REFERENCES

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A NEW *ENCEPHALITOZOON* SPECIES (MICROSPORIDIA) ISOLATED FROM THE LUBBER GRASSHOPPER, *ROMALEA MICROPTERA* (BEAUVOIS) (ORTHOPTERA: ROMALEIDAE)

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ABSTRACT: We describe a new microsporidian species, *Encephalitozoon romaleae* n. sp., isolated from an invertebrate host, the grasshopper *Romalea microptera*, collected near Weeks Island, Louisiana, and Jacksonville, Florida. This microsporidian is characterized by specificity to the gastric caecae and midgut tissues of the host and a life cycle that is nearly identical to that of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*. Mature spores are larger (3.97 × 1.95 μm) than those of other *Encephalitozoon* species. Polar filament coils number 7 to 8 in a single row. Analysis of the small subunit (SSU) rDNA shows that *E. romaleae* fits well into the *Encephalitozoon* group and is a sister taxon to *E. hellem*. This is the first *Encephalitozoon* species that has been shown to complete its life cycle in an invertebrate host.

Species of *Encephalitozoon* (Microsporidia) infect a variety of vertebrate animals, including humans (reviewed by Didier and Bessinger, 1999). The most frequently identified microsporidian human pathogens are *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi*, accounting for more than 90% of human infections (Didier, Didier, Friedberg et al., 2006).

Encephalitozoon-like microsporidian species have been reported to occur in invertebrates only twice, in both cases in association with a mammal. Ribeiro and Guimaraes (1998) observed an *Encephalitozoon* sp. infecting ticks that had fed on rabbits. Although rabbits are commonly infected with *E. cuniculi* (Canning and Lom, 1986), the authors did not report whether the rabbit was infected with this pathogen. Ribeiro and Passos (2006) observed an *Encephalitozoon*-like microsporidian infecting a tick found feeding on a horse known to be infected with *Babesia caballi*. The horse died and was not tested for microsporidiosis. Neither microsporidian isolate was sequenced, but the latter isolate was reported to conform to the morphological description of *E. cuniculi*.

We recently isolated an undescribed microsporidian from a laboratory colony of the lubber grasshopper *Romalea microptera* (Beauvois) (=*guttata* (Houttuyn) (Otte, 1995) at Illinois State University (ISU). First exposure of the colony to the pathogen was apparently in July 2001 when adult *R. microptera* grasshoppers that were field-collected near Weeks Island, Louisiana, were introduced into the ISU colony. The pathogen infects the gastric caecae and, to a lesser extent, the midgut tissues, and was noted to have a developmental cycle in host cells that is highly unusual for entomopathogenic microsporidia. Spores are passed with the feces, and transmission is achieved when uninfected individuals consume spore-contaminated food (Johny et al., 2008). The pathogen seems to cause low-to-moderate mortality in the ISU *R. microptera* colony. Here, we describe the new species and provide data on the phylogenetic relationship of this microsporidian with congeneric microsporidian species described from vertebrate animals.

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MATERIALS AND METHODS

Host insects

The ISU *R. microptera* laboratory colony was originally established in 1996 from animals collected from Copeland, Florida, and is periodically augmented by the addition of field-collected specimens from the same location. Additional grasshoppers collected from various localities, including Weeks Island, Louisiana, were periodically established at ISU, but they were maintained separately in the laboratory. The insects are group-reared in large screen cages; fed Romaine lettuce leaves supplemented with oatmeal, wheat bran, and various fresh vegetables; and maintained under a 14:10-hr L:D photoperiod (Matuszek and Whitman, 2001). Although the rearing rooms are maintained at 26–29 C, heat lamps allow individuals to seek their preferred temperature (~35 C) during the day. Despite the use of standard hygiene procedures (Matuszek and Whitman, 2001), including the bimonthly sterilization of cages and maintenance of insects in age- and population-specific cages, virtually 100% of laboratory grasshoppers from all localities became infected with the microsporidian pathogen after its unintentional introduction to the ISU colony.

In addition to the colony insects, *R. microptera* grasshoppers were collected from 4 sites in the Weeks Island (Iberia Parish), Louisiana, area in July 2006. Of 37 Weeks Island grasshoppers dissected, infected individuals were recovered from 3 sites (Table I). Infected host insects used in these studies included both individuals from the ISU colony and field-collected individuals. In August 2008, another infected *R. microptera* population was discovered in Jacksonville, Florida. This microsporidian isolate was sequenced to determine its relationship with the other 2 isolates.

Microscopic studies

Freshly excised internal organs of infected *R. microptera* were examined using phase contrast microscopy at ×400 and ×1,000. Midgut and gastric caecae tissues of infected insects were fixed in 100% methanol, stained with Giemsa (Vávra and Maddox, 1976), and examined using bright field microscopy. Fresh spores (n = 30) were immobilized in immersion oil (Hazard et al., 1981) and measured using an Image Splitting Eyepiece (Vickers Instruments, Ltd., Malden, Massachusetts); Giemsa-stained spores (n = 30) were also measured. Because uninfected *R. microptera* were not available, no timed studies were performed to evaluate early development of the microsporidian; however, all developmental forms described for *Encephalitozoon* species were observed in the study insects.

For transmission electron microscopy, small samples of infected tissues were fixed for 1 hr at 4 C in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.4; post-fixed in 1% aqueous osmium tetroxide (v/w); and en bloc-stained with 1% uranyl acetate. The material was dehydrated through an ascending acetone series after which samples were embedded in Spurr's resin. Thick sections (1.0 μm) stained with methylene blue were observed using light microscopy to determine the infected sites. Ultrathin sections were post-stained with methanolic uranyl acetate followed by lead citrate and were observed and photographed at

TABLE I. Field survey for the microsporidian *Encephalitozoon romaleae* in grasshopper hosts.

