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Journal of Invertebrate Pathology 91 (2006) 168–182
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Development, ultrastructure, natural occurrence, and molecular characterization of *Liebermannia patagonica* n. g., n. sp., a microsporidian parasite of the grasshopper *Tristira magellanica* (Orthoptera: Tristiridae)

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Received 3 December 2005; accepted 25 January 2006

Abstract

A new microsporidium, *Liebermannia patagonica* n. gen., n. sp., is described from midgut and gastric caecum epithelial cells of *Tristira magellanica*, an apterous grasshopper species of southern Patagonia, Argentina. *L. patagonica* is diplokaryotic, apansporoblastic, homosporous, and polysporoblastic. Transitional (from merogony to sporogony) stages and sporonts of *L. patagonica* were surrounded by host rough endoplasmic reticulum. The ovocylindrical spores measured $2.9 \pm 0.09 \times 1.2 \pm 0.04$ μm (fresh, $n = 50$), and they had an isofilar polar filament of only three coils and a cluster of tubules instead of a classical posterior vacuole. Prevalence was high (up to 80.6%) at the type locality for the four years sampled. Maximum likelihood, neighbor joining, and maximum parsimony analyses of the small subunit rDNA all placed *L. patagonica* (Accession No. DQ 239917) in one clade with *Orthosomella operophterae*.

Keywords: Argentina; Patagonia; Microsporidia; Orthoptera; *Tristira*; Ultrastructure; Host–parasite relationships; Molecular phylogeny

1. Introduction

The microsporidia, spore-forming unicellular eukaryotes that only live as intracellular parasites of other eukaryotes and were traditionally regarded as protozoa, are now considered highly specialized and reduced organisms with fungal affinities (Fast and Keeling, 2005; Keeling and Fast, 2002). Although microsporidia are known from almost all animal groups and some protists, the most common hosts are crustaceans, insects, and fish (Canning and Vávra, 1983; Larsson, 1999). In spite of the role of microsporidia in the natural regulation of insect populations (Solter and Becnel, 2000), relatively little is known about the diversity of microsporidia associated with species of Orthoptera, many of which are agricultural pests of considerable importance worldwide (Jago, 1998). Such a paucity of knowledge is more evident when compared with the state of knowledge available on microsporidia affecting other groups of insects, such as Diptera, Lepidoptera, or Coleoptera (Becnel and Andreadis, 1999). Only 18 species of microsporidia have been recorded from Orthoptera worldwide (Table 1). During surveys of grasshopper and locust pathogens in Argentina, a microsporidium was discovered and isolated from individuals of *Tristira magellanica* Bruner, 1900, an apterous species that belongs to Tristiridae, a unique family of South American grasshoppers with unknown relationships

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doi:10.1016/j.jip.2006.01.003

Table 1
Known species of microsporidia from Orthoptera

Species	Type host	Distribution	References
Heterovesicula cowani	*Anabrus simplex* (Tettigoniidae)	USA	Lange et al. (1995)
Johenrea locustae	*Locusta migratoria* (Acrididae)	Madagascar	Lange et al. (1996)
Microsporidium italicus	*Calliptamus italicus* (Acrididae)	Russia	Issi and Krylova (1987)
Microsporidium sp.	*Gryllodes laplatae* (Gryllidae)	Argentina	Lange (1987a)
Nosema asiaticus	*Oedaleus asiaticus* (Acrididae)	Mongolia	Wen (1996)
Nosema chorthippi	*Chorthippus albomarginatus* (Acrididae)	Russia	Issi and Krylova (1987)
Nosema cuneatum	*Melanoplus confusus* (Acrididae)	USA	Henry (1971); Streett and Henry (1987)
Nosema maroccanus	*Dociostaurus maroccanus* (Acrididae)	Uzbekistan	Issi and Krylova (1987)
Nosema montanae	*Melanoplus packardii* (Acrididae)	USA	Wang et al. (1991)
Nosema pyrgomorphae	*Pyrgomorpha cognata* (Pyrgomorphida)	Cape Verde, Senegal	Toguebaye et al. (1988); Lange et al. (1992)
Nosema sp.	*Chorthippus brunneus* (Acrididae)	Spain	Hernández-Crespo et al. (2001)
Nosema trilophidiae	*Trilophidia annulata* (Acrididae)	Mongolia	Wen and Li (1993)
Paranosema (*Nosema*) *grylli*	*Gryllus bimaculatus* (Gryllidae)	Turkmenistan	Sokolova et al. (2003)
Paranosema (*Nosema*) *locustae*[a]	*Locusta migratoria* (Acrididae)	South Africa, North America, India, Argentina, China	Canning (1953); Henry and Oma (1981); Lange (2003a,b); Whitlock and Brown (1991); Shrivastava and Bhanotar (1985); Yan (1998); Sokolova et al. (2003)
Perezia dichroplusae	*Dichroplus elongatus* (Acrididae)	Argentina	Lange (1987b)
Tubulinosema (*Nosema*) *acridophagus*	*Schistocerca americana* (Acrididae)	US	Henry (1967); Streett and Henry (1993); Franzen et al. (2005)
Undescribed sp	*Acrida turrita* (Acrididae)	Benin	Moutairou et al. (1993)
Vairimorpha sp ?	*Pediodectes* spp. (Tettigoniidae)	USA	Henry (1986)

[a] Slamovits et al. (2004) proposed the transfer of *Nosema locustae* to the genus *Antonospora*, erecting the new combination *A. locustae*. However, we consider the earlier assignment by Sokolova et al. (2003) of *N. locustae* to the genus *Paranosema* more appropriate as justified by Sokolova et al. (2005).

to other Orthoptera (Cigliano, 1989). The following is a description of the new microsporidium, for which the name *Liebermannia patagonica* n. g., n. sp. is proposed.

2. Materials and methods

2.1. Field studies, sampling, and light microscopy

Grasshopper sampling was conducted on rangeland of typical Patagonian steppes (Cabrera and Willink, 1973) in early December of four years (1998–2000, 2003) for one site (Río Mitre, 30 km SW of Calafate, in the vicinity of "Parque Nacional Los Glaciares", southwestern Santa Cruz province) and for one year (1995) at another site (Talagapa plateau, 60 km North of Gan Gan, northern Chubut province). Grasshoppers collected at Río Mitre were older nymphs and adults of the tristirids (Tristiridae: Tristirinae) *T. magellanica* ($n = 675$) and *Bufonacris bruchi* Brancsik ($n = 39$), and older nymphs of the Oedipodine (Acrididae: Oedipodinae) *Trimerotropis pallidipennis* Burmeister ($n = 59$). At Talagapa, all grasshoppers collected were *Bufonacris claraziana* (Saussure) ($n = 315$), another tristirid that is closely related to *T. magellanica* (Cigliano, 1989).

For pathogen detection, each grasshopper was ventrally dissected in order to remove samples of organs and tissues that were examined as fresh mounts under phase-contrast microscopy. After dissection and removal of samples, each grasshopper was then homogenized whole in a tissue grinder, and fresh mounts of homogenates were examined to either confirm absence of infection or initiate preparation of spore suspensions for transmission experiments (Undeen and Vávra, 1997). Fresh mounts were also prepared from feces of grasshoppers suspected of being infected.

Two types of preparations were used for light microscopy: fresh mounts with distilled water or one-quarter-strength Ringer's solution (Poinar and Thomas, 1984) and methanol-fixed smears. The latter were stained either with Giemsa (Wang et al., 1991) or Calcofluor White M2R (Didier et al., 1995) or doubled stained with Calcofluor and DAPI to visualize nuclei (all reagents from Sigma, St. Louis, MO).

Spore suspensions were prepared from infected tissues following the homogenization procedure used by Henry et al. (1973). A hemocytometer was used for counting spores in suspensions (Undeen and Vávra, 1997). Laboratory-reared third instars of the melanopline grasshoppers (Acrididae: Melanoplinae) *Ronderosia bergi* (Stål) ($n = 50$) and *Baeacris punctulatus* (Thunberg) ($n = 50$) were fed with either 10^5 or 10^7 spores per insect according to previous protocols (Habtewold et al., 1995; Lange et al., 2000).

2.2. Transmission electron microscopy

For transmission electron microscopy (TEM), samples of infected tissues were fixed for 1 h at 4 °C in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% aqueous OsO_4 (w/v), and en bloc stained with 1% uranyl acetate. Dehydration was through an ascending acetone series after which samples were embedded in Spurr's resin. For general histology, semithin (0.5–1 μm) sections were mounted on slides, stained with methylene blue (Loginov et al., 1987), and viewed under bright field optics of a Nikon Eclipse E—600 microscope.

