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ORIGINAL ARTICLE

Fine structure of the microsporidian parasite (Holomycota: Microsporidia) found in the freshwater tardigrade *Grevenius pushkini* (Tardigrada: Isohypsibioidea)

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Summary

Proliferative stages and spores of a microsporidian parasite were identified in the freshwater tardigrade *Grevenius pushkini* (Tardigrada: Isohypsibioidea). Infection with microsporidia was confirmed by transmission electron microscopy (TEM). Parasites were detected in hypodermal cells, storage cells within the body cavity, muscle cells, epithelial gonadal cells, germ cells, and nurse cells within the gonad cavity. The typical stages of the microsporidian life cycle were observed, including meronts, sporonts, sporoblasts, and spores. Early proliferative stages developed in direct contact with the host cell's cytoplasm, while sporoblasts, immature, and mature spores were enclosed within voluminous vacuoles. The mature spores were elongated and contained a single nucleus. The isofilar polar filament formed 7–9 coils, arranged in a single row. The exospore was ornamented with a thick layer of densely packed prismatic elements, displaying a honeycomb-like structure in tangential sections. The spore wall showed an interruption at the anterior pole of the spore, positioned above the polar sac-anchoring disc complex. In this area, the spores displayed a nipple-like protrusion filled with loose amorphous material and bounded with an electron-dense envelope, distinctive from any other spore wall layer. The unique morphological features of the discovered microsporidian parasite differentiate it from any known microsporidian species, warranting its classification as a new species of microsporidia, designated as *Microsporidium papillum* sp. nov. This study provides the first detailed morphological description of a microsporidia found in tardigrades, and represents the second documented case of tardigrade-invading microsporidia described by TEM, highlighting Tardigrada as a new group in the list of microsporidian host taxa.

Key words: Microsporidia, Tardigrada, ultrastructure

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Introduction

Tardigrada is a group of microscopic multicellular animals widely distributed in nature (Nelson et al., 2018). Known for their cryptobiotic capabilities, they are able to enter an anhydrobiotic state to survive desiccation. This group is currently considered the present-day descendants of the extinct Cambrian "Lobopoda" group, which is thought to have given rise to the mega-diverse phylum Arthropoda (Jørgensen et al., 2018). Despite the wealth of the modern data on tardigrade morphology and biology, this group remains underexplored. One of the least studied aspects of tardigrade biology is their symbiotic relationships with prokaryotic and eukaryotic microorganisms (Nelson et al., 2018). Tardigrades have been observed to be infected with fungi, bacteria, and viruses (Vecchi et al., 2016 and references therein). Reports of microsporidian invasions in tardigrades are scarce and primarily rely on light microscopic observations (Marcus, 1929; Petersen, 1951; Weglarska, 1970). Some of these observations are subject to doubt (e.g., the so-called X-bodies, as noted by Nelson et al., 2018). So far, the only publication describing the microsporidian invasion in tardigrades using transmission electron microscopy (TEM), is the paper by Rost-Roszkowska et al. (2013). The latter study mainly focused on describing the presumed mechanisms of the host reaction to the invasion of midgut epithelial cells, briefly mentioning the presence of parasites in other tardigrade tissues but lacking detailed images of the parasite. Our finding of microsporidian proliferative stages and spores in *Grevenius pushkini* Tumanov, 2003 (Tardigrada: Isohypsibioidea) represents the second documented case of tardigrade-invading microsporidia confirmed by TEM studies and the first one, specifically dedicated to the microsporidian parasite.

Material and methods

Sampling and tardigrade isolation

Samples of the bottom sediments (sand with leaf litter and debris) were collected from Serdobolskiy pond in the park of St. Petersburg State Forestry University (59.994949; 30.332382) in May 2023. Tardigrades were extracted from fresh samples by washing them through two sieves (Tumanov, 2018). The content of the finer sieve was examined under a Leica M205C stereo microscope, and tardigrade specimens were isolated using a glass pipette. Infected specimens were recognised by the presence of an unusual matte-whitish coloration of the body and reduced mobility. Among the 12 animals obtained, a single infected specimen of *Grevenius pushkini* was found. It was examined and documented using a Leica DM2500 microscope equipped with a Nikon DS-Fi3 digital camera with NIS software and fixed for TEM studies.

