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SHORT COMMUNICATION

A Microsporidian Infection in Phoronids (Phylum Phoronida): *Microsporidium phoronidi* n. sp. from a *Phoronis embryolabi*

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ABSTRACT

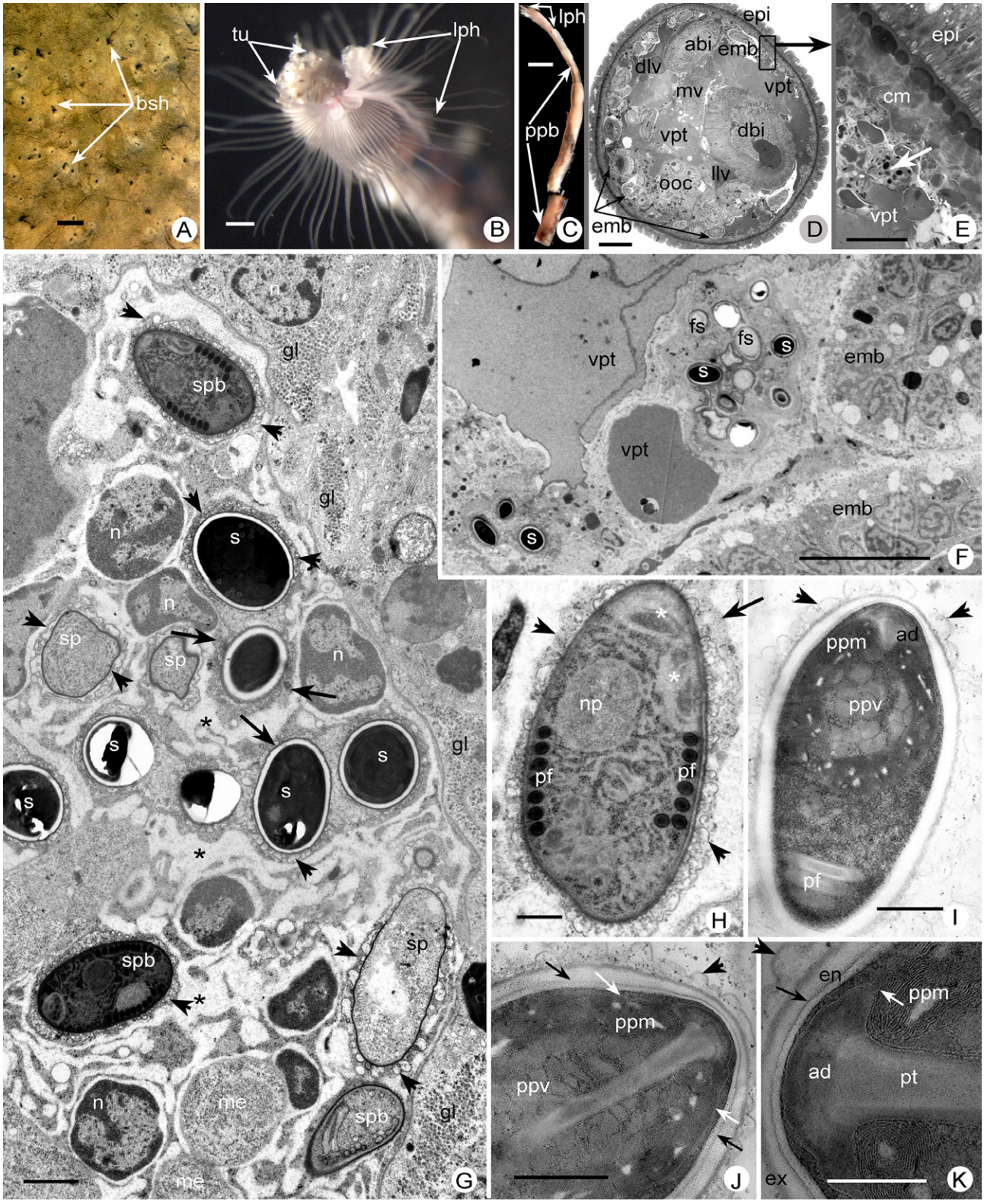
Microsporidia-like spores (2.0–3.0 × 1.3–1.5 μm) were discovered upon examination of histological sections taken from *Phoronis embryolabi* Temereva, Chichvarkhin 2017 found inhabiting burrows of shrimps *Nihonotrypeae japonica* (Decapoda, Callianassidae) from the Sea of Japan, Russia. Ultrastructural examination of spores revealed one nucleus and a uniform polar filament of 7–11 coils. Representatives of the phylum Phoronida have never been recorded as hosts of microsporidia. Parasites developed in vasoperitoneal tissue and caused formation of multinucleate syncytia. Basing on unique host and fine morphology, we assign the novel finding to *Microsporidium phoronidi* n. sp. and place provisionally in the collective genus *Microsporidium*.

