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Description and phylogeny of *Zelenkaia trichopterae* gen. et sp. nov. (Microsporidia), an aquatic microsporidian parasite of caddisflies (Trichoptera) forming spore doublets

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ABSTRACT

Two novel microsporidia infecting the fat body tissues in larvae of two hosts, *Halesus digitatus* and *Micropterna sequax* (Trichoptera, Limnephilidae), were investigated using light and electron microscopy and rDNA sequence analyses. The molecular and morphological characters of these isolates warrant creation of a new microsporidian genus, *Zelenkaia* gen. n., with two species, one named herein. Developmental stages of *Zelenkaia* spp. have single nuclei. In sporogony, a plasmodium with four nuclei gives rise by rosette-like budding to two pairs of uninucleate sporoblasts, each within a thin-walled, subpersistent sporophorous vesicle. Sporoblasts and mature spores adhere temporary together, forming doublets oriented in parallel, within the sporophorous vesicle. Spores are long-oval and uninucleate, and those of the type species *Z. trichopterae* measure 10.3 × 3.5 µm and have 24–25 polar filament coils. Phylogenetic analysis based on rDNA places *Zelenkaia* spp. within the aquatic clade of microsporidia and, more specifically, in the clade containing some microporidia from amphipod hosts.

1. Introduction

With more than 1300 described species in 172 genera, microsporidia are very common parasitic organisms (Cali and Takvorian, 2011). They are almost exclusively parasites of animals, with crustaceans and insects being their most frequent hosts. Many microsporidia, primarily those infecting terrestrial hosts, are transmitted among susceptible conspecific hosts by ingestion of infective spores.

It is of fundamental interest, however, that some microsporidian species, uniquely from aquatic invertebrates such as crustaceans and insects, have spores that are not orally infective for conspecific hosts (Vávra, 1964b; Vávra and Larsson, 1994; Vávra et al., 2005; Hyliš et al., 2007; Wolinska et al., 2011).

Microsporidia, parasitizing caddisflies are of special interest to the study of the evolution of the Phylum Microsporidia for several reasons. (1) Trichopteran lifecycles include aquatic (larval) and terrestrial (imaginal) stages. (2) Trichopteran larvae harbor microsporidia that produce mature spores that are not infective for the original host (Heilveil et al., 2001; Hyliš et al., 2007). (3) Phylogenetically, microsporidia from trichopteran hosts are related to the Amblyosporidae clade, many representatives of which have two-host lifecycles (Vossbrinck et al., 2004; Becnel et al., 2005; Andreadis, 2005, 2012). These observations support the assumption that some extinct or extant trichopteran microsporidia may also require an intermediate host to complete their life cycle. In addition, the order Trichoptera is closely related to Lepidoptera, an insect order hosting many genera and species of Microsporidia. So studies on trichopteran parasites might shed light on the origin and diversification of terrestrial microsporidia groups.

Representatives of 10 microsporidian genera and 24 species have been identified in Trichoptera (Larsson, 1995; Canning and Vávra, 2000; Hyliš et al., 2007). This paper describes the morphology and molecular phylogeny of two species of caddisfly microsporidia that possess unique structural characters and represent a new genus.

2. Materials and methods

2.1. Origin of isolates

Two microsporidian isolates with morphologically similar mature spores, designated as isolates iMS1 and iMS2, were discovered infecting trichopteran larvae in Bulgaria in June 2001. iMS1 was found in one living larva of *Halesus digitatus* Schrank, 1781 (Trichoptera, Limnephilidae) in a small temporary stream near the village Levishte, Bulgaria (43°07′59.14″ North; 23°46′04.62″ East).

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iMS2 was found in several dead larvae of *Micropterna sequax* McLachlan, 1875 (Trichoptera, Limnephilidae) in a small pond near the village Dragichevo, Bulgaria (42°38′28″ North; 23°09′0.58″ East).

2.2. Examination of infected host tissues: scanning and transmission electron microscopy

Fresh host tissues infected with iMS1, a suspension of necrotic and lysed tissues with iMS2, and tissue smears of both organisms stained with Giemsa (Sigma® Diagnostic Accustain) were examined under light microscopy. Spores were immobilized using the agar method (Vávra, 1964a) and were measured ($n = 50$) with an Image Splitting Eyepiece (Vickers Instruments Ltd.) (Vávra and Maddox, 1976). For field emission scanning electron microscopy (FESEM), an aqueous suspension of fresh purified spores of iMS1 was rapidly frozen in liquid nitrogen, and then was examined in a non-coated state in a JEOL JSM-7401F scanning electron microscope. For transmission electron microscopy (TEM), iMS1 infected adipose tissues were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4 in the same buffer. Fixed tissue was dehydrated through an ascending ethanol and acetone series and embedded in Araldite – Poly/Bed® 812 mixture. Thin sections were cut on a Reichert–Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined and photographed using a JEOL JEM-1011 electron microscope. Fine structure measurements were performed using a Megaview III camera and analySIS 3.2 software (Soft Imaging System®).

2.3. rDNA sequences; phylogenetic analysis

DNA was isolated from fresh purified spores of iMS1 and iMS2 according to the protocol of Hyliš et al. (2005). Primers ss530f: ls580r (Weiss and Vossbrinck, 1999) were used to amplify the small subunit (in part), ITS region, and large subunit rDNA (in part). The 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 with 50–100 ng of DNA, 25 pmol of each primer, 1 unit Taq polymerase (TAKARA BIO INC. Otsu, Shiga, Japan) and buffer/dNTP (TaKaRa) according to manufacturers instructions. PCR products were separated using 1% agarose gel electrophoresis, extracted from the gel, purified using the DNeasy Tissue Kit® (QIAGEN, Germantown, Maryland, USA), cloned (TOPO TA Cloning Kits®, Invitrogen, Carlsbad, California, USA) and sequenced on an automatic sequencer (Beckman CEQ 2000 XL). The sequences were aligned using the ClustalX program (Thompson et al., 1997), gaps and ambiguously aligned regions were omitted from further analyses.

