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Morphology and taxonomy of the microsporidium *Liebermannia covasacrae* n. sp. from the grasshopper *Covasacris pallidinota* (Orthoptera, Acrididae)

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ABSTRACT

During a survey for grasshopper pathogens in Argentina in 2005–2006, individual *Covasacris pallidinota* from halophylous grasslands in Laprida, Buenos Aires province were found to be infected with a microsporidium. Infection was restricted to the salivary gland epithelial cells. The microsporidium produced ovocylindrical spores averaging 2.6 ± 0.28 × 1.4 ± 0.12 μm (range 2.2–3.4 × 1.1–1.7 μm), which resembled in size and shape the spores of *Liebermannia patagonica* and *L. dichroplusae*, two recently described species that also parasitize Argentine grasshoppers. The life cycle of the microsporidium included the formation of polynucleate, diplokaryotic, moniliform, merogonial plasmodia wrapped in flattened cisterns of the host endoplasmic reticulum (ER). Plasmodia divided to produce diplokaryotic cells. The latter underwent elongation, dissociation of diplokarya counterparts, vacuolization, dismantling of the host ER envelope, and deposition of electron-dense material outside the plasma membrane. The resultant binucleate sporogonial plasmodia divided into two uninucleate sporoblasts, which eventually transformed into spores. Uninucleate spores contained a lamellar polaroplast, embraced by an elongated polar sac, anchoring disc, 3–5 polar filament coils, and a cluster of anastomizing tubules (sporoblast trans-Golgi, posterosome) at the posterior end. Sequence similarity of the SSU rDNA of the newly discovered microsporidium (Genbank accession no. EU709818) to *L. patagonica* and *L. dichroplusae* was 99% and 97%, respectively, suggesting that the three species belong to one genus. All three species fell into one clade in SSU rDNA-based phylogenetic trees produced by neighbor joining, maximum parsimony, and maximum likelihood analyses with 100% statistical support. We assign the name *Liebermannia covasacrae* to this microsporidium. It can be easily differentiated from both congeners by host species, tissue tropism, type of sporogony, and several features of morphology. Comparison of the three *Liebermannia* spp. demonstrates that the nuclear phase (dikaryotic versus monokaryotic spores) and type of sporogony (polysporous versus disporous) may vary in closely related species.

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1. Introduction

Natural pastures in the Pampas region of Argentina have been the focus of intense concern of agricultural scientists, botanists, and ecologists (Torrusio et al., 2002). The drastic reduction and alteration of these unique halophylous communities are due to extensive livestock grazing and crop production, but occasional outbreaks of grasshoppers also have contributed to deterioration of the grasslands and crops in the Pampas in recent decades (Cigliano et al., 2003; Torrusio et al., 2002). The reasons for great fluctuations of grasshopper population densities in outbreak versus non-outbreak periods remain unknown.

Previous surveys for grasshopper biotic mortality agents in the Pampas during 1997–2003 identified two microsporidia. The introduced species *Paranosema (Nosema) locustae* Canning infects as many as 16 species of grasshoppers inhabiting the Pampas, mostly in the subfamily Melanoplinae. *Liebermannia (Perezia) dichroplusae* Lange, a native species, apparently infects only the melanopline *Dichroplus elongatus* (Lange, 2003), normally the most common and harmful grasshopper in the area (Cigliano et al., 2003). In subsequent surveys during 2005–2006, individual *Covasacris pallidinota* grasshoppers were infected with microsporidian spores

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resembling in size and shape those of *L. dichroplusae* and *L. patagonica,* a microsporidium that parasitizes the grasshopper *Tristira megellanica* in Patagonia (Sokolova et al., 2006, 2007). Light and electron microscopy suggested that the unknown microsporidium was a new species similar to the two *Liebermannia* species. SSU rDNA-based phylogenetic analyses revealed close genetic relationships among the three species, suggesting that all of them belong to one genus.

In this paper we describe a new species, *Liebermannia covasacrae*, and compare structural and molecular characteristics of the three congeners.

2. Materials and methods

Grasshopper sampling and microsporidia detection. Grasshoppers were sampled in a halophylous grassland site (37°45′11.4″S, 60°44′34.1″W) located in Laprida county, southern Buenos Aires province, in the southern Pampas region. *C. pallidinota* is usually associated with halophylous communities (Carbonell et al., 2006). Nymphs and adults ($n = 404$) were collected by the sweep net method (Larson et al., 1999).

Immediately after collection, grasshoppers were taken to the laboratory where they were either frozen at −32 °C or examined by ventral, longitudinal dissection. For detection of microsporidia, frozen samples were thawed and examined by the homogenization method (Henry et al., 1985). Fractions of whole insect homogenates were observed as fresh preparations under phase contrast optics at 400–1000×. For determination of tissue tropism, organs and tissues from dissected grasshoppers were separated, smeared on slides, and also examined by phase contrast microscopy. Infection was restricted to salivary glands, therefore, only these organs were used to acquire DNA extractions from spores and for the examination of pathogen life cycles and ultrastructure by light and transmission electron microscopy.