RECENTLY, a new species of phoronids (Phylum Phoronida) *Phoronis embryolabi* Temereva, Chichvarkhin 2017 was described from the burrows of benthic shrimps *Nihonotrypeae japonica* (Decapoda, Callianassidae), inhabiting shallows of the inlet Thihaya Zavod of the Vostok Bay, Sea of Japan, Russia (Temereva and Chichvarkhin 2017). Upon examination of histological sections, electron-dense spore-like inclusions within the vasoperitoneal tissue observed in one animal attracted our attention. Ultrastructural analysis revealed infection with a microsporidium. The Phoronida is one of the smallest phyla of invertebrates. It includes as few as 14 valid species; all are marine inhabitants, mostly with worldwide distribution (Emig 1979; Temereva and Nekludov 2017). Phoronids possess biphasic life cycle that includes plankton larval and benthic adult stages (Emig 1982; Temereva 2009; Temereva and Malakhov 2015). Adult phoronids are commensals of invertebrates: bivalves, gastropods, cirripedians, sponges, and cnidarians, and live in a tube that is produced by the epidermal gland (Emig 1982). Phoronids have never been recorded as hosts of microsporidia. However, at least seven species of microsporidia were described in fresh water representatives of the Bryozoa (Canning et al. 1997, 2002; Desser et al. 2004; Morris and Adams 2002; Morris et al. 2005), a sister taxon to the Phoronida (Nesnidal et al. 2013). The discovery of a microsporidium in phoronids suggests new potential routes of transmission of these parasites among marine hosts. Unfortunately, no DNA material was available from the phoronid microsporidium. Basing on a unique host and fine morphology, we describe the novel finding as a new species placing it in a collective genus *Microsporidium* until it is molecularly characterized, and name *Microsporidium phoronidi* n. sp.

MATERIAL AND METHODS

Habitat and sampling

Adult phoronids were collected in July 2015 in the inlet Tihaya Zavod, Vostok Bay, Sea of Japan. The phoronid habitat is characterized by fluctuation of the salinity from 11 to 32.5‰. Adult phoronids live as commensals in the burrows of the shrimp *Nihonotrypaea japonica*. These shrimps make borrows at shallows, in a substratum composed of silt and sand (Selin 2015). Burrow holes are located at the depth of



60 cm and are well visible under water (Fig. 1A). Adult phoronids with sediment were removed from the burrows of the shrimp with a vacuum pump, which was driven into the burrow holes. Tubes with animals were separated from sediment by washing through a sieve with 2-mm openings. The tubes were collected in sea water, and live animals were photographed with a digital camera mounted on a Leica MZ12.5 stereomicroscope.