Transmission electron microscopy

To ensure immediate fixation of intracellular parasites within the cuticle-covered multicellular animal, a pre-fixation protocol with osmium tetroxide was used. The infected tardigrade was prefixed in 1% OsO4 prepared in 0.1M phosphate buffer (pH 7.2–7.4) for 1 hour at room temperature (RT), washed for 3×20 min in the same buffer at RT (Hayat, 1981). The sample was fixed in a mixture of 2.0% glutaraldehyde and 0.8% formaldehyde prepared in 0.1M phosphate buffer for 48 hrs at +4 °C; with subsequent 3×20 min washes in the same buffer at RT. Postfixation was performed with 2.5% glutaraldehyde for 24 hrs at +4 °C, followed by washing for 3×20 min in the same buffer at RT. Subsequently, the sample was further postfixed with 2.0% osmium tetroxide in distilled water for 1h at +4 °C, washed for 3×5 min in water at RT, and dehydrated through a graded ethanol series and final dehydration in 100% acetone. The blocks were embedded in SPI-PON 812 resin (SPI Suppliers, USA) according to the manufacturer's instructions. Sections were cut using a Leica Ultracut 7 ultramicrotome. Semi-thin sections were stained with Epoxy Tissue Stain (Electron Microscopy Sciences, USA, cat. 14950). Thin sections were double-stained with a 2.0% aqueous solution of uranyl acetate and Reynolds' lead citrate. All samples were examined with a JEOL JEM-1400 (JEOL Ltd., Japan) electron microscope at 80 kV.

Results and discussion

Pathogenesis

The infected tardigrade specimen exhibited an unusual matte-whitish coloration of the body and reduced mobility. Examination under a light microscope revealed significantly enlarged (hyper-trophied) body cavity cells (storage cells) containing numerous rounded bodies (spores) in the