Grasshopper species	Location	Date	Stage	No. examined	No. infected
Romalea microptera					
Athens, Georgia	33°57′N, 83°24′W	August 2004	Adult	11	0
Jacksonville, Florida	30°16′52″N, 81°(18–21)′W	September 2008	Adult	11	11
Orlando, Florida	28°40′N, 81°13′W	September 2007	Adult	2	0
20 km N Copeland, Florida	26°9′N, 81°21′W	July 2003	Adult	7	0
Copeland, Florida	25°57′N, 81°21′W	July 2003	Adult	15	0
3 km E Ochopee, Florida	25°51′N, 81°15′W	July 2003	Adult	14	0
		March 2004	Third	19	0
		April 2004	Fifth	10	0
		June 2004	Adult	10	0
		March 2005	Third	5	0
		April 2005	Fifth	10	0
Shark Valley, Florida	25°45′N, 80°46′W	July 2003	Adult	18	0
Anhinga Trail, Florida	25°23′N, 80°35′W	July 2003	Adult	29	0
Paurotis Pond, Florida	25°15′N, 80°52′W	July 2003	Adult	10	0
Weeks Island, Louisiana	29°50′N, 91°47′W	July 2003	Adult	7	1
	29°52′N, 91°47′W	August 2003	Adult	9	5
	29°53′31″N, 104°38′32″W	July 2006	Adult	13	0
	29°51′36″N, 91°47′11″W	July 2006	Adult	7	3
	29°52′31″N, 91°47′03″W	July 2006	Adult	10	2
	29°50′53″N, 91°47′17″W	July 2006	Adult	7	1
Barataria, Louisiana	29°59′N, 90°21′W	July 2008	Adult	11	0
Taeniopoda eques					
Wilcox, Arizona	32°14′N, 109°49′W	September 2003	Adult	10	0
Douglas, Arizona	31°24′N, 109°29′W	September 2003	Adult	5	0
Rodeo, New Mexico	31°50′N, 109°01′W	September 2008	Adult	2	0
Brachystola magna					
Rodeo, New Mexico	31°50′N, 109°01′W	September 2008	Adult	10	0

an accelerating voltage of 80 kV with an H600 electron microscope (Hitachi, Tokyo, Japan).

DNA isolation, amplification, sequencing, and sequence analysis

Tissues isolated from infected ISU colony insects and grasshoppers collected from Louisiana and Florida were homogenized in a glass tissue grinder, filtered through tightly woven synthetic material, centrifuged to pellet the spores and washed several times by resuspending the pellet in deionized water and centrifuging. DNA was prepared by grinding a cleaned spore pellet in deionized water on a Genogrinder 2000 (SpexCertiprep, Metuchen, New Jersey). The macerated spores were heated at 95 C for 10 min and centrifuged. Two microliters of this preparation was used for amplification of the small subunit rRNA gene using primers 18F and 1492R (Weiss and Vossbrinck, 1999). The polymerase chain reaction products were either purified using Microcon-100 (Amicon Bioseparations, Bedford, Massachusetts) or sequenced unpurified. Sequencing was performed on an ABI3730 genetic analyzer (Applied Biosystems, Foster City, California) at the Iowa State University DNA Facility (Ames, Iowa) using standard sequencing protocols. The Florida isolate was sequenced at the Keck Center, University of Illinois (Urbana, Illinois) on a 3730XL capillary sequencer (Applied Biosystems). Both strands of the gene were sequenced using primers 18F, 530F, 1047R, and 1492R (Weiss and Vossbrinck, 1999). Contigs were assembled using the sequence editor Sequence Assembler (Applied Biosystems) and imported into MacClade 4.06 (Maddison and Maddison, 2003) to perform the alignment. The sequences were visually aligned to an existing microsporidian sequence database.

The analysis of the phylogenetic placement of *E. romaleae* was performed in a 2-step process. First, to confirm the generic placement of *E. romaleae* and to identify potential outgroups for a more detailed examination of the relationship between *E. romaleae* and the other *Encephalitozoon* species, we included a broad sampling of microsporidian species (Table II). Species were selected to span the major lineages of Microsporidia for which the small subunit rRNA gene has been sequenced. Where possible, sequences of the type species for the genus were selected. Taxa used for the second stage of the analysis are also listed in Table II.

Parsimony and neighbor joining (NJ) were the primary methods used for phylogenetic analysis. A heuristic search criterion was used for parsimony analysis, incorporating 1,000 random stepwise addition replicates and tree bissection-reconnection branch swapping. For NJ analysis Kimura 2-parameter and HKY85 models of base substitution were assumed. Support for the various nodes was performed using parsimony and NJ bootstrap analysis. For parsimony bootstrap analyses, 1,000 replicates were performed under the same heuristic search options as described above. Similarly the NJ bootstrap analyses were performed assuming the models above. All analyses were performed using PAUP version 4.0b10 (Swofford, 2001). Regions of uncertain homology (hypervariable regions) were excluded from the analyses.

Field collections of microsporidia-infected *R. microptera*

Between 2003 and 2008, we examined 235 specimens of *R. microptera* collected in the field from Florida, Georgia, and Louisiana (Table I). Tissue smears from each insect were prepared from freshly dissected gastric caecae and midgut tissues and examined using light microscopy (×400). We also examined individuals of the closely related grasshopper species *Taeniopoda eques* (n = 17) and *Brachystola magna* (n = 10) (Romaleidae) that were collected in Arizona and New Mexico (Otte, 1995; Stauffer and Whitman, 2007).

DESCRIPTION

Encephalitozoon romaleae n. sp.

Diagnosis (light microscopy): Development only in epithelial cells of midgut and gastric caecae. Proliferative stages primarily spherical in

TABLE II. Microsporidian taxa used in phylogenetic analyses.