For TEM thin sections were contrasted with lead citrate and examined with Hitachi H—300 and JEM 100CX microscopes at 80–100 kV.

2.3. DNA sequencing

DNA isolation, amplification, and sequencing were accomplished as previously described (Vossbrinck et al., 1998, 2004). Suspension of 10^8spores/ml in distilled water prepared as described above for transmission experiments had been stored at −20° since 2003. Approximately 1 ml of thawed spore suspension was spun in a 1.5-ml microcentrifuge tube and resuspended in 150 µl of TAE (40 mM Tris–acetate, 2 mM EDTA) buffer. One hundred and fifty milligrams of glass beads (0.5 mm in diameter) were added to the spore suspension, and the tube was shaken in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 50 s and then heated at 95 °C for 3 min.

One to five microliters of the TAE/ruptured spore suspension was removed and used as a template in a standard PCR (Milks et al., 2004). The 16S rRNA gene was amplified in a Labsystems thermocycler using V1f–1492r pairs of primers (see Vossbrinck et al., 1998, 2004; Weiss and Vossbrinck, 1999) under the following conditions: denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 90 s, with a 5-min extension at 72 °C.

PCR products were separated on an ethidium–bromide stained 2% agarose gel, and the desired bands were extracted with the aid of a Zimoclean Gel DNA Recovery Kit (Zimo Research, Orange, CA). The isolated DNA fragments were sequenced at the Molecular Medicine Laboratory at Louisiana State University, School of Veterinary Medicine, with the following microsporidian primers (Vossbrinck et al., 2004):

V1f (18f) (5′-CAC CAG GTT GAT TCT GCC TGA C-3′); 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′); 350f (5′-CCA AGG A(T/C)G GCA GCA GGC GCG AAA-3′); 350r (5′-TTT CGC GCC TGC TGC C(G/A)T CCTTG-3′); 530f (5′-GTG CCA GC(C/A) GCC GCG G-3′); 530r (5′-CCG CGG C(T/G)G CTG GCA C-3′); 1047r (5′-AAC GGC CAT GCA CCA C-3′); 1061f (5′-GGT GGT GCA TGG CCG-3′). The resulting DNA sequences were assembled with the DNAssist program Version 1.02. The consensus alignment was deposited in GenBank under Accession No. DQ 239917.

2.4. Phylogenetic analysis

Sequences with maximum similarity to our isolate were obtained from GenBank by a BLAST search. Sixteen sequences belonging to the microsporidian genera *Orthosomella*, *Glugoides*, *Vittaforma*, *Cystosporogenes*, *Endoreticulatus*, and *Pleistophora* produced the highest scores in the BLAST search. Only sequences belonging to species parasitizing insects were included into final analyses. *Vittaforma corneae* from immuno-deficient humans and *Glugoides intestinalis* from a cladoceran, *Daphnia pulex*, were excluded. Close evolutionary relationships between *Vittaforma* and the *Endoreticulatus*/*Cystoporogenes* group have been demonstrated and discussed previously (Baker et al., 1995; Frankenhuyzen van et al., 2004; Silveira and Canning, 1995), as was the position of *G. intestinalis* as a sister taxon to the *Cystosporogenes–Endoreticulatus* group (Vossbrinck and Debrunner-Vossbrinck, 2005). Partial SSU rRNA sequence similarity between *G. intestinalis* and a representative of the *Cystosporogenes*/*Endoreticulatus* group most likely reflects their common origin from an ancestral microsporidium with a complex life-cycle, which eventually evolved into separate groups of species developing in insects and in freshwater invertebrates (i.e., cladocerans) (Baker et al., 1997; Sokolova et al., 2006; Vossbrinck and Debrunner-Vossbrinck, 2005). SSU rDNA sequences of *Nosema bombycis*, *Thelohania solenopsae*, *Systenostrema alba*, *Paranosema locustae*, and *Paranosema grylli* were introduced into the analyses to determine their relationships with the newly described microsporidium. Accession numbers of the sequences included in the analyses, and orders of insect hosts are given in Table 2.

Sequences were aligned with the CLUSTAL X program (Thompson et al., 1997) without any additional changes and were trimmed at the 3′-end to a final length of 1300 characters including gaps. A zygomycete fungus, *Basidiobolus ranarum* (Fungi: Zygomycetes) (Accession No. D29946), was selected as an outgroup. Aligned sequences were analyzed by neighbor joining (NJ), maximum parsimony (MP),

Table 2
Microsporidia species, their hosts, and accession numbers for 13 SSU rRNA sequences usedin thephylogenetic analyses

Microsporidium	Host	Accession No.
Cystosporogenes legeri	*Lobesia botrana* (Lepidoptera)	AY233131
Cystosporogenes operophterae	*Operophtera brumata* (Lepidoptera)	AJ303320
Endoreticulatusschu bergi	*Choristoneura fumiferana* (Lepidoptera)	L39109
Endoreticulatus bombycis	*Bombyx mori* (Lepidoptera)	AY009115
Endoreticulatus sp.	*Lymantria dispar* (Lepidoptera)	AY502945
Liebermannia patagonica	*Tristira magellanica* (Orthoptera)	DQ 239917
Nosema bombycis	*Bombyx mori* (Lepidoptera)	AB125662
Orthosomella operophterae	*Operophtera brumata* (Lepidoptera)	AJ 302317
Paranosema grylli	*Gryllus bimaculatus* (Orthoptera)	AY305325
Paranosema locustae	*Locusta migratoria* (Orthoptera)	AY305324
Pleistophora bombycis	*Bombyx mori* (Lepidoptera)	D85500
Systenostrema alba	*Aeshna sp.* (Odonata)	AY953292
Thelohania solenopsae	*Solenopsis invictae* (Hymenoptera)	AF134205

and by a maximum likelihood (ML) algorithm with PAUP*, version 4.0 (Swofford, 2002). A GTR + G model of nucleotide substitution was suggested as a best-fit one by likelihood ratio tests and AIC criteria in Modelrest 3.6 (Posada and Crandall, 1998) and its settings were applied to ML analyses. Bootstrap values for all tree-building methods were obtained from 100 resamplings. Manipulations of trees were carried out with Tree-View, version 1.6.6. Sequence comparison in the form of a data matrix was calculated by Kimura-2 parameter analysis (PAUP*, version 4.0).

3. Results

3.1. General observations

The microsporidium was found in adults and nymphs of *T. magellanica*. The other three grasshopper species collected (*Bufonacris clarazianus*, *B. bruchi*, and *T. pallidipennis*) did not harbor the microsporidium. No obvious external signs or symptoms of infection were evident. Prevalence was high for all four years [80.6% ($n = 151$) in 1998; 70% ($n = 40$) in 1999; 53.8% ($n = 300$) in 2000; and 58.8% ($n = 204$) in 2003]. Spores were commonly present in the feces of infected *T. magellanica*. There was no evidence of progressive infection in the melanopline grasshoppers inoculated, *R. bergi* and *B. punctulatus*.

3.2. Light microscopy

All presporulation stages observed had paired nuclei (i.e., diplokaryotic). The most commonly observed stages were rounded binucleate and tetranucleate cells (Figs. 1A–D). Proliferation of the parasite at this stage of development was probably by binary fission of tetranucleate cells. The nuclear arrangement of stages in sporulation could not

