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Globulispora mitoportans n. g., n. sp., (Opisthosporidia: Microsporidia) a microsporidian parasite of daphnids with unusual spore organization and prominent mitosome-like vesicles

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ABSTRACT

The microsporidian parasite *Globulispora mitoportans*, n. g., n. sp., infects the intestinal epithelium of two species of daphnids (Crustacea: Cladocera). Mature spores are thin-walled and possess a novel type of polaroplast with a conspicuous part consisting of globules that occupies a large part of the spore volume. Both developmental stages and the spores possess large, electron-lucent vesicles enveloped by a double membrane and filled with an internal web of filamentous material, corresponding structurally to microsporidian mitosomes. The SSU rRNA phylogeny places *Globulispora* into a specific "*Enterocytospora*-like" clade, part of a large "non-enterocytozoonidae" clade, grouping a heterogenous assemblage of microsporidia infecting almost exclusively insects and crustacea.

1. Introduction

Microsporidia are obligatory intracellular eukaryotic parasites of animals and some protists, characterized by possessing within their spores an evaginable tube ("polar filament", "polar tube", "invasion tube", "injection tube") through which the spore contents are injected into cells of their hosts during spore germination (Vávra and Lukeš, 2013). The origin of microsporidia is not fully understood, but recent data indicate that microsporidia are related to fungi, either as a sister group of the "lower fungi" clade Cryptomycota, (James et al., 2013), as clade within Cryptomycota itself (Keeling, 2014), or as members of the newly proposed superphylum Opisthosporidia, which unites Microsporidia, Cryptomycota and algal parasites Aphelida, and represents a phylogenetic sister clade to the "true" fungi (Karpov et al., 2014). Microsporidia are ubiquitous organisms with more than 1500 species described in about 200 genera (Vávra and Lukeš, 2013; Becnel et al., 2014). Arthropods (Crustacea and Insecta) are their main hosts (Becnel and Andreadis, 2014; Stentiford and Dunn, 2014), but many microsporidia occur in other animal phyla, vertebrates included. Humans are hosts of several vertebrate-specific microsporidian species, but occasionally can be infected by non-specific microsporidia infecting invertebrates or unknown hosts (Snowden, 2014).

Because microsporidia are among the most common single-cell animal parasites, their origin and basal phylogeny are particularly interesting from the evolutionary point of view, but neither are well known. It is only recently that understanding of the phylogeny has increased (Keeling, 2014; Vávra and Lukeš, 2013) and it has been suggested that microsporidia may have originated in an aqueous environment. Microsporidia of aquatic invertebrates (especially those filtering water, e.g. the daphniids) are, thus, a group that may provide clues to the origin of these unique organisms. A microsporidium recently reported infecting the gut epithelium of a species of water flea (Crustacea, Branchiopoda), is claimed to represent a basal organism in the microsporidia tree, closest to the common ancestor at the Cryptomycote *Rozella*-Microsporidia branching point. This organism possesses a large "mitochondrium-like structure with a double membrane", an organelle that is atypical both in structure and biochemical functions to mitosomes of other microsporidia (Haag et al., 2014). Here, we report the isolation of another microsporidian parasite from the gut epithelium of two daphnid species, which differs from other microsporidia described from the same host-tissue. This isolate is unique in structure among the

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microsporidia and represents a new genus. The large and structurally complex mitosome-like vesicles in the cells contribute to the understanding of microsporidian mitosome structural diversity.

2. Material and methods

2.1. Collection of specimens and microscopy

Infected daphnids, *Daphnia pulex* (Leydig, 1860), and *Simocephalus vetulus* (Müller, 1776), were collected in 2013 in a large, nearly permanent forest marsh, near Běleč, Central Bohemia region, Czech Republic (50°3′12.240″N, 14°1′4.221″E). Although the infection was frequent in *D. pulex* (about 5% infected), only three infected *S. vetulus* were found. Infected hosts and the parasite were examined by routine methods of light microscopy (LM) (Vávra and Maddox, 1976; Becnel, 2012) including spore immobilization on agar, negative staining using Burri Bacteriology Ink and measurement of spores using QuickPHOTO MICRO 3.0 (Promicra). For electron microscopy (TEM) the standard techniques and instruments described in Refardt et al. (2008) were used. Observations requiring high-resolution were made using Jeol 2010 200 kV electron microscope equipped with Gatan camera Orius SC 1000. Five infected specimens of *D. pulex* and two of *S. vetulus* were examined by TEM.

