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

Yuliya Y. Sokolova [a,b,1,*], Carlos E. Lange [c], Yanina Mariottini [d], James R. Fuxa [a]

[a] *Laboratory of Insect Pathology, Department of Entomology, Louisiana State University, 404 Life Sciences Bldg., Baton Rouge, LA 70803-1710, USA*
[b] *Institute of Cytology, Department of Cytology of Unicellular Organisms, Russian Academy of Sciences, 4 Tikhoretsky Ave., 194064 St. Petersburg, Russia*
[c] *Comisión de Investigaciones Científicas (CIC) de la Provincia de Buenos Aires, Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT La Plata CONICET – UNLP, Calle 2 # 584, La Plata (1900), Argentina*
[d] *Centro de Estudios Parasitológicos y de Vectores (CEPAVE), CCT La Plata CONICET – UNLP, Calle 2 # 584, La Plata (1900), Argentina*

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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

* Corresponding author. Present address: Tulane National Primate Research Center, Division of Microbiology, 18703 Three Rivers Road, Covington, LA 70433, USA. Fax: +1 985 871 6248.
E-mail addresses: ysokolov@tulane.edu, jumicro@yahoo.com (Y.Y. Sokolova).
[1] Address after May 30 2009: Institute of Cytology, Department of Cytology of Unicellular Organisms, Russian Academy of Sciences, 4 Tikhoretsky Ave., 194064 St. Petersburg, Russia. Fax: +7 (812) 2971034.

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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

Table 1
Hosts and GenBank accession numbers of the SSU rDNA sequences of 15 microsporidian species used in the phylogenetic analyses.

#	Microsporidium	Host	GenBank accession no.
1	*Amblyospora connecticus*	*Aedes cantator* (Insecta, Diptera)	AF025685
2	*Amblyospora excrucii*	*Acantocyclops vernalis* (Crustacea, Copepoda)	AY090044
3	*Encephalitozoon hellem*	*Homo sapiens* (Mammalia, Primates)	AF338366
4	*Endoreticulatus schubergi*	*Choristoneura fumiferana* (Insecta, Lepidoptera)	L39109
5	*Enterocytozoon salmoni*	*Oncorynchus tshawytsca* (Teleostei, Salmoniformes)	U10883
6	*Euplotespora binucleata*	*Euplotes woodruffi* (Ciliophora, Spirotrichea)	DQ675604
7	*Heterovesicula cowani*	*Anabrus simplex* (Insecta, Orthoptera)	EU275200
8	*Liebermannia covasacrae*	*Covasacris pallidinota* (Insecta, Orthoptera)	EU709818
9	*Liebermannia dichroplusae*	*Dichroplus elongatus* (Insecta, Orthoptera)	EF016249
10	*Liebermannia patagonica*	*Tristira magellanica* (Insecta, Orthoptera)	DQ239917
11	*Nosema bombycis*	*Bombyx mori* (Insecta, Lepidoptera)	L39111
12	*Orthosomella operophtera*	*Operophtera brumata* (Insecta, Lepidoptera)	AJ302316
13	*Ovavesicula popilliae*	*Popillia japonica* (Insecta, Coleoptera)	EF564602
14	*Paranosema grylli*	*Gryllus bimaculatus* (Insecta, Orthoptera)	AY305325
15	*Paranosema locustae*	*Locusta migratoria* (Insecta, Orthoptera)	AY305324
16	*Vairimorpha necatrix*	*Trichoplusia ni* (Insecta, Lepidoptera)	Y00266

values for all tree-building methods were obtained from 100 re-samplings. Trees were manipulated with Tree-View, version 1.6.6. A pairwise sequence comparison in the form of a data matrix was calculated by the Kimura-2-Parameter analysis built in PAUP*, version 4.0 (Swofford, 2002).

3. Results

3.1. Light microscopy

Prevalence of infection was 36.1%. Refractive ovocylindrical spores were the predominant stage in homogenates and smears prepared from infected salivary glands. Occasionally elongated moniliform plasmodia (Fig. 1a, arrow) and sporonts (twice as large as spores) (Fig. 1b) were seen in fresh smears. Unfixed spores averaged $2.6 \pm 0.28 \times 1.4 \pm 0.12$ μm ($n = 40$; range 2.2–3.4 × 1.1–1.7 μm). Patterns of staining with Trichrome and Giemsa were typical for microsporidia (Fig. 1b and c). Mature spores stained poorly with Giemsa; immature spores and sporoblasts clearly displayed one nucleus. In addition to spores, large (6–8 μm in diameter) diplokaryotic cells were not uncommon in the Giemsa-stained smears (Fig. 1c).

Infection was restricted to the epithelial cells of salivary glands and was distributed evenly throughout the cells. Intracellular stages were concentrated around the nuclei of infected cells, surrounded by a zone of spores (Fig. 1d). Infected cells were slightly larger than uninfected ones and their nuclei occasionally displayed invaginations of the nuclear membrane. The epithelial columnar cells of the salivary glands from infected insects were heavily loaded with spores and developmental stages. Goblet cells were not infected with the pathogen.