Species	Host	GenBank	Reference
First stage analysis			
Pleistophora typicalis	*Myoxocephalus scorpius*	AF044387	Nilsen et al., 1998
Glugea anomala	*Gasterosteus aculeatus*	AF044391	Nilsen et al., 1998
Ichthyosporidium sp.	*Leiostomus xanthurus*	L39110	Baker et al., 1995
Kabatana takedai	*Oncorhyncus masu*	AF356222	Nilsen et al., 2001
Spraguea lophii	*Lophius americanus*	AF033197	Hinkle et al., 1997 (ds*)
Ameson michaelis	*Callinectes sapidus*	L15741	Zhu, Wittner, Tanowitz, et al., 1993
Orthosomella operophterae	*Operophtera brumata*	AJ302317	Canning et al., 2001
Vittaforma corneae	*Homo sapiens*	L39112	Baker et al., 1995
Endoreticulatus schubergii	*Lymantria dispar*	L39109	Baker et al., 1995
Enterocytozoon bieneusii	*H. sapiens*	L07123	Zhu, Wittner, Tanowitz, Kotler et al., 1993
Ordospora colligata	*Daphnia* spp.	AF394529	Refardt et al., 2002
Encephalitozoon cuniculi	*Oryctolagus cuniculus*	L39107	Baker et al., 1995
Vairimorpha necatrix	*Pseudaletia unipuncta*	Y00266	Vossbrinck et al., 1987
Nosema bombycis	*Bombyx mori*	L39111	Baker et al., 1995
Bryonosema tuftyi	*Plumatella* sp.	AF484693	Canning et al., 2002
Janacekia debaisieuxi	*Simulium* sp.	AJ252950	Cheney et al., 2000
Tubulinonosema (*Nosema*, *Visvesvaria*) *acridophagus*	*Schistocerca americana*	AF024658	Pienazek et al., 2005 (ds)
Gurleya daphniae	*Daphnia* spp.	AF439320	Refardt et al., 2002
Amblyospora californica	*Culex salinarius*	U68473	Baker et al., 1997
Parathelohania anophelis	*Anopheles quadrimaculatus*	AF027682	Baker et al., 1998
Flabelliforma montana	*Phlebotomus ariasi*	AJ252962	Cheney et al., 2000
Flabelliforma magnivora	*Daphnia magna*	AY649786	Refardt, 2004 (ds)
Weiseria palustris	*Cnephia ornithophilia*	AF132544	Adler et al. (ds)
Tritrichomonas foetus (Trichomonadida)-outgroup	*Box taurus*	M81842	Chakrabarti et al., 1992
Giardia lamblia (Diplomonadida)-outgroup	Not stated	M54878	Sogin et al., 1989
Second stage analysis			
Vittaforma corneae	*H. sapiens*	L39112	Baker et al., 1995
Endoreticulatus schubergii	*L. dispar*	L39109	Baker et al., 1995
Enterocytozoon bieneusi	*H. sapiens*	L07123	Hartskeerl et al., 1995
Ordospora colligata	*D. magna*	AF394529	Refardt et al., 2002
Encephalitozoon intestinalis	*H. sapiens*	L39113	Baker et al., 1995
Encephalitozoon hellem	*H. sapiens*	L39108	Baker et al., 1995
Encephalitozoon cuniculi	*O. cuniculus*	L39107	Baker et al., 1995
Encephalitozoon lacertae	*Mabuya perrotetii*	AF067144	Koudela et al., 1998
Encephalitozoon romaleae n. sp. (laboratory isolate)	*Romalea microptera*	EU502838	This study
Encephalitozoon romaleae n. sp. (field isolate)	*R. microptera*	EU502839	This study

* ds = direct submission, unpublished.

shape. Earliest stages small and uninucleate (Fig. 1A), undergoing karyokinesis to form binucleate meronts (Fig. 1B, D). Formation of globular plasmodia with 4, 6, 8, or more nuclei (Fig. 1D). Larger plasmodia with simultaneously occurring incipient cleavages (fragmentations) in periphery of cytoplasm (Fig. 1E, F). Developing sporonts with fusiform appearance (Fig. 1C, G). Primarily rounded meronts attached to interfacial envelope (inner periphery of vacuolar membrane) (Xu et al., 2006) in proliferative phase of development (Fig. 1H). Asynchronous development within vacuole, proliferative stages on periphery and sporulating stages located elsewhere (Fig. 1I). Spherical vacuoles varying in size, 11–35 μm (Fig. 1J), and containing 16 to many spores (Fig. 1K). Spores measuring (mean ± SE) 3.97 ± 0.23 by 1.95 ± 0.08 μm in fresh preparations (n = 30) (Fig. 1L) and 3.09 ± 0.15 by 1.81 ± 0.1 μm in Giemsa-stained smears (n = 30).

Ultrastructure: Nuclear configuration never diplokaryotic. Meronts rounded, uninucleate (Fig. 2A) and binucleate (Fig. 2E) with double nuclear membrane (Fig. 2A). Meronts with large rounded nuclei, a few cisternae of endoplasmic reticulum, and dense cytoplasm with abundance of ribosomes. Limiting membrane appearing as double entity with 2 unit membranes in intimate association (Fig. 2B). Progressive separation of parasite cytoplasmic membrane and vacuole envelope resulting in development of electron lucent episporontal space between 2 membranes (Fig. 2C, D), growing larger as parasite proliferates (Figs. 2F, 3B). After separation of membranes, electron dense material appearing on outer face of parasite membranes; parasite cells simultaneously elongating (Fig. 3A, B). Fusiform stages detaching from interfacial envelope and forming sporonts (Fig. 3C, D). Sporonts distinguishable from proliferate forms by thickened envelopes, more dense cytoplasm, elongated shape, and numerous membrane profiles inside cytoplasm (Fig. 3C, D). Sporont plasma membranes invaginated, suggesting production by polysporous sporogony of ribbon-like sporogonial plasmodia (Fig. 3D, E; see also Fig. 1G, I). Vacuoles with large, highly variable numbers of stages and spores, occupying significant volume of host cell cytoplasm (Fig. 3E). Host cell cytoplasm and organelles sometimes crowded to cytoplasmic membrane. Mature spores (Fig. 4C) too dense for complete analysis but internal details

