

Short Communication

Phylogenetic relationships of *Heterovesicula cowani*, a microsporidian pathogen of Mormon crickets, *Anabrus simplex* (Orthoptera: Tettigoniidae), based on SSU rDNA-sequence analyses

Yuliya Y. Sokolova^{a,c,*}, Carlos E. Lange^b, James R. Fuxa^a

^a *Laboratory of Insect Pathology, Department of Entomology, Louisiana State University AgCenter, 404 Life Sciences Building, Baton Rouge, LA 70803-1710, USA*

^b *Comisión de Investigaciones Científicas (CIC) de la provincia de Buenos Aires, Centro de Estudios Parasitológicos y de Vectores (CEPAVE), UNLP – CONICET, Calle 2 # 584, La Plata (1900), Argentina*

^c *Institute of Cytology, Russian Academy of Sciences, 4 Tikhoretsky Avenue, 194064 St. Petersburg, Russia*

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Abstract

The microsporidium *Heterovesicula cowani*, discovered in 1985, was initially identified as *Vairimorpha* sp. because it produces two types of spores: *Nosema*-like diplokaryotic spores and *Thelohania*-like mononuclear meiospores. However, light and electron microscopy studies revealed characters that did not fit any known microsporidian genera, and a new monotypic genus *Heterovesicula* was erected. The goal of this study was to test the validity of the genus *Heterovesicula* by molecular characterization of *H. cowani* and to assess its phylogenetic relationships to other microsporidia from insects. DNA from spores stored at $-32\text{ }^{\circ}\text{C}$ since 1992 was isolated and PCR-amplified with V1-1492 primers to obtain a partial small subunit ribosomal RNA gene sequence of 1165 bp, which was submitted to GenBank (Accession No. EU275200). Neighbor joining, maximum parsimony and maximum likelihood analyses performed against 18 microsporidia sequences, placed *H. cowani* as a sister taxon to the *Nosema*–*Vairimorpha* clade. The consensus of these analyses suggests that the *Heterovesicula*–*Nosema*–*Vairimorpha* group forms a dichotomy with the *Encephalitozoon* spp. branch. Other microsporidia parasitizing Orthoptera fell into two unrelated (or distantly related) lineages of terrestrial microsporidia: the *Liebermannia* spp. branch forms a dichotomy with *Orthosomella operophterae* within the *Endoreticulatus*–*Orthosomella*–*Liebermannia* group; and the *Paranosema* spp. branch clusters together with the *Tubulinosema*–*Systemostrema* lineage. The minimum pairwise distance in Kimura-2-Parameter analysis among 18 analyzed sequences was 0.37, which supports well the generic status for *Heterovesicula*. The obtained phylogenetic trees suggest that *H. cowani* is related to the *Vairimorpha necatrix* group, but not to other insect microsporidia producing octospores. © 2008 Elsevier Inc. All rights reserved.

Keywords: Microsporidia; Orthoptera; *Heterovesicula cowani*; Tettigoniidae; Mormon cricket; Molecular taxonomy

1. Introduction

The microsporidium *Heterovesicula cowani*, a pathogen of adipose tissue of Mormon crickets, *Anabrus simplex*,

was discovered in 1985, and was initially tentatively identified as *Vairimorpha* sp. (MacVean and Capinera, 1991). Later extensive studies by light and electron microscopy revealed characters that did not fit any known microsporidian genera, and a new monotypic genus *Heterovesicula* was erected (Lange et al., 1995). A salient morphological character that distinguishes *H. cowani* from other dimorphic microsporidia is that both spore types (diplokaryotic, *Nosema*-like and uninucleate, *Thelohania*-like) occur within sporophorous vesicles. Uninucleate spores occur in groups

* Corresponding author. Address: Laboratory of Insect Pathology, Department of Entomology, Louisiana State University AgCenter, 404 Life Sciences Building, Baton Rouge, LA 70803-1710, USA. Fax: +1 225 578 1643.