3.2. Ultrastructure

The earliest life cycle stages that were observed were diplokaryotic meronts (2.2–3.8 μm in diameter). Some of them were in the interphase marked by the presence of the nucleolus inside the nucleus (Fig. 2a) and some were undergoing mitosis. Delayed plasmotomy resulted in formation of an elongated, moniliform merogonial plasmodia consisting of 2–4 cells that were interconnected by cytoplasmic bridges (Fig. 2b). Each cell contained one diplokaryon. Individual meronts and merogonial plasmodia were surrounded by host cell mitochondria (Fig. 2c) and wrapped in a layer of flattened cisterns of endoplasmic reticulum (Fig. 2a and d). Plasmodia divided into separate cells and underwent elongation and dissociation of diplokarya counterparts (Fig. 2e–g). This process was synchronized with the deposition of a thin electron-dense envelope outside the plasma membrane. This envelope eventually replaced the layer of flattened cisterns (Fig. 2g). The dissociation of nuclei marked the onset of sporogony. A binucleate, elongated, sporogonial plasmodium (Fig. 3a) was the most abundant stage in sections of infected salivary glands with the exception of poorly preserved mature spores. Each plasmodium eventually divided into two slightly elongated sporoblasts (Fig. 3b). Immediately following plasmodium division, a voluminous vacuole occupied the apical part of the cell (Fig. 3c). The sporont Golgi apparatus, a cluster of anastomizing tubules, was located in the vicinity of the nucleus toward the posterior end (Fig. 3b). During the course of

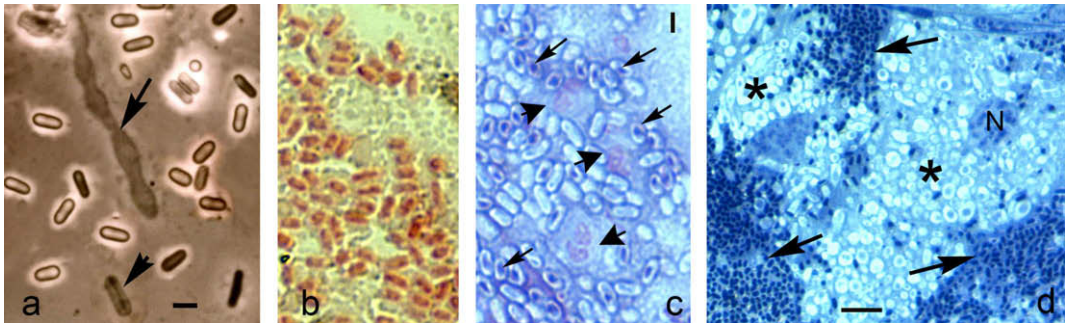

Fig. 1. Light microscopy of *Liebermannia covasacrae*. a, phase contrast, spores and stages of the microsporidium on a fresh smear from an infected salivary gland. Arrow points to the elongated moniliform merogonial plasmodium; arrowhead indicates sporont; b, methanol-fixed and Trichrome-stained spores; c, Giemsa-stained uninucleate spores (thin arrows) and diplokaryotic stages (arrowheads); d, Methylene blue-stained hemi-thin section through heavily infected cells: stages (asterisk) are concentrated in the central part of the cell around the nucleus, whereas spores (arrows) occupy the cell periphery. Scale bars: a–c, 2 μm; d, 10 μm.