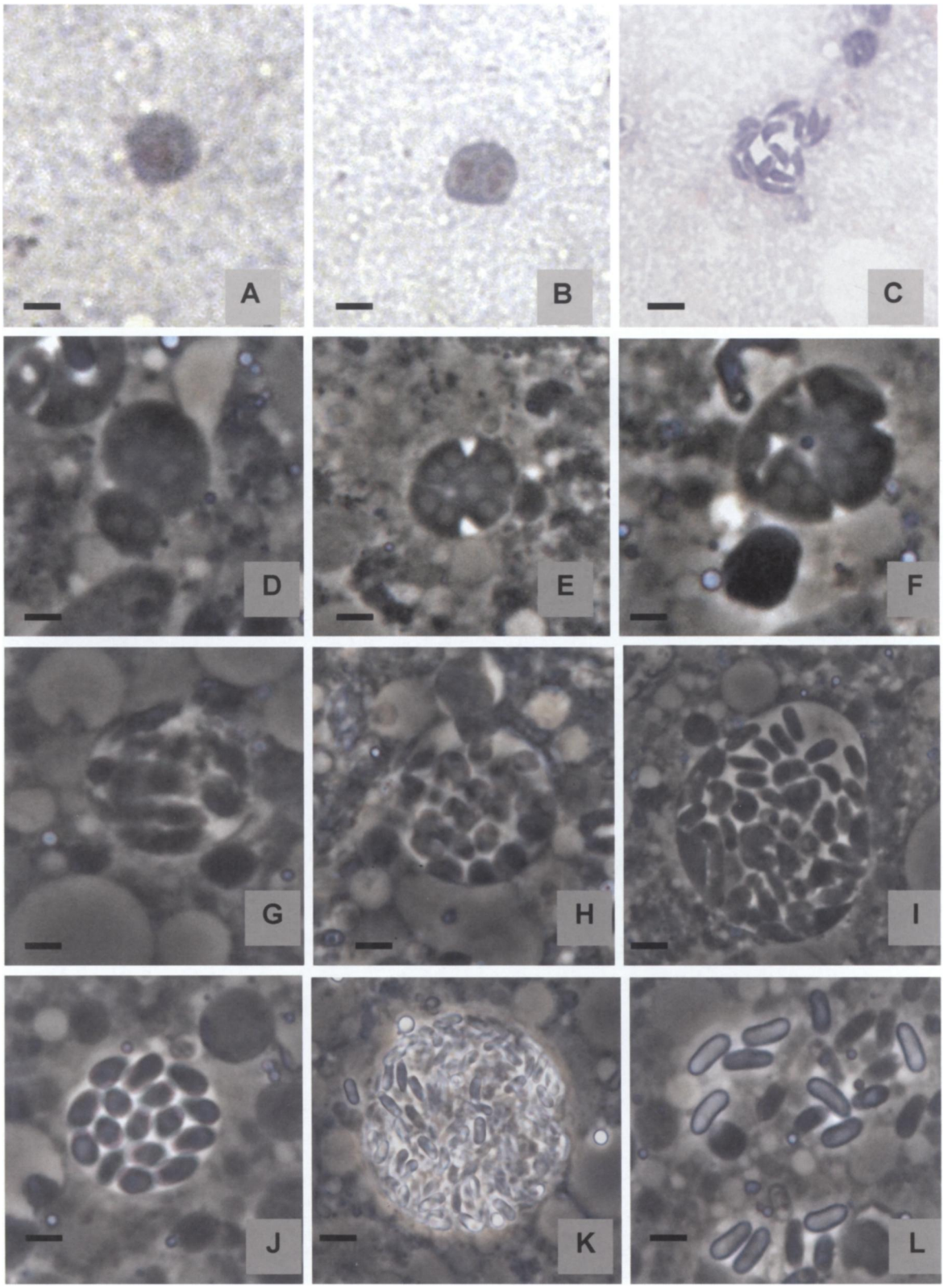

FIGURE 1. Giemsa stains and phase contrast light microscopy (×1,000) of developing stages and sporulation of *Encephalitizoon romaleae*. (**A**) Early monokaryotic meront; bar = 1.85 μm. (**B**) Karyokinesis produces a binucleate form; bar = 1.6 μm. (**D**) Binucleate stage and plasmodium with multiple nuclei; bar = 2.5 μm. (**E–F**) Cleavages from among plasmodia within the vacuolar envelope; bar = 2.5 μm (E) and 2.3 μm (F). (**C, G, H**) Formation of fusiform sporonts; bar = 5 μm (C), 2.5 μm (G), and 5 μm (H). (**G**) Meronts continue to develop along the interfacial envelope (inner periphery of the vacuolar envelope). (**I**) Asynchronous development continues as sporulation begins; bar = 6 μm. (**J**) Small spore-filled vacuole; bar = 5 μm. (**K**) Large vacuole with mature spores; bar = 6.5 μm. (**L**) Mature spores; bar = 3.25 μm.

