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Microsporidia *Alfvenia sibirica* sp. n. and *Agglomerata cladocera* (Pfeiffer) 1895, from Siberian microcrustaceans and phylogenetic relationships within the "Aquatic outgroup" lineage of fresh water microsporidia

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ABSTRACT

Here we report on two microsporidia from freshwater crustaceans collected during the ongoing survey for microsporidia in the river Karasuk and adjacent waterbodies (Novosibirsk region, Western Siberia). The first species parasitized hypoderm and fat body of a cyclopid *Cyclops* sp. (Maxillopoda, Copedoda) and produced oval spores, measured 2.0 × 3.6 μm (fixed) enclosed individually or in small groups in fragile sporophorous vesicles (SVs). We describe it here as *Alfvenia sibirica* sp. n. The second species infected the same tissues of a cladoceran *Daphnia magna* (Branchiopoda, Phyllopoda). Its spores were pyriform, 2.3 × 4.0 μm (fixed), and resided in relatively persistent SVs in groups of 8–16. This species was identified as a Siberian isolate (Si) of *Agglomerata cladocera* (Pfeifer) because ultrastructurally it was hardly distinguishable from *A. cladocera* recorded from England from the same host (Larsson et al., 1996). *A. cladocera* (Si) shared 99% SSU rDNA sequence similarity to *Binucleata daphniae* from *D. magna* collected in Belgium (Refardt et al., 2008). The major outcome of our work is that we present molecular (SSUrDNA) characterization coupled with EM description, for representatives of two genera, *Alfvenia* (encompasses 3 described so far species) and *Agglomerata* (7 species), which allowed us to place these two genera on the phylogenetic tree. We also summarized the literature data on *Alfvenia* and *Agglomerata* spp., and provided their comparative morphological analysis. These two genera belong to so called "Aquatic outgroup" (Vossbrinck et al., 2004), a poorly resolved lineage, a sister to Amblyosporidae. This lineage probably includes majority of fresh water forms of microsporidia, most of which remain undescribed. SSUrDNA-based phylogenetic analysis and analysis of hosts suggest that diversification within the "Aquatic outgroup" could have been connected with the host switch from dipterans or copepods to cladocerans that had occurred in some ancestral *Amblyospora*-related lineage(s).

1. Introduction

During the ongoing survey for microsporidia in the river Karasuk and adjacent water bodies in Western Siberia we discovered two microsporidia species that infect freshwater crustaceans. The first species was isolated from a cyclopid *Cyclops* sp. (Maxillopoda, Copedoda) and morphologically resembled representatives of the genus *Alfvenia* (Vidtmann and Sokolova, 1995). The second species infected a cladoceran *Daphnia magna* (Branchiopoda, Phyllopoda) and shared structural similarities with *Agglomerata cladocera* (Larsson et al., 1996). Representatives of both genera were recorded previously from Europe. The initial goal of the research was to describe fine morphology of the discovered microsporidia, identify the species, and determine their relationships with other taxa of the phylum Microsporidia by comparative ultrastructural and SSU rDNA-based phylogenetic analyses. Obtaining SSU rDNA sequences for representatives of two genera *Alfvenia* and *Agglomerata*, allowed us to place these two genera on the phylogenetic tree within the so-called "Aquatic outgroup" (sensu Vossbrinck et al., 2004), a poorly resolved lineage, a sister to Amblyosporidae. This lineage probably includes majority of fresh water forms of microsporidia, most of which remain undescribed. We also provide a comparative morphological analysis of the representatives of the genera *Alfvenia* and *Agglomerata*.

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2. Material and methods

2.1. Sampling

The crustaceans were collected in the lake Krotova Lyaga belonging to the river Karasuk basin, Karasuk District of Novosibirsk region, South-Western Siberia (56°32′N, 29°31′E) in May and June in 2013 and 2014. In May 2013, water fleas *D. magna* were abundant in the lake, with significant proportion of organisms exhibiting pathology characteristic for microsporidiosis. In June 2014, a single heavily infected adult of *Cyclops* sp. was collected. Transformation of its habitus due to infection did not allow identification of species. The smears of infected crustaceans were immediately fixed with methanol, for consequent light microscopy (LM) examination. Infected animals were cut in halves. One half was placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer to be processed further for electron microscopy, another – in 95% ethanol to be used for DNA extraction. Samples were stored at 4 °C until further processing.

2.2. Electron microscopy

For transmission electron microscopy (TEM), in about 2 weeks after fixation, the samples were cut in smaller pieces, transferred to fresh portion of fixative for 2 h, washed in 0.1 M cacodylate buffer supplemented with 5% sucrose, post fixed in 2% osmium tetroxide, dehydrated in ascending ethanol series, transferred to propylene-oxide, and embedded in Epon-Araldite. Thick (0.5–1 μm) sections were stained with Methylene blue, examined and photographed under Zeiss Axioplan microscope equipped with Olympus DP73 digital camera and CellSens (Version 510) software. Thin (70–80 nm) sections were stained with uranyl acetate and Reynolds' lead citrate end examined in JEOL JEM 1011 transmission electron microscope equipped with HAMAMATSU ORCA-HR digital camera (Tokyo, Japan).