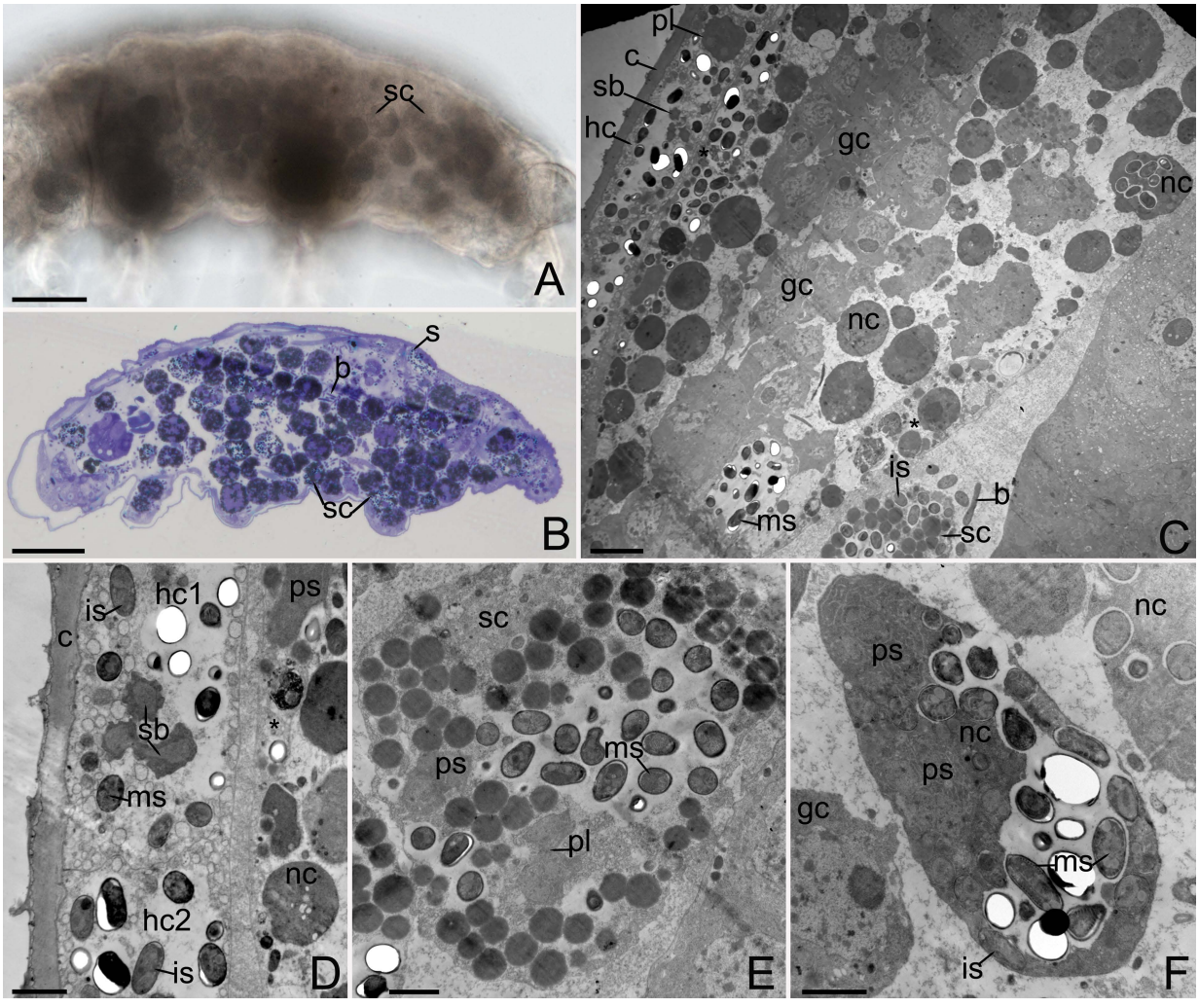

Fig. 1. Microsporidian infection in the tardigrade *Grevenius pushkini*. A – Live infected tardigrade under the light microscope, bright field. Storage cells within the body cavity are hypertrophied; B – longitudinal semi-thin section through the infected tardigrade revealing numerous spores and proliferative stages of microsporidia in hypodermal cells and storage cells, along with distinctive accumulations of bacteria not typical of healthy tardigrades; C – ultra-thin sagittal section through the tardigrade with infected hypodermal cells, the epithelial gonadal cell, germ cells, nurse cell within the gonad cavity and storage cell within the body cavity; D – hypodermal cells with immature and mature spores and sporoblasts of parasite; epithelial gonadal cell (*asterisk*) with the proliferative stages; E – spores developing within voluminous vacuoles and sporogonial plasmodia of the parasite within the cytoplasm of the storage cell; F – numerous microsporidian spores developing within the voluminous vacuoles and proliferative stages in the cytoplasm of a nurse cell. *Abbreviations*: s – parasite spores within hypodermal cells, b – bacteria aggregates, c – cuticle, hc – hypodermal cell, is – immature spore, ms – mature spore, nc – nurse cell, pl – plasmodium, ps – proliferative stage, sb – sporoblast, sc – storage cell, gc – germ cell. Scale bars: A–B – 50 μm, C – 5 μm, D–F – 2 μm.

cytoplasm. The cell surface appeared distorted due to the accumulation of spores, giving the cells a knobby appearance (Fig. 1, A). Microsporidian infection was confirmed by TEM in hypodermal cells, storage cells, muscle cells, epithelial gonadal cells, germ cells, and nurse cells in the gonad (Fig. 1, B–F). All typical stages of microsporidian life cycle were observed, including meronts, sporonts, sporoblasts, and spores. The development of parasites within a single cell was asynchronous; all stages of the life cycle could be observed in an individual infected host cell (Fig. 1, D–F). Early

proliferative stages developed in direct contact with the cytoplasm of host cell while sporoblasts, immature, and mature spores were enclosed within the voluminous vacuoles. While the origin of these vacuoles is unknown, it can be inferred from general knowledge of microsporidian developmental patterns and life cycles that they are surrounded by a membrane of parasitic origin, therefore representing sporophorous vesicles. Interestingly, late sporogonial stages and spores were frequently found together within the same sporophorous vesicle in our specimens.