E-mail address: jsokolova@lsu.edu (Y.Y. Sokolova).

of eight within fusiform, persistent interfacial envelopes, while binucleate spores are present in variable numbers inside non-persistent interfacial envelopes. The goal of this study was to test the validity of the genus *Heterovesicula* by molecular characterization of *H. cowani* and to assess its phylogenetic relationships to microsporidia from other Orthoptera and other insects.

2. Materials and methods

2.1. Source of spores

Spores used in the analyses were obtained from Mormon crickets experimentally infected in 1992 while conducting work leading to the description of the pathogen (Lange et al., 1995). Spore suspensions for DNA isolation were prepared in double distilled water from whole-insect homogenates by filtration and centrifugation (Undeen and Vavra, 1997); the suspensions were stored at -32°C in double distilled water since 1992. Before DNA isolation the suspensions were tested by light microscopy for spore visualization and checking the quality of their preservation. Unfixed spores were observed under phase contrast optics, and methanol-fixed and Giemsa- or Trichrome-stained smears were examined in bright field. For visualizing nuclei, fresh or methanol-fixed smears were stained with DAPI (10 μM solution in phosphate-buffered saline) or by Ethidium bromide solution (5×10^{-3} mg/ml). Spores were measured and photographed under a Nikon Eclipse E-600 Microscope equipped with the fluorescence unit and a Metaview digital camera and software (MetaView, 1998, Meta Imaging Series 4.5. Universal Imaging Corporation, West Chester, PA).

2.2. DNA sequencing

After thawing, the spore suspension was immediately transferred into guanidine buffer (4.2 M guanidine thiocyanate, 50 mM Tris-HCl, pH 7.6, 10 mM EDT, 25% lauryl sarcosinate) and stored until use at $+4^{\circ}\text{C}$ for 12–48 h. Spores were spun down and re-suspended in 150 μl of TAE buffer (0.04 M Tris acetate, 0.01 M EDTA) and bead-beaten in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) at maximum speed for 1 min. After the sample was heated in a thermoblock for 10 min at 95°C , the supernatant (“crude extract”) was directly used as a DNA template for PCR amplification (Vossbrinck et al., 2004). Alternatively, Phenol-Chloroform-Isoamyl alcohol extraction followed by alcohol precipitation was applied to the sample after bead beating. The primers for PCR amplification were VI (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 VI, 530r (5'-CCG CGG C(T/G)G CTG GCA C-3'), 530f (5'-GTG CCA GC (G/A) GCC GCG G-3'); 1061f (5'-GGT GGT GCA TGG CCG-3'), and 1492r (Vossbrinck et al., 2004; Weiss and Vossbrinck, 1999). These primers pro-

duced overlapping sequences that were assembled with Chromas. Pro. 1.34 software (<http://www.technelysium.com.au/ChromasPro.html>). Samples obtained by the two above mentioned techniques—“crude” DNA extracts and phenol-chloroform isolated DNA—were subjected to PCR amplification. Bands of about 1400 bp were excised from 2% agarose gel; DNA was extracted from gels with a Zymoclean DNA recovery kit (Zymo Research, CA). PCR amplification and sequencing was performed at least twice for each DNA sample to avoid sequence errors and to clarify ambiguities. Two vouchers of spore suspensions originating from different infected insects were tested as described above. All reagents used in the study, unless designated otherwise, were from SIGMA (St. Louis, MO).

