The Journal of Eukaryotic Microbiology

Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Establishing a New Species *Encephalitozoon pogonae* for the Microsporidian Parasite of Inland Bearded Dragon *Pogona vitticeps* Ahl 1927 (Reptilia, Squamata, Agamidae)

Yuliya Y. Sokolova[a,b], Kanako Sakaguchi[c] & Daniel B. Paulsen[c]

a Microscopy Center, School of Veterinary Medicine, Louisiana State University, Baton Rouge 70803, Louisiana, USA
b Institute of Cytology, Russian Academy of Sciences, St. Petersburg 194064, Russia
c Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge 70803, Louisiana, USA

Keywords
herpetology; intracellular parasitism; Lacertilia; Microsporidiosis; molecular phylogeny; reptilian diseases; ultrastructure.

Correspondence
Y. Sokolova, Microscopy Center, Department of Comparative Biological Sciences, School of Veterinary Medicine, Louisiana State University, 1909 Skip Bertman Drive, Baton Rouge 70803, LA, USA
Telephone/FAX number: +1225-578-9899;
e-mail: sokolova@lsu.edu

Received: 7 December 2015; revised 6 January 2016; accepted January 6, 2016.

doi:10.1111/jeu.12296

ABSTRACT

The microsporidium parasitizing Inland Bearded Dragons *Pogona vitticeps*, and developing primarily in macrophages within foci of granulomatous inflammation of different organs, is described as a new species *Encephalitozoon pogonae*. Establishing the new species was based on sequencing the ITS-SSUrDNA region of the ribosomal gene and consequent SSUrDNA-inferred phylogenetic analyses, as well as on comparison of pathogenesis, host specificity, and ultrastructure among *Encephalitozoon* species and isolates. The new species is closely related to *E. lacertae* and *E. cuniculi*. Analysis of the literature suggests that this microsporidium has been reported previously as an unidentified microsporidian species or isolate of *E. cuniculi* and may represent a common infection in bearded dragons. All stages of *E. pogonae* develop in parasitophorous vacuoles. Uninucleate spores on methanol-fixed smears measured 2.1 × 1.1 μm, range 1.7–2.6 × 0.9–1.7 μm; on ultrathin sections spores measured 0.8–1.1 × 1.8–2.2 μm. Ultrastructural study revealed 3–6 polar filament coils, a mushroom-shaped polar disk, and a polar sac embracing half of the volume occupied by the lamellar polaroplast. In activated spores, polar filament everted eccentrically. The overall morphology and intracellular development of *E. pogonae* were similar to other *Encepahalitozoon* spp. We also review the existing data on microsporidia infecting reptiles.

MICROSPORIDIA in reptiles have been reported rarely (Snowden 2014). However, this fact may not reflect actual prevalence but rather result from biased sampling. Most cases of microsporidiosis have been reported from animals kept in terrariums, like bearded dragons or colubrid snakes (Table 1), suggesting that wild reptiles have not been yet studied as potential hosts for microsporidia.

In 2014, microsporidian infection was identified postmortem in *Pogona vitticeps*, which had been brought to the Veterinary Teaching Hospital of the LSU School of Veterinary Medicine for examination (Sakaguchi et al. 2015). The pathogen was initially identified as *Encephalitozoon cuniculi* basing on similarity of morphology, ITS sequence, and disease symptoms to those of the *P. vitticeps* parasite described recently in Europe as a new genotype of *E. cuniculi* (Richter et al. 2013). However, further analyses reported herein, suggests that the *P. vitticeps* microsporidium (hereafter in the text, tables and figures *Encephalitozoon* ex. *Pogona*), a specialized parasite of reptiles, deserves the status of a separate species. The goal of this paper was to justify the erection of the new species for the *Encephalitozoon* ex. *Pogona* based on novel ITS-SSUrDNA-sequence information and associated phylogenetic analyses, as well as on comparison of pathogenesis, host specificity, and fine morphology among *Encephalitozoon* species and isolates. We also review the existing data on microsporidia infecting reptiles that have never been summarized before.

MATERIAL AND METHODS

Animal, clinical findings, and specimen preparation

A 10-mo-old, female bearded dragon, weighing 191.4 g, was presented as an emergency to the Louisiana state University Zoological Medicine Service. The animal was

Table 1. Records on microsporidia infections in reptiles

Microsporidia genus and species 1	Host species (infra-order and/or family), common name 2	Spore morphology, tissue tropism, and pathogenesis 3	References, country 4
Microsporidiosis in lizards (Reptilia, Squamata, Lacertilia)			
Encephalitozoon cuniculi	*Pogona vitticeps* (Iguania, Agamidae), Inland Bearded Dragon	Oval spores 1.4–2.1 × 0.9–1.2 μm; in pv; 5–6 pfc; in macrophages in granulomatous inflammation in liver, adrenal glands, ovaries, disseminated	Richter et al. 2013; Austria
E. lacertae	*Podacris muralis* (Lacertoidea, Lacertidae), Common Wall Lizard	Ellipsoid curved spores 3.5 × 1.5 μm; in PVs; > 7pfc; restricted to the gut epithelium	Canning 1981; GB
E. lacertae	*Mabuya perrotetii* (Scincomorpha), African Skink	Ellipsoid curved spores 2.3–3.5 × 1–1.5 μm; in pv; 5–7 pfc; restricted to the gut epithelium	Koudela et al. 1998; Czech Republic
Encephalitozoon ex. *Pogona*	*Pogona vitticeps* (Iguania, Agamidae)	Oval spores 1.7–2.6 × 0.9–1.7 μm; in pvs; 3–5.5 pfc; in macrophages in granulomatous inflammation in ovaries, adrenal glands, tarsal joint	Herein, USA
Unidentified microsporidium	*Pogona vitticeps* (Iguania, Agamidae)	Oval spores; in pvs; 2.0–2.5 × 1.0–1.1 μm; 6 pfc; in macrophages in granulomatous inflammation in adrenal gland, ovaries, liver, disseminated	Jacobson et al. 1998; USA
Unidentified microsporidium	*Pogona vitticeps* (Iguania, Agamidae)	Spores within foci of granulomatous yolk sacculitis and placentitis in hatchlings	Mitchel and Garner 2011; USA
Unidentified microsporidium	*Pogona vitticeps* (Iguania, Agamidae)	Gram+ spores in macrophages in granulomatous inflammation in pelvic limb	Strunk and Reavill 2011; USA
Unidentified microsporidium	*Intellagama leueurii* (Iguania, Agamidae), Water Dragon	Gram+ spores in groups; Acid Fast+ cysts in muscles and macrophages; myonecrosis, granulomatous inflammation	(Reece and Hartley 1994) in Ladds 2009; Australia
Unidentified microsporidium	*Uroplatus henkeli* (Gekkota), Leaf-tailed Gecko	Gram+ spores in muscles, diffused myositis, "intralesional organisms"	(Boyd and Gregory 2001) in Ladds 2009; Guyenot and Naville 1924; USA
Unidentified microsporidium	*Nephrurus amyae* (Gekkota), Centralian Knob-tailed Gecko	Ovaries were replaced by masses of presumed microsporidia in macrophages	(Reece and Hartley 1994) in Ladds 2009; Australia
Microsporidiosis in snakes (Reptilia, Squamata, Serpentes)			
Heterosporis anguillarum	*Thamnophis sirtalis* (Colubridae) Garter Snake	Macrospores 5.5–6.8 × 2.0–4.8 μm, 29–42 pfc; Microspores 2.7–3.5 × 1.8–2.4,8 μm, 11 pfc; > 50 spores in sporophorocyst; muscles and disseminated	Richter et al. 2014; Austria
Pleistophora[a] (*Glugea*) *danilewskyi*	*Natrix (Tropidonotus) natrix* (Colubridae) Grass Snake	Pansporoblast gives rise to multiple spores; in intercostal and spinal muscles	(Debasieux, 1919) in Canning et al. 1964; France
Pleistophora (Glugea) danilewskyi/ Microsporidium (Glugea) ghigi	*Natrix (Tropidonotus) natrix* (Colubridae) Grass Snake	Spores 3–4/2–3 μm long; 16–100 spores in pansporoblasts; cysts in muscles and connective tissue; hyperparasites of cestodes/trematodes	Guyenot and Naville 1924; Sprague 1977; Italy