Figure 1 *Microsporidium phoronidi* n. sp. form *Phoronis embryolabi* from the Sea of Japan. (**A**) *Phoronis embryolabi* live as a commensal in burrows of shrimps *Nihonotrypaea japonica*: numerous holes of burrows are visible under a thin layer of water. The photograph is courtesy of Nikolai Selin (Vladivostok, Russia). (**B**) Live *P. embryolabi* viewed from the top of the lophophore. (**C**) Side view of fixed *P. embryolabi* (from Temereva and Chichvarkhin 2017). The posterior portion of the body is filled with embryos and vasoperitoneal tissue. (**D**) Transverse thick section of the posterior portion the body of *P. embryolabi* stained with Methylene blue. The region of vasoperotoneal tissue infected with microsporidia is framed. (**E**) Enlarged portion of (**G**) showing microsporidia spores (arrow). (**F**) Thin section through the infected vasoperitoneal tissue with two groups of spores residing in vicinity of embryos. Some spores are empty, that is, they have "fired" their polar filament. (G) Vasoperitoneal tissue of *P. embryolabi* infected with *M. phoronidi* n. sp. displays multinucleate syncytia heavily loaded with microsporidian developmental stages. Black asterisks mark electron-lucid areas formed presumably due to glycogen depletion associated with the parasite proliferation. Uninfected vasoperitoneal cells are extremely rich in glycogen granules. Long arrows indicate the borders of sporophorous vesicles, presumably originated from merogonial or sporogonial plasmodia. Arrowheads point to foam-like ornamentation on the envelopes of sporonts and sporoblasts. (**H**) A sporoblast or young spore with primordial polar filament coils, one nucleus, and precursors of other elements of the extrusion apparatus (asterisks). Exospore is decorated with foam-like membranous sheath (arrowheads). Endospore is poorly developed. Black arrow indicates the border of the sporophorous vesicle. (**I**) A spore demonstrating bipartite polaroplast, relatively thick endospore, and membranous decorations of the exospore (arrowheads). (**J, K**) Ultrastructure of the apical parts of spores. Endospore is separated in two parts by a thin electron-dense layer (black arrows). The anchoring disk is covered by the polar sac (white arrows) and forms a continuum with manubrial part of the polar filament. Polaroplast is composed of a layer of thin membranes (ppm) and layer of broad vesicles (ppv). Membrane ornamentation of the exospore is seen as an undulating membrane (arrowheads). abi = ascending branch of intestine; ad = anchoring disk; bsh = holes of shrimp burrows; cm = circular muscles; dbi = descending branch of intestine; dlv = dorsolateral blood vessel; emb = embryos; epi = epithelium; ex = exospore; en = endospore; fs = fired spores; llv = left lateral blood vessel; lph = lophophore; me = meront; mi = mitochondria; mv = median blood vessel; n = host nucleus; n = nucleus; np = parasite nucleus; ooc = oocytes; ppb = posterior portion of the body; p = sporont; pf = polar filament; ppm = part of a polaroplast composed of layers of thin membranes; ppv = part of polaroplast composed of broad vesicles; s = spore; s = spores of the microsporodium; sp = sporont; spb = sporoblast; tu = tube; vpt = vasoperitoneal tissue. Scale bars: A, 5 sm; B, 0.1 mm; C, 100 μm; E, 20 μm; F, 10 μm; G, 1 μm; H–J, 500 nm; K, 250 nm.

Transmission electron microscopy

For TEM, tubes with adult phoronids inside were fixed without dissection, at 4 °C in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 0.1 M NaCl. Then, the animals were peeled from the tubes and dissected. Parts of the bodies were then washed several times in 0.05 M cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in ethanol followed by an acetone series and were then embedded in EMBed-812. Thick (500 nm) and thin (60 nm) sections were cut with a Leica UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Thick sections were stained with methylene blue, observed with a Zeiss Axioplan 2 microscope, and photographed with an AxioCam HRm camera. Thin sections were stained with uranyl acetate and lead citrate and observed with the JEOL JEM 100B electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Host species, tissue tropism, signs of pathology