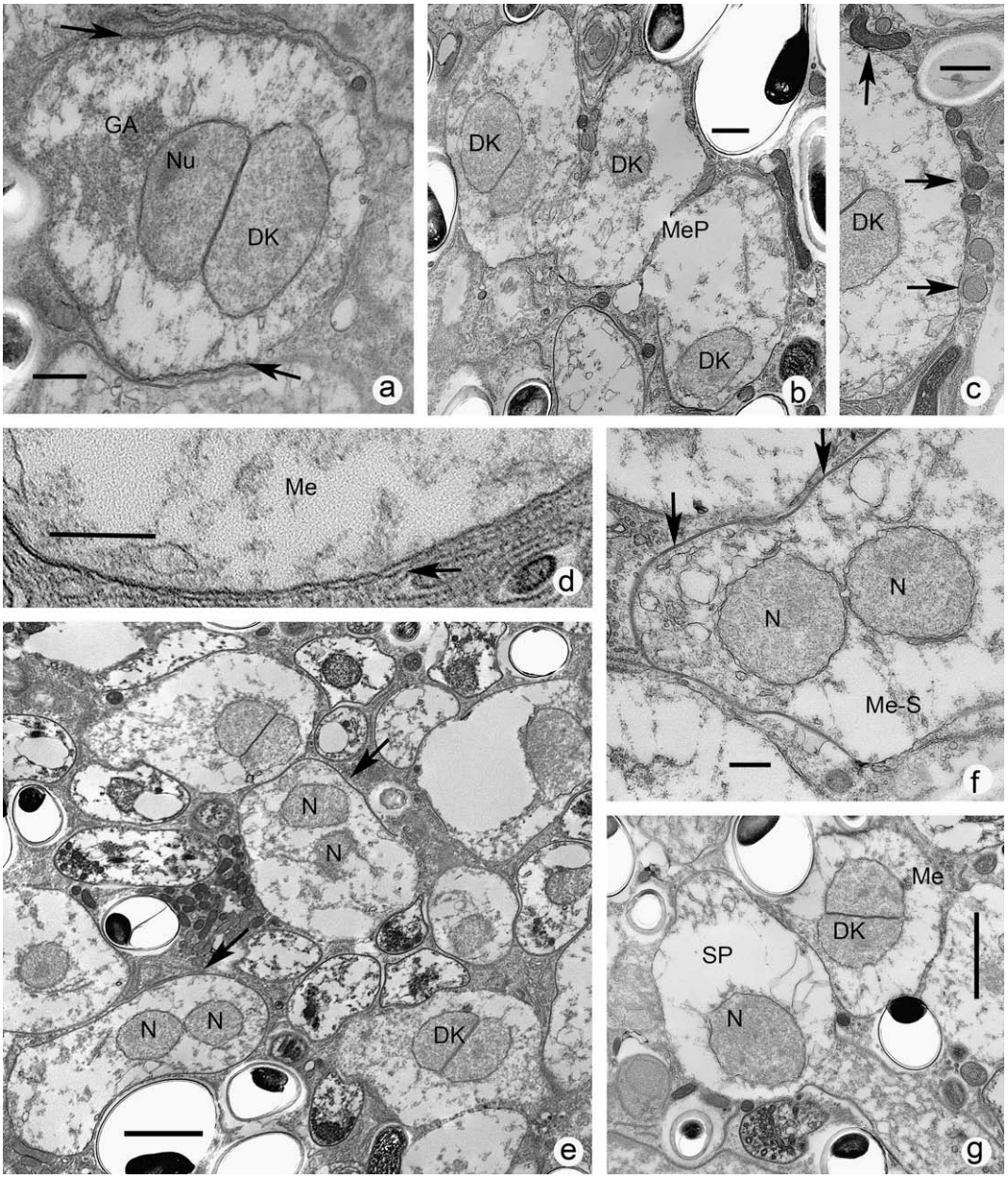

Fig. 2. Electron microscopy of *Liebermannia covasacrae*: merogony and transition to sporogony. (a) Diplokaryotic meront (merogonial plasmodium) surrounded by a layer of flattened ER cisterns (arrows); (b) Merogonial plasmodium with three diplokarya; (c) Meront surrounded by host mitochondria (arrows); d, meront, arrow points to the layer of flattened cisterns of the host endoplasmic reticulum surrounding the parasite cell; e, section through an infected cell filled with the parasites; dissociation of the members of diplokarya and thickening of the plasma membrane (arrows) mark transition to spogony; (f) Meront–sporont transitional stage: two nuclei are separated, arrows indicate deposition of electron-dense envelope outside the plasma membrane; (g) Diplokaryotic meront and sporogonial plasmodium with an individual nucleus are in the view. GA – Golgi apparatus; DK – diplokaryon; Me – meront or merogonial plasmodium; N – individual nucleus; Nu – nucleolus; SP – sporont/sporogonial plasmodium; Spb – sporoblast. Scale bars: a–d, f–g, 500 nm; e, 2 μm.

morphogenesis, at least part of the tubular cluster transformed into the polar filament, while the apical vacuole gave rise to the polar sac and polaroplast (Fig. 3c and d). The transition to sporogony was accompanied by gradual shrinkage of the cytoplasm and condensation of the nucleoplasm. Sporogonial plasmodia and early sporoblasts were nearly two times thinner than meronts, only 1.5–1.6 μm wide, whereas the sporoblasts with advanced signs of morphogenesis were only 1.2–1.3 μm wide. The diameter of individual nuclei decreased from 1.2–1.7 μm in meronts to 0.6–0.8 μm in sporoblasts. Mature uninuclear spores in ultrathin sections measured 1.5–2.5 × 0.6–1.1 μm. They contained a lamellar polaroplast embraced by an elongated polar sac, an anchoring disc, 3–5 polar filament coils, and the remnants of the sporoblast trans-Golgi (posterosome) consisting of anastomising tubules of lesser diameter than the polar filament (Fig. 3e and f). The multilayered undulating exospore was about 30 nm thick and an electron-lucid endospore – approximately 90 nm thick, except for the apical end, which was only 40–45 nm thick. A plasma membrane was observed as a line under the endospore.