(continued)

Table 1 (continued)

Microsporidia genus and species 1	Host species (infra-order and/or family), common name 2	Spore morphology, tissue tropism, and pathogenesis 3	References, country 4
Pleistophora atretii	*Atretium schistosum* (Colubridae), Asian water snake, Olive Keelback	Spores oval, 4.5–5.4 × 1.8–2.5 μm, 320–360 spores in pansporoblast; cysts up to 1 mm adjacent to vertebral column	Narasimhamutri et al. 1982; India
Unidentified microsporidium	*Thamnophis sirtalis* (Colubridae) Common Garter Snake	Spores broadly oval, > 16 spores within pansporoblast membrane; micro- and macrospores; no cysts, in muscles	Gardiner et al. 1968, USA
Unidentified microsporidium	*Pelamus platura* (Elapidae) Yellow-bellied Sea Snake	Gram+ organisms; Acid Fast+ cysts in muscles and macrophages; myonecrosis, granulomatous inflammation	(Reece and Hartley 1994) in Ladds 2009; Australia
Microsporidiosis in rynchocephalids (Reptilia, Rynchocephalia)			
Pleistophora sp.	*Sphenodon punctatus* (Sphenodontidae) Tuatara	Pyriform spores 4.0 × 2.2 μm; 16–100 spores in pansporoblast; in disintegrated muscle fibers; myositis of tongue and skeletal muscles	Liu and King 1971; USA
Microsporidiosis in crocodiles (Reptilia, Crocodilia)			
Encephalitozoon hellem[b]	*Crocodylus johnstoni* (Crocodillidae) Freshwater Crocodile	Broadly oval spores 5–6 × 2–3 μm; 20–26 pfc; no pv; in hepatocytes and phagocytes; no gross pathology recorded	Scheelings et al. 2015, Australia

pv, parasitophorous vacuole; pfc, polar filament coils.

[a]We use here the senior synonym "*Pleistophora*, Gurley 1893" (Becnel et al. 2014) instead of "*Plistophora,* Labbe 1899" occasionally used in original publications.

[b]Identification is likely erroneous, see the text.

lethargic and died shortly. At necropsy, tissue samples were fixed in 10% neutral buffered formalin and either placed in cassettes, processed through an alcohol series, cleared in xylene, and embedded in paraffin, or submitted for electron microscopy (EM). Samples from the cardiac mass were stored at −20 °C.

Light microscopy

The sections (5–7-μm thick) were stained with hematoxylin and eosin (HE), Gram, Giemsa and Gomori's methenamine silver. Smears prepared from formalin-fixed tissues were treated with methanol for 5 min, air-dried, and stained with modified Trichrome Blue, or BactidropTM Calcoflour White (Remel, Lenexa, KS) as previously described for fresh stool smears (Sokolova et al. 2011a, b). Excitation/emission ratio for fluorescent microscopy of Cacofluor White-stained smears was 365/435 nm. "Thick" (0.5–1 μm) sections prepared from Epon-Araldite - embedded tissues (see below) were stained with modified Methylene blue (Sokolova and Fuxa 2008), examined and photographed with a Zeiss Axioplan microscope equipped with an Olympus DP73 (Olympus Corporation, Tokyo, Japan) digital camera.

Transmission and scanning electron microscopy

Approximately 1 mm^3 pieces of formalin-fixed periovarian/adrenal tissues, were excised and subjected to transmission (TEM) and scanning (SEM) electron microscopy according standard protocols. Briefly, samples were further fixed in a mixture of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodilate buffer for 2 h, washed several times in the same buffer supplemented with 5% sucrose. For TEM, samples were postfixed in 1% osmium for 1 h, washed in water, dehydrated in an ascending ethanol series and propylene oxide, and embedded in Epon-Araldite. Blocks were sectioned with an Ultratome Leica EM UC7. Thin, 70–80 nm, sections were stained with uranyl acetate and lead citrate and examined in JEOL JEM 1011 microscope with the attached HAMAMATSU ORCA-HR digital camera. For SEM, specimens were fixed with glutaraldehyde only, dehydrated through the ethanol series, followed by exchanging ethanol with CO_2 in Polaron E3000 Standard Critical Point Drier. Dried samples were mounted on 13 mm aluminum mount specimen stubs covered with carbon adhesive tabs, sealed with colloidal silver paste, coated with Gold/Palladium in EMS 550X Sputter Coater for 4 min to achieve the thick-

ness of coating 20–25 nm., and examined in Fei Quanta 200 ESEM in a high vacuum mode at 20 kV. All reagents for EM were from EMS Chemicals (Fort Washington, PA).

DNA isolation

Infected heart tissue was stored at −20 °C for about 3 wks before being used for DNA isolation. DNA was isolated by Zymo Research Quick-DNA™ Universal Kit (Zymo Research, Irvine, CA) using Solid Tissue Protocol with modifications. Briefly, 20-mg pieces of heart tissue with visible whitish lesions were thawed in a water bath at 37 °C. Infection with microsporidia was confirmed by examining the Calcofluor White-stained smears under a fluorescent microscope. The tissue samples were digested in 200 μl of Solid Tissue Buffer contained 20 mg/ml Proteinase K for 4 h at 55 °C. To release DNA content from spores, 200 mg of 0.1 mm glass beads were added to Eppendorf tubes with the samples. Tubes were shaken with a Bullet Blender™24 bead-beater (Next Advance, Inc., Averill Park, NY) for 90 s at maximal speed. Tubes were placed on a hot plate at 95 °C for 3–5 min, and then transferred to ice. Beads and debris were spin down by centrifuged at 12,000 *g*. The supernatants (about 150 μl for each sample) were subjected to DNA isolation in strict accordance with the manufacturer's protocol. The DNA was eluted in 35 μl of DNA elution buffer and was used as a template in downstream polymerase chain reactions (PCRs).

PCR and sequencing

The SSU rRNA sequence was amplified by PCR with 1–3 μl of DNA eluate using either OneTaq® Quick-load® master mix (New England Biolabs, Inc., Ipswich, MA) or PureTaq™ Ready-To-GO™ PCR beads (GE Healthcare, Buckinghamshire, UK). For amplification of SSUrDNA V1 (5′—CAC CAG GTT GAT TCT GCC TGA C—3′) and 1492r (5′—GGT TAC CTT GTT ACG ACT T-3′) primers were used (Vossbrinck et al. 2004). The PCR cycle in this case included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles with a denaturation at 95 °C, 30 s, annealing at 45 °C, 60 s, and elongation at 72 °C, 120 s, and a final extension at 72 °C for 10 min. Internal transcribed spacer (ITS) with flanking regions of the small (SSU) and large (LSU) subunits of the ribosomal operon were amplified by MSP3 forward (GGA ATT CAC ACC GCC CGT C(A,G)(C,T) TAT) and MSP2A reverse (TCA CTC GCC GCT ACT) primers (Katzwinkel-Wladarsch et al. 1996). The ITS cycle comprised initial denaturation at 95 °C for 5 min, 36 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 1 min, and elongation at 72 °C for 2 min, followed by final elongation for 10 min. The expected amplicon sizes were approximately 1,200 and 350 base pairs (bps), for V1f-1492r and MSP3-MSP2A regions, respectively.

Amplicons were loaded onto a 2% agarose gel, and bands of the expected size were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD). The purified PCR bands were sequenced with the Applied BioSystems BigDye Terminator technology (version 3.1) and the resulting chromatogram was obtained by a Beckman Coulter Seq 8000 DNA. The primers for sequencing were V1, 530r (5′—CCG CGG C(T/G) G CTG GCA C—3′), 530f (5′—GTG CCA GC (G/A) GCC GCG G), 1061f (5′—GGT GGT GCA TGG CCG—3′), 1492r (Vossbrinck et al. 2004; Weiss and Vossbrinck 1999), MSP3 and MSP2A. These primers produced overlapping sequences that were assembled with ChromasPro. 1.34 software (http://www.technelysium.com.au/ChromasPro.html). Direct PCR amplification and sequencing were performed twice for two DNA samples from two different pieces of infected heart tissue.