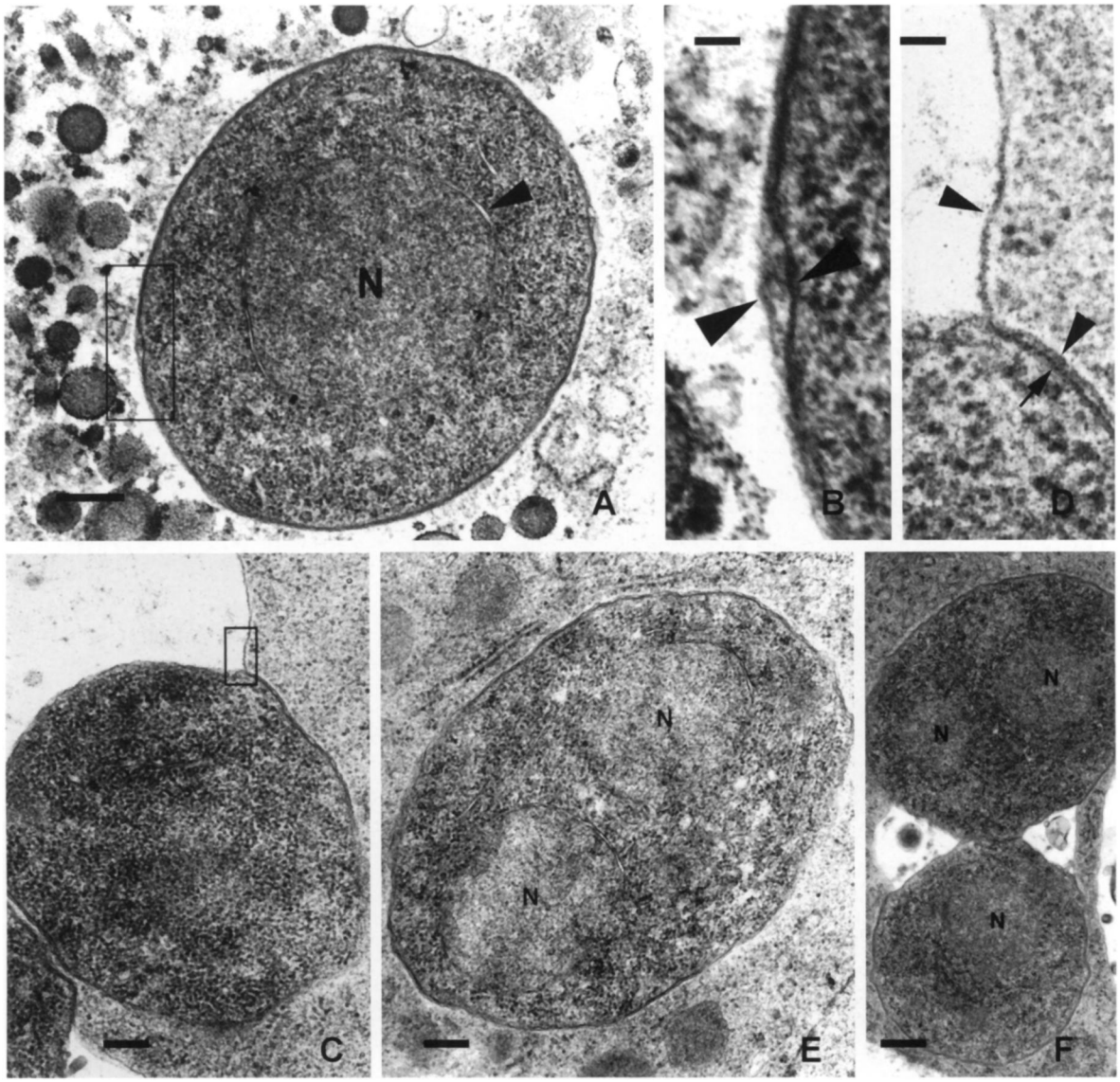

FIGURE 2. Transmission electron micrographs of early developmental stages of *Encephalitozoon romaleae*. (**A**) Early meront with initial formation of vacuolar envelope; bar = 0.5 μm. (**B**) Magnification of meront plasma membrane in contact with interfacial envelope; bar = 0.22 μm. (**C**) Meront attached to interfacial envelope; bar = 0.4 μm. (**D**) Junction of interfacial envelope and meront plasma membrane, magnified from Figure 4c; bar = 0.04 μm. (**E**) Binucleate meront, most likely the first karyokinesis; bar = 0.35 μm. (**F**) Cytokinesis of meronts within vacuolar envelope; karyokinesis without cytokinesis in upper body; bar = 0.70 μm. N = nucleus.

congruent with internal structures observed in immature or young spores (Fig. 4A, B). Spores centrally located within vacular envelope; large nucleus; short, isofilar polar filament with 7–8 coils arranged in a single layer; anterior extrusion apparatus; abundance of ribosomes; reduced posterior vacuole. Polar filament cross-sections with electron lucent center, at least 3 outer layers with alternating thin electron dense rings sandwiching thicker electron lucent layers. Polaroplast seems lamellar (Fig. 4B). Mature spores ovocylindrical and uninucleate (Fig. 4C).

Molecular analyses: SSU rDNA sequence-based phylogenetic analyses place *E. romaleae* within *Encephalitozoon* (Fig. 5). All analyses performed with this data matrix indicate that that *E. romaleae* is sister taxon of *E. hellem* (Fig. 6).

Taxonomic summary

Type host: Lubber grasshopper, *Romalea microptera* (Beauvois) (=*guttata* (Houttuyn)) (Orthoptera, Romaleidae) (Otte, 1995).

Site of infection: Gastric caecae and midgut tissues.

Type locality: Weeks Island, Louisiana (area approximately 29°51′36″N, 91°47′11″W).

Collection data: In sites where the microsporidian was recovered, 6 infected of 16 collected in 2003 (Louisiana), 6 of 37 in 2006 (Louisiana), and 11 of 11 in 2008 (Florida).

Transmission: Horizontal, via feces.

Deposition of type specimens: Live spores stored in the liquid nitrogen collection at Illinois Natural History Survey, University of Illinois, accessions 2004-D (laboratory isolate, ISU), 2006-A (field collection, Weeks Island, Louisiana), and 2008-B (field collection, Jacksonville, Florida); stained slides archived in the Illinois Natural History Survey microsporidian slide collection. SSU rDNA sequences deposited in GenBank, accessions EU502838 (laboratory colony) and EU502839 (field collection, Weeks Island, Louisiana). Pinned specimens of the host *R. microptera* deposited in the Sam Houston State University Insect Collection, accession SHSU-05001.

Etymology: Denotes generic name of the host.