Proliferative stages

The earliest proliferative stage observed was the merogonial bi- and multinucleate plasmodia, characterised by electron-lucent cytoplasm filled with numerous ribosomes and large nuclei containing electron-dense patches of chromatin located at the periphery. A notable feature was the close association of the plasma membrane of merogonial plasmodia with the host mitochondria (Fig. 2, A). Late uninucleate meronts exhibited a rather electron-dense cytoplasm and prominent rough endoplasmic reticulum (Fig. 2, B). Dividing and binucleate cells with even denser cytoplasm and initial signs of cell envelope thickening were observed, likely representing early sporonts (Fig. 2, C–D). Division occurred through intranuclear closed pleuromitosis involving centriolar plaques and intranuclear microtubules (Fig. 2, C). Subsequent steps led to the formation of a sporogonial plasmodium characterized by electron-dense cytoplasm and the light accumulation of electron-dense material (primordial exospore) on the outer surface of the plasma membrane. It is worth noting that in the studied tardigrade parasite, the thickening of the plasma membrane during primordial exospore formation in the sporonts was not as pronounced as in most other species of microsporidia. Plasmodia were divided by a rosette-like plasmotomy, resulting in the separation of sporoblasts. The whorl-like cluster of membranes located at the center of the rosette likely served as a membrane source for the ongoing growth and separation of sporoblasts (Fig. 2, E).

Immature and mature spores

The immature spores displayed a completely formed spore wall with well-developed exospore and nearly imperceptible endospore. Precursors of the anchoring disk and polar filament were visible within the developing spore (Fig. 3, A, B). The cytoplasm contained numerous ribosomes and transparent 'channels', likely representing endoplasmic reticulum cisternae.

Mature spores, when viewed in cross-sections, appeared generally oval, sometimes elongated. They were monokaryotic (Fig. 3, D) and measured approximately 2.0–2.5 μm in length and 0.8–1.0 μm in width in longitudinal sections. The spore wall of immature and mature spores did not differ; apparently, it was formed during the early steps of sporogenesis. The spore wall consisted of a thick exospore, and a thin, nearly undeveloped electron-lucent endospore (Fig. 3, D–F). The exospore measured approximately 35 nm in thickness and comprised two distinct layers. The basal layer was very thin and electron-dense. The upper layer of the exospore was composed of tightly packed prismatic elements, forming a honeycomb-like structure in transversal sections (Fig. 3, B, D, F). The outermost part of these elements was denser and, in the best sections, appeared as a very fine line outlining the exospore. Above this layer, there was an arrangement of loose filamentous material. However, it was challenging to ascertain whether this material originated from the spore wall or the host cell (Fig. 3, D). The exospore enveloped the entire spore except for the anterior pole just above the polar sac-anchoring disc complex. The basal layer of the exospore was discontinuous in this area, and the material from the outer exospore layer detached from the cell membrane, forming a bulge filled with transparent material. At this area, the cell membrane formed a nipple-like protrusion with a denser (likely filamentous) conical underlying basement (Fig. 3, A–E). This protrusion measured around 160 nm in diameter; the exospore material covering the "nipple" was thinner and less uniformly structured than in other areas of spore surface.

We did not notice the presence of the upper prismatic layer of primordial exospore at the surface of late sporonts, whereas in immature spores, the spore wall had already reached full maturity (Fig. 3, A). The morphogenesis of internal structures of the spore, namely the organelles of the extrusion apparatus, occurred in immature spores with a fully developed spore wall. Such early maturation of the spore wall is quite unusual.