3.3. SSU rDNA-based phylogenetic relationships

The consensus SSU rRNA sequence of the new microsporidium had 1243 base pairs with 58.6% GC content (GenBank accession no. EU709818). The evolutionary distance calculated by Kimura 2-parameter distance matrix analysis between the novel sequence and the sequences of *L. patagonica* and *L. dichroplusae* was as low as 0.01 and 0.03, respectively. The sequence closest to *Lieberannia*

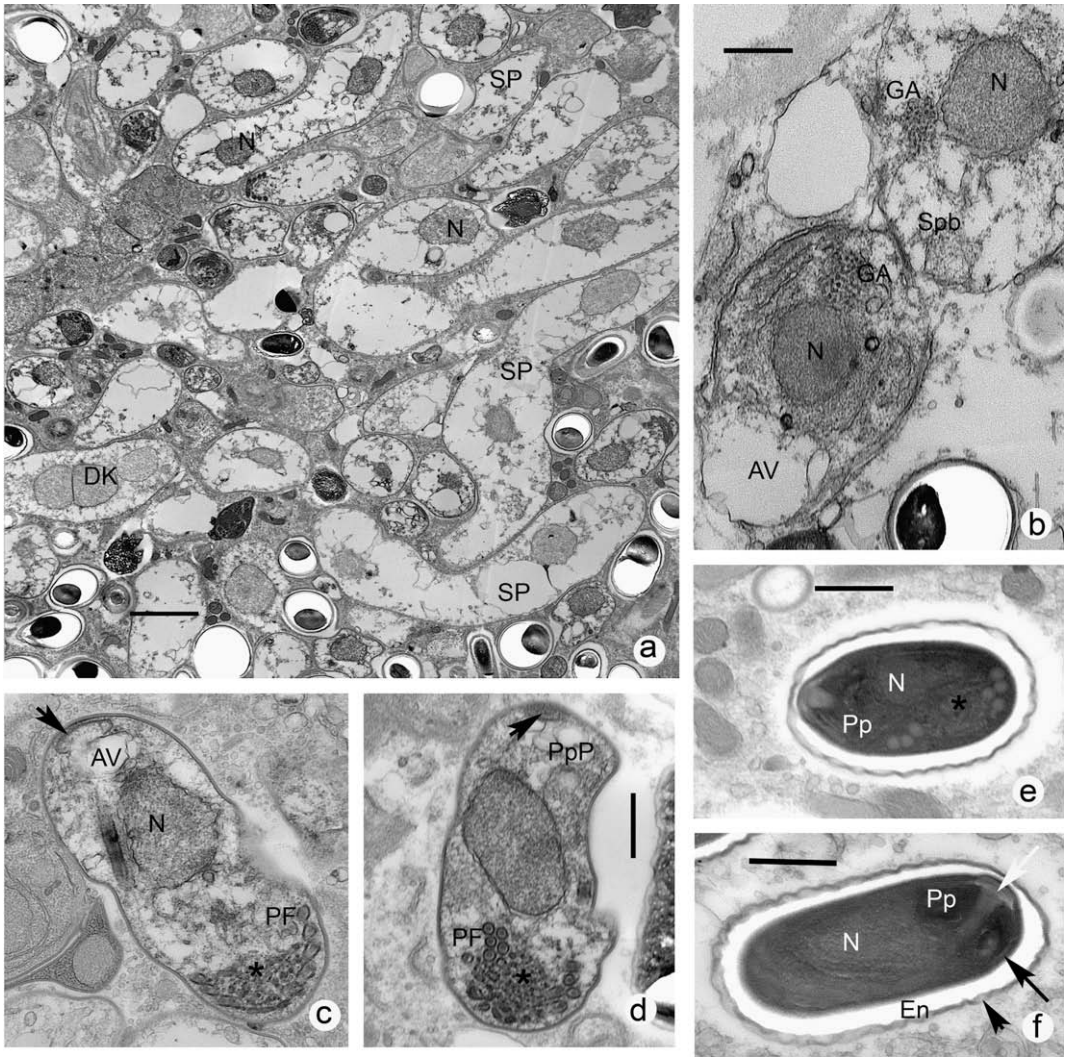

Fig. 3. Electron microscopy of *Liebermannia covasacrae*: sporogony. (a) Sporogonial plasmodia with two individual nuclei; (b) Two sporoblasts formed after division of the sporogonial plasmodium; presence of the prominent Golgi apparatus and large vacuole at the apical end indicate the beginning of morphogenesis of the extrusion apparatus; (c–d) Sporoblasts; apical vacuole transforms into precursors of the polar cap (arrows) and polaroplast, trans-Golgi tubular cluster (asterisk) gives rise to the polar filament. (e–f) Uninucleate mature spores with a few polar filament coils, posterosome (asterisk) at the posterior end, and the multilayered undulating exospore (arrowhead). Apical zone of the spore includes the anchoring disc attached to the basal part of the polar filament (white arrow) and the polar sac (black arrows), which embraces the compact polaroplsat. AV – apical vacuole; GA – Golgi apparatus; DK – diplokaryon; En – endospore; N – individual nucleus; PF – polar filament; PpP – primordial polaroplast; Pp – polaroplast; SP – sporont/sporogonial plasmodium; Spb – sporoblast. Scale bars: a, 2 μm; b–f, 500 nm.