2.3. Phylogenetic analysis

Small subunit ribosomal RNA gene (SSU rDNA) sequences of *Encephalitozoon* spp. showed maximum identity to the novel sequence in BLAST search; thus two of them (Accession Nos. L39107 and AF 338366) were included in analyses. The sequences were compared with those of microsporidia from orthopterans, (*Liebermannia patagonica* [Accession No. DQ 239917], *Liebermannia dichropluseae* [Accession No. EF 016249], *Paranosema locustae* [Accession No. AY 305324], and *P. grylli* [Accession No. AY 305325]; and with microsporidia of insects with octosporous sporogony (*Systemostrema alba* [Accession No. 61969671]; *Thelohania solenopsae* [Accession No. AF134205], *Vairimorpha necatrix* [Accession No. Y 002666] and two octospore-producing species from mosquitoes *Amblyospora connecticus* [Accession No. AF 025685] and *Parathelohania anopheles* [Accession No. AF027682]). In addition, sequences were added to represent the major genera of terrestrial insect microsporidia: *Nosema bombycis* (Accession No. L 39111), *Tubulinosema ratisbonensis* (Accession No. AY 695845), *Anncaliia meligethi* (Accession No. AY 894423), *Endoreticulatus schubergi* (Accession No. L 39109), *Orthosomella operophtera* (Accession No. AJ302316), and *Antonospora scoticae* (Accession No. AF024655).

All sequences were trimmed from the 5'-end beginning with the last nucleotide of the VI 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) (Accession No. D29946), was selected as an outgroup. The resultant alignment was analyzed by neighbor joining (NJ), maximum parsimony (MP), and by a 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 one by likelihood ratio tests and a TIM + I + G model—by AIC criteria in Modeltest 3.6 (Posada and Crandall, 1998); the settings of the former 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. 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 and discussion

Spores of both types were present in the samples: diplokaryotic *Nosema*-like spores and monokaryotic octospores, assembled in clusters of eight inside fusiform sporophorous vesicles (SVs) (Fig. 1), which allowed unambiguous identification of the species as *H. cowani* (Lange et al., 1995). Most of the spores contained intact nuclei visualized by DAPI and ethidium bromide staining (data not shown). Unfixed diplokaryotic spores were ovocylindrical in shape, ranged from 4.5 to 5.2 $\mu\text{m} \times 1.7$ –2.4 μm , and averaged $4.72 \pm 0.047 \mu\text{m} \times 1.96 \pm 0.047 \mu\text{m}$ (average \pm SE, $n = 15$); while the slightly pyriform octospores ranged from 2.8 to 3.4 $\mu\text{m} \times 1.6$ –1.9 μm , and averaged $3.13 \pm 0.103 \mu\text{m} \times 1.84 \pm 0.078 \mu\text{m}$ ($n = 10$). SVs ranged from 16.2 to 18.2 $\mu\text{m} \times 3.8$ –4.3 μm and averaged $17.37 \pm 0.314 \mu\text{m} \times 4.04 \pm 0.079 \mu\text{m}$ ($n = 6$). The sizes of unfixed spores and SVs measured in this study (after 15 years of storage in the freezer) were about 10–13% less than the one of the fresh spores and SVs given in the original description (Lange et al., 1995). This discrepancy might be caused by natural shrinkage due to water loss in the process of storage or inconsistency of the methods of spore measuring. Fixed spores averaged as follows: diplokaryotic spores, $4.20 \pm 0.080 \times 1.92 \pm 0.060 \mu\text{m}$ ($n = 28$); octospores, $3.06 \pm 0.049 \times 1.94 \pm 0.003 \mu\text{m}$ ($n = 28$); SV, $15.95 \pm 0.285 \times 3.75 \pm 0.092 \mu\text{m}$ ($n = 10$).

The unambiguous consensus sequence submitted to Genbank (Accession No. EU275200) was 1165 nucleotides long and contained 43.3% of GCs.

BLAST search did not reveal much similarity between the novel SSU rDNA sequence and any other sequence deposited in GenBank. The closest sequences with maximum bit scores (200–235) and identities (80–85% for 74–76% of query coverage) were those of the *Encephalitozoon* spp.