Phylogenetic analyses

For the SSU rRNA phylogenetic analysis, 19 sequences were retrieved from Genbank (see Table S1 for Accession numbers). The selected sequences included seven sequences of all four known ITS genotypes of *E. cuniculi* isolated from various mammalian hosts and geographic localities, two—of *Encephalitozoon intestinalis* from humans, three—of *Encephalitozoon hellem* from humans and birds, two of *Encephalitozoon romaleae* from field and laboratory populations of lubber grasshoppers; and one—of *Encephalitozoon lacerate* from an African skink. Four microsporidia species chosen as sources for outgroup sequences, belonged to the two major supertaxa of terrestrial microsporidia (Vossbrinck and Debrunner-Vossbrinck 2005). "Clade 4" to which *Encephalitozoon* spp. belong, was represented by *Mockfordia xantocoecilia* from barklice, a sister taxon to the *Encephalitozoon* clade (Sokolova et al. 2010), and two human microsporidia, *Vittaforma corneum* and *Enterocytozoon bieneusi*; "Clade 2"—by *Paranosema grylli* from crickets. The SSUrDNA region of the novel sequence was aligned with abovementioned sequences by Muscle (MEGA 5.05), with default parameters (Edgar 2004). The final datasets resulted in 1,202 informative positions. Pairwise genetic distances were calculated by the Kimura-2 parameter method with a gamma distribution (Tamura et al. 2011). The alignments were subjected to phylogenetic reconstructions by maximum likelihood (ML) and maximum parsimony (MP) using MEGA 5.05 (Tamura et al. 2011). The ML phylogenetic analyses were based on the Hasegawa-Kishino-Yano model with a discrete Gamma distribution model (HKY + G) of nucleotide substitution (Hasegawa et al. 1985) as suggested by Modeltest. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Felsenstein 1985; Nei and Kumar 2000). Bootstrap tests were limited by 1,000 replications for both analyses.

RESULTS

Histopathology, tissue tropism, and light microscopy

The pathogenesis of the microsporidium was associated with granulomatous inflammation observed in the right tar-

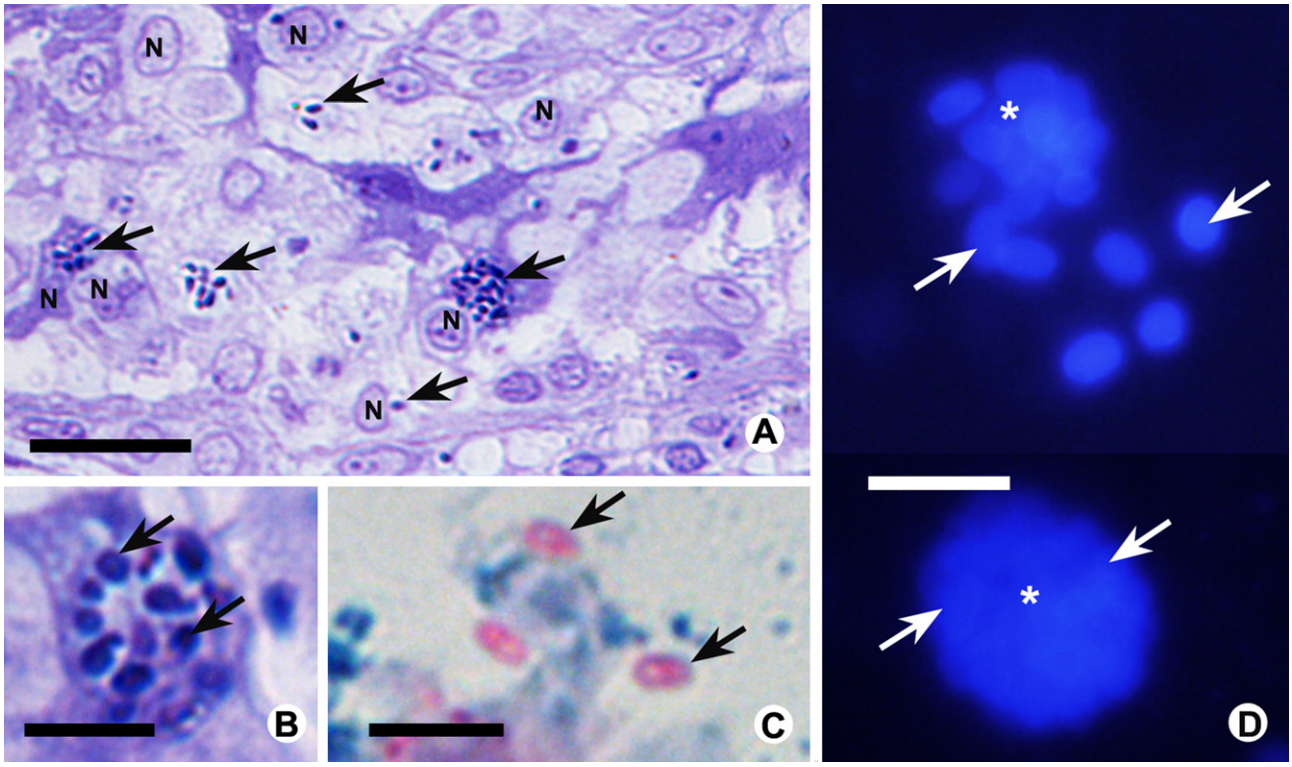

Figure 1 *Encephalitozoon* ex. *Pogona*: light microscopy. (**A**). 500-nm-thick sections through a granuloma stained with Methylene blue. Arrows indicate microsporidian spores within macrophages. N, macrophage nucleus. (**B**) A group of spores (arrows) in a parasitophorous vacuole, at higher magnification. (**C**) Spores (arrows) on methanol-fixed smears stained with Trichrome. (**D**) Spores (arrows) and parasitophorous vacuoles (asterisks) with spore stained with Calcofluor White. Scale bars: A, 20 μm; B–D, 5 μm.

sal joint, heart, and adrenal glands, which were subjected to the most severe pathological transformation. A bilateral mass located in vicinity of ovaries was composed of granulomatous inflammation and completely effaced the adrenal glands. A large granuloma was also seen attached to the heart. The granulomatous inflammation was composed of central core of numerous heterophils (type of granulocytes abundant in blood of birds and reptiles) surrounded by layers of macrophages and multinucleated giant cells. A few macrophages and multinucleated cells within the layers were infected with microsporidia. Spores inside the host cells were often seen grouped together (Fig. 1A, B). Infection was not observed inside intestinal epithelium cells; only rare spores were visible in the gut lumen (not shown).

On methanol-fixed smears stained with Trichrome or Calcofluor White prepared from thawed infected tissues, oval spores of typical for *Encephalitozoon* spp. size, shape, and staining pattern were seen (Fig. 2C, D). Spores measured 2.1 ± 0.03 × 1.1 ± 0.03 μm, and the range was 1.7–2.6 × 0.9–1.7 (n = 38).

Electron microscopy

Scanning electron microscopy revealed oval spores with smooth surface clustered together within parasitophorous vacuoles (PVs) (Fig. 2A). Preservation of fine structure of infected tissues on ultrathin sections was poor due to improper fixation with 10% formalin. Majority of infected macrophages contained large PVs with numerous spores (Fig. 2B–D). At the same time, macrophages with PVs enclosing a single parasite cells were not uncommon as well. Individual PVs never contained stages other than mature spores (Fig. 2D), occasionally activated or firing.