spp. was that of *O. operopterae*, which diverged from *Liebermannia* spp. by 0.19–0.20. The other sequences analyzed were only distantly related to the novel sequence, with divergences ranging from 0.31 to 0.57 (Table 2). Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) phylogenetic analyses placed the new microsporidium in one clade with *Liebermannia* spp. with 100% statistical support (Fig. 4a). Inside this clade the novel sequence formed a dichotomy with *L. patagonica.* General tree topologies produced by all three algorithms were similar and in good accord with previously published data (Fokin et al., 2008; Vossbrinck and Andreadis, 2007; Vossbrinck and Debrunner-Vossbrinck, 2005). The *Amblyospora* spp. clade formed an outgroup (“aquatic outgroup”), (Vossbrinck and Debrunner-Vossbrinck, 2005) opposed to other sequences, which all belong to parasites of terrestrial insects with the one exception of *E. binucleata,* which parasitizes a ciliate. Our analyses confirmed clustering of the *E. binucleata* sequence with those of genera *Endoreticulatus* and *Cystosporogenus* (Fokin et al., 2008), comprised predominantly of parasites of Lepidoptera. *O. papillae,* a parasite of Japanese beetle, in all our analyses fell into one clade with *Paranosema spp.* with 100% bootstrap support in agreement with Vossbrinck and Andreadis (2007), suggesting relatedness of the microsporidia with extremely dissimilar developmental characters. In all trees, the *Paranosema–Ovavesicula, Orthosomella–Enterocytozoon–Endoreticulatus–Euplotespora*, and *Liebermannia* clades were united in one superclade separated from the *Nosema–Vairimorpha–Encephalitozoon–Heterovesicula* clade. The only discrepancy among analyses was the position of the *Paranosema–Ovavesicula* clade: in the NJ tree this clade formed a dichotomy with the *Orthosomella–Liebermannia* branch; on PS and ML trees it produced a separate lineage (Fig. 4a and b).

4. Discussion

Our placement of the microsporidium from *C. pallidinota* into the genus *Liebermannia* is well substantiated. It is based on similarity of SSU rDNA sequences, related orthopteran hosts, parasitism of the epithelia of alimentary tract, similarity of shape and structure of spores, and developmental stages (Table 3).

Table 2
Kimura 2-parameter distance matrix.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Ac[a]	–																
2	Ae	0.09	–															
3	Br	0.34	0.36	–														
4	Vn	0.42	0.44	0.50	–													
5	Nb	0.42	0.43	0.50	0.15	–												
6	Hc	0.44	0.44	0.52	0.32	0.32	–											
7	Ench	0.43	0.45	0.52	0.33	0.36	0.33	–										
8	**Lc**	**0.43**	**0.46**	**0.57**	**0.49**	**0.53**	**0.45**	**0.41**	–									
9	Lp	0.44	0.47	0.57	0.51	0.54	0.48	0.42	**0.01**	–								
10	Ld	0.43	0.45	0.55	0.49	0.52	0.45	0.42	**0.03**	0.04	–							
11	Oo	0.43	0.45	0.54	0.47	0.50	0.46	0.39	**0.19**	0.20	0.19	–						
12	Ploc	0.44	0.47	0.61	0.56	0.63	0.54	0.47	**0.33**	0.34	0.33	0.31	–					
13	Pgr	0.44	0.46	0.61	0.56	0.62	0.53	0.47	**0.33**	0.35	0.33	0.32	0.03	–				
14	Ovp	0.52	0.53	0.64	0.61	0.65	0.54	0.52	**0.37**	0.40	0.38	0.36	0.28	0.29	–			
15	Ends	0.46	0.47	0.54	0.42	0.45	0.44	0.42	**0.31**	0.31	0.29	0.25	0.39	0.38	0.44	–		
16	Eupb	0.43	0.44	0.49	0.38	0.39	0.41	0.38	**0.35**	0.36	0.34	0.30	0.49	0.48	0.51	0.18	–	
17	Ents	0.43	0.46	0.55	0.44	0.46	0.42	0.40	**0.31**	0.33	0.31	0.26	0.44	0.44	0.49	0.28	0.27	–

[a] 1 – Ac, *Amblyospora connecticus*; 2 – Ae, *A. excrucii;* 3 – Br, *Basidiobolus ranarum* (outgroup); 4 – Vn, *Vairimorpha necatrix*; 5 – Nb, *Nosema bombycis*; 6 – Hc, *Heterovesicula cowani;* 7 – Ench, *Encephalitozoon hellem*; **8 – Lc, *Liebermannia covasacris* n. sp.**; 9 – Lp, *Liebermannia patagonica*; 10 – Ld, *Liebermannia dichroplusae;* 11 – Oo, *Orthosomella operophtera*; 12 – Pl, *Paranosema locustae*; 13 – Pg, *Paranosema grylli*; 14 – Op, *Ovavesicula popilliae;* 15 – Ends, *Endoreticulatus schubergi*; 16 – Eupb, *Euplotespora binucleata;* 17 – Ents, *Enterocytozoon salmoni*.

The differences among the three closely related congeners (Fig. 5, Table 3) are more interesting to address, because they reflect specific adaptations that drive divergence evolution of microsporidian species.