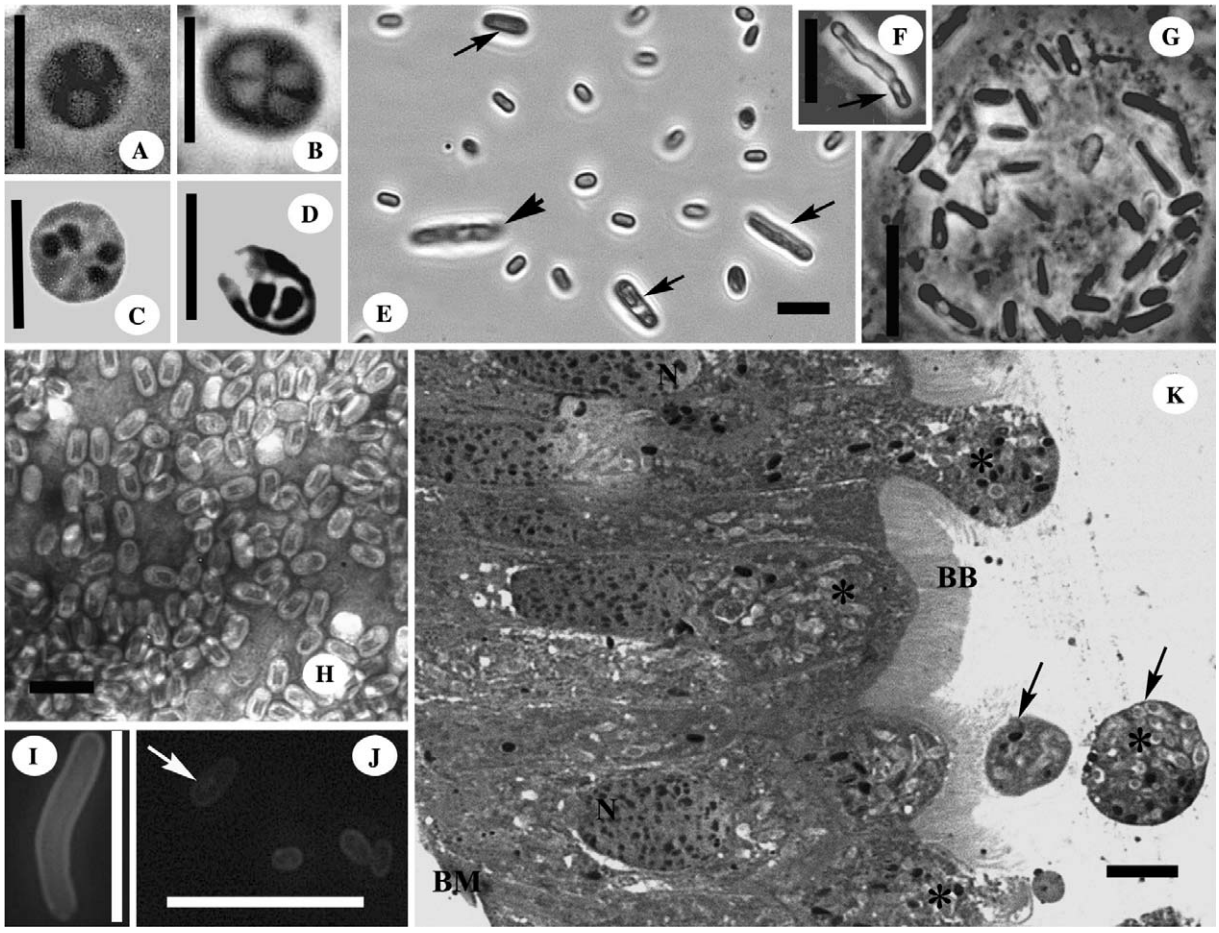

Fig. 1. Developmental stages and spores of *L. patagonica* n. sp., n. gen. under light microscopy. (A–D) Rounded, diplokaryotic presporulation stages. (E) Suspension of spores prepared from infected insects, contains occasional sporonts at different stages of development (arrows). Arrowhead indicates the elongated sporont ready to produce at least three sporoblasts, thus demonstrating polysporoblastic sporogony. (F) Sporont, from which the sporoblast is budding off (arrow). (G) Clump of spores and sporoblasts, presumably degenerating inside the phagosome (see also Fig. 5). (H) A smear from the infected tissue contains mainly mature uniform ovoid spores. (I) Elongated sporont. (J) Ovocylindrical small spores and more elongated sporont with an obvious diplokaryon inside (arrow). (K) Semi-thin section through midgut columnar cells heavily infected with stages and spores (asterisks), and portions of infected cells (arrows) in the lumen of the midgut. (A, B, and E–G), Fresh mounts; (C, D, and H), Giemsa stained smears; (I and J), Calcofluor-DAPI, fluorescent microscopy; (K), Methylene blue stained hemithin sections (0.1 μm). BB, brush border; BM, basement membrane; N, host nucleus. (A–E, and H), bar = 5 μm; (F, G, and I–K), bar = 10 μm.

be resolved at the light-microscopy level. Sporonts were remarkably long (Figs. 1E, F, and I). Sporoblasts appeared to have been budded off sequentially from long sporonts (Figs. 1E and F). Sporulation stages were often surrounded by transparent spaces, as if enclosed inside interfacial envelopes (as defined by Sprague et al., 1992) containing large and variable numbers of parasites (Fig. 1G). Mature spores (Figs. 1E and H) were ovocylindrical, sometimes slightly curved. Live spores measured $2.9 \pm 0.09 \times 1.2 \pm 0.04$ μm (mean ± SE, $n = 50$). The envelopes of elongated sporonts (not only of spores, as in most microsporidian species) contained chitin, based on their staining with Calcofluor (Figs. 1I and J).

The epithelial cells of the midgut and gastric caecae were the sites of infection by the microsporidium. Entire host cells or parts of cells, heavily loaded with stages and spores, were frequently observed in the lumen of midguts (Fig. 1K). Distinction of infected and uninfected tracts of alimentary canals under the dissecting microscope was not possible due to a lack of gross signs of disease. Spores and sporoblasts tended to localize at the apical region of intestinal epithelial cells, while presporulation stages were normally in the median region of the cell at the level of the nucleus. The basal area of cells was free of infection. Early presporulation stages (presumably meronts), but never spores, were abundant in undifferentiated cells inside crypts (data not shown).

3.3. Electron microscopy

All observed stages were diplokaryotic, and development was in direct contact with the host cell cytoplasm.

3.4. Meronts

Meronts were roundish cells with homogenous cytoplasm, numerous free ribosomes, and few membrane structures (Figs. 2A and B). Their diameter ranged from 1. 8 to 4.6 μm and averaged 2.8 ± 0.30 μm ($n = 9$). They were surrounded by host cell mitochondria and sparse cisternae of host endoplasmic reticulum (ER). Presumably, after a certain number of divisions meronts transformed into the next proliferative stage, sporonts (Figs. 2C–J).

3.5. Sporonts

Transition to sporogony was marked by the following features: (1) clearing of cytoplasm, which often appeared at this stage as more electron transparent than in meronts (Figs. 2C–E); (2) appearance of intracellular membrane structures: coated vesicles 60–120 nm in diameter associated with the spindle plaques (Figs. 2F and G), and rough endoplasmic reticulum (RER) (Fig. 2D); (3) prominent pores in the nuclear envelope (Figs. 2C, D, and H), which were hardly distinguishable in meronts; (4) enclosure of the parasite by host cell RER cisternae (Figs. 2C, D, and H); followed by (5) assemblage of an electron-dense envelope. Elongated cisternae of host RER formed an additional external layer around the parasite's plasmalemma (Fig. 2D), which was not the case in the previous (Fig. 2B) or subsequent (Figs. 3 and 4) life-cycle stages. Eventually, the patches of electron dense material were deposited along the plasmalemma (Figs. 2C and D) until they formed a continuous solid envelope (Figs. 2K and 3 A). Coating of the parasite plasma membrane with electron dense material coincided with the emergence of tubular-like secretions in the host cytoplasm (Fig. 2K). In transverse sections (Figs. 2C–E), sporonts were similar in size to meronts, but in longitudinal sections sporonts appeared to be not rounded but elongated and often contained 2–3 diplokarya (Figs. 2I and J). Sporonts were variable in size (3.0–7.2 × 1.5–2.8 μm) and measured an average 5.48 ± 0.391 ($n = 13$) × 2.04 ± 0.104 ($n = 16$) μm. Sporonts proliferated by closed intra-nuclear mitosis (Fig. 2H) (Raikov, 1982). Spindle plaques were always coupled with 3–5 coated vesicles, mentioned above, and were located in the parasite cytoplasm close to the nuclear envelope (Figs. 2F–H). These vesicles eventually moved away from the nuclear envelope to the periphery of the cell (Fig. 2G) to initiate formation of the extrusion apparatus in the subsequent stages of sporogony.

3.6. Sporoblasts

In many cells, formation of a solid electron-dense layer occurred simultaneously with polarization of the cell and development of a Golgi organelle, which in sporoblasts was composed of three parts: a cluster of small tubules, a tubular network, and a distal region with maturing polar filament precursors (Figs. 3B–D). Eventually, the electron-dense spore envelope became a three-layered structure (Fig. 3D). Sporoblast measurements ranged 1.2 –2.9 × 2.0–3.9 μm and averaged 3.19 ± 0.391 ($n = 5$) × 1.70 ± 0.189 ($n = 9$) μm.