The phoronid host *P. embryolabi* grows in dense settlements inside shrimp borrows. The lophophore (Fig. 1B,C) is extended from the tube and exposed into the burrow lumen, whereas the posterior part of the body (Fig. 1C) is hidden within the tube and submerged into the wall of the burrow. The unique feature of *P. embryolabi* is the development of embryos and early larvae inside the mother organism (Temereva and Malakhov 2016). Because the fertilization is internal, all stages of development from the terminal oocytes to early larvae occur in the body and can be recognized on thick sections (Fig. 1D). The embryos develop in the posterior part of the body, which is occupied by special vasoperitoneal tissue (Fig. 1D–F) that nurses the developing oocytes (Temereva 2017) and embryos (Temereva and Malakhov 2016). The vasoperitoneal cells arise from mesodermal cells of the trunk lining, which compose the walls of the blood vessels, and form follicles around oocytes (Temereva et al. 2011). In the vasoperitoneal epithelial layer, individual cells are connected with each other and with developing oocytes via desmosomes. In *P. embryolabi*, vasoperitoneal cells are extremely rich in glycogen granules and contain well-developed synthetic apparatuses (Temereva 2017). Microsporidia-like spores were discovered within the vasoperitoneal tissue surrounding the embryos and developing oocytes (Fig. 1E–G and Fig. S1A). The infection was discovered in only one of three examined animals and was barely visible in the light microscope (Fig. 1E). No signs of gross pathology were detected upon visual examination of the infected animal or on histological sections. Electron microscopy revealed that infected cells formed multinucleate syncytia filled with the parasite cells resembling meront, sporont, sporoblasts, and spore stages of microsporidia (Fig. 1G and Fig. S1B–G). Manipulation of the host cell cycle including induction of multinucleate syncytia formation is quite common for microsporidia (Williams et al. 2014). For example, *Schroedera airthreyi* parasitizing freshwater bryozoan *Plumatella* sp. induced syncytial growth of the body wall epithelial cells that eventually detached and floated in the coelomic cavity (Morris et al. 2005). In *P. embryolabi*, syncytia loaded with parasites, contained numerous electron-lucid areas (Fig. 1G), presumably due to glycogen depletion associated with the parasite proliferation. Inside some syncytia, discharged spores were seen (Fig. 1F and Fig. S1B). This observation suggests that autoinfection and/or infection of embryos

developing in close vicinity may occur, thus indicating potential involvement of vertical transmission in the parasite life cycle. Low pathogenicity implied by the absence of pathology signs is characteristic for vertical route of microsporidia dissemination as well (Vizoso and Ebert 2005).

The microsporidium life cycle stages

Meronts developed in direct contact with the host cytoplasm were surrounded by a smooth membrane and were occasionally arranged in chains (Fig. 1G and Fig. S1C). Nuclei and other organelles could be hardly distinguished in their homogenous cytoplasm. We speculate that while merogonial plasmodia proliferate and grow, they restructure vasoperitoneal epithelium layer and transform it in a syncytium in a way described recently for *Nematocida parisi* infecting intestine of *Caenorhabditis elegans* (Troemel 2016). Sporonts, sporoblasts, and spores were often seen on sections submerged into an amorphous substance of moderate electron density surrounded by a membrane (Fig. 1G). These sporophorous vesicles (SVs) could originate from either mother merogonial or sporogonial plasmodia, in which sporonts had formed endogenously. Sporonts could be recognized by thicker envelopes (Fig. 1G and Fig. S1D). Envelopes of sporonts, sporoblasts, and young spores were ornamented with foam-like membranous sheath that likely was derived by delamination of the plasmalemma of either the merogonial or sporogonial plasmodium. Upon spore maturation, this episporal foam-like ornamentation became less conspicuous (Fig. 1G–I). Thus, its function is associated probably with metabolic needs of the developing parasites. In contrast, episporal structures in most freshwater and marine microsporidia serve to increase buoyant of spores and are more conspicuous at the spore stage, like in *Trichonosema algonquuinisi* from freshwater bryozoans (Desser et al. 2004). Host mitochondria concentrated in vicinity of the SV envelopes enclosing spores (not shown). The ultrastructure of spores of the novel organism was typical for "higher microsporidia" (Vávra and Larsson 2014) including species described so far from Bryozoa (Canning et al. 2002; Morris et al. 2005). Spores on sections measured 2.0–3.0 × 1.3–1.5 μm. They possessed one nucleus and a polar filament coiled in 7–11 uniform coils arranged in one row (Fig. 1H, I). The envelope was composed of the 30–45 nm-thick exospore of moderate electron density and electron-transparent endospore 80–120 nm thick (about 40 nm at the apical end). The endospore was separated by a thin electron-dense layer in two parts (Fig. 1J,K). The mushroom-shaped polar disk was covered by a polar sac and formed a continuum with the apical uncoiled part of the polar filament (Fig. 1J,K). Polaroplast included the region of tightly packed membranes that enclosed the zone of broad vesicles (Fig. 1I–K). Exospore of mature spores was ornamented with a waving membranous layer, the remnant of the foam-like membranous sheath, observed at the earlier stages.