The NJ, MP and ML analyses, performed against 18 microsporidian sequences (Fig. 2), placed *H. cowani* as a sister taxon to the *Nosema*–*Vairimorpha* clade. To assess the robustness of the tree in one of our analyses we included six additional sequences: *Encephalitozoon hellem* [Accession No. L19070]; *Septata intestinalis* [Accession No. L39113]; *Encephalitozoon lacerate* [Accession No. AF067144]; *Vairimorpha lymantriae* [Accession No. AF033315]; *Vairimorpha imperfecta* [Accession No. AJ131646]; and *Nosema portugal* [Accession No. AF033316]. These did not alter the basal position of the *Heterovesicula* branch in relation to the *Vairimorphal*–*Nosema* clade, and they did not change the general topology of the NJ, MP, and ML trees. We did not include these sequences into the final analyses in order to avoid overloading the tree (Fig. 2) with redundant information.

The *Heterovesicula*–*Nosema*–*Vairimorpha* group forms a dichotomy with the *Encephalitozoon* spp. branch. Other microsporidia parasitizing Orthoptera fell into two additional unrelated (or distantly related) lineages of terrestrial microsporidia: the *Liebermannia* spp. branch forms a dichotomy with *Orthosomella operophtherae* within the *Endoreticulatus*–*Orthosomella*–*Liebermannia* group; the *Paranosema* spp. branch clusters with the *Tubulinosema*–*Systemostrema* lineage. It is noteworthy that the host of *H. cowani*, *Anabrus simplex* (family Tettigoniidae, superfamily Tettigoniioidea, suborder Ensifera) also is not closely related to the host species of other orthopteran microsporidia that have been characterized molecularly to date: *Gryllus bimaculatus* (Gryllidae, Grylloidea, Ensifera), the host of *Paranosema grylli*; *Locusta migratoria* (Acrididae, Acridoidea, Caelifera), the host of *P. locustae*; *Dichroplus elongatus* (Acrididae, Acridoidea, Caelifera), the host of *L. dichroplusae*; and *Tristiria magellanica* (Tristiridae, Acridoidea, Caelifera), the host of *L. patagonica*. However, even the current incomplete data suggest that colonization of orthopterans occurred independently by different microsporidian lineages. Relatedness between nosemas, the parasites of lepidopterans, and heterovesiculids, the parasites of katydids, might have an ecological explanation. Katydids, unlike other Ensifera (for example,

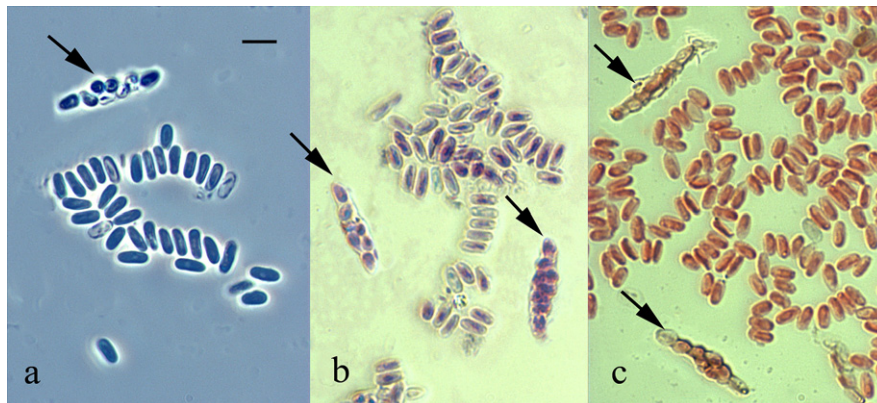


Fig. 1. Spores of *Heterovesicula cowani*. a, unfixed spores; b, methanol-fixed and Giemsa-stained spores; c, methanol-fixed and trichrome-stained spores. Arrows indicate fusiform sporophorous vesicles with eight spores inside. Bar = 5 μm .