3.7. Spores

Mature spores (Figs. 4A–C) possessed a comparatively thin exospore (13–50 nm thick) with weakly differentiated layers and an endospore about 120 nm thick (except for the apical region where it was about 60 nm), underlined by the plasma membrane. Mature spores had a short polar filament of only three coils (Figs. 4A and B). The polar filament was of the isofilar type, revealing a "standard" internal structure with a core surrounded by several concentric layers of varying electron density (Figs. 4B and B′). A classical posterior vacuole was never observed. Its place was occupied by a tubular network (Figs. 4A, B and D), similar to the one observed in sporoblasts as a part of the Golgi complex. Diplokarya in spores were surrounded by several layers of rough endoplasmic reticulum, seen on sections like strands of membrane-lined ribosomes (Figs. 4D and E). The anterior part of the extrusion apparatus, the polar disk, polar sac, and lamellar polaroplast, were of typical structure (Vávra and Larsson, 1999) (Figs. 4A and

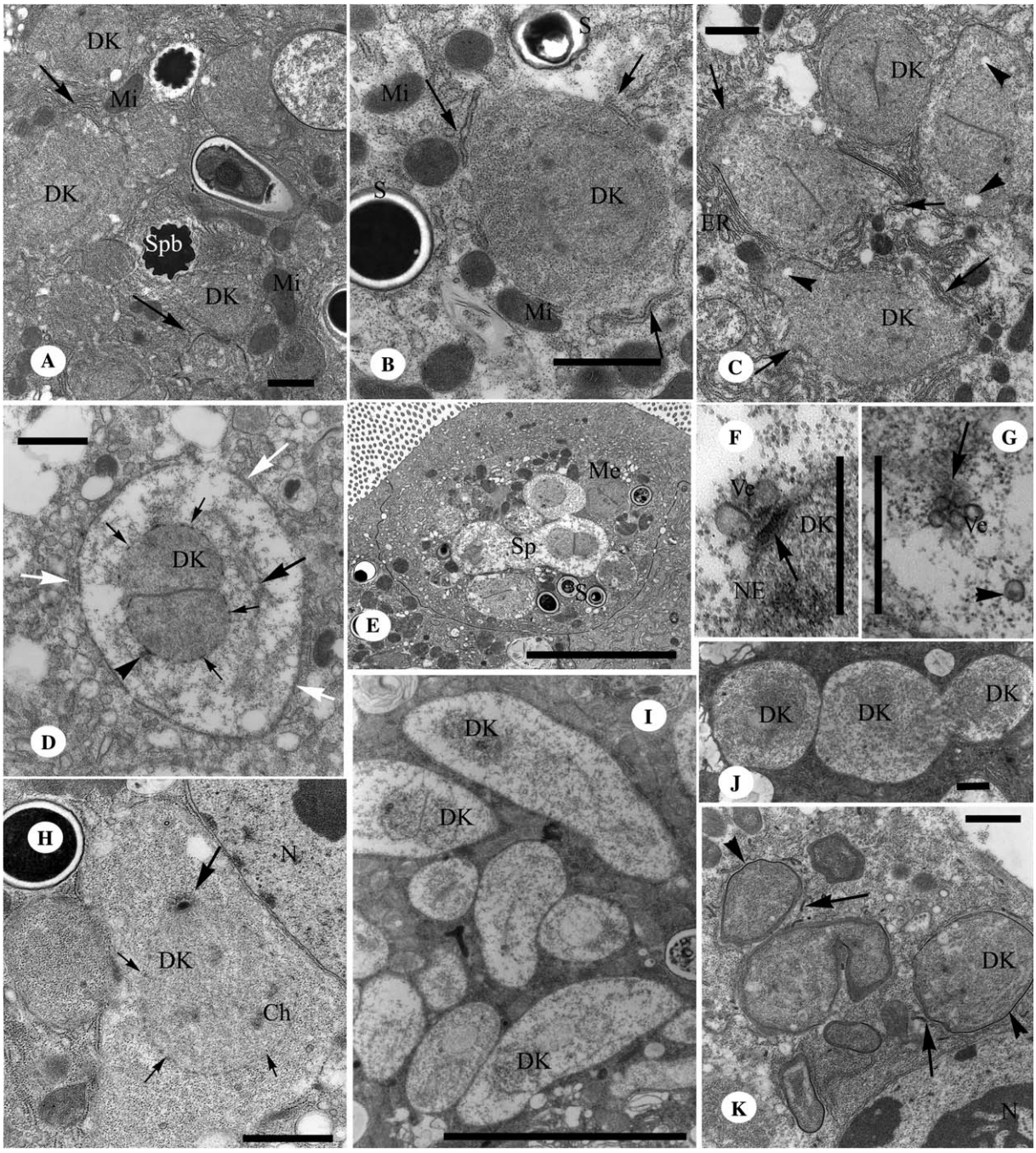

Fig. 2. Transmission electron microscopy of meronts and transitional to sporogony stages of *L. patagonica* n. gen. n. sp. (A) Meronts inside the enterocyte are surrounded by host cell mitochondria and endoplasmic reticulum (ER) (arrow). (B) Meront at higher magnification: ER cisternae (arrow) are grouping in close proximity to the parasite cell, but they do not form a uniform layer. (C) Transition to sporogony: host ER cisternae tend to embrace the whole parasite surface (arrow); cytoplasm looks more electron lucid compared to the previous stage due to presence of small vacuoles (arrowheads). (D) Sporont with electron lucid cytoplasm and distinguishable intracellular membrane structures. Coated vesicles are associated with a spindle plaque (arrowheads), and ER (big black arrows). Nuclear envelope has prominent nuclear pores (small black arrow). Elongated and flattened cisternae of host ER form an additional external layer around parasite plasmalemma (white arrows). (E) Infected enterocyte at lower magnification: small spores, giant electron-transparent multinuclear sporonts (sporogonial plasmodia) and electron dense meronts are in the view. (F and G) Spindle plaques (arrows) are coupled with 3–5 coated vesicles. One of the vesicles (arrowhead) moved away from the nuclear envelope. (H) Sporonts proliferating by closed mitosis. The spindle plaque with adjacent vesicles is indicated by a larger arrow, nuclear pores by smaller arrows. (I and J) Elongated sporonts (sporogonial plasmodia) with 2–3 diplokarya tightly packed by host ER cisternae. (K) Eventually, electron dense envelope assembled around sporonts (arrowheads). Coating of the parasite plasma membrane with electron dense material coincided in time with emergence of tubular-like secretions in host cytoplasm (arrows). Ch, chromosomes; DK, diplokaryon; ER, endoplasmic reticulum; Me, meront; Mi, mitochondria; N, host cell nucleus; NE, nuclear envelope; S, spore; Sp, sporont; Spb, sporoblast; Ve, spindle plaque associated vesicle; (A–D, G, H, J, and K), bar = 1 μm; (E), bar = 5 μm; (F), bar = 0.5 μm; (I), bar = 10 μm.