A few prespore stages were noticed on the periphery of PVs contacting PV membrane (Fig. 2D, E). Most prespore stages were destroyed during the fixation procedure, or were poorly preserved (Fig. 2D). Remnants of mitochondria were regularly seen adjacent to SV membrane, often in the region of contact with a developing parasite (Fig. 2B, D). Spores on thin sections (Fig. 2F–I) measured 0.8–1.1 × 1.8–2.2 μm. They had one nucleus and a large posterior vacuole that often collapsed during processing causing characteristic shrinkage in central parts of spores (Fig. 2F, G, I). Number of polar filaments coils ranged from 3 to 5.5 with a mean of 3.94 ± 0.1 (n = 32) and median of 4. The anchoring disk was mushroom-shaped. The polar sac embraced approximately half of the space occupied by the lamellar polaroplast (Fig. 2F). The straight region of the polar filament, about 1 μm long, traversed the long axis of the spore (Fig. 2H). Spore envelopes varied in thickness from 44 to 80 nm and were composed of the plasma membrane (8–12-nm thick), endospore (26–49 nm), and exospore (10–19 nm). Some spores within SVs displayed

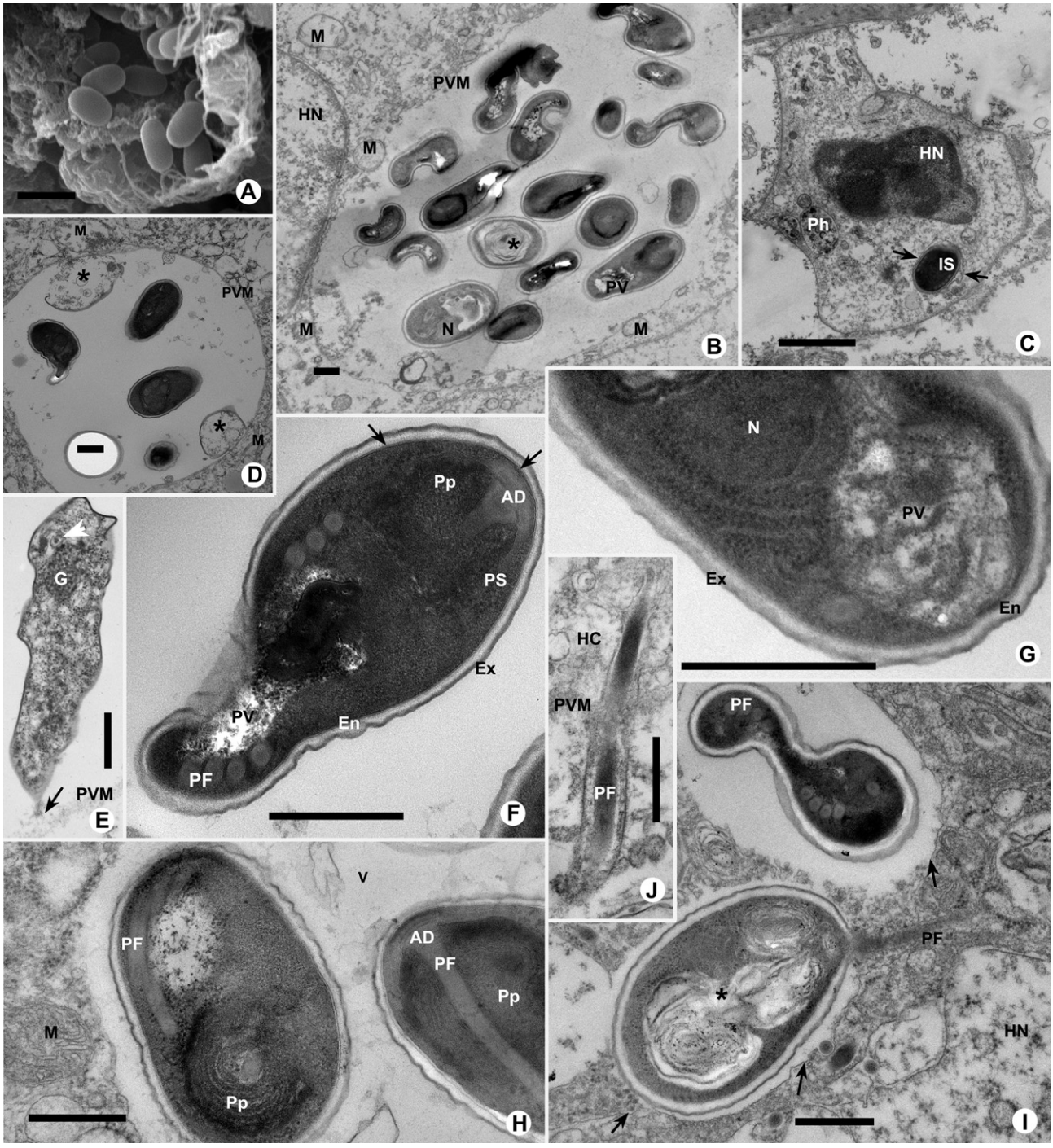

Figure 2 *Encephalitozoon* ex. *Pogona*: electron microscopy (EM). (**A**) Scanning EM image of oval spores of *Encephalitozoon* sp. with smooth surface falling out of a fractured parasitophorous vacuole. (**B–I**) Transmission EM. (**B**) Section through parasitophorous vacuole with numerous spores. Asterisk marks a firing spore. (**C**) An individual spore (presumably phagocityzed) within a membrane-bound vacuole (arrows) in the cytoplasm of the macrophage. (**D**) Section through parasitophorous vacuole: two poorly preserved prespore stages (asterisks) contact the membrane of the parasitophorous vacuole. (**E**) Sporoblast connected with the membrane of parasitophorous vacuole (arrow). Polar tube (arrowhead) morphogenesis occurs in Golgi cisternae. (**F–H**) Sections through mature spores with standard sets of organelles. (**I**) Section through parasitophorous vacuole in vicinity of host cell nucleus. The spore marked with an asterisk is in the process of firing. (**J**) Everted polar filament passing through the membrane of parasitophorous vacuole. AD, anchoring disk; En, endospore; Ex, exospore; G, Golgi complex; HC, host cytoplasm; HN, host nucleus; IS, individual spore; M, mitochondria; N, parasite nucleus; Ph, phagosome; Pp, polaroplast; PV, posterior vacuole; PVM, parasitophorous vacuole membrane. Scale bars: A, C, 2 μm; B, D–J, 500 μm.

Table 2. Comparison of spore morphology among three isolates of *Encephalitozoon* ex. *Pogona* and five *Encephalitozoon* spp.

Species/Isolate	Number of polar filament coils	Width × length, of fixed spores: range (and means if available), μm	Average width/ length ratio	References
Encephalitozoon ex. Pogona[a]	6	1.8–2.9 × 0.8–1.8	–	Jacobson et al. 1998
Encephalitozoon ex. Pogona[b]	5–6	1.4–2.1 × 0.9–1.2	–	Richter et al. 2013;
Encephalitozoon ex. *Pogona*	3–5.5	1.7–2.6 × 0.9–1.7 (2.1 × 1.1)	0.52	This study
E. cuniculi	5–7	2.0–3.0 × 1.0–1.5 (2.5 × 1.5)	0.60	Visvesvara et al. 1999; Ghosh et al. 2014
E. intestinalis	4–7	2.0–2.5 × 1.0–1.2 (2.0 × 1.2)	0.60	Ghosh et al. 2014
E. hellem	4–9	2.0–2.5 × 1.0–1.5 (2.0 × 1.0)	0.50	Didier et al. 1991;
E. romaleae	7–8	(3.0 × 1.8)	0.60	Lange et al., 2009
E. lacertae	5–7	2.5–3.5 × 1.0–1.5 (2.7 × 1.2)	0.44	Koudela et al.,1998; Canning 1981

[a]In the original publication *Microsporidium* sp. (Jacobson et al., 1998).
[b]*E. cuniculi* (Richter et al. 2013).

irregular membrane whorls characteristic for the spores in the process of firing (Fig. 2B, I). The polar filament everted eccentrically (Fig. 2I). Occasionally, discharged polar filaments were seen inside PVs; some penetrated inside the host cytoplasm through the PV membrane (Fig. 2J).