For Scanning electron microscopy (SEM), specimens were fixed with glutaraldehyde only, dehydrated through the ethanol series, followed by exchanging ethanol with CO_2 in Polaron E3000 Standard Critical Point Drier. Dried samples were mounted on 13 mm aluminum mount specimen stubs covered with carbon adhesive tabs, sealed with colloidal silver paste, coated with Gold/Palladium in EMS 550X Sputter Coater for 4 min to achieve the thickness of coating 20–25 nm., and examined in Fei Quanta 200 ESEM in a high vacuum mode at 20 kV. All reagents for LM were from SIGMA-ALDRICH (St. Louis, MO), and for EM – from EMS Chemicals (Fort Washington, PA).

2.3. DNA sequencing

Ethanol-fixed samples were transferred to new tubes and left to air dry. After ethanol evaporation, samples were homogenized with a plastic pestle in 100 μl lysis buffer (2% cetrimonium bromide, 1.4 M NaCl, 100 mM EDTA and 100 mM Tris–Cl (pH 8.0)). Another 500 μl of lysis buffer containing 0.2% β-mercaptoethanol and 10 μl proteinase K (20 mg mL^{-1}) were added to each tube. The samples were then incubated at 65 °C for 3 h. DNA was extracted routinely with phenol-chloroform (Sambrook et al., 1989) and re-suspended in 50 μl UHQ water. The small subunit (SSU) rRNA gene was amplified by the 18f-1047r primer set (Weiss and Vossbrinck, 1999).

PCR was run using MyCycler (Bio-Rad) in 20 μl volume containing 10 μl tenfold-diluted DNA template; 2 μl PCR buffer with 25 mM $MgCl_2$; 1 μl dNTPs, 0.25 mM; 0.5 μl Taq-polymerase (2500 U mL^{-1}, Sileks, Russia); 1 μl of each forward and reverse 10 μM primers (Evrogen, Russia). Initial denaturation was carried out at 92 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 30 s, and final extension at 72 °C for 10 min. The PCR products of about 900 bp long were gel-purified and sequenced in both directions using ABI Prism 3500 (Applied Biosystems). The obtained sequence reads were manually corrected and assembled using BioEdit software (Hall, 1999).

For host identification, additional PCRs were performed using primer sets LepF1:LepR1 (Hebert et al., 2004) specific for COI region of a broad range of Metazoa, and f1300:ITS2-28S specific for rRNA in Eukarya (Navajas et al., 1998). The conditions for reactions were as described above. COI region was successfully amplified for the water flea. The obtained sequence was 98–100% identical to those available in Genbank for *Daphnia magna* and below 96% for other *Daphnia* species. This allowed host identification as *D. magna*, supporting the routine identification with the field guide. Genomic DNA extracted from the infected cyclopid could not be amplified with the host-targeted primers. The genus (*Cyclops*) was identified using the conventional field guide (Tsalolihin, 1995).

2.4. Phylogenetic analysis

For the SSU rDNA phylogeny 19 sequences belonging to microsporidia were retrieved from Genbank. The source of sequences selected for the analyses, included twelve closest matches in BLAST search and 6 outgroup taxa: (i) two *Amblyospora* species of the Clade 1 (*sensu* Vossbrinck and Debrunner-Vossbrinck, 2005); (ii) three taxa of "Marinosporidia" infecting marine and freshwater crustaceans, and (iii) *Bacillidium* and *Janacekia* representing another group of aquatic microsporidia (Clade 5, Vossbrinck and Debrunner-Vossbrinck, 2005). *Paramicrosporidium vanellae* from the super taxon Cryptomycota (James et al., 2013) (known also as Rozellomycota (Corsaro et al., 2014)), the closest fungi relative of Microsporidia (Corsaro et al., 2014), was used as a non-microsporidian outgroup. The sequences were aligned with Muscle (MEGA 5.05), with default parameters (Edgar, 2004). The final datasets resulted in 605 informative positions. Pairwise genetic distances were calculated by the Kimura-2 parameter method with a gamma distribution 1 (Tamura et al., 2011). The alignments were subjected to phylogenetic reconstructions by maximum likelihood (ML) using MEGA 5.05 (Tamura et al., 2011) and Bayesian inference using MrBayes 3.2 (Ronquist et al., 2012). ML phylogenetic analyses used GTR + G model of nucleotide substitution (Nei and Kumar, 2000) as suggested by Modeltest, with 1000 bootstrap replications. MrBayes was run for 1,000,000 generations and every 1000th generation was sampled. The first 25% of samples were discarded as burn-in, parameter values were summarized, and a consensus tree was constructed. Standard deviation of split frequencies, which estimates the precision of the clade probabilities, reached 0.007 after 1,000,000 generations.

3. Results

3.1. The microsporidium isolated from Cyclops sp.

3.1.1. Tissue tropism and light microscopy

The hypoderm and fat body of the singe infected copepod were loaded with mature spores (Fig. 1a), which substituted host tissues and were partly washed out during processing. Muscle and intestine were free from infection. On methanol-fixed smears only mature spores were identified. The spores measured 2.0 ± 0.04 × 3.6 ± 0.04 μm, ranging 1.7–2.3 × 3.1–4.0 μm (n = 20).