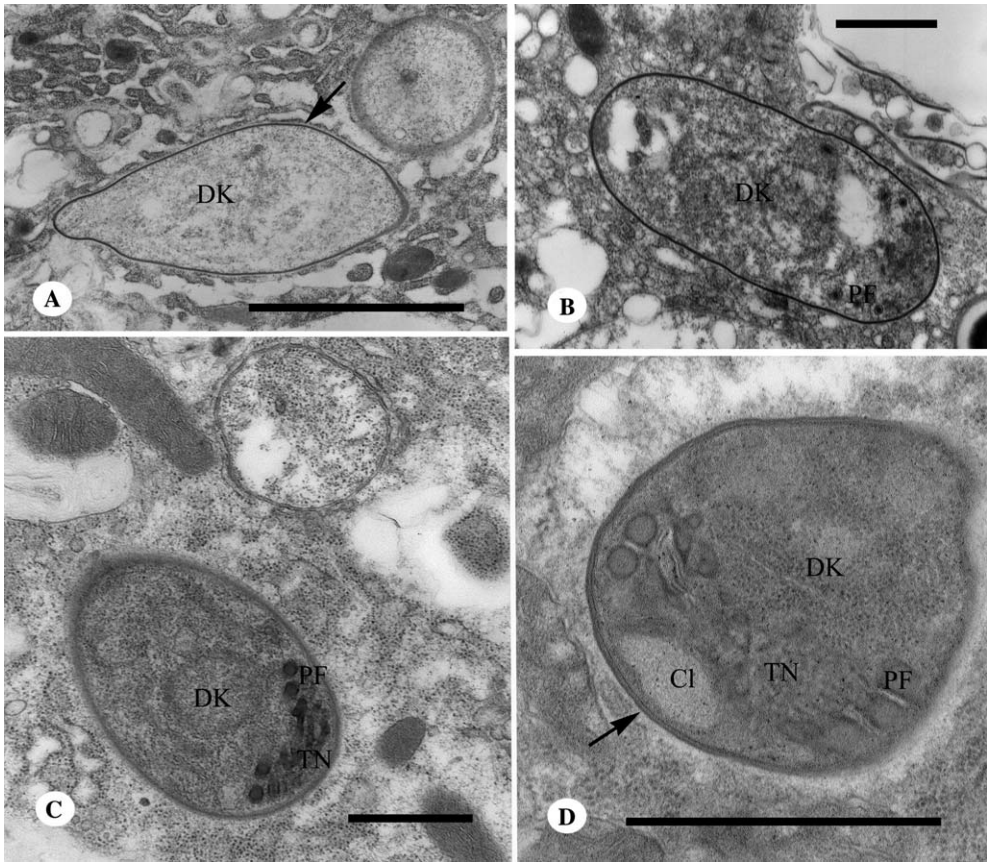

Fig. 3. Transmission electron microscopy of sporoblasts/early spores of *L. patagonica* n. gen., n. sp. (A) Late sporont or early sporoblast; a continuous electron dense envelope encloses the parasite cell, still covered with host cell endoplasmic reticulum (arrow). Note that host cell endoplasmic reticulum does not cover the surface of the parasite any more. (B and C) Sporoblasts at different stages of maturation. (D) Sporoblast or young spore with three-layered cell wall (arrow) and a Golgi organelle, composed of three parts: cluster of small tubules, tubular network, and a compartment containing polar filament coils. Cl, cluster of small vesicles; PF, polar filament precursors; TN, tubular network, other abbreviations as on Fig. 2. (A), bar = 5 μm; (B–D), bar = 1 μm.

C). The polar filament had a long straight part, about a half to two-thirds of the total spore length. The angle of tilt of the anterior filament coil to the long axis of the spore was about 70°. The anterior (more voluminous) part of the polaroplast was composed of tightly and regularly arranged membranes, while the posterior was comprised of loosely packed membranes (Fig. 4C). All spores, regardless of the infection status (i.e., early or light, advanced or heavy), had the same morphological characteristics. Intact spores (those with the "complete set" of internal features) were relatively rare in the sections. Instead we observed numerous spores activated for discharge, or at different steps of polar filament extrusion (Figs. 4F–L). In many of these "stimulated" spores we observed a voluminous spongy structure composed of coated membrane profiles resembling the "posterosome," as termed by Weiser and Žižka (1975). This structure was located in the posterior end of the spore where the posterior vacuole is placed in most other microsporidian species (Figs. 4G, I, and F). Typical "exfilamentation" of polar tubes was never seen, but we observed the contents of the spore pouring out through the opening in the apical region of the envelope (Figs. 4J–L).

The TEM observations also revealed that adjacent cells within the epithelial layer were heavily loaded with parasites (Fig. 5A). Analysis of ultrathin sections showed that tracheole cells attached to the midgut epithelium also occasionally contained parasites (Fig. 5B). In the infected host cells, lysosomes were frequently observed in the vicinity of intact and discharged spores (Fig. 5C). Both fired and intact spores, but not sporonts, were phagocytized by host cells and were seen at different stages of digestion inside typical phagosomal compartments (Figs. 5D–F). Thus, what appeared as interfacial envelopes under light microscopy were actually phagosomes with partially digested spores.

3.8. SSU-rDNA sequence and phylogenetic analysis

The sequence of 1157-bp length with 57.0% GC was deposited in the GenBank under Accession No. DQ239917. Twelve SSU-rDNA sequences, including six sequences with the highest BLAST scores—those of the *Nosema* type species, *N. bombycis*, and the five species parasitizing different insect orders, including two species from orthopteran hosts (Table 1)—were aligned with the novel sequence and analyzed as described above. The novel SSU-rDNA sequence

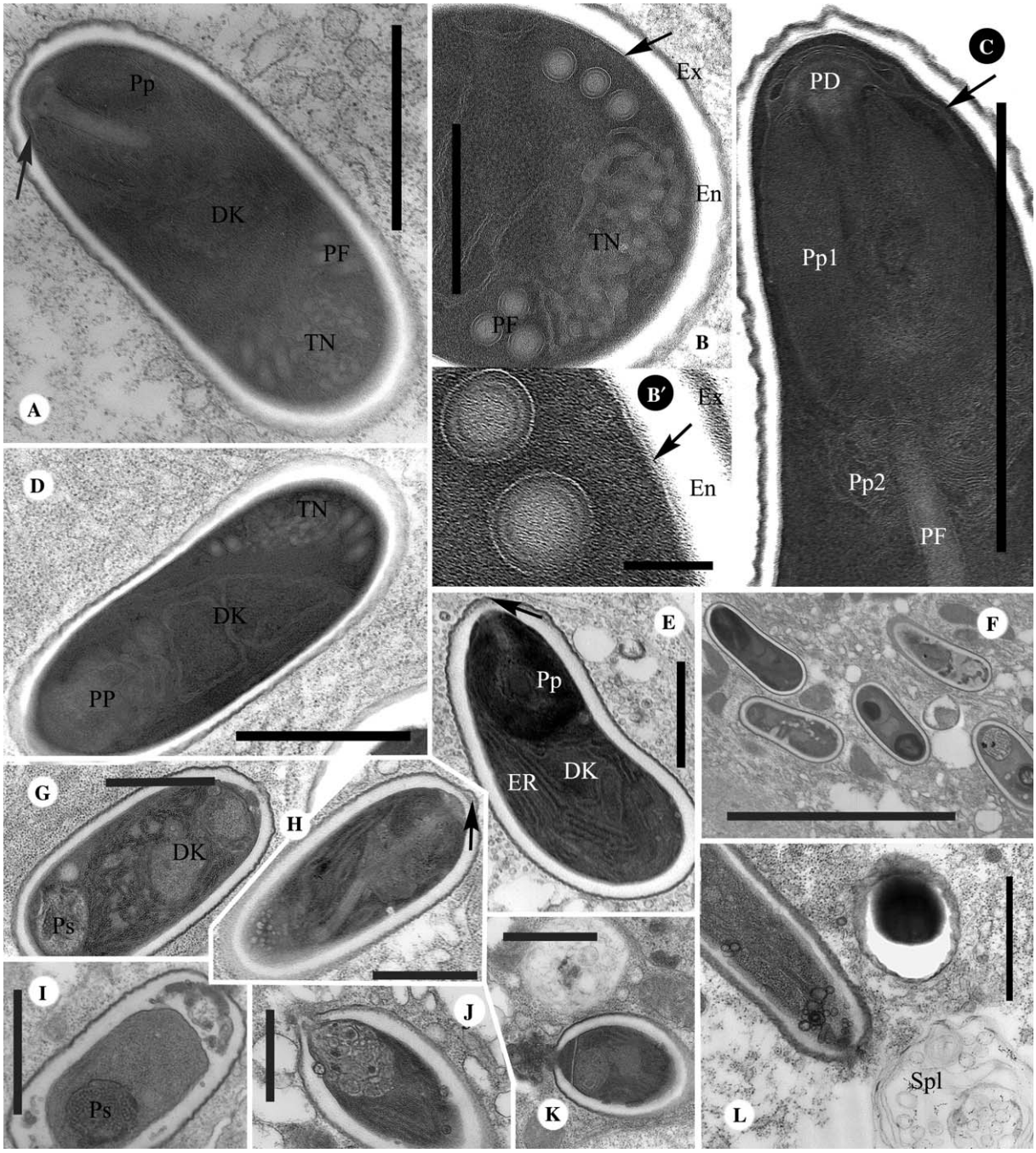

Fig. 4. Transmission electron microscopy of spores of *L. patagonica* n. gen., n. sp. (A) Longitudinal section through the spore displaying internal structure, typical for microsporidian spore, except for the extremely short polar filament (3 coils) and the tubular network, placed at the spore posterior end instead of a posterior vacuole. An arrow indicates the polar sac. (B, B′) Transverse section through the posterior end of the spore: three polar filament coils show internal structure consisting of several concentric layers of various electron density (B′); a tubular network, which rarely can be seen in mature spores of other microsporidia species, is well developed. More or less uniform exospore, endospore, and plasma membrane (arrow) can be seen. (C) Anterior end of the spore, showing the organization of the polaroplast and anchoring apparatus of the polar filament. Polar sac is indicated by the arrow. (D) Longitudinal section through the spore displaying two nuclei in diplokaryotic arrangement. (E) Centrally located diplokaryon is surrounded by rough ER, strands of membrane-lined ribosomes. The electron-dense polaroplast and evagination of the exospore at the anterior end indicate that this spore has been stimulated to discharge and probably is already in the process of exfilamentation. (F–I) Spores at different steps of polar filament extrusion. Arrows indicate evagination of the exospore at the anterior end. (J–L) Spore content pours out through the opening (arrow) in the apical region of the envelope. DK, diplokaryon; En, endospore; ER, endoplasmic reticulum; Ex, exospore; TN, tubular network; PD, polar disk; PF, polar filament; PP, polaroplast; PP1, anterior part of polaroplast; PP2, posterior part of polaroplast; Ps, posterosome; Spl, sporoplasm. (A, C–E, and G–L), bar = 1μm; (B), bar = 0.5μm; (B′), bar = 125 nm; (F), bar = 5 μm.