Sequenced-based phylogenetic analyses

The consensus sequence of 1,535 bp length was deposited in Genbank under Accession # KR99831. It included the complete SSU (positions 1–1,299) and ITS (1,300–1,342) regions, and a part of LSU (1,343–1,535).

ITS region included 2 + 2 GTTT repeats, which were preceded and separated by the GTTTT repeat to produce the sequence: 5′GTTTTGTTTGTTTGTTTTGTTTGTTT3′. This motif does not match any of 4 known ITS genotypes of *E. cuniculi*, or other *Encephalitozon sp.*, except for the reported *E. cuniculi* isolated from the same reptile, *P. vitticeps* (Richter et al. 2013).

Distance matrix analysis based on alignment of the recovered SSUrDNA sequence with 19 orthologues from GenBank displayed minimal pairwise divergence values ("evolutionary distances") ranged from 0.0101 to 0.0109 between the novel microsporidium and *E. cuniculi* isolates from a rabbit, a mouse, a fox, a dog, and humans (2 isolates) (Table 2). At the same time the distances separated these *E. cuniculi* isolates from each other were essentially less and ranged from 0.0008 to 0.0025.

SSUrDNA-inferred phylogenetic analyses by Maximum Parsimony (MP) and Maximum Likelihood (ML) produced trees of nearly identical topologies, well consistent with previously published phylogenies (Fig. 3). All *Encephalitozoon* spp. sequences clustered in a well-supported clade that displayed dichotomy with the *M. xanthcoecilia* sequence. *Vittaforma corneae* and *E. bieneusi* clustered together and emerged as a sister lineage to the *Encephalitozoon* group within the "Clade 4" (sensu (Vossbrinck and Debrunner-Vossbrinck 2005). The *E. cuniculi* branch split in three groups: the (i) *E. hellem-E. romaleae*, (ii) *E. intestinalis* and (iii) *E. cuniculi-E. lacertae-Encephalitozoon* ex. *Pogona* lineages. In ML analysis the *E. cuniculi* branch fell in dichotomy with *Encephalitozoon laceratae*, and the novel isolate displayed a basal position to the *E. cuniculi-E. lacerate* branch. In MP tree *Encephalitozoon* ex. *Pogona* appeared as a sister to the *E. cuniculi* group, whereas *E. lacertae* branch took a basal position. The node support for *E. lacertae* and *E. cuniculi* grouping in ML tree was low (58%), so the phylogenetic relationships among the *E. laceratae*, *Encephalitozoon* ex. *Pogona*, and *E. cuniculi* branches remain unresolved.

DISCUSSION

Encephalitozoon sp. infecting bearded dragon is a new species

Analysis of the literature suggests that microsporidiosis in bearded dragon may be a common disease (Jacobson et al. 1998; Richter et al. 2013; Scheelings et al. 2015; Table 1). The records, mostly coming from veterinarians providing medical care for this popular pet, are incomplete and focused more on the disease than on the pathogen. They are often published in poorly available proceedings of professional veterinarian meetings. However, even these incomplete records allow the conclusion that the etiological agent of all cases of *P. vitticeps* microsporidiosis (*Pv* microsporidiosis) is a microsporidium producing small spores of the size of a bacterium within macrophages in granulomatous lesions of different organs, commonly the liver, heart, adrenal glands, ovaries, and joints. The pathological signs of *Pv* microsporidiosis are consistent among all described cases, including our observations, and are summarized in Table 1. Neither of the records mentioned the presence of other pathogens or any accompanying health problems, so an opportunistic, incidental nature of the disease is unlikely. Moreover, there is strong evidence

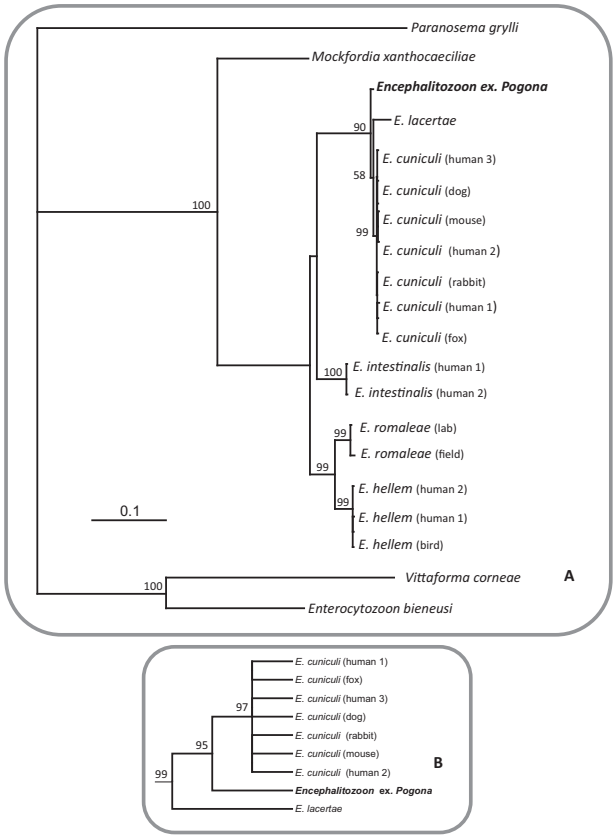

Figure 3 Relationships of *Encephalitozoon* ex. *Pogona* with other microsporidia suggested by SSU-rDNA-inferred phylogenetic analysis by Maximum Likelihood (ML) (**A**) and Maximum Parsimony (MP) (**B**). The final dataset included 20 sequences and 1,202 informative positions. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown at the nodes. (**A**) In ML tree *Encephalitozoon* ex. *Pogona* branches as a sister taxon to the dichotomy *E. cuniculi* isolates complex—*E. lacertae*. Bootstrap support for the nodes is shown only if > 50%. Scale bar corresponds to 0.1 substitutions per site. (**B**) Fragment of the MP tree presented as a cladogram. *Encephalitozoon* ex. *Pogona* clusters with *E. cuniculi* complex of isolates, and the sister *E. lacertae* branch takes a basal position. The branches with less than 50% bootstrap support are shown as polytomies.

that in addition to well documented horizontal transmission, *Pv* microsporidiosis can be transmitted transovarially. Microsporidia spores were detected within foci of granulomatous yolk sacculitis and placentitis in hatching bearded dragons obtained from a breeder with a documented history of microsporidiosis and evaluated immediately posthatching (Mitchel and Garner 2011). Hence, the microsporidium causing granulomatous inflammation in *Pogona* vitticeps can be characterized by a specific reptilian host, distinctive pathogenesis, and by the likely presence of both alimentary and transovarian routes of transmission. It is possible, although, that the host range of this microsporidium is not limited to bearded dragons: the pathogenesis and signs described for microsporidiosis in Centralian knob-tailed gecko *Nephrurus amyae,* another lizard of Australian origin, is very similar to the symptoms of *Pv* microsporidiosis (Reece and Hartley, 1994 in Ladds 2009).

Among the published records, there are two comprehensive descriptions, including electron microscopy study, of the etiological agent of *Pv* microsporidiosis. These descriptions are based on series of cases of *Pv* microsporidiosis reported in the United States (Jacobson et al. 1998) and Europe (Richter et al. 2013). Overall comparisons including morphology, fine structure, and pathology, among three descriptions (by Jacobson et al. (1998), Richter et al. (2013), and herein, strongly indicates that all researchers were dealing with the same species of the genus *Encephalitozoon* (Table 2). Unfortunately, statistical comparison of spore parameters among isolates was not feasible. Presence of the unique ITS repeat in the European isolate of *P. vitticeps* microsporidium (Richter et al. 2013) and in the parasite identified in Louisiana in this research confirms genetic identity of both isolates.

The microsporidium found in Bearded Dragons had not been identified to the generic level (Jacobson et al. 1998; Mitchel and Garner 2011; Strunk and Reavill 2011) until Richter et al. (2013) attributed it to *E. cuniculi* basing on similarity of spore morphology, development in parasitiphorous vacuole and the close (97%) similarity of its 34-bp-long Internal Transcribed Spacer (ITS) region of the ribosomal gene (rDNA).