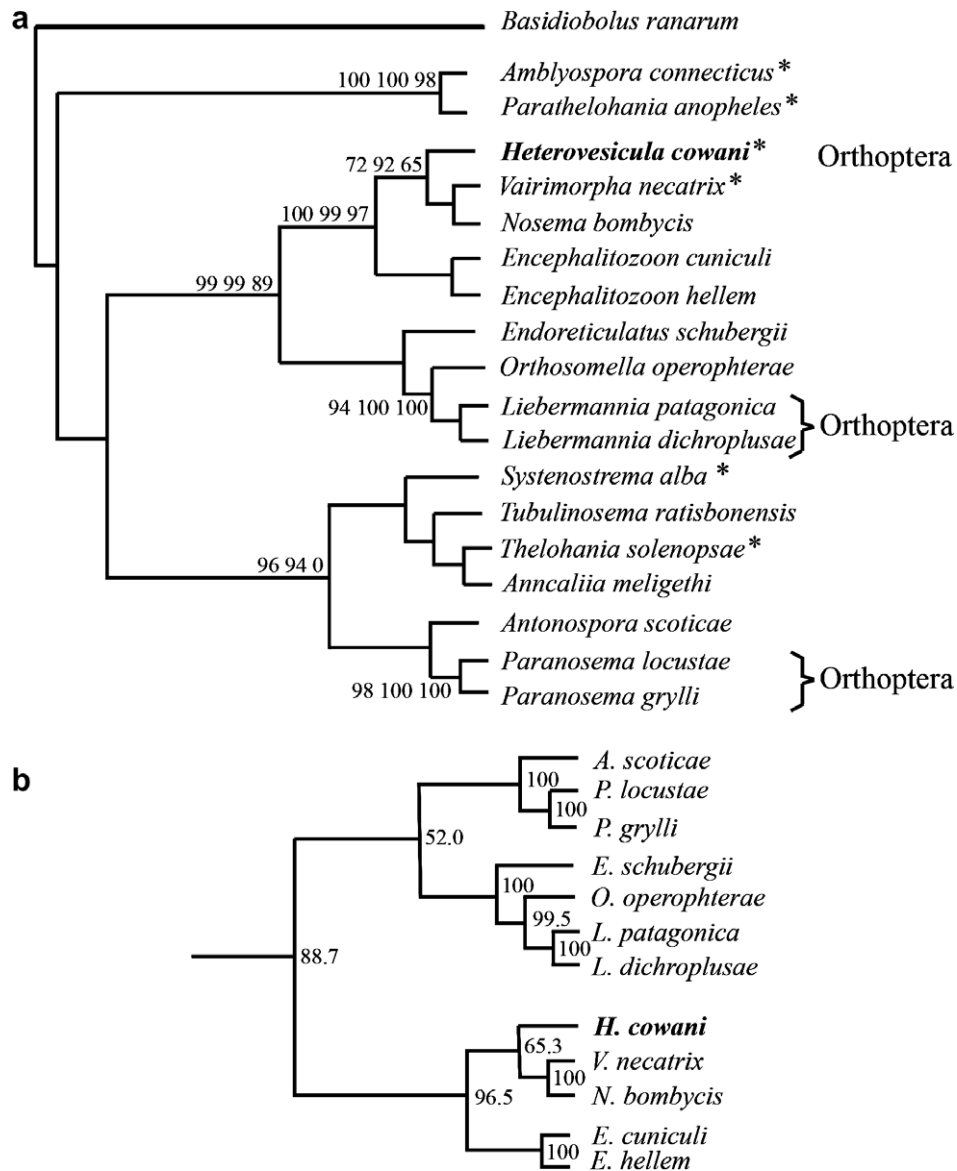


Fig. 2. Phylogenetic relationships of *Heterovesicula cowani* with 15 microsporidia from insects and two *Encephalitozoon* spp. (a) Tree topology produced by maximum likelihood (ML) and neighbour-joining (NJ) analyses. The *Heterovesicula-Nosema-Vairimorpha* and *Encephalitozoon* spp. branches form a dichotomy that in turn forms one clade. Other microsporidia parasitizing Orthoptera fell into two unrelated (or distantly related) lineages of terrestrial microsporidia. *H. cowani* is related to *Vairimorpha necatrix* but not to other microsporidia with octosporous sporogony (marked with asterisk). The first, second and third numbers at nodes are bootstrap values for the node support in ML, NJ and PS analyses, respectively, all in 100 replicates. Support is indicated only if less than 100 in at least one of the analyses. (b) Part of the PS tree of dissimilar topology: the *Antonosporea-Paranosema* branch clusters together with the *Endoreticulatus-Operophthera-Liebermannia* lineage, though the heuristic bootstrap value for this clade on the most parsimonious tree is as low as 52.

the Gryllidae), evolved as a group associated with vegetation at all stages of their development (Belwood, 1990; Sharov, 1968) and thus shared the habitat with lepidopterans, perhaps allowing parasites to transfer between two groups.

As a rule, pairwise distances of about 10–15% separate sequences of representatives of closely related genera (Sokolova et al., 2003, 2007). Hence, 37–39% of sequence divergence with the closest sequences, the ones of *E. hellem*, *V. necatrix* and *N. bombycis*, not only proves the sound generic status of *Heterovesicula* (Table 1) but also likely supports the idea that *H. cowani* belongs to a separate family-level taxon, *Heterovesiculidae*, as was suggested by the

authors of the species based on morphological traits (Lange et al., 1995).

The position of *H. cowani* on the phylogenetic trees suggests that it is related to the *Vairimorpha necatrix* group but not to other insect microsporidia with octosporous sporogony found practically in all microsporidia lineages (Vossbrinck and Debrunner-Vossbrinck, 2005) (Fig. 2a). Microsporidia that infect insects and produce octosporous are obviously not monophyletic. The potential to produce octospores (meiospores) is probably a fundamental feature of all microsporidia. It is based on peculiarities of their nuclear cycle (Flegel and Pasharawipas, 1995); thus, the

Table 1
Comparison of SSU rDNA sequences of 18 microsporidia: pairwise distances (bottom diagonal) and percentage of similarity (top diagonal) obtained through Kimura-2-Parameter analysis