Analysis was carried out using distance, maximum parsimony and maximum likelihood methods. The distance matrix was calculated using the LogDet paralinear model with the portion of invariable sites included as estimated in the maximum likelihood search. Maximum Parsimony (MP) trees were constructed using PAUP 4b10 (Swofford, 2000) with TBR as a branch-swapping method and 1000 bootstrap replicates. Maximum likelihood trees were constructed by PHYML program (Guindon and Gascuel, 2003), using the GTR model for nucleotide substitutions with discrete gamma distribution in 4 + 1 categories; all parameters (gamma shape, proportion of invariants) were estimated from the dataset. Multiple datasets for ML bootstrap analyses were prepared using SeqBoot (PHYLIP 3.6.3; Felsenstein, 2001). ML bootstrap support was computed in 300 or 1000 replicates using PHYML program with the TN93 model for nucleotide substitutions and one category of sites with a TI/TV ratio estimated from the data set.

Table 1
Species list of microsporidian SSU rDNA sequences included in the phylogenetic analysis, hosts from which they were obtained, taxonomic classification of hosts and GenBank accession numbers.

Organism	Host	Host taxonomic classification	GenBank Acc. No.
Amblyospora bracteata	*Odagamia ornata*	Insecta, Diptera, Simuliidae	AY090068
Amblyospora ferocious	*Psorophora ferox*	Insecta, Diptera, Culicidae	AY090062
Berwaldia schaefernai	*Daphnia galeata*	Crustacea, Cladocera, Daphniidae	AY090042
Episeptum circumscriptum	*Hydropsyche incognita, H. siltalai*	Insecta, Trichoptera, Hydropsychidae	DQ864440
Episeptum pseudoinversum	*Sericostoma personatum*	Insecta, Trichoptera, Sericostomatidae	DQ864441
Episeptum trichoinvadens	*Potamophylax cingulatus*	Insecta, Trichoptera, Limnephilidae	DQ864439
Gurleya daphniae	*Daphnia pulex*	Crustacea, Cladocera, Daphniidae	AF439320
Gurleya vavrai	*Daphnia longispina, D. pulex*	Crustacea, Cladocera, Daphniidae	AF394526
Hazardia milleri	*Culex quinquefasciatus*	Insecta, Diptera, Culicidae	AY090067
Hazardia sp.	*Anopheles crucians*	Insecta, Diptera, Culicidae	AY090066
Larssonia obtusa	*Daphnia pulex*	Crustacea, Cladocera, Daphniidae	AF394527
Marssoniella elegans	*Cyclops vicinus*	Crustacea, Copepoda, Cyclopidae	AY090041
Microsporidium sp. Angskar 21	*Daphnia longispina*	Crustacea, Cladocera, Daphniidae	EU075350
Microsporidium sp. BKES1 CAL	*Odontogammarus calcaratus*	Crustacea, Amphipoda, Eulimnogammaridae	FJ756018
Microsporidium sp. BKES1 KES	*Pallaseopsis kessleri*	Crustacea, Amphipoda, Pallaseidae	FJ756019
Microsporidium sp. BKES1 LAT	*Brandtia latissima latior*	Crustacea, Amphipoda, Acanthogammaridae	FJ756020
Microsporidium sp. BLAP2	*Acanthogammarus lappaceus*	Crustacea, Amphipoda, Acanthogammaridae	FJ756026
Microsporidium sp. BLAT20	Brandtia latissima lata	Crustacea, Amphipoda, Acanthogammaridae	FJ756071
Microsporidium sp. BSEI1 LAC	*Gammarus lacustris*	Crustacea, Amphipoda, Gammaridae	FJ756154
Microsporidium sp. CRANA	*Crangonyx* sp.	Crustacea, Amphipoda, Crangonyctidae	AJ966721
Microsporidium sp. MIC1	*Daphnia galeata*	Crustacea, Cladocera, Daphniidae	FJ794862
Microsporidium sp. Ripley Pond I	*Daphnia pulicaria*	Crustacea, Cladocera, Daphniidae	EU075355
Microsporidium sp. Turtle Lake	*Daphnia pulicaria*	Crustacea, Cladocera, Daphniidae	EU075357
Octosporea muscaedomesticae	*Musca domestica, Phormia regina*	Insecta, Diptera, Muscidae/Calliphoridae	FN794114
Paraepiseptum plectrocnemiae	*Plectrocnemia conspersa*	Insecta, Trichoptera, Polycentropodidae	DQ864438
Paraepiseptum polycentropi	*Hydropsyche fulvipes, Polycentropus flavomaculatus*	Insecta, Trichoptera, Hydropsychidae/Polycentropodidae	DQ864437
Parathelohania anophelis	*Anopheles quadrimaculatus*	Insecta, Diptera, Culicidae	AF027682
Parathelohania obesa	*Anopheles crucians*	Insecta, Diptera, Culicidae	AY090065
Senoma globulifera	*Anopheles messeae*	Insecta, Diptera, Culicidae	DQ641245
Trichotuzetia guttata	*Cyclops vicinus*	Crustacea, Copepoda, Cyclopidae	AY326268
Vairimorpha sp.	*Solenopsis richteri*	Insecta, Hymenoptera, Formicidae	AF031539
Zelenkaia sp. (=iMS 2)	*Micropterna sequax*	Insecta, Trichoptera, Limnephilidae	EF537881
Zelenkaia trichopterae (=iMS 1)	*Halesus digitatus*	Insecta, Trichoptera, Limnephilidae	EF537879