Morphological analysis is not very helpful in distinguishing among *Encephalitozoon* species. Staining patterns are similar for all *Encephalitozoon* spp. The spores of *E. romaleae* and *E. laceratae* are larger, whereas the spore size ranges of *E. cuniculi, E. intestinalus, and E. hellem* overlap (Table 2). Statistical values like Mean, Median, Standard Error, Standard Deviation, and Number of measurements (N) that would allow significant comparison among species, for most species/isolates are not available. At the same time, comparison of length/width (l/w) ratio applied to the average values provided in papers, allow differentiation of the *Encephalitizoon* ex. *Pogona* (l/w = 0.52) from *E. cuniculi* and *E. intestinalis* (l/w = 0.60) but not from *E. hellem* (Table 2). However, this characteristic is not robust and cannot be used for differential diagnosis. Ultrastructural data were even less informative, especially given that all research on reptilian microsporidia was performed on 10% formalin-fixed material obtained postmortem, with poorly preserved ultrastructure. Among ultrastructural characters, only the number of polar filament coils could be used as a specific characteristic. The number of polar filament coils in the *P. vitticeps* microsporidium varied from 3 to 5.5, and averaged in 4. Richter et al. (2013) mentioned 5–6 coils, and Jacobson et al. (1998) 6. However, the numbers of spores that were examined by previous authors were not indicated. Other species of the genus *Encephalitozoon* demonstrated either more numerous coils (*E. romaleae,* 7–8), or the range of numbers extended to the larger side (*E. hellem* 4–9; *E. intestinalis*, 4–7; *E. cuniculi*, 5–7) as compared with the species studied herein, 3–6 (Table 2). This characteristic, as

Table 3. SSU-rDNA-inferred pairwise evolutionary distances among *Encepalitozoon* spp.[a]

	Species (origin)	1	2	3	4	5	6	7	8	9
1	*Encephalitozoon* ex *Pogona*									
2	*E. cuniculi* (dog)	0.0101								
3	*E. cuniculi* (fox)	0.0101	0.0008							
4	*E. cuniculi* (rabbit)	0.0109	0.0017	0.0008						
5	*E. cuniculi* (mouse)	0.0109	0.0008	0.0017	0.0008					
6	*E. lacerate* (lizard)	0.0237	0.0238	0.0237	0.0237	0.0238				
7	*E. intestinalis* (human)	0.0912	0.0973	0.0969	0.0978	0.0980	0.1028			
8	*E. hellem* (bird)	0.1085	0.1109	0.1105	0.1114	0.1116	0.1088	0.0827		
9	*E. romaleae* (grasshopper)	0.1018	0.1042	0.1038	0.1048	0.1049	0.1030	0.0827	0.0393	

[a]Distance matrix was estimated by Kimura 2-parameter model.

the previous one, suggesting a difference, is not adequately robust, and cannot be used to differentiate the species from congeners.

Aside from morphological concerns, it is very unlikely that the same species would parasitize poikilothermic and warm-blooded vertebrates, which are evolutionary so distinct from each other. This consideration was used by Canning (1981) to support the distinction of *E. lacertae, another E. cuniculi*—related species parasitizing lizards (Canning 1981; Koudela et al.1998).

SSU-r DNA-inferred phylogenetic analyses strongly suggest that *Encephalitozoon* sp. ex. *Pogona* forms a separate, well-supported lineage within the *Encephalitozoon* branch (Fig. 3A, B). Pairwise evolutionary distances separating the *P. vitticeps* microsporidium from *E. cuniculi* isolates (0.0101–0.0109) are about 10 times greater than the distances separating genotypes of *E. cuniculi* (0.0008–0.0017), or isolates of *E. hellem* (0.0008) and *E. intestinalis* (0.0008–0.0017). They are comparable to the distances between *E. lacertae* and *E. cuniculi* (0.0237–0.0238) (Tables 3, S2). Hence, molecular taxonomy tools suggested the separate species (or subspecies) status for *P. vitticeps* rather than the status of an *E. cuniculi* genotype.

Basing on the mentioned above considerations we establish here a new species *Encephalitozoon pogonae* n. sp. for the microsporidium that causes systemic microsporidiosis associated with infection of macrophages, in the foci of granulomatous inflammation in different organs in a reptilian host, the Inland Bearded Dragon *P. vitticeps*.

Microsporidia parasitizing reptiles

In the chapter "Microsporidia in vertebrates" of the most recent and comprehensive review on the group—"Microsporida: Pathogens of Opportunity" (Anonymus 2014), the section on microsporidia from amphibians and reptiles is limited to one sentence saying that microsporidiosis in these groups has been rarely reported, and a table that includes four records on microsporidiosis in reptiles of a total of ten records for both groups (Snowden 2014). Indeed, reptilian microsporidiosis was not in the focus of attention of research microsporidiologists. At the same time, studies on microsporidiosis in reptiles are intriguing from the evolutionary point, given that all three contemporary groups of "higher vertebrates"—Reptilia, Mammalia, and Aves, constitute a single clade and share a common "basal amnyote" ancestor (Donoghue and Benton 2007). In particularly, studies on distribution of microsporidia among reptiles may shed light on evolutionary history and diversification of species specialized to parasitism in vertebrates hosts, like most of *Encephalitozoon* spp. These evolutionary considerations and scarcity of information forced us to undertake a more intensive search for the evidence of microsporidian distribution among reptiles. Reptiles are popular pets and common zoo residents, and as such are regular patients of veterinary clinics. Of a total of eighteen records on microsporidiosis in reptiles (Table 1), fourteen represent case studies or brief reports of practicing veterinarians published in veterinary journals or meeting proceedings. Some of these records report infection of two and more animals. Interestingly, microsporidia were detected in three of four major groups of reptiles (Squamata, Rynchocephalia, and Crocodilia (Table 1) and were absent, to our knowledge, only in Testudines (turtles, terrapins, and turtoises).

Infection of tuataras, amazing "live fossils," with *Pleistophora*-like microsporidium, was likely acquired in the Bronx Zoo shortly after stressful transportation of animals from New Zealand to New York City (Liu and King 1971). Opportunistic features of microsporidiosis and cross-infectivity of microsporidian spores among snakes and lizards species were recorded in the Baltimore Zoo. It was demonstrated that *Pleistophora*-type spores were constantly shed in the feces of 19 species of snakes and a lizard, all of which died from acute cryptosporidiosis (Graczyk and Cranfield 2000).

Microsporidian infection in Australian freshwater crocodiles was identified as *E. hellem,* based on 97% homology of a 264 bp fragment of SSUrDNA. However, morphological and ultrastructural features presented in the paper (Scheelings et al. 2015; Fig. 2), namely (i) spores of approximately 5–6 × 2-μm size, (ii) development without sporophorous vacuole, (iii) and up to 26(!) polar filament coils, are not compatible with the species (*E. hellem*

Didier et al. 1991; or even the genus (*Encephalitozoon* Levaditi, Nicolau, and Schoen 1923) diagnoses (Canning and Vavra 2000; Didier et al. 1991). Interestingly that the crocodile microsporidium sequence shared > 99% homology and clustered on the phylogenetic tree with sequences from microsporidia parasitizing captive birds in South Australia and New South Wales. Crocodiles phylogenetically are closely related to birds, in fact more closely than to other reptiles (Modesto and Anderson 2004), and thus may share microsporidian species derived from a parasite of the ancestral reptile.