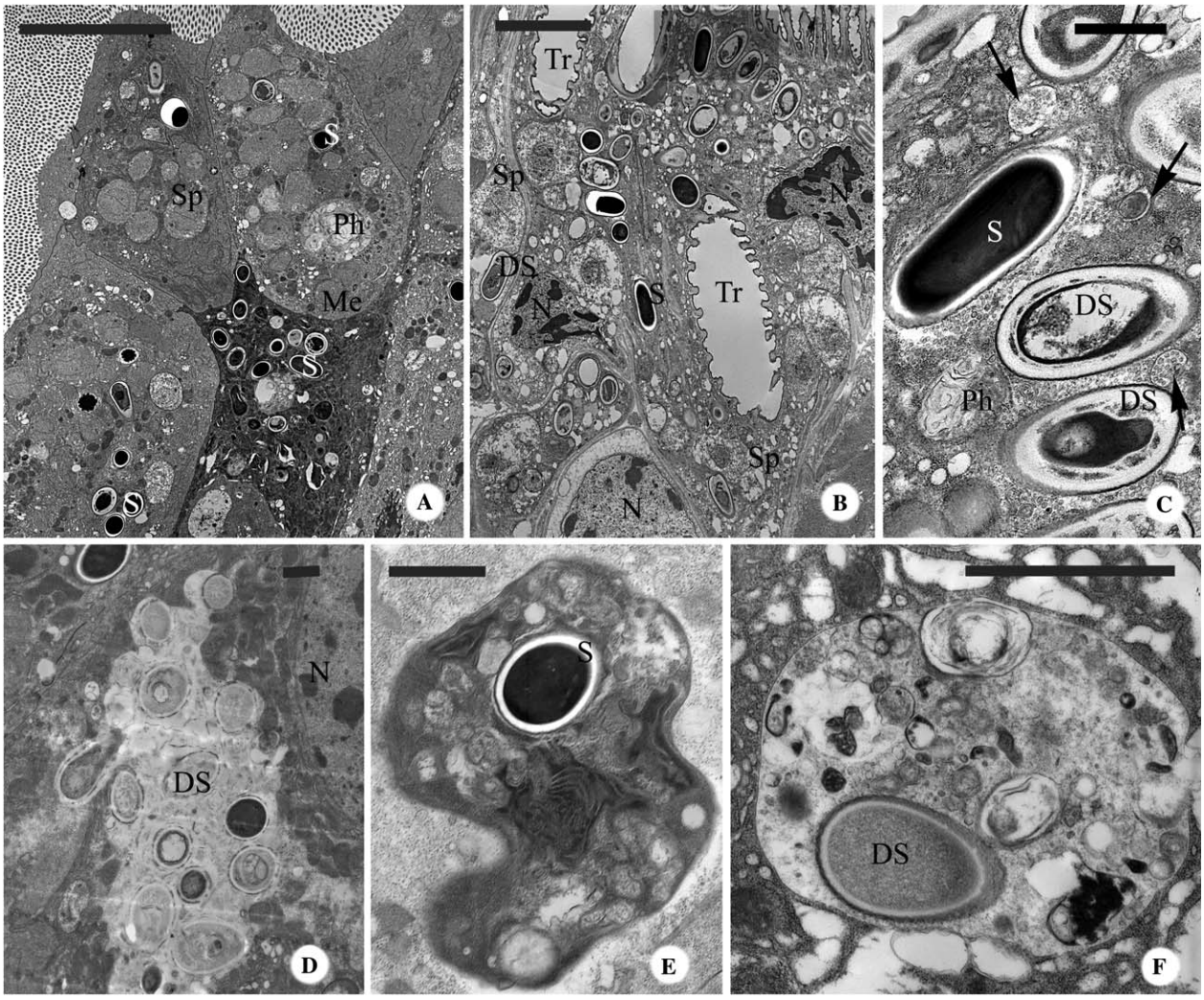

Fig. 5. Tissue tropism and interactions with host cell of *L. patagonica* n. gen., n. sp. in *T. magellanica*. (A) Heavily infected midgut epithelium cells. (B) Section through the infected tracheole. (C) Enlarged portion of the previous figure. Lysosomes (arrows) occur in nearly every spore seen. (D–F) Spores at different stages of digestion inside the phagosomes. DS, discharged spores; Me, meront; N, host cell nucleus; Ph, phagosome; S, spore; Sp, sporont; Tr, lumen of a tracheole. (A), bar = 10 μm; (B), bar = 5 μm; (C–F), bar = 1 μm.

had low similarity with *N. bombycis* (29.9%), as well as with the octosporous microsporidia *T. solenopsae* and *S. alba* (39 and 36%, respectively). It had greater resemblance to two species from Orthoptera (about 57%) and exhibited maximal similarity with *Orthosomella operophterae*, a microsporidium from a winter moth, *Operophtera brumata* (78.9%) (Table 3). In the phylogenetic trees inferred from SSU-rDNA sequence analysis performed by ML, NJ, and PS algorithms, the new species clustered together with *O. operophtera* and formed a sister taxon to the *Endoreticulatus–Cystosporogenes* group. Bootstrap values for all branches were above 70% in the ML, NJ, and PS trees (Fig. 6).

3.9. Taxonomy summary

3.9.1. Liebermannia n. gen

Nuclei are in diplokaryotic arrangement throughout the life-cycle. Development is in direct contact with the host cell cytoplasm. Transitional (from merogony to sporogony) stages and sporonts of *L. patagonica* are surrounded by host rough endoplasmic reticulum. One morphological spore type is produced. Several spores are produced sequentially from an elongated sporont. Spore extrusion apparatus of typical structure, except for small number of polar filament coils and undeveloped posterior vacuole, at least in spores located inside the host cells. Infects primarily epithelial cells.

3.9.2. Liebermannia patagonica n. sp

Type host: Tristira magellanica Bruner, 1900 (Orthoptera: Tristiridae: Tristirinae).

Type locality: Rio Mitre, 30 km SW of Calafate, in the vicinity of "Parque Nacional Los Glaciares", soutwestern Santa Cruz province, southern Patagonia region, Argentina.

Infection site: Epithelial cells of midgut and gastric caecum.

Transmission: Unknown. Liberation of spores through faeces and persistent high field prevalences suggest per os horizontal transmission.

Table 3
Comparison of SSU-rDNA sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal) obtained by Kimura 2-parameter analysis

	Pl	*Pg*	*Ts*	*Sa*	*Lp*	*Oo*	*Esp*	*Eb*	*Es*	*Psp*	*Cl*	*Co*	*Nb*
Pl	—	96.6	49.9	43.8	56.8	60.2	55.4	55.1	55.8	55.5	49.2	48.6	28.8
Pg	0.034	—	48.4	43.1	57.7	60.9	56.1	55.7	56.5	56.4	50.3	49.7	29.6
Ts	0.501	0.516	—	60.9	39.6	44.1	48.3	47.4	48.6	48.1	48.3	47.1	34.7
Sa	0.562	0.569	0.391	—	36.1	40.4	45.0	44.1	44.7	44.6	43.4	41.8	41.5
Lp	0.432	0.423	0.604	0.639	—	78.9	67.1	66.9	67.1	67.3	65.8	63.4	29.9
Oo	0.398	0.391	0.559	0.596	0.211	—	73.8	73.6	73.6	73.8	72.5	71.3	33.7
Esp	0.446	0.439	0.517	0.550	0.329	0.262	—	99.3	99.5	99.2	88.4	87.5	38.2
Eb	0.449	0.443	0.526	0.559	0.331	0.264	0.007	—	99.5	99.2	88.2	87.3	38.4
Es	0.442	0.435	0.514	0.553	0.329	0.264	0.005	0.005	—	99.4	88.7	87.8	34.7
Psp	0.445	0.436	0.519	0.554	0.327	0.262	0.008	0.008	0.006	—	88.3	87.4	38.2
Cl	0.508	0.497	0.517	0.566	0.342	0.275	0.116	0.118	0.113	0.117	—	98.2	35.7
Co	0.514	0.503	0.529	0.582	0.366	0.287	0.125	0.127	0.122	0.126	0.018	—	34.6
Nb	0.712	0.704	0.653	0.585	0.701	0.663	0.618	0.616	0.653	0.618	0.643	0.654	—

For full species names see Table 1.