Sequence analyses suggest that the novel species is indeed closely related to *L. dichropluse* (97% sequence similarity in the BLAST search and 0.03 of sequence divergence in the Kimura 2-parameter distance matrix test) and to *L. patagonica* (94% and 0.01, respectively), and that the three species probably diverged only recently. However, the diplokaryotic nuclear condition throughout the life cycle of *L. patagonica* versus nuclear dissociation prior to sporogony in *L. covasacrae* (Fig. 5) justifies placement of these microsporidia into different subclasses (Sprague et al., 1992), or at least into different genera. Life cycles of both *L. dichroplusae* and *L. covasacrae* include nuclear dissociation (Fig. 5), but the judging from sequence analyses they are probably more distantly related than *L. covasacrae* and *L. patagonica* (Table 2, Fig. 4). *L. patagonica* and *L. covasacrae* share several structural and developmental characters, such as a small number of polar filament coils, the structure of merogonial plasmodia, and the close association of certain parasite stages with host ER (Table 3, Fig. 5).

Tree topologies (Fig. 4 a and b) demonstrated a basal position of *L. dichroplusae* relative to *L. covasacrae–L. patagonica* dichotomy, and a longer branch (corresponding to the evolutionary distance) led to *L. patagonica* than to *L. covasacrae*. It allows us to assume that *L. dichroplusae* may be more similar to the ancestral form, than the two other congeners, and that *L. patagonica* may be the most derived of the three species. Indeed, the life cycle with dihaplophase/haplophase transition is likely a plesiomorphic feature among major microsporidian groups (Vossbrinck and Debrunner-Vossbrinck, 2005). Thus, the haplophase could have been eliminated from the *L. patagonica* life cycle during the process of evolution.

Numerous insect microsporidia reside in tissues of the alimentary tract and about 20% specifically infect midgut epithelium (Sprague et al., 1992). Among those are the ancient chytridiopsids of the genera *Buxtehudea, Chytridioides* and *Chytridiopsis*. The fact that the representatives of the first two genera infect ancient hexapods belonging to the Thysanura (and several millipedes (class Diplopoda)) supports the idea that gut parasitism was characteristic of the initial steps in microsporidia–insect host relationships, and that avoiding the unfavorable and unstable environment of the digestive system became the key evolutionary strategy of microsporidia parasitizing insects (Issi, 1986). Some species (*Buxtehudea, Chytridioides, Chytridiopsis, Cystosporogenes, Endoreticulatus, Flabelliforma, Hessea, Octosporea* spp.) reside in more or less robust parasitophorous vacuoles or their structural analogues to sustain phago-lysosomal digestion (Becnel and Andreadis, 1999; Canning and Vavra, 2000; Sprague et al., 1992). Others (representatives of majority of existing genera) have escaped to other tissues and organs, such as Malpighian tubules, salivary glands, fat body, and hypodermis. A few including several *Nosema* spp. and *Edhazardia aedes,* preserved a stage of gut parasitism (“early spores” sequence) in the initial stages of the life cycle (Becnel et al., 1989; Maddox et al., 1999; Sprague et al., 1992). Gut parasitism by *Nosema apis,* a specialized pathogen of honey bee colonies, may be considered as an “ultimate” result of this evolutionary tendency. The only species outside the genus *Nosema*, that develops inside the gut without interfacial envelopes is *O. lambdinae*, a parasite of the spring hemlock looper *Lambdina athasaria* (Andreadis et al., 1996). Interestingly, this species as well as its congener *O. operopthterae*, the parasite of salivary glands of the winter moth *Operophtera brumata*, are closely related to *Liebermannia* spp. based on structural characters (Andreadis et al., 1996; Canning et al., 1985) and SSU rDNA sequence divergence (Table 2). It is noteworthy that none of the midgut parasites exhibited meiosis; most of them are monokaryotic throughout the life cycle, and only *Hessea squamata* and *Nosema* spp. are diplokaryotic (Sprague et al., 1992), at least at the “gut-parasitizing” stage.

Liebermannia spp., like *Orthosomella* spp., probably evolved as specialized parasites of the alimentary tract. *L. patagonica* was adapted to parasitizing midgut epithelium, which demanded considerable simplification and shortening of the life cycle to survive and successfully multiply in short-lived midgut cells (Sokolova et al., 2006). Wrapping the most vulnerable presporogenic stages in host cell ER, a feature shared with *L. covasacrae*, may have helped to protect the parasite against the aggressive phago-lyzosome system of the midgut cells (Sokolova et al., 2006). *L. dichrpolusae* and *L. covasacrae* subsequently might have evolved to infect Malpighian tubules and salivary glands, respectively, niches which provided less antagonistic and more stable environments, while allowing spore dissemination with feces or saliva, an obvious advantage for perorally transmitted pathogens. These species developed their own specific characteristics but preserved a life cycle with dihaplophase/haplophase transition.