The microsporidium described here from *P. embryolabi* can be distinguished from all other species of the phylum Microsporidia by developing in a phoronid host, and by the presence of foam-like ornamentation on the surfaces of sporogony stages. The novel species can be distinguished from microsporidia infecting bryozoans, taxonomically the closest host group to phoronids, by producing mononucleate spores and infecting marine hosts.

Future studies should focus on the re-isolation of the microsporidium and its molecular characterization to reveal phylogenetic relations to other microsporidia. The comparative molecular phylogenetics of microsporidia parasitizing ectoprocts and phoronids might also help resolving enigmatic phylogenetic relations of the Bryozoa and Phoronida within the Lophophorata clade (Hausdorf et al. 2010; Jang and Hwang 2009; Kocot et al. 2017; Nesnidal et al. 2013).

TAXONOMIC SUMMARY

Phylum: Microsporidia Balbiani 1882
Family: *insetrae sedis*
Genus: *insetrae sedis* (temporally assigned into a holding genus *Microsporidium* Balbiani 1884)

Microsporidium phoronidi n. sp. Sokolova, Temereva.

Diagnosis

Type host. *Phoronis embryolabi* Temereva, Chichvarkhin 2017 (Phoronida). Accession numbers for molecular barcoding of the host: KY643691 (COI gene); KY643695 (18SRNA gene).

Type locality. The inlet Tihaya Zavod of the Vostok Bay of the Sea of Japan, Russia, burrows of shrimps *Nihonotrypeae japonica* (Decapoda, Callianassidae).

Cell and tissue tropism. Vasoperitoneal tissue surrounding embryos.

Signatures of the pathogenesis. No signs of gross pathology. Infected cells form multinucleate syncytia; depletion of glycogen and other nutrients from the host cell cytoplasm is marked by electron-transparent regions around spores and proliferative stages.

Interfacial envelopes. Meronts reside in direct contact with the host cell cytoplasm. Some spores develop in sporophorous vesicles-like compartments presumably derived from merogonial or sporogonial plasmodia.

Life and nuclear cycle. Spores are monokaryotic, number of nuclei in other stages is unknown. Life cycle likely includes autoinfection. Low pathogenicity, development in vicinity to embryos, and presence of empty spores suggest vertical route of dissemination.

Meronts. Shapeless cells of moderate electron density surrounded by a smooth membrane, often arranged in chains.

Sporonts. Elongated cells surrounded by electron-dense envelope ornamented with a prominent foam-like membranous sheath.

Sporoblasts and young spores. Oval, contain precursors of polar filament coils. Exospore is ornamented with a prominent foam-like membranous sheath.

Spores. Spores on sections measure 2.0–3.0 × 1.3–1.5 nm, with one nucleus and an isofilar polar filament making 7–11 turns arranged in one row. The envelope is

100–170 nm thick, composed of a 30–45 nm-thick exospore and endospore 80–120 nm thick (c. 40 nm at the apical end of spores). The mushroom-shaped polar disk covered by the polar sac forms a continuum with the uncoiled part of the polar filament. Polaroplast includes a region of tightly packed membranes that enclosed the zone of broad vesicles. Exospore of mature spores is ornamented with undulating overlapping membranes, the remnants of the foam-like membranous sheath, observed at the previous stages.

Etymology. The species name is after the host genus name.

Type material: Grids with thin sections, slides with semithin sections, and images of thin sections, labeled *E1–E5; F1–F2; K1–K2* (grids), 02.01–02.03 (the thin sections), 11-02-2016, 18-04-2016, 24-03-2016 (images) are stored in the collection of E.T., Moscow State University.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Ultrastructure of *Microsporidium phoronidi* n. sp. form *Phoronis embryolabi*.