The polar sac-anchoring disk complex exhibited a canonical mushroom-like appearance, which was easily discernible in both immature and mature spores (Fig. 3, A–D). The polaroplast was composed

Fig. 2. Fine structure of proliferative stages of *Microsporidium papillum* sp. nov. infecting the tardigrade *Grevenius pushkini*. A – Merogonial plasmodium characterised by electron-transparent cytoplasm filled with numerous ribosomes and by large nuclei with electron-dense patches of chromatin located at the periphery; B – late uninucleate meronts; C – division of an early sporont showing the final step of intranuclear closed pleuromitosis and cytokinesis. Centriolar plaque and intranuclear microtubules are seen; D – early binucleate sporont; E – rosette-like division of a sporogonial plasmodium resulting in formation of the sporoblasts with an electron-dense envelope (primordial exospore). *Abbreviations*: cp – centriolar plaque, d – electron-dense inclusions (granules inside the storage cells), er – endoplasmic reticulum, m – host mitochondria, mc – membrane cluster, mt – intranuclear microtubules, n – parasite nucleus, ex – primordial exospore. Scale bars: A, C, E – 500 nm; B, D – 1 μm.

of rare, loosely arranged lamellae (Fig. 3, D). The isofilar polar tube displayed 7–9 coils in a single row (Fig. 3, C, D). The pronounced posterior vacuole was frequently observed to be bi- or tripartite (Fig. 3, C, D).

We have observed interesting structures in the cytoplasm of developing spores. In some sections, we noticed a series of electron-dense lamellae emerging from the central region of the spore and ending in a rounded thickening near the spore wall (Fig. 3, B). This observation prompts us to hypothesize that this may represent a specific stage in the formation of the polar filament, although further observations are essential to confirm our interpretation of these images. Additionally, within some immature spores (Fig. 3, E), we documented a distinctive labyrinthi-