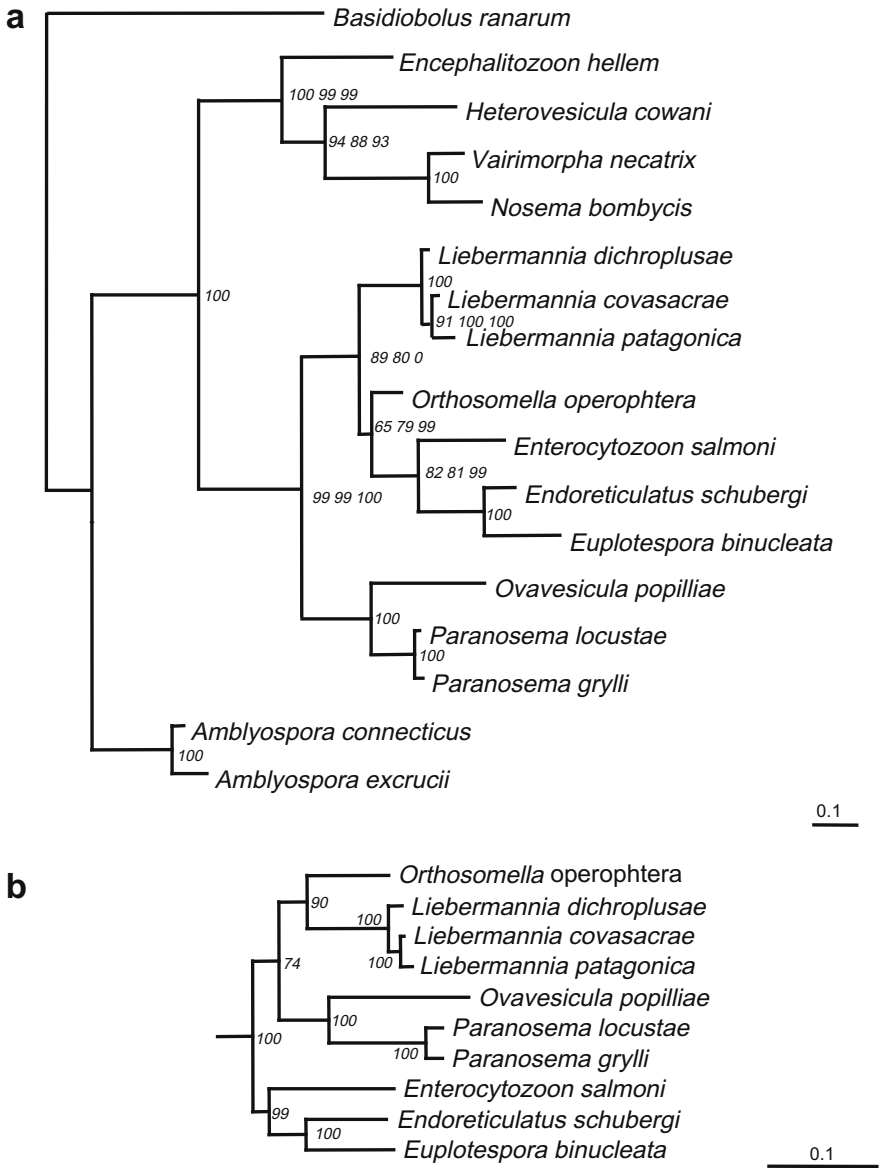

Fig. 4. SSU rDNA-based phylogenetic relationships of *Liebermannia covasacrae* with 16 other microsporidia. (a) – Maximum likelihood tree; (b) – part of the neighbor – joining tree demonstrating different placement of the *Ovavesicula–Paranosema* clade. The first, second, and third numbers at nodes are bootstrap values for the node support in ML, NJ, and MP analyses, respectively, all in 100 replicates; one number at the node means that all values equaled 100. Branch lengths represent evolutionary distance. The scale bar represents one substitution per 10 nucleotides.

Thus accumulating evidence is leading to a conclusion that adaptation to parasitizing different parts of the host alimentary tract played a key role in speciation within such microsporidian genera as *Liebermannia* and *Orthosomella*, similar to that suggested for many genera of protistan and metazoan parasites of digestive systems of various vertebrates and invertebrates (Roberts and Janovy, 2005). Comparison of the three *Liebermannia* spp. also demonstrates that the nuclear phase (dikaryotic versus monokaryoic spores) and type of sporogony (polysporous versus disporous) can vary in closely related species.

5. Taxonomic summary

5.1. L. covasacrae n. sp.

Type host: C. pallidinota (Bruner, 1900) (Orthoptera: Acrididae: Acridinae).

Type locality: Laprida (37°45'11.4″S, 60°44'34.1″W), Buenos Aires province, Argentina.

Infection site: Columnar cells of the salivary gland epithelium.

Transmission: Unknown; salivary glands as the only infection site suggest peroral horizontal transmission.

Merogony: By binary fission of rounded diplokaryotic meronts, which give rise to elongated moniliform merogonial plasmodia consisting of 2–4 cells with diplokarya interconnected by cytoplasmic bridges. Presporulation stages are enveloped in a layer of host endoplasmic reticulum.

Transition to sporogony: Rounded or elongated cells surrounded by electron-dense material result from plasmotomy of sporogonial plasmodia; they undergo elongation and dissociation of the diplokaryon.

Sporogony: Elongated sporogonial plasmodia with two monokaryons covered with a thin layer of electron-dense matrix; plasmodia produce sporoblasts by binary fission.

Table 3
Comparative characteristics of three *Liebermannia* spp.

