocnemiae (AY880955)
Paraepiseptum polycentropi (AY880951)
Marssoniella elegans (AY090041)
Vairimorpha sp (AF031539)
Multilamina teevani (KC990122)
Alfvenia sibirica (KT950766)
Microsporidium sp. RBS Van13 (KC111791)
Octosporea muscaedomesticae (FN794114)
Zelenkaia trichopterae (EF537878)
Novothelohania ovalae (JF826419)
Parathelohania anophelis (AF027682)
Takaokaspora nipponicus (KF110991)
Amblyospora cinerei (AY090059)
Amblyospora rugosa (HM594266)
Hyalinocysta chapmani (AF483838)
Andreanna caspii (EU664450)
Vavraia culicis (XR552272)
100/100/1.0
100/100/1.0
57/68/0.93
100/100/1.0
97/96/1.0
93/100/1.0
100/100/1.0
58/-/0.94
95/68/1.0
55/-/0.84
65/84/0.89
-/-/0.73
62/-/0.89
70/79/0.95
-/-/0.81
-/-/0.74
-/56/0.79
-/76/0.60
98/98/1.0
-/97/0.55
99/94/1.0
96/97/1.0
99/100/1.0
100/100/1.0
-/81/0.5
100/100/1.0
77/97/1.0
71/90/0.73
100/97/1.0
96/100/1.0
F
E
D
C
B
A
0.1

Fig. 19. The woodland pool near Přerov nad Labem, Central Bohemia Region, Czech Republic (50°167′N, 14°810′E), the type habitat of *Lanatospora costata* sp. n.

of the "aquatic outgroup" of Vossbrinck *et al.* 2004, represented in Fig. 18 by well supported clades B–F. Three microsporidia infecting copepods occur in the "aquatic outgroup", however only one, *Trichotuzetia guttata* from oocytes and other tissues of *Cyclops vicinus*, is member of a sister clade to *Lanatospora* (clade E, Fig. 18). The other two species (*Marssoniella elegans*, parasite of ovarial tissue of *Cyclops vicinus* (clade D, Fig. 18) and *Alfvenia sibirica* (clade C, Fig. 18), parasite of the hypoderm and fat body of *Cyclops* sp.) are widely separated from *Lanatospora* and have no mutual phylogenetic relationships (Fig. 18). Such phylogeny discrepance is interesting, as *Alfvenia sibirica* was reported to have a complex, multilayer exospore (Sokolova *et al.* 2016), very slightly reminiscent of the exospore of *Lanatospora*.

In general, relatively few microsporidia from copepods have been sequenced. Most of the sequenced microsporidia species infecting copepods belong to the genera *Amblyospora*, *Hyalinocysta*, *Parathelohania*, *Duboscqia* and *Trichoctosporea*, which are dimorphic or polymorphic species infecting mosquitos and have copepods as intermediate hosts (Bjørnson and Oi 2014, Simakova *et al.* 2011). These microsporidia form a defined *"Amblyospora-Parathelohania-Hyalinocysta"* clade, sister to the "aquatic outgroup" superclade (Fig. 18).

Other microsporidia from copepods are spread over the complete microsporidia phylogeny and are well outside of the aquatic outgroup (part of clade I in Vossbrinck, Debrunner-Vossbrinck 2005): *Paranucleospora* (syn. *Desmozoon*) (Clade IV), and *Facilispora* (Clade III) are fish parasites with a copepod morph (Jones *et al.* 2012), *Mrazekia macrocyclopis* Issi *et al*., 2010 belongs to microsporidia nested within the clade IV.

TAXONOMIC SUMMARY

Lanatospora costata sp. n.

Developmental stages: round meronts (8–10 µm), with single or two large (2.2–3 µm), isolated nuclei. Round sporonts (10 µm), with four smaller (2–3 µm) nuclei divide by rosette-like budding into 4-8 sporoblast and spores. Spores egg-shaped, 4,7 × 2,7 µm with single nucleus, small posterior vacuole. Shape of spores well preserved in dry smears, spores negatively stained measure 4,4 × 2.5 µm.

Spore: enclosed in a semipersistent sporophorous vesicle ensheating loosely the spore. Spore with a thick transparent endospore (70–80 nm), single nucleus, lamellar polaroplast of closely parallel membranes, occupying about 1/3 of the spore length and isofilar polar filament with 8–9 isofilar coils in a single row. Exospore built of a dark layer (50 nm), extending into a complex system of branched ribs with inner substructure, stretching to a distance of 160–200 nm from the spore surface and forming basket-like armor at the spore surface.

Transmission mode: not examined.

Type host, tissue infected and habitat: *Megacyclops viridis* (Jurine, 1820), connective and adipose tissue. Woodland pool in the vicinity of the town Přerov nad Labem, Central Bohemia Region, Czech Republic (50°167′N, 14°810′E).

Deposition of types: slides with Giemsa and Burri-ink stained spores are deposited in the International Protozoan Type Slide Collection, Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA, Accs. USNM Nos. (1421569, 1421570) and in respective slide collections of Jiří Vávra and Miroslav Hyliš at the Department of Parasitology and at Laboratory of Electron Microscopy, Faculty of Science, Charles University in Prague, Czech Republic. SSU rRNA gene sequence was deposited at GenBank Acc. No. KX832080.

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