Presporulation stages: Rounded binucleate and tetranucleate cells. Proliferation by binary fission.

Transition to sporogony: Stages become enclosed by host rough endoplasmic reticulum, show vacuolated cytoplasm, vesicles associated with a spindle plaque and parasite endoplasmic reticulum, prominent nuclear pores, and different levels of progression in the assemblage of an electron-dense envelope.

Sporogony: Stages are enclosed by host rough endoplasmic reticulum or coated with an electron-dense material of increasing thickness, proliferate by closed mitosis and start elongation until they form unusually long, multinucleate sporoblast-mother cells that sequentially bud off sporoblasts.

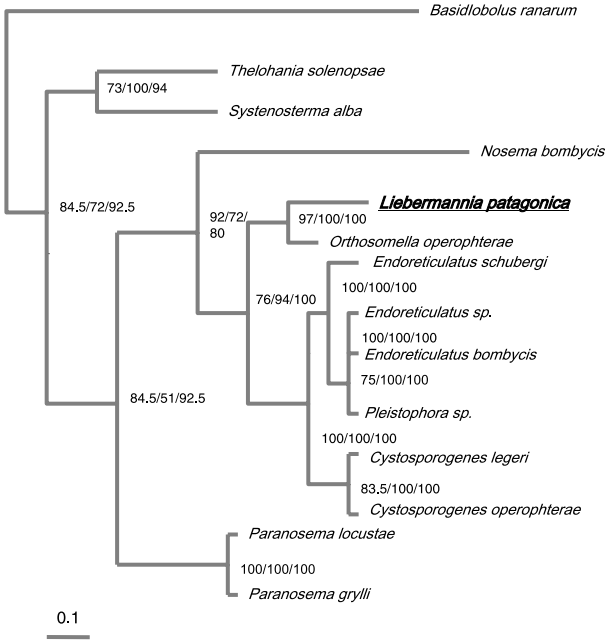

Fig. 6. Phylogenetic tree based on small subunit (SSU) rDNA derived by maximum likelihood (ML) analysis showing the relationship of*L. patagonica* with other microsporidia. *L. patagonica* clusters together with *O. operophtera* and forms a sister taxon to the *Endoreticulatus–Cystosporogenes* group. The *Paranosema*, *Thelohania–Systenostrema*, and *Nosema bomycis* brunches cluster as an unrelated or distantly related groups. Phylogenetic tree constructed by neighbor joining (NJ) and maximum parsimony (MP) algorithms revealed similar topologies. The first, second and third numbers at the node are bootstrap values for ML, NJ, and MP correspondently (all in 100 replicates).

Spore: Binucleate, ovocylindrical (sometimes slightly curved), $2.9 \pm 0.09 \times 1.2 \pm 0.04\,\mu m$ (fresh, mean ± SE, $n = 50$). Thin exospore (13–50 nm thick) with weakly differentiated layers, endospore about 120 nm thick. Isofilar polar filament with three turns. Posterior vacuole is revealed as network of tubular structures.

Type specimens: Type material (Giemsa-smears and EM blocks) will be designated and deposited in the collections at the "Center for Parasitological Studies and Vectors (CEPAVE)", La Plata National University, Argentina, and also sent to the International Protozoan Type Slide Collection at the Smithsonian Institution, Washington, DC.

We include SSU rRNA gene partial sequence under GenBank Accession No. DQ 239917 to the species diagnosis.

Etymology The generic name is after Dr. Jose Liebermann, who was a prominent Argentine acridologist who conducted early observations of the Tristiridae. The specific epithet is derived from Patagonia, the region South of the Colorado River in Argentina, in the Southern part of which *T. magellanica* occurs.

4. Discussion

4.1. Comparison of L. patagonica with microsporidia from orthopterans

This is the first report of a microsporidium parasitizing an orthopteran species of the family Tristiridae. All previously known microsporidia from Orthoptera were in the families Acrididae (13 species of microsporidia), Gryllidae (2), Pyrgomorphidae (1), or Tettigonidae (2) (Table 1).

The new microsporidium can not be assigned to any of the currently recognized genera of insect microsporidia, because it is diplokaryotic (i.e., intimately paired nuclei throughout the life-cycle), apansporoblastic (i.e., develop-

ment in direct contact with the host cell cytoplasm), presumably homosporous (i.e., presence of a single morphological type of spore), and polysporoblastic (i.e., several sporoblasts as final division products of a sporont), (Becnel and Andreadis, 1999; Canning and Vávra, 1983; Larsson, 1999). Among the known microsporidia from Orthoptera, *L. patagonica* in *T. magellanica* has some resemblance to species in the genera *Nosema*, *Paranosema*, *Antonospora*, and *Tubulinosema* (Franzen et al., 2005; Henry, 1967, 1971; Issi and Krylova, 1987; Lange et al., 1992; Slamovits et al., 2004; Sokolova et al., 2003; Streett and Henry, 1987, 1993; Wang et al., 1991; Wen, 1996; Wen and Li, 1993). These all are diplokaryotic, apansporoblastic, and homosporous. However, while the species of the genera *Nosema*, *Antonospora*, *Paranosema*, and *Tubulinosema* are disporoblastic (i.e., two sporoblasts are produced as final division products of a sporont), *L. patagonica* is polysporoblastic. In addition to this differential characteristic, which is a major character traditionally used in microsporidian taxonomy at the generic level (Sprague et al., 1992), another fact that strongly suggests a generic status different from *Nosema*, *Paranosema*, or *Tubulinosema* for *L. patagonica* is the lack of susceptibility of grasshopper species in the subfamily Melanoplinae (melanoplines). The known North American orthopteran microsporidia—*Nosema cuneatum*, *Nosema montanae*, *Tubulinosema acridophagus*, and *P. locustae*—all infect melanopline grasshopper hosts (Bomar and Lockwood, 1993; Erlandson et al., 1985; Habtewold et al., 1995; Henry, 1969; Lange, 2003a,b; Wang et al., 1991).

At the specific level, several other important characters, like spore size, number of polar filament coils, and tissue/organ specificity, are unique to *L. patagonica* when compared with the *Nosema*, *Paranosema*, or *Tubulinosema* pathogens of Orthoptera (see Sokolova and Lange, 2002, for a comparative list of characters). At 2.9×1.2 μm, *L. patagonica* has the smallest spore size and the lowest number of polar filament coils, only three. Although other species of microsporidia of Orthoptera, such as *T. acridophagus*, *Nosema asiaticus*, *N. cuneatum*, *Nosema maroccanus*, *Nosema pyrgomorphae*, and *Nosema trilophidiae*, may infect tissues of the alimentary canal, *L. patagonica* is the only one that exclusively or primarily develops in the epithelium of the midgut and gastric caecum. Prior to the discovery of *L. patagonica*, only two other microsporidia were known to be associated with grasshoppers in Argentina (Lange, 2003a, Table 1): *Perezia dichroplusae* and *P. locustae*. *P. dichroplusae*, a parasite of the Malpighian tubules of *Dichroplus elongatus* in the Pampas region of central Argentina, has a diplokaryotic merogony followed by a haplokaryotic sporogony via nuclear dissociation, with final production of uninucleate and larger (mean: 3.5 by 1.5 μm) spores with a maximum of 8 polar filament coils (Lange, 1987b). *P. locustae*, established in grasshopper communities of the Pampas after its introduction from North America as a biocontrol agent, is a parasite of the adipose tissue of numerous grasshopper species that has larger spores (mean: 5.2×2.8 μm) with 15–18 polar filament coils (Sokolova et al., 2003; Sokolova and Lange, 2002). SSU rDNA-based phylogenetic analysis shows that *L. patagonica* might be distantly related to the *Paranosema* clade (Fig. 5). The percentage identity of the *L. patagonica* sequence with those of both *Paranosema* species is higher (about 57%) than with *T. solenopsae* (39.6%), *S. alba* (36.1%), or *N. bombycis* (29.9%) sequences (Table 3).