Species	Characters							
	Host	Type locality	Tissue tropism	Meronts[a]	Stages, transitional to sporogony[b]	Sporonts[c]	Sporoblasts	Spore: shape; size (μm); nucleus; PF[d]; PP
Liebermannia dichroplusae	*Dichroplus elongates* Acrididae Orthoptera	Grasslands, Buenos Aires province, Argentina	Malpighian tubules epithelium	Not wrapped in the layer of host ER	Not associated with host ER; 1–2 DKs undergoing nuclear dissociation, or 2–4 MKs	2–4 MKs; sporonts are not wrapped in the layer of host ER	MK; produced by multiple fission of moniliform sporogonial plasmodia	Ovocylindrical; 1.6–6.7 × 1.0–2.7; MK; 6–8 coils, isofilar; lamellar, uniform
Liebermannia patagonica	*Tristira magellanica* Tristiridae Orthoptera	Steppes, Southern Patagonia, Argentina	Midgut and gastric caecum epithelium	Wrapped in the layer of host ER	Loosely surrounded by host ER; 1–2 DKs	2–4 DKs; sporonts are wrapped in the layer of host ER	DK; produced by multiple fission of moniliform sporogonial plasmodia	Ovocylindrical; 1.9–3.9 × 0.8–1.6; DK; 3 coils, isofilar; lamellar, uniform
Liebermannia covasacrae	*Covasacris pallidinota* Acrididae Orthoptera	Grasslands, Buenos Aires province, Argentina	Salivary gland epithelium	Wrapped in the layer of host ER	Loosely surrounded by host ER; 1 DK undergoing nuclear dissociation	2 MKs; sporonts are not wrapped in the layer of host ER	MK; produced by binary fission	Ovocylindrical; 2.2–3.4 × 1.1–1.7; MK; 3–5 coils, isofilar; lamellar, uniform

[a] Merogony in all three species by binary fission of roundish cells with 2–4 DKs.
[b] Transitional stages in all three species are rounded or elongate cells with patches of electron-dense material on the cell surface.
[c] Sporonts/sporogonial plasmodia in all three species are elongate cells surrounded by electron-dense layer.
[d] Abbreviations: DK – diplokaryon; MK – monokaryon; ER – host endoplasmic reticulum; PF – polar filament; PP – polaroplast.

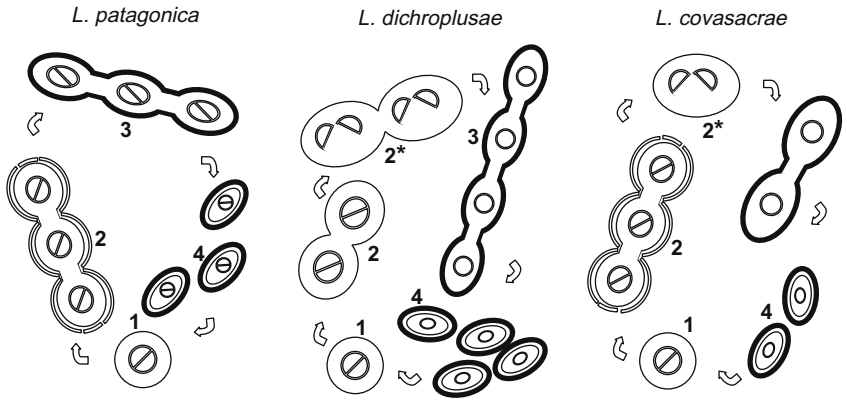

Fig. 5. Life cycle variations of three *Liebermannia* spp. 1 – Meronts; 2 – merogonial plasmodia; 2* – dissociation of nuclei (transition from merogony to sporogony); 3 – sporogonial plasmodia; 4 – spores.

Spores: Ovocylindrical spores 2.6 ± 0.28 × 1.4 ± 0.12 μm (n = 40) and ranging from 2.2 to 3.4 × 1.1–1.7 μm, with individual nuclei, lamellar polaroplast, and 3–5 coils of isofilar polar filament.

Type specimens: Type material (Giemsa and Trichrome-stained smears, EM blocks, frozen samples of isolated DNA), have been deposited in the Center for Parasitological Studies and Vectors (CEPAVE), La Plata National University, Argentina, in the collection of Microsporidia at the Institute for Plant Protection, Russian Academy of Agricultural Sciences, St. Petersburg (Dr. Issi's collection); paratypes are kept at the private collection of the senior author.

Etymology: The specific epithet is derived from the generic name of the host.

We include SSU rRNA gene partial sequence under GenBank accession no. EU709818 in the species characterization.

5.2. Synopsis of the genus Liebermannia

Liebermannia patagonica Sokolova et al. (2006), type species.
Liebermannia dichroplusae Sokolova et al. (2007).
Liebermannia covasacrae n. sp. Sokolova, Lange, Mariottini & Fuxa.

5.3. Differential diagnosis of L. covasacrae n. sp

The species can be differentiated from *L. patagonica* by host species, tissue tropism, production of monokaryotic spores, and disporous sporogony, and from *L. dichroplusae* by host species, tissue tropism, presence of multinucleate merogonial plasmodia enveloped into the host cell rerticulum, and disporous sporogony (Table 3).

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