Light microscopy. Salivary glands from infected individual insects were smeared on slides and observed directly (phase contrast, 400–1000×) or dried and then fixed with 100% methanol. Alternatively, glands from several individuals were processed together in distilled water. After homogenization in a grinder (10 s), several drops of this suspension containing spores and stages were deposited on to slides that were then air-dried and fixed with methanol. The fixed slides were stained with Giemsa (Sigma, Saint Louis, MO) or with the Trichrome Blue (Remel, Lenexa, KS) and observed in bright field. Spores were photographed with a Nikon Eclipse E-600 digital camera. Spore measurements were taken with imaging software (MetaView. 1998, Meta Imaging Series 4.5. Universal Imaging Corporation, West Chester, PA).

Transmission electron microscopy (TEM). Infected salivary glands were fixed for 1 h at 4 °C in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% aqueous OsO_4, and en block stained with uranyl acetate. Dehydration was through an ascending alcohol series and acetone. Before the final incubation in 100% acetone, the samples were exposed to a saturated solution of lead acetate in 1:1 alchohol/acetone for 1 h (Elliott, 2007). Samples were embedded in Spurr's resin. Thin sections were examined and digitally photographed under a JEOL-JEM–1011 electron microscope without additional staining. For general histology, 1 μm sections were mounted on slides, stained with Methylene blue, and viewed by light microscopy.

DNA isolation and sequencing. Spore suspensions for DNA isolation were prepared in double distilled water from slightly homogenized salivary glands isolated from infected grasshoppers. The suspensions were stored at −32 °C until use (1–3 months). Before DNA isolation, the suspensions were thawed and checked by light microscopy for quality of spore preservation. Then the spore suspension was immediately transferred into guanidine buffer (4.2 M guanidine thyocyanate, 50 mM Tris–HCl, pH 7.6, 10 mM EDT, 25% lauryl sarcosinate) and stored at +4 °C for 12–76 h. Spores were spun down and re-suspended in 150 μl of TAE buffer (0.04 M Tris acetate, 0.01 M EDTA), bead-beaten in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) at maximum speed for 1 min, and heated in a thermoblock for 10 min at 95 °C. Afterwards the supernatant (crude extract) was used directly as a DNA template for PCR amplification (Vossbrinck et al., 2004). Alternatively, the crude extracts were subjected to phenol–chloroform–isoamylalcohol extraction followed by alcohol precipitation. The primers for PCR amplification were V1 (5′-CAC CAG GTT GAT TCT GCC TGA C-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′); the primers for sequencing were V1, 530r (5′-CCG CGG C(T/G)G CTG GCA C-3′), 530f (5′-GTG CCA GC (G/A) GCC GCG G), 1061f (5′-GGT GGT GCA TGG CCG-3′), and 1492r (Vossbrinck et al., 2004; Weiss and Vossbrinck, 1999). These primers produced overlapping sequences that were assembled with Chromas. Pro. 1.34 software (http://www.technelysium.com.au/ ChromasPro.html). Crude DNA extracts and phenol–chloroform isolated DNA were subjected to PCR amplification. Bands of about 1400 base pairs were excised from 2% agarose gel; DNA was extracted from gels with a Zymoclean DNA recovery kit (Zymo Research, CA). Direct PCR amplification and sequencing were performed at least twice for each DNA sample. Two vouchers of spore suspensions originating from different insect groups were tested as described above. All reagents used in the study, unless designated otherwise, were from SIGMA (St. Louis, MO).

Phylogenetic analysis. Sixteen microsporidian sequences were obtained from the NCBI GenBank database (for accession numbers and host species see Table 1). Small subunit ribosomal RNA gene (SSU rDNA) sequences of *L. dichroplusae*, *L. patagonica*, *Orthosomella operophterae*, *Endoreticulatus schubergi*, and *Enterocytozoon salmoni* showed maximum identity to the novel sequence in a BLAST search. Sequences of *Heterovesicula covani, Paranosema locustae*, and *P. grylli* were included in the analyses to determine relationships of the new species with other microsporidia infecting Orthoptera. To determine its relationship with the *Nosema–Encephalitozoon* clade, sequences of *Nosema bombycis, Vairimorpha necatrix*, and *Encephalitozoon hellem* were included, as well as two *Ampblyospora* spp. for comparison with the "aquatic outgroup" (Vossbrinck et al., 2004). We also included the sequence of *Ovavesicula popillae*, known to be related to the *Paranosema* clade (Vossbrinck and Andreadis, 2007), to better clarify the *Paranosema* clade position, which remains poorly resolved at this time (Sokolova et al., 2007, 2008). Finally, we added the sequence of the very recently described *Euplotespora binucleata* (Fokin et al., 2008), the sole available SSU rDNA sequence from a microsporidium infecting ciliates, to confirm its clustering with *E. schubergi*, which was claimed by Fokin et al. (2008).

All sequences were trimmed from the 5′-end beginning with the last nucleotide of the V1 universal primer and at the 3′-end to a final length of 1300 characters including gaps. They were aligned with the CLUSTAL X program (Thompson et al., 1997) without additional changes. A zygomycete fungus, *Basidiobolus ranarum* (Fungi: Zygomycetes) (GenBank accession no. D29946), was selected as an outgroup. The resultant alignment was analyzed by neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) algorithms with PAUP*, version 4.0 (Swofford, 2002). A TrN+I+G model of nucleotide substitution was suggested as a best-fit by Hierarchical Likelihood Ratio tests and a TIM+I+G model by Akaike Information Criteria in Modeltest 3.6 (Posada and Crandall, 1998). Settings of the first model were applied to the ML analyses presented in this paper; application of TIM+I+G model settings did not change the ML tree topology but slightly (±5%) shifted the bootstrap support values (not shown). Bootstrap