2.2. DNA isolation, PCR, and SSU rDNA sequencing

DNA was isolated from a single infected *D. pulex* female according to a protocol of Andreadis et al. (2013) in which, however, host tissues and spores were homogenized together. Single specimen isolation was selected in order to avoid possible contamination by other microsporidia present in the habitat. The specimen was placed in 0.5 ml microvial tube with equal volumes of 0.5 mm/0.1 mm (1:1) glass beads (BioSpec Products), 150 µl STE buffer (Fluka, BioUltra, pH 7.8) and was shaken in a Mini-Beadbeater (Biospec Products) for 30 s at maximum speed. Part of the mixture containing spore debris and spores was controlled by light microscope to ascertain that a single microsporidian species was present, another part was immediately incubated at 95 °C for 5 min and centrifuged at 14,000 g for 5 min. Supernatant was removed and 3 µl were used for PCR. Primers 18f and 1492r (Weiss and Vossbrinck, 1999) were used to amplify the SSU rDNA. PCR reaction (95 °C for 2 min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min., 72 °C for 2 min; and 72 °C for 10 min) was conducted in a total volume of 25 µl, containing 25 pmol of each respective primer and GoTaq® Green Master Mix (Promega), according to manufacturer instructions. PCR product was separated using 1% agarose gel electrophoresis, extracted from the gel, purified using the DNeasy Tissue Kit® (QIAGEN) and prepared for automated sequencing with primers 18f, 530r, 530f, 1047r, 1061f and 1492r (Weiss and Vossbrinck, 1999) and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an 3130XL Genetic Analyzer (Applied Biosystems). Three PCR products were read.

2.3. Alignments and phylogenetic analysis

Sequences were aligned using MAFFT v6.626b (Katoh et al., 2005) with the E-INS-i multiple alignment method and default parameters. Alignment was cross-checked using SEAVIEW v3.2 (Galtier et al., 1996). Alignment included our novel microsporidian sequence and available GenBank sequences of representative closely related microsporidia. *Vavraia culicis* was set as the outgroup. Phylogenetic trees were calculated from the sequence alignment using maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP). ML analysis was done in RAxML v7.0.3 (Stamatakis, 2006) with a GTR + Γ model. MP was calculated in PAUP* v4.0b10 (Swofford et al., 2001) with a heuristic search, random addition of taxa and Ts:Tv = 1:2. Bootstrap support was calculated from 500 replicates in ML and 1000 replicates in MP analysis. BI was done using MrBayes v3.0 (Ronquist and Huelsenbeck, 2003) with the GTR + Γ model of evolution. Initially, 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 determine the burn-in period. The burn-in period was set to 10%, i.e. 1000 initial trees.

2.4. Topology tests

TreeGraph v2.0.47-206 beta (Stöver and Muller, 2010) was used to generat alternate topologies for the topological tests. Designed topologies in Newick format were specified in the assumption block and the data with ML parameters (the same generated by RAxML program for ML analysis) were executed in PAUP* to generate likelihood scores for each constrained tree. Resulted per-site log likelihood scores were analyzed for significant differences in CONSEL v6.1 (Shimodaira and Hasegawa, 2001), using three likelihood-based tests: approximately unbiased (AU), Kishino-Hasegawa (KH), and Shimodaira-Hasegawa (SH).

3. Results

3.1. Light microscopy (LM)

The parasite infects the upper to middle part of the midgut epithelium of its hosts and the gastric ceca (Figs. 1 and 4). Diffuse infiltration of the epithelium by parasites was typical (Fig. 1). Infected cells contained masses of parasite spores, ovoid to oval in shape (2.5 × 1.6 µm fresh, 2.5 × 1.5 µm on dry, negatively stained smears, $n = 10$). Spores occurred singly, not grouped (Fig. 2) and their shape was well preserved in dry, negatively stained smears (a diagnostic character, see Fig. 3).

3.2. Electron microscopy (TEM)

Even at low power TEM allowed discrimination of the characteristic oval spores of the parasite interspersed with developmental stages (Figs. 4 and 5). At higher magnification, the developmental stages were shown either as small, irregularly lobed ribbon-like sporogonial plasmodia with several isolated nuclei or uninucleate cells originating by plasmotomy of plasmodia (Figs. 6–9). The cytoplasm contained a dense population of ribosomes, most of them free, some associated with endoplasmic reticulum cisternae (Fig. 6). The plasma membrane of developmental stages was covered with a 20 nm thick, fuzzy granular layer of medium density (Fig. 10), indicating that all developmental stages observed belonged to the sporogony sequence. No merogonial stages were observed. All developmental stages were in direct contact with host cell cytoplasm.

A conspicuous structure in the developmental stages was one or several large electron-lucent vesicles containing irregularly dispersed membranous material. These vesicles typically adhered to the nuclei (Figs. 8 and 9). Vesicles were variable in size (evidently due to the plane of section), measuring from 300 × 80 nm to 1500 × 100 nm. These vesicles divided when the nucleus divided evidenced by a long extension connecting the respective divided nuclei (Fig. 7). At higher magnification the vesicles were observed to be enveloped by a double membrane and contained a loose web of filamentous material of medium density and sometimes clump