	<i>Hc</i> ^a	<i>Pl</i>	<i>Pg</i>	<i>As</i>	<i>Ts</i>	<i>Am</i>	<i>Tr</i>	<i>Sa</i>	<i>Vn</i>	<i>Nb</i>	<i>Ec</i>	<i>Eh</i>	<i>Lp</i>	<i>Ld</i>	<i>Oo</i>	<i>Es</i>	<i>Ac</i>	<i>Pa</i>
<i>Hc</i>	—	40,8	41,0	35,2	45,8	41,8	45,0	41,7	61,9	60,5	58,5	62,6	50,3	53,3	52,9	54,4	45,3	46,0
<i>Pl</i>	0,59	—	96,6	91,2	48,0	49,3	47,9	44,2	40,6	34,1	50,3	46,0	55,6	57,6	57,8	55,2	40,0	46,2
<i>Pg</i>	0,59	0,03	—	90,6	48,2	50,2	47,3	43,2	40,7	34,3	50,6	46,1	56,3	58,3	57,5	56,4	40,4	47,8
<i>As</i>	0,65	0,09	0,09	—	50,7	50,2	50,4	47,8	31,5	25,2	47,6	45,2	49,9	52,7	54,2	48,3	40,8	38,7
<i>Ts</i>	0,54	0,52	0,52	0,49	—	84,7	75,6	60,8	48,5	46,3	37,2	39,8	41,3	43,2	43,3	48,4	45,8	40,9
<i>Am</i>	0,58	0,51	0,50	0,50	0,15	—	72,1	56,1	43,9	43,3	36,2	37,0	39,9	41,9	46,1	47,7	42,7	40,5
<i>Tr</i>	0,55	0,52	0,53	0,50	0,24	0,28	—	58,7	49,5	46,9	37,2	39,2	44,6	45,8	46,2	49,4	44,2	44,8
<i>Sa</i>	0,58	0,56	0,57	0,52	0,39	0,44	0,41	—	45,5	47,9	33,6	40,0	35,7	34,6	36,4	41,3	42,1	38,9
<i>Vn</i>	0,38	0,59	0,59	0,69	0,51	0,56	0,50	0,55	—	82,3	59,8	64,8	48,1	50,4	51,0	56,6	44,9	37,6
<i>Nb</i>	0,39	0,66	0,66	0,75	0,54	0,57	0,53	0,52	0,18	—	54,6	60,1	46,3	48,0	50,3	52,6	44,6	36,9
<i>Ec</i>	0,41	0,50	0,49	0,52	0,63	0,64	0,63	0,66	0,40	0,45	—	88,3	57,2	58,4	63,4	59,0	44,2	43,1
<i>Eh</i>	0,37	0,54	0,54	0,55	0,60	0,63	0,61	0,60	0,35	0,40	0,12	—	56,1	55,9	58,5	58,0	44,7	43,1
<i>Lp</i>	0,50	0,44	0,44	0,50	0,59	0,60	0,55	0,64	0,52	0,54	0,43	0,44	—	96,3	80,0	70,4	45,1	44,5
<i>Ld</i>	0,47	0,42	0,42	0,47	0,57	0,58	0,54	0,65	0,50	0,52	0,42	0,44	0,04	—	80,4	71,2	45,9	46,6
<i>Oo</i>	0,47	0,42	0,43	0,46	0,57	0,54	0,54	0,64	0,49	0,50	0,37	0,42	0,20	0,20	—	75,3	46,3	49,5
<i>Es</i>	0,46	0,45	0,44	0,52	0,52	0,52	0,51	0,59	0,43	0,47	0,41	0,42	0,30	0,29	0,25	—	42,5	46,5
<i>Ac</i>	0,55	0,60	0,60	0,59	0,54	0,57	0,56	0,58	0,55	0,55	0,56	0,55	0,55	0,54	0,54	0,58	—	70,7
<i>Pa</i>	0,54	0,54	0,52	0,61	0,59	0,59	0,55	0,61	0,62	0,63	0,57	0,57	0,55	0,53	0,51	0,54	0,29	—

^a Abbreviations used: *Hc*, *Heterovesicula covani*; *Pl*, *Paranosema locustae*; *Pg*, *P. grylli*; *As*, *Antonosporea scoticae*; *Ts*, *Thelohania solenopsae*; *Am*, *Anncaliia meligethi*; *Tr*, *Tubulinosema ratisbonensis*; *Sa*, *Systemostrema alba*; *Vn*, *Vairimorpha necatrix*; *Nb*, *Nosema bombycis*; *Ec*, *Encephalitozoon cuniculi*; *Eh*, *E. hellem*; *Lp*, *Liebermannia patagonica*; *Ld*, *L. dichrophusae*; *Oo*, *Orthosomella operophterae*; *Es*, *Endoreticulatus schubergi*; *Ac*, *Amblyospora connecticus*; *Pa*, *Parathelohania anopheles*.

presence or absence of octospores should not be used as a discriminating character for taxa of any rank.

The NJ, ML and MP analyses produced identical tree topologies, except for the position of the *Antonosporea–Paranosema* branch, which clustered with the *Endoreticulatus–Operophtera–Liebermannia* in the MP tree, and with the *Systemostrema–Tubulinosema–Anncaliia* groups in the NJ and ML trees. Because the *Antonosporea–Paranosema* branch had weak statistical support—the heuristic bootstrap value on the shortest tree was only 52 (Fig. 1b)—the topology suggested by the NJ and ML analyses more likely reflects the real relations of this group to other terrestrial microsporidia.

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