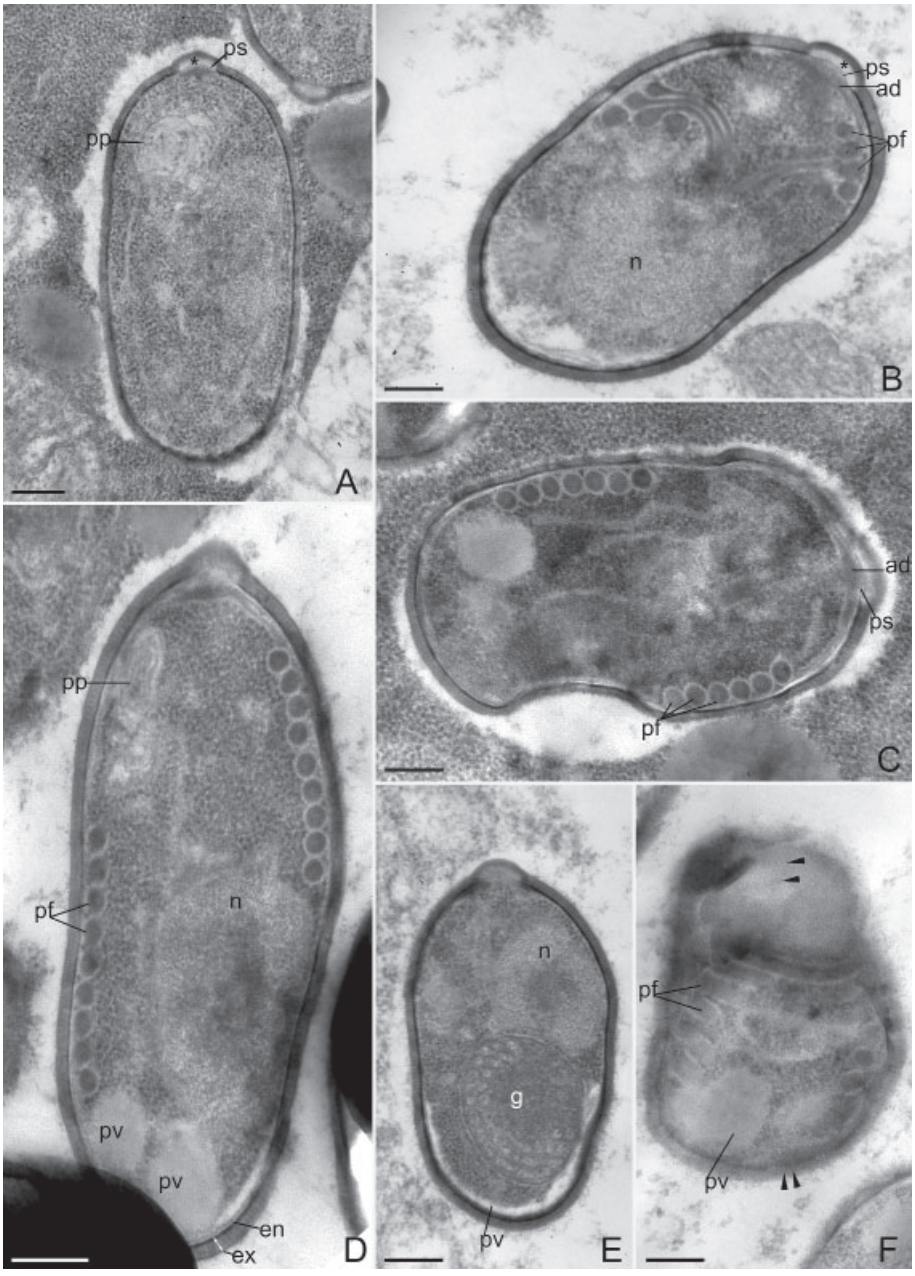

Fig. 3. Fine structure of immature and mature spores of *Microsporidium papillum* sp. nov. infecting the tardigrade *Grevenius pushkini*. A – Immature spore displaying a fully formed spore wall and nipple-like structure (*asterisk*) above the polar sac-anchoring disk complex (PS-AD). Precursors of polaroplast lamellae are visible; B – immature spore showing the polar filament in development. The spore wall, the PS-AD complex and a characteristic nipple-like structure (asterisk) are apparently fully formed; C – nearly mature spore exhibiting well-developed spore structures and organelles of the invasion apparatus, including the PS-AD complex and polar filament; D – longitudinal section through the mature spore revealing nine coils of the polar filament, loosely arranged polaroplast lamellae, a relatively large nucleus, a bipartite posterior vacuole, and a spore wall with a well-defined exospore; E – the Golgi tubular meshwork in the immature spore; F – tangential section illustrating the honeycomb-like structure of the upper layer of exospore, composed of tubular elements. Individual "tubes" are indicated by black arrowheads. *Abbreviations*: ps – polar sac, pp – polaroplast, pf – polar filament, ad – anchoring disc, en – endospore, ex – exospore with an upper layer composed of tubular elements in a honeycomb-like arrangement, pv – posterior vacuole, g – tubular meshwork of Golgi apparatus, n – nucleus. Scale bars: A, D–E – 250 nm, B–C, F – 200 nm.

ne structure, likely representing the Golgi tubular meshwork, which is believed to be involved in the formation of precursors of the coiled polar filament.

Microsporidia were observed in various cell types of *G. pushkini*, although they were not detected in the midgut epithelial cells (though it is worth noting that not all the midgut was completely sectioned and examined). This contrasts with the only other known microsporidian parasite found in tardigrades, specifically in *Isohypsibius granulifer granulifer*, where the parasite was identified within the midgut epithelial cells of the dorsal midgut surface. Notably, the epithelial cells of the ventral surface and the crescent-like cells were free of parasites. The gonads, storage cells, and epidermis were also reported to be infected with microsporidia, although visual documentation was not provided (Rost-Roszkowska et al., 2013).