Most frequently microsporidian infections were reported in lizards (reptiles of the suborder Lacertilia) and snakes (suborder Serpentes), two sister groups in the order Squamata. Among ten records of microsporidiosis in lizards, five were reported from Inland Bearded Dragons, and were likely caused by *E. pogonae,* as was discussed above. Another *Encephalitozon* species, *E. lacerate,* was reported to infect two reptiles: a Common Wall Lizard, *Podacris muralis,* and an African Skink, *Mabuya perrotetii*. Of note, the reptilian encephalitozoonids include two species, each characterized by specific tissue tropisms, in the same manner as mammalian microsporidia *E. intestinalis and E. cuniculi*. *E. lacertae* has evolved as a parasite of enterocytes, while *E. pogonae* primarily parasitizes macrophages. Two cases of microsporidiosis characterized by diffused myonecrosis and myositis in an Australian Water Dragon *Intellagama leueurii*, and a Leaf-tailed Gecko, *Uroplatus henkeli*, were rather associated with a *Pleistophora*-like microsporidium, given different symptoms of infections and presence of "cysts" in muscles (Table 1), although the scarcity of information does not allow reliable conclusions.

Five of six cases of microsporidiosis in snakes were observed in colubrids (Colubridae family, grass, and water snakes): the European Grass Snake, *Natrix natrix*; Garter Snake, *Thamnophis sirtalis*, native to North America; and Asian Water Snake, *Atretium schistosum* (Table 1). The recent study by Richter et al. (2014) is the only one that included electron microscopy and molecular analysis. It demonstrated that microsporidiosis in a Garter Snake affected primarily muscles and was caused by *Heterosporis anguillarum* (Hoshina) (Lom et al. 2000). The sizes and structure of dimorphic spores, life cycle and pathology were identical to ones described in *H. anguillarum* from the type host, the Japanese eel, *Anguilla japonica* (Lom et al. 2000). It is likely that three other cases of microsporidian infections in colubrids in France (Debasieux, 1919 in Canning et al. 1964), in Italy (Guyenot and Naville 1924), and in the USA (Gardiner et al. 1968) can be attributed to the same or related species. All these studies mention "pansporoblasts" (Canning et al. 1964; Gardiner et al. 1968), "cysts" (Guyenot and Naville 1924), or "sporophorocysts" (Richter et al. 2014) with multiple (16–100) spores, characteristic for genera *Pleistophora* and *Heterosporis* (Kent et al. 2014). Gardiner et al. (1968) and Guyenot and Naville (1924) also indicated simultaneous presence of two types of spores (indicative of *Heterosporis* spp.), described in the latter study as two different species, *Glugea danilewskyi*, and *Glugea ghigii* (Guyenot and Naville 1924). These species were transferred later to genera *Pleistophora* and *Microsporidium*, respectively (Sprague 1977; Becnel et al. 2014). Infection in an Asian Water Snake was probably associated with another intramuscular *Pleistophora*-like microsporidium, because it was reported to produce larger numbers of spores (320–360) within pansporoblasts (Narasimhamutri et al. 1982). An unidentified microsporidium described in a yellow-bellied sea snake, *Pelomus platura* (family Elapidae) developed in muscles and produced "Acid Fast-positive cysts" (Reece, Hartley, 1994 in Ladds 2009), and might also belong to *Pleistophora* or related genera of fish microsporidia. Thus, all reported cases of microsporidiosis in snakes were caused by *Pleistophora*-like microsporidia (Table 2). Of note, all these snakes are ecologically connected with water. For several groups of microsporidia, it was previously shown that shared water habitat facilitated host switches through common food chains (Sokolova et al. 2015; Vossbrinck and Debrunner-Vossbrinck 2005) and parasites (Freeman et al. 2003; Freeman and Sommerville 2011; Nylund et al. 2010). "*G. danilewskyi*" and "*G. ghigii*" were described also as hyperparasites of trematodes and cestodes, which commonly infested Grass Snakes (Guyenot and Naville 1924). So, both routes might have played a role in switching hosts of fish microsporidia to water-associated snakes and vice-versa.

Further studies with the application of advanced morphological and molecular methods will fill in the gaps in the knowledge on the already known species mentioned in this review, discover more diversity among microsporidia parasitizing reptiles, and reveal their evolutionary and ecological associations with the taxa infecting other vertebrates and invertebrates.

TAXONOMIC SUMMARY

Phylum Microsporidia Balbiani 1882
Family: incertae sedis
Genus: *Encephalitozoon* Levaditi, Nicolau and Schoen 1923

ENCEPHALITOZOON POGONAE N. SP.

Diagnosis

Type host: *Pogona vitticeps* Ahl 1927 (Reptilia, Squamata, Lacertilia, Agamidae), of Australia origin, distributed worldwide as a pet
Cell and tissue tropism: macrophages and other phagocytic cells in granulomatous inflammation of multiple organs
Associated pathology: granulomas of varible size (up to 3.5 cm in diameter) in different organs, primarily in adrenal glands, gonads, heart and joints.
Interfacial envelopes: development of all stages within parasitophoros vacuole
Meronts: not described
Sporonts and sporoblasts: contact the membrane of the parasitophorous vacuole; their structure not described

Spores: methanol-fixed spores on smears measured 2.1 ± 0.03 × 1.1 ± 0.03 μm, range 1.7–2.6 × 0.9–1.7 μm (n = 38); on ultrathin sections—0.8–1.1 × 1.8–2.2 μm; uninucleate; 3–6 polar filament coils (median = 4, mean = 3.94 ± 0.1, n = 32) and mushroom-shaped polar disk; polar sac embraces half of the volume occupied by lamellar polaroplast; straight region of the polar filament about 1 μm long traversing the long axis of the spore; polar filament everts eccentrically in fired spore.

Etymology. The species name indicates the genus of the type host.

Gene sequences. The ribosomal DNA sequence, deposited to Genbank under Accession # KR99831 composed of SSU (positions 1–1299); ITS (1300–1342) and partial LSU (1343–1535) regions, is a part of the species description.

Type material. Slides with methanol-fixed smears of infected heart and ovaries, paraffin blocks and sections stained with Hematoxylin-Eosin, Gram, and Giemsa, and hemithin sections of Epon-Araldite-embedded infected tissues are deposited in the slide collection of the Department of Pathobiological Sciences SVM LSU, in the microsporidia collection of the Institute of Plant Protection, St. Petersburg, Russia (Prof. Issi's collection), and in the collection of the corresponding author (YS). Embeddings, grids with thin sections, and images of thin sections, labeled "*E. pogonae*" are stored in the collection of YS.

Differential diagnosis of Encephalitozoon pogonae

Encephalitizoon pogonae can be differentiated from morphologically similar *E. cuniculi*, *E. intestinalis*, and *E. hellem* by the parasitism of a reptilian host and by signatures of pathogenesis. It can be differentiated from another reptilian pathogen, *E. lacerate*, morphologically by smaller spore size and less elongates spores, and by parasitizing phagocytic cells, but not enterocytes like *E. lacertae. E. pogonae* differs from *E. romaleae* morphologically and by parasitizing a vertebrate host. The presence of unique repeat 5′GTTTTGTTTGTTTGTTTTGTTTGTTT3′ within ITS region of SSUrDNA allows distinguishing the novel species from congeners and *E. cuniculi* isolates at the molecular level.

ACKNOWLEDGMENTS

We acknowledge use of Core Microscopy Center at Department of Comparative Biomedical Sciences, School of Veterinary Medicine Louisiana State University at Baton Rouge. The authors are indebted to Elizabeth Didier and Liza Bowers (Tulane National Primate Research Center) for sharing *Encephalitozoon* primers and helpful suggestions on PCR protocols.

LITERATURE CITED

Anonymus. 2014. Microsporidia: Pathogens of Opportunity, 1st ed. Weiss, L. & Becnel, J. J. eds, Wiley Blackwell, Oxford, UK,709P.

Becnel, J. J., Takvorian, P. M. & Cali, A. 2014. Appendix A. Checklist of available generic names for microsporidia with type species and type host. *In:* Weiss, L. & Becnel, J. J. (eds), Microsporidia: Pathogens of Opportunity, 1st ed. Wiley Blackwell, Oxford, UK. p. 671–684.

Canning, E. U. 1981. *Encephalitozoon lacerate* n.sp., a microsporidian parasite of the lizard *Podacris muralis*. "Parasitological Topics", Society of Protozoology Special Publications No 1.57-64.