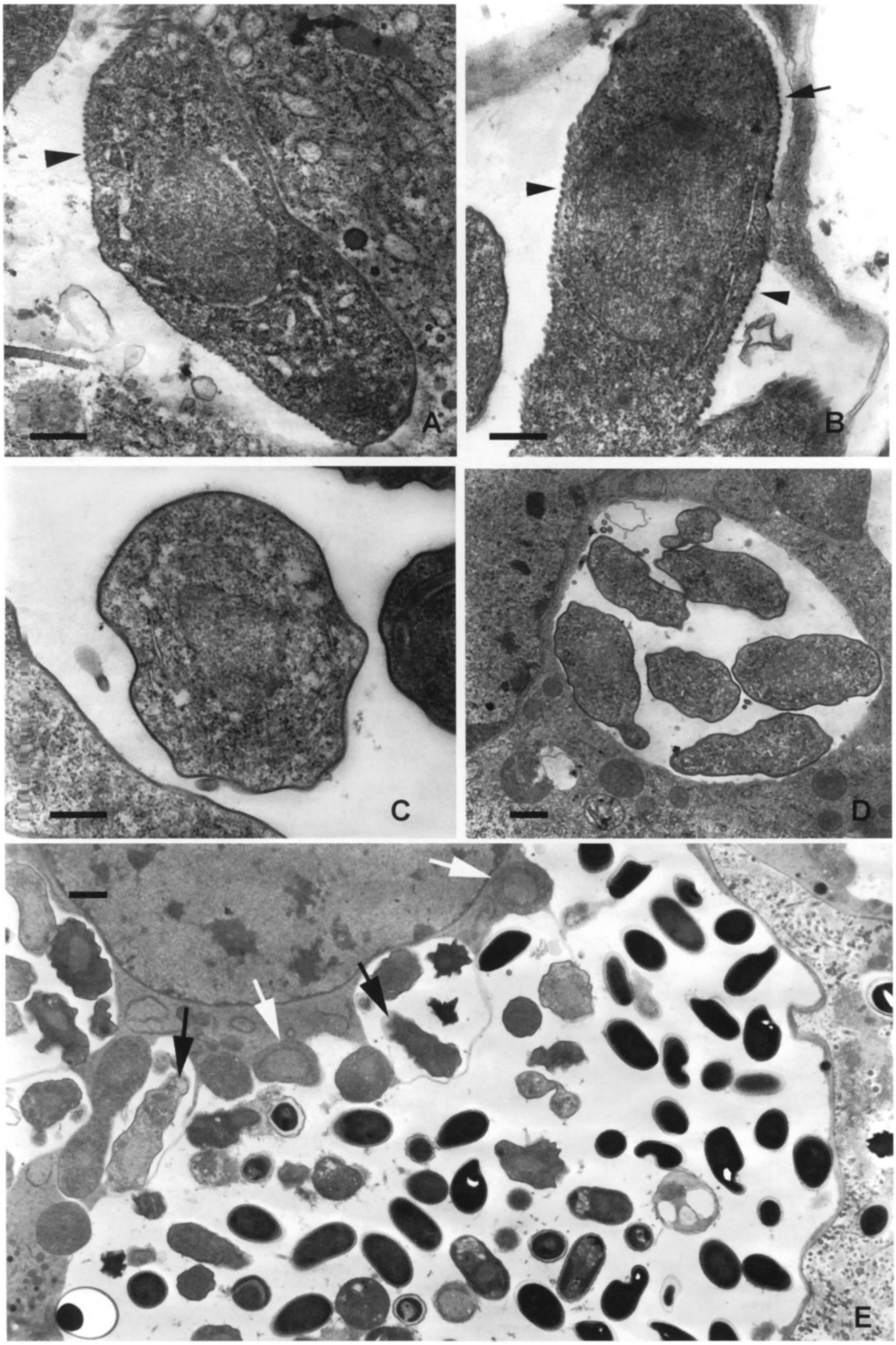

FIGURE 3. Sporulation cycle of *Encephalitozoon romaleae*. (**A**) Elongation and separation of parasite plasma membrane from interfacial envelope as sporont matures, and electron-dense deposits on surface of sporont cytoplasmic membrane (arrowhead); bar = 0.55 μm. (**B**) Further separation of sporont (arrow) and deposition of electron dense material (arrowheads); bar = 0.50 μm. (**C**) Cytoplasmic membrane thickens as sporont matures; bar = 0.62 μm. (**D**) Fusiform sporonts within vacuolar envelope; bar = 1.0 μm. (**E**) All developmental stages, including meronts (white arrows) and dividing fusiform sporont (black arrows) within vacuolar envelope; bar = 2.0 μm.

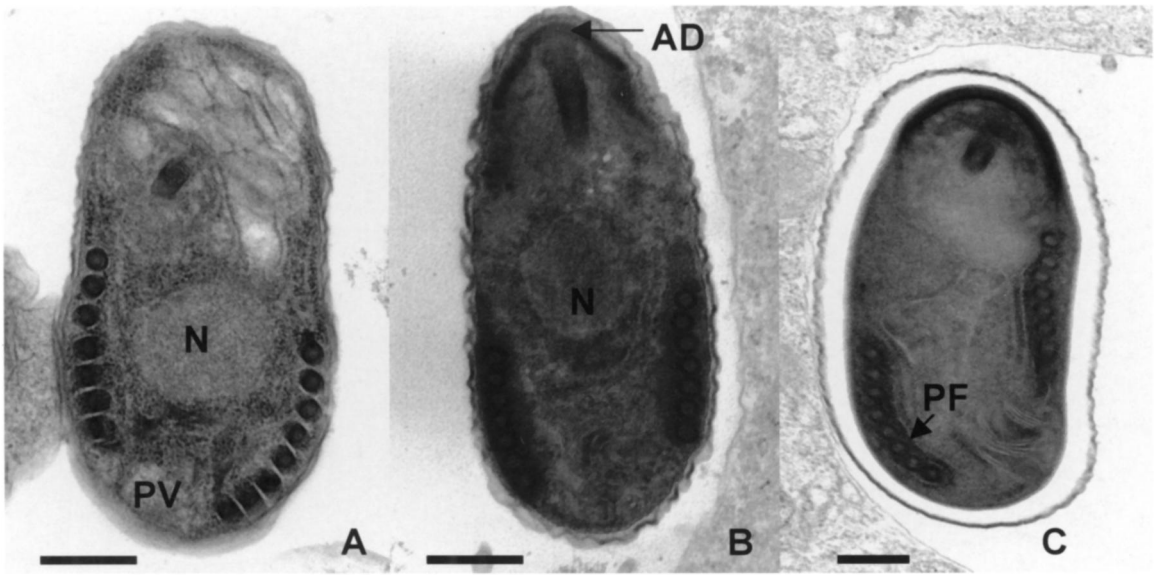

FIGURE 4. Sporoblasts and spores of *Encephalitozoon romaleae*. (**A**) Sporoblast; bar = 1 μm. (**B**) Immature spore; bar = 1 μm. (**C**) Mature spore; bar = 0.5 μm. AD = anchoring disk; N = nucleus; PF = polar filament; PV = polar vacuole.

Remarks

All 4 currently described *Encephalitozoon* species, *E. cuniculi* (type species), *E. hellem*, *E. intestinalis*, and *E. lacertae*, are remarkably homogenous in terms of morphological features and developmental pattern (Table III) (Sprague and Vernick, 1971; Pakes et al., 1975; Canning, 1981; Canning and Lom, 1986; Didier, Didier, Orenstein, and Shadduck, 1991; Didier, Didier, Friedberg et al., 1991; Cali et al., 1993; Koudela et al., 1998; and Visvesvara et al., 1999). In fact, distinguishing these species solely on the basis of morphology and development in the host tissues is problematic. The life cycle and morphology of the microsporidian isolated from *R. microptera* clearly corresponds with the characterization of *Encephalitozoon* sp. as provided by Vávra and Larsson (1999) and Visvesvara et al. (1999) and shows the following above-mentioned species-level features: it is monomorphic (homosporous) and monokaryotic (haplokaryotic), has proliferative stages surrounded by delicate membranes closely associated with the plasma membrane, and has polysporoblastic sporogony within a vacuole. The morphological characters that we identified in our study and that are among those traditionally used to differentiate microsporidian species, including spore size and shape and the number of polar filament coils in the spore, do not conclusively demonstrate a separate specific status. The new microsporidian does, however, produce the largest mature spores of the *Encephalitozoon* species.

Due to the apparent paucity of morphological characters for defining species of *Encephalitozoon*, we used molecular data to evaluate divergence of *E. romaleae* from the other members of the genus and assess its assignment as a new species. Outgroup species *Vittaforma corneae*, *Endoreticularus schubergii*, *Enterocytozoon bieneusi*, and *Ordaspora colligata* were selected for a more detailed analysis of *Encephalitozoon* species. *Vairimorpha necatrix* and *Nosema bombycis* were not used as outgroups because the extreme reduction of the hyper-variable regions would have eliminated several informative characters at this taxonomic level. The results placing *E. romaleae* as the sister taxon of *E. hellem* were highly supported (100% in all analyses) by bootstrap analysis (Fig. 6). It is also clear from sequence divergence (Table IV) that this species is indeed different from all known *Encephalitozoon* species; *E. romaleae* differs from the more distantly related *Encephalitozoon* species by 9–11% and from *E. hellem* by 4.4%, which exceeds the difference observed between the other closely related sister group relationships in the genus, e.g., *E. lacerate* and *E cuniculi* differ by 3.4%.