The study by Rost-Roszkowska et al. (2013) describing microsporidia from *I. granulifer granulifer* lacks detailed information on the ultrastructure of the spore, making a direct comparison between the two microsporidian parasites found in tardigrades unfeasible. It can only be noted that both parasites exhibit the development of sporogonial stages and spores within vacuoles. In the case of the microsporidia from *I. granulifer granulifer*, early sporonts were also observed within vacuoles, with each one being enclosed in its own individual vacuole. These sporont-containing vacuoles likely progressed to form sporophorous vesicles with the spores. We did not observe such individual vacuoles around sporonts in the studied organism. Furthermore, the microsporidia from *I. granulifer granulifer* lack the characteristic nipple-like structure at the apical end of the spore, as well as the honeycomb-like arrangement of prismatic exospore elements on the spore surface. Additionally, the species identified in *I. granulifer granulifer* displays a noticeably more developed and thicker endospore. Based on these distinctions, we can confidently assert that these two microsporidian parasites represent different species.

A structure similar to a nipple-like protrusion was also observed in the spores of *Microsporidium epithelialis* infecting the oligochaeta *Tubifex* sp., as described by Oumouna et al. (2000). Furthermore, this parasite displays a well-developed ornamentation of exospore. However, *M. epithelialis* differs in having spores enclosed within individual vacuoles, possessing a smaller number of polar filament coils, and exhibiting a well-developed endospore.

The studied species displays a distinctive vertical arrangement of the exospore, composed of tightly packed prismatic elements. Though noteworthy, this feature is not uncommon. Among microsporidia infecting aquatic hosts, the exospore is frequently multilayered and adorned with various ornamentations, resulting in the formation of spore wall exhibiting the distinctive genus- or species-specific traits (Vavra, 1963; Voronin, 1986; Bronnvall and Larsson, 1995; Vavra and Larsson, 2014).

In May 2023, only one specimen of the studied tardigrade species was found, and it was infected. Subsequently, in the same location a month later, 10 more specimens were isolated, yet none displayed any signs of infection. Due to the limited sample size, we were unable to sequence the SSU rRNA gene or gather other molecular data. As a result, we are providing solely a morphological description of the identified microsporidian parasite. While contemporary standards accepted within the community of microsporidiologists advocate for the inclusion of molecular data in a comprehensive species description, a new species can still be delineated within the non-taxonomic complex group *Microsporidium* based on a distinct set of morphological characteristics. This approach is being employed here for the newly identified microsporidium. The parasite infecting the tardigrade *Grevenius pushkini* exhibits morphological features that differentiate it from any known microsporidian species that warrant its classification as a new species of microsporidia, *Microsporidium papillum* sp. nov.

Taxonomic summary

Microsporidium papillum sp. nov.

Diagnosis. Monomorphic, monokaryotic throughout life cycle. Presporogonial stages in direct contact with the cytoplasm of host cell. Sporoblasts and spores within the voluminous vacuoles. Sporogony by rosette-like budding. Spores are oval, ca 2.0-2.5 × 0.8-1.0 μm in size. The isofilar polar filament with 7–9 coils, arranged in a single row. The exospore is ornamented with a thick layer of densely packed prismatic elements. The endospore is remarkably thin, almost undeveloped. Nipple-like protrusion ('papilla') at the anterior pole of the spore. The spore wall is fully formed since the early steps of sporogenesis. The polaroplast is composed of rare, loosely arranged lamellae. The pronounced bi- or tripartite posterior vacuole.

Sites of infection. Hypodermal cells, storage cells within the body cavity, muscle cells, epithelial

gonadal cells, germ cells and nurse cells within the gonad cavity.

Type host. *Grevenius pushkini* (Tardigrada: Isohypsibioidea).

Type locality. Serdobolskiy pond in the park of St. Petersburg State Forestry University (59.994949; 30.332382).

Type material. Epon embedded tardigrades for TEM are stored at the Department of Invertebrate Zoology, Faculty of Biology, Saint Petersburg University.

Images of fixed infected tardigrades are kept at the image collection of the same department.

Etymology. The species name alludes to the 'papilla', the characteristic nipple-like protrusion at the anterior pole of the spore.

Our finding once again demonstrates that Tardigrade is to be added to the already very broad list of microsporidian host taxa (Bojko et al., 2022). The molecular and biological aspects of this new host-parasite system deserve further attention.

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