Canning, E. U., Elkan, E. & Trigg, P. I. 1964. *Plistophora myotrophica* spec. nov., causing high mortality in the common toad *Bufo bufo* L. with notes on the maintenance of *Bufo* and *Xenopus* in the laboratory. *J. Protozool.*, 11:157–166.

Canning, E. U. & Vavra, J. 2000. Microsporidia. *In:* Lee, J. J., Leedale, G. F. & Bradbury, P. (eds), The Illustrated Guide to the Protozoa, 2nd ed. Society of Protozoologists, Lawrence Kansas. p. 39–126.

Didier, E. S., Didier, P. J., Friedberg, D. N., Stenson, S. M., Orenstein, J. M., Yee, R. W., Tio, F. O., Davis, R. M., Vossbrinck, C., Millichamp, N. & Shadduck, J. A. 1991. Isolation and characterization of a new human microsporidian, *Encephalitozoon hellem* (n. sp.), from three AIDS patients with keratoconjunctivitis. *J. Infect. Dis.*, 163:617–621.

Donoghue, P. C. J. & Benton, M. J. 2007. Rocks and clocks: calibrating the Tree of Life using fossils and molecules. *Trends Ecol. Evol.*, 22:424–431.

Edgar, R. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.*, 32:1792–1797.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39:783–791.

Freeman, M. A., Bell, A. S. & Sommerville, C. 2003. A hyperparasitic microsporidian infecting the salmon louse, *Lepeophtheirus salmonis*: an rDNA-based molecular phylogenetic study. *J. Fish Dis.*, 26:667–676.

Freeman, M. A. & Sommerville, C. 2011. Original observations of *Desmozoon lepeophtherii*, a microsporidian hyperparasite infecting the salmon louse *Lepeophtheirus salmonis*, and its subsequent detection by other researchers. *Parasit. Vectors*, 4:231.

Gardiner, G., Fayer, R. & Dubey J. P. 1968. An Atlas of Protozoan Parasites in Animal Tissues. U.S. Department of Agriculture. Agriculture handbook No 651. Springfield, VA. 12–13.

Ghosh, K., Schwartz, D. & Weiss, L. M. 2014. Laboratory diagnosis of microsporidia. *In*: Weiss, L. & Becnel, J. J. (eds.), Microsporidia: Pathogens of Opportunity, 1st edn. Wiley Blackwell, Oxford, UK. p. 421–456.

Graczyk, T. K. & Cranfield, M. R. 2000. *Cryptosporidium serpentes* oocysts and microsporidian spores in feces of captive snakes. *J. Parasitol.*, 86:413–414.

Guyenot, E. & Naville, A. 1924. *Glugea encyclometrae* n.sp. et *G. ghigii* n.sp. parasites de platodes et leur development dans l'hote vertebre (*Tropidonotus natrix* L.). *Rev. Suisse Zool.*, 31:75–115.

Hasegawa, H. M., Kishino, H. & Yano, T. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. *J. Mol. Evol.*, 22:160–174.

Jacobson, E. R., Green, D. E., Undeen, A. H., Cranfield, M. & Vaughn, K. L. 1998. Systemic microsporidiosis in inland bearded dragons (*Pogona vitticeps*). *J. Zoo Wildl. Med.*, 29:315–323.

Katzwinkel-Wladarsch, S., Lieb, M., Heise, W., Loscher, T. & Rinder, H. 1996. Direct amplification and species determination of microsporidian DNA from stool specimens. *Tropical Med. Int. Health*, 1:373–378.

Kent, M. L., Shaw, R. W. & Sanders, J. L. 2014. Microsporidia in fish. *In:* Weiss, L. & Becnel, J. J. (eds), Microsporidia: Patho-

gens of Opportunity, 1st ed. Wiley Blackwell, Oxford, UK. p. 493–520.
Koudela, B., Didier, E. S., Rogers, L. B., Modrý, D. & Kučerová, Š. 1998. Intestinal microsporidiosis in African skink *Mabuya perrotetii*. *Folia Parasitol.*, 45:149–155.
Ladds, P. 2009. Pathology of Australian Native Wildlife. CSIRO Publishing, Collingwood, Australia.
Lange, C. E., Johny, S., Baker, M. D., Whitman, D. W. & Solter, L. F. 2009. A new *Encephalitozoon* species (Microsporidia) isolated from the lubber grasshopper, *Romalea microptera* (Beauvois) (Orthoptera: Romaleidae). *J Parasitol.*, 95:976–986.
Liu, S. K. & King, F. W. 1971. Microsporidiosis in the tuatara. *J. Am. Vet. Med. Assoc.*, 159:1578–1582.
Lom, J., Dykova, I., Wang, C. H., Lo, C. F. & Kou, G. H. 2000. Ultrastructural justification for the transfer of *Pleistophora anguillarum* Hoshina, 1959 to the genus *Heterosporis* Schubert, 1969. *Dis. Aquat. Organ.*, 43:225–231.
Mitchel, M. & Garner, M. (2011) Microsporidiosis in bearded dragons: a case for vertical transmission. 18th Annual Conference of the Association of Reptilian and Amphibian Veterinarians. Seatle: 123.
Modesto, S. P. & Anderson, J. S. 2004. The phylogenetic definition of reptilia. *Syst. Biol.*, 53:815–821.
Narasimhamutri, C. C., Kalavati, C. & Ahamed, S. N. 1982. A new microsporidian, *Pleistophora atretii* sp.n. from the Fresh water snake, Atretium Schistosum Gunter. *Acta Protozool.*, 21:127–137.
Nei, M. & Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.
Nylund, S., Nylund, A., Watanabe, K., Arnesen, C. E. & Karlsbakk, E. 2010. *Paranucleospora theridion* n. gen., n. sp. (Microsporidia, Enterocytozoonidae) with a life cycle in the salmon louse (*Lepeophtheirus salmonis*, Copepoda) and Atlantic salmon (*Salmo salar*). *J. Eukaryot. Microbiol.*, 57:95–114.
Richter, B., Csokai, J., Graner, I., Eisenberg, T., Pantchev, N., Eskens, H. U. & Nedorost, N. 2013. Encephalitozoonosis in two Inland bearded dragons *(Pogona vitticeps)*. *J. Comp. Pathol.*, 148:278–282.
Richter, B., Graner, I. & Csokai, J. 2014. *Heterosporis anguillarum* Infection in a Garter Snake (*Thamnophis sirtalis*). *J. Comp. Pathol.*, 150:332–335.
Sakaguchi, K., Sokolova, Y. Y., Higbie, C., Nevarez, J. G. & Paulsen, D. B. 2015. Microsporidiosis in A Bearded Dragon (*Pogona Vitticeps*). 2015 ACVP / ASVCP / STP Combined Annual Meeting. Minneapolis: D-30.
Scheelings, T. F., Slocombe, R. F., Crameri, S. & Hair, S. 2015. *Encephalitozoon hellem* infection in a captive juvenile freshwater crocodile (*Crocodylus johnstoni*). *J. Comp. Pathol.*
Snowden, K. F. 2014. Microsporidia in higher vertebrates. *In:* Weiss, L. & Becnel, J. J. (eds), Microsporidia: Pathogens of Opportunity, 1st ed. Wiley Blackwell, Oxford UK. p. 469–491.
Sokolova, O. I., Demyanov, A. V., Bowers, L. S., Didier, E. S. & Sokolova, Y. Y. 2011a. On the use of FTA® technology for collection, archieving, and molecular analysis of microsporia dna from clinical stool samples. *Tsitologiya*, 53:911–914. *(in Russian, English summary)*
Sokolova, O. I., Demyanov, A. V., Bowers, L. C., Didier, E. S., Yakovlev, A. V., Skarlato, S. O. & Sokolova, Y. Y. 2011b. Emerging microsporidian infections in Russian HIV-infected patients. *J. Clin. Microbiol.*, 49:2102–2108.
Sokolova, Y. Y. & Fuxa, J. R. 2008. Biology and life-cycle of the microsporidium *Kneallhazia solenopsae* Knell Allan Hazard 1977 gen. n., comb. n., from the fire ant *Solenopsis invicta