4.2. Support of developmental and ultrastructural characters for the position of L. patagonica on the SSU rDNA-inferred phylogenetic tree

Interestingly, *L. patagonica* displays maximum structural resemblance with the genus *Vittaforma*, which is also diplokaryotic, apansporoblastic, homosporous, and polysporoblastic. Moreover, *V. cornea* produces elongated sporonts similar to ones of *L. patagonica*. However, besides being a human pathogen and having spores of different size, shape, and morphology, all stages of *Vittaforma* species, including spores, "are individually enveloped by a cisternae of host endoplasmic reticulum studded with ribosomes" (Silveira and Canning, 1995); only sporonts of *L. patagonica* were enclosed in host endoplasmic reticulum. In published phylogenetic trees constructed from SSU-rDNA sequences (Canning et al., 2002; Frankenhuyzen van et al., 2004; Vossbrinck and Debrunner-Vossbrinck, 2005) as well as in our analyses (data not shown), *Vittaforma* falls in the same clade with the genera *Cystosporogenes* and *Endoreticulatus*. High sequence similarity and the phylogenetic analyses indicated comparatively close relations between *L. patagonica* and the *Cystosporogenes*/*Endoreticulatus* clades (Fig. 5). All representatives, except *Vittaforma*, of the compact *Cystosporogenes*/*Endoreticulatus* group (87–99% of sequence identity among group members, Table 3; also see trees in Frankenhuyzen van et al., 2004; Vossbrinck and Debrunner-Vossbrinck, 2005) parasitize Lepidoptera and are monokaryotic throughout the life-cycle.

Orthosomella operophterae is the species with which *L. patagonica* shares maximum sequence similarity (78.9%). The genus *Orthosomella* (formerly *Orthosoma*) was erected in 1983 for the microsporidium parasitizing winter moths, *O. brumata* (L.), that formerly had been described as *Nosema operophtera* (Canning, 1960; Canning et al., 1983, 1991). Currently, two species, *O. prumata* and *O. lambdina* from the spring hemlock looper *Lambdina athasaria*, have been assigned to this genus. Both of them were studied by light and electron microscopy (Andreadis et al., 1996; Canning et al., 1985, 1983); their morphological and developmental characters are summarized by Andreadis et al. (1996). The major difference between *Orthosomella* species a *L. patagonica* is the presence of unpaired nuclei throughout the whole life-cycle. Also, *Orthosomella* species have larger numbers of polar filament coils (6–7) and different spore shapes. However, there is some resemblance between *L. patagonica* and *Orthosomella* species. All three species primarily parasitize epithelial cells, and *O. lambdinae* and

L. patagonica nearly exclusively infect gut cells. All three species developed in direct contact with host cell cytoplasm. *O. operophterae* meronts were closely associated with host cell endoplasmic reticulum in the same way as sporonts of *L. patagonica*. Actually, we would classify "meronts" of Canning et al. (Figs. 31–32 in Canning et al., 1983), as a meront–sporont transitional stage or early sporonts, based on the lucid cytoplasm and well-developed internal membrane structures. The structures of elongated multinuclear sporonts were very similar in all three species, except for the diplokaryotic nuclei in *L. patagonica*. These sporonts either divided into diplokaryotic sporoblasts in *L. patagonica*, or in split into binucleate cells which then gave rise to uninucleate sporoblasts *O. operophterae*, or transformed directly into sporoblasts and then into a chain of spores in *O. lambdinae* (Andreadis et al., 1996). The posterior vacuoles in *O. lambdinae* and *L. patagonica* spores both displayed posterosome-type structures.

SSU-rDNA sequence analysis confirmed the inference, initially based on structural and developmental data, that the microsporidium discovered in *T. magellanica* is a new species, which cannot be assigned to any of the existing microsporidian genera. This sequence analysis determined the position of the new species on the microsporidian phylogenetic tree and revealed the relations of *L. patagonica* with the genus *Orthosomella*. Certain structural resemblance between *L. patagonica* and two *Orthosomella* species proved the consistency of sequence and morphological data. We also speculate that the diplokaryotic vs. monokaryotic arrangement of nuclei might be less important for distinguishing microsporidian taxons of supergeneric ranks than the characters reflecting the fine interactions of the parasite with the host cell, such as microtubular incrustations of the meront surface in tubulinosematids (Franzen et al., 2005) or close association with host cell endoplasmic reticulum as in the *Orthosomella*/*Liebermannia*/*Cystosporogenes*/*Endoreticulatus* clade.

4.3. Host–parasite relationships

Although there are exceptions (Hernández-Crespo et al., 2001; Lange and De Wysiecki, 1999; Streett and Henry, 1984), microsporidia of Orthoptera occur normally at relatively low prevalences in nature. At least three characteristics of the *T. magellanica*/*L. patagonica* system might explain the persistent high field prevalences: the limited dispersal capabilities of the host due to its aptery and its aggregation behavior (i.e., patchy occurrence); the continuous liberation of spores through feces (i.e., a permanent source of infective units); and the apparent low virulence of the microsporidium, which affects enterocytes, that are generally short-lived and constantly replaced (Billingsley and Lehane, 1996; Smith, 1968). It is generally accepted that the high regenerative capacity of the epithelium of the digestive tract compensates to some extent for cell destruction (Vávra and Larsson, 1999).

Judging from our own observations, enterocyte replacement appears to be a particularly active process in *T. magellanica*. A microsporidium propagating in such an unstable environment should somehow accomplish two goals: to multiply and produce spores for transmission quickly enough to avoid untimely elimination, and to escape attack from the phagolysosomal system. We believe *L. patagonica* has evolved ways to deal with these limiting factors. Our study suggests that the microsporidium has a simplified and short life-cycle. No traces of sexual processes (i.e., synaptonemal complexes, nuclear dissociation) were observed. The initial sporoplasm–meront phase appears reduced. Since meronts were extremely rare on smears and sections, they presumably transform into sporonts after the very first division of the sporoplasm. Sporonts, which form elongated plasmodia, appear to be the major trophic and proliferative stage. Sporonts are also the only stage that is surrounded by host ER. Such a feature, although not unique to *L. patagonica* (Brooks et al., 1988; Canning and Curry, 2004; Silveira and Canning, 1995), probably allows the parasite to avoid attack by host lysosomes at the most crucial stage of development. By the time sporoblasts are produced, they eventually loose the defensive layer of the host ER, and it is at this point that they appear to become susceptible to lysosome attack. Nearly every spore was accompanied by one or several primary lysosomes. The secondary lysosomes (phagolysosomes or digestive vacuoles) were observed to be full of spores in both intact condition and fired. We speculate that interaction with lysosomes might serve as a stimulus to sporoplasm discharge in *L. patagonica*. Intact (unfired) spores were seldom seen in sections. Most spores were fired or stimulated to firing. Interestingly, the posterior vacuole in intact and "stimulated" *L. patagonica* spores resembled the "posterosome" (Weiser and Žižka, 1975), the component of the Golgi complex found in late sporonts and sporoblasts of other microsporidia. The posterosome has also been referred to as "prominent tubular network, the microsporidian late Golgi compartment" (Takvorian and Cali, 1994), "Golgi vesicles" (Vávra and Larsson, 1999)", or a cluster of small tubules"(Sokolova et al., 2003). Also, small number of polar filament coils might indicate under-development of the extrusion apparatus, as if the parasite gains the ability for spore firing at a premature ("juvenile") stage. The process of "discharge" that we observed in sections can hardly be called "polar tube exfilamentation," but only a pouring of the sporoplasm through an opening at the anterior end of the exospore. It is possible that the "apical bursting" was an artifact due to improper fixation. On the other hand, this phenomenon was observed consistently in material from three independent fixations, and it has not been recorded previously for other microsporidia fixed in a similar way. Such a mechanism could conceivably serve for autoinvasion of the same cell. It is unclear how the infection of other cells and tissues occurs, and how spores (abundant in feces), may infect other individual hosts. Perhaps, the spores functioning in horizontal transmission continue maturation in

the environment after the infected cells shed off the epithelial layer.

Apparently, *L. patagonica* evolved remarkable adaptations to the unstable environment of the intestinal epithelium cells of *T. magellanica*, demonstrating once more the evolutionary and ecological plasticity of microsporidia.

Acknowledgments

The work was supported partly by Russian Foundation for Basic Research (Grant # 03-04-49629) and the "Consejo Nacional de Investigaciones Cientificas y Tecnicas" (CONICET) of Argentina (Grant PIP # 2062).

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