The laboratory and field sequences differ by a single base in the small subunit rRNA coding region. The sequence of the Florida isolate is identical to the Louisiana isolate with the exception of a possible 1-bp insertion at approximately position 63. The isolate requires further study but is considered to be the same species.

DISCUSSION

Encephalitozoon cuniculi, the first microsporidian to be recognized as a pathogen of mammals, was first observed in rabbits (Wright and Craighead, 1922) and was described by Levaditi et al. (1923). This species has a broad mammalian host range and most microscopic analyses in the 1980s suggested that infections in both immunologically compromised and competent human patients were caused by *E. cuniculi*. Didier, Didier, Friedberg et al. (1991), however, used biochemical and antigenic methods to describe *Encephalitozoon hellem* found in several acquired immunodeficiency syndrome patients. *Encephalitozoon cuniculi* and *E. hellem*, the latter also recovered from several avian species, are morphologically similar and are distinguished only by antigenic, biochemical, or nucleic acid analysis (Didier, Didier, Orenstein, and Shadduck, 1991; Didier, Didier, Friedberg et al., 1991). A third species, *Encephalitozoon* (*Septata*) *intestinalis* (Orenstein, Dieterich, and Kotler, 1992; Orenstein, Tenner et al., 1992), which has ultrastructural similarities to other members of the genus, has been recovered primarily from humans (Didier, Didier, Orenstein, and Shadduck, 1999) and from domestic animals (Bornay-Llinares et al., 1998). A fourth species, *Encephalitozoon lacertae*, was recovered from a lacertid lizard (Canning and Lom, 1986) and African skinks (Koudela et al., 1998) and is most closely related to *E. cuniculi* (Koudela et al., 1998).

The lubber grasshopper, *Romalea microptera*, ranges across the southeastern United States from the southern tip of Florida, north to South Carolina and Tennessee, and west to eastern Texas. This species is flightless and sedentary, and the different populations are relatively genetically isolated (Mutun and Borst, 2004), leading to considerable morphological, physiological, ecological, and life history variation among different geographic

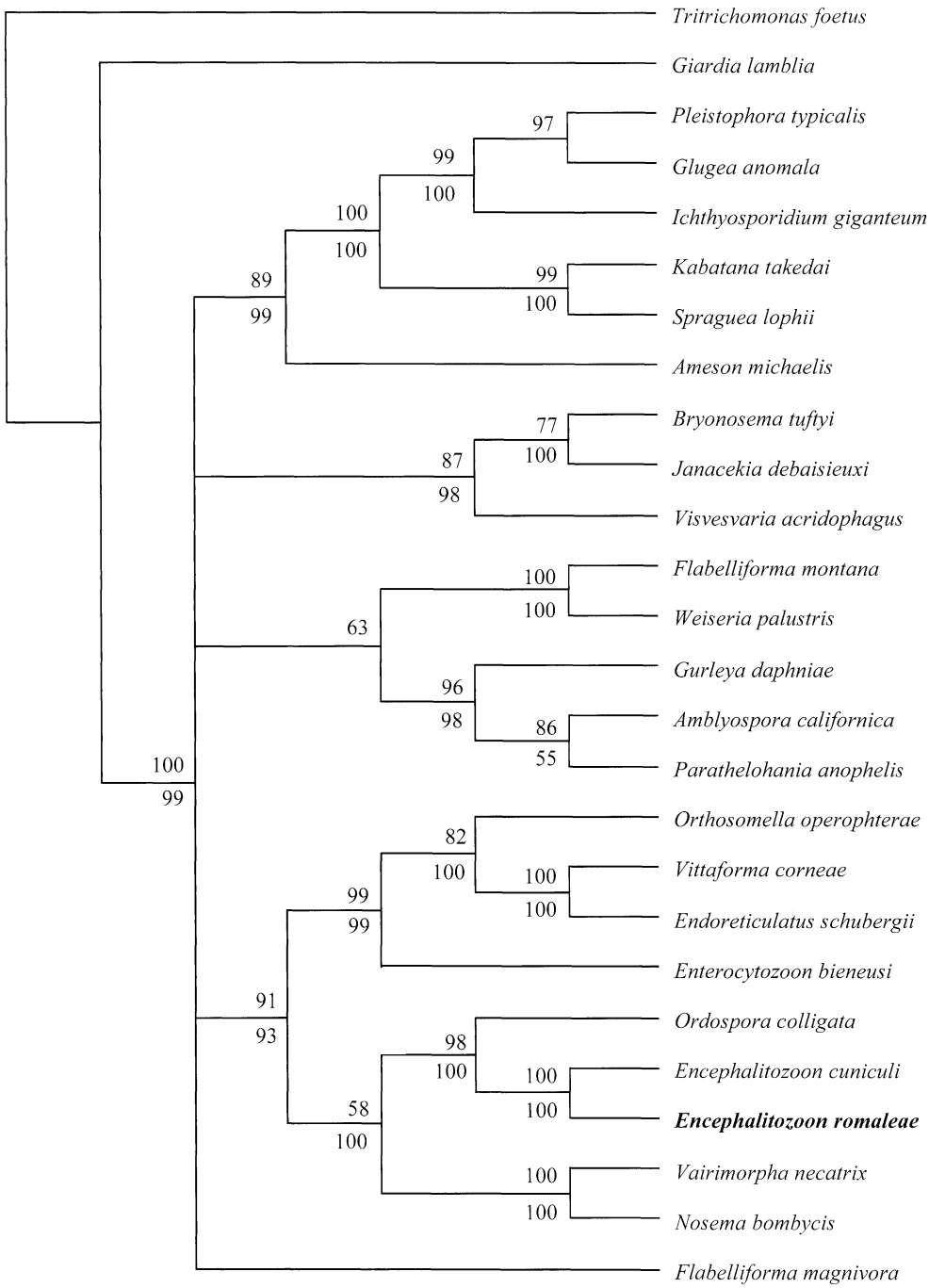

FIGURE 5. Maximum Parsimony bootstrap tree of broad sampling of microsporidian taxa using *Tritrichomonas foetus* and *Giardia lamblia* as outgroups. Numbers above and below the branches represent parsimony bootstrap and neighbor joining bootstrap support, respectively. Placement of *E. romaleae* highlighted.

populations (Hatle et al., 2002, 2004; Homeny and Juliano, 2007; Huizenga et al., 2008). Field surveys have detected the microsporidian pathogen infecting *R. microptera* only at Weeks Island, Louisiana, and Jacksonville, Florida. No infections were detected in grasshoppers collected from 10 additional sites in Florida, Georgia, and Louisiana (n = 171), nor were infections found in 17 *Taeniopoda eques* and 10 *Brachystola magna* grasshoppers collected from Arizona and New Mexico (Table I).

In the laboratory, the virulence of the microsporidium and the gross pathology (lethargy, anal and oral discharge, and death of the host) seemed to vary among the different *R. microptera* populations, but all exposed individuals in the laboratory colonies developed infections. Colony grasshoppers originating from southern Florida showed no symptoms of infection other than presence of microsporidia in feces and tissue. *Romalea microptera* collected from Athens, Georgia, and Weeks Island, Louisiana,

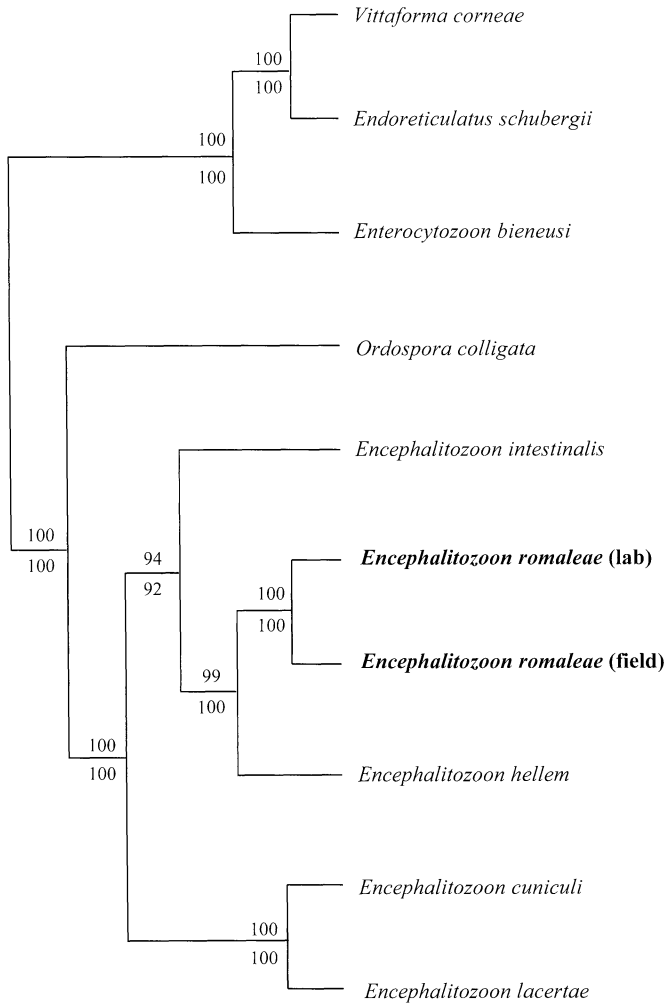

FIGURE 6. Maximum Parsimony bootstrap tree of *Encephalitozoon* matrix. *Vittaforma corneae*, *Endoreticularus schubergii*, *Enterocytozoon bieneusi*, and *Ordospora colligata* are used as outgroups. Numbers above and below the branches represent parsimony bootstrap and neighbor joining bootstrap support, respectively.

TABLE IV. Percent sequence difference among the five *Encephalitozoon* species.

Taxon	1	2	3	4	5
1. *E. intestinalis*	—				
2. *E. cuniculi*	10.063	—			
3. *E. lacertae*	10.903	3.392	—		
4. *E. romaleae*	9.379	10.846	11.118	—	
5. *E. hellem*	9.154	10.935	11.088	4.447	—

became lethargic, secreted brown fluid from the mouth and anus, and quickly died. Our observations on virulence and pathology are, however, anecdotal, and it is possible that a co-occurring unrecognized pathogen, such as a virus, produced some of the observed pathologies.

Although reared in separate cages, all wild-caught, uninfected *Taeniopoda eques* and *Brachystola magna* grasshoppers became infected with the microsporidian pathogen within 6 days of being brought to ISU and placed in the rearing room with the *R. microptera* colony. These species exhibited more extreme symptoms than those observed in *R. microptera*, including rapid mortality, which can be an indication of a nontarget host response to microsporidian infection (Solter et al., 1997). No infections were produced in *Melanoplus femurrubrum* (De Geer) (Orthoptera: Acrididae), *Gryllodes sigillatus* (F. Walker) (Orthoptera: Gryllidae), *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), *Aedes albopictus* (Skuse) (Diptera: Culicidae), or *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) fed freshly produced mature spores.

Despite the morphological similarity to other *Encephalitozoon* species, we believe that the molecular evidence, larger average spore size, presence of electron-dense material on the surface of the sporonts, and unusual invertebrate host association is sufficient to assign this microsporidian species status.

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TABLE III. Comparison of spore morphology in species of *Encephalitozoon*.

Species	Spore shape	No. polar filament coils	Spore size (μm) (length × width) Fresh	Spore size (μm) (length × width) Fixed	Reference
E. cuniculi	Ovoid, ellipsoid	3–8	2.5 × 0.5–1.0	1.8–2.4 × 1.0–1.5*	Sprague et al., 1992 Visvesvara et al., 1999 Canning and Vávra, 2000
E. hellem	Ovocylindrical	4–9		2.0–2.5 × 1.0–1.5*	Didier, Didier, Friedbery et al., 1991
E. intestinalis	Ovocylindrical	4–7		2.0 × 1.2* 1.2–2.4 × 0.9–1.2†	Cali et al., 1993 Del Aguila et al., 1998
E. lacertae	Elongate ellipsoid Slightly curved	7	3.5 × 1.5	2.7 × 1.2†	Canning, 1981 Canning and Lom, 1986
E. romaleae n. sp.	Ovocylindrical	7–8	3.97 × 1.95	3.01 × 1.81†	This study

* Transmission electron microscopy.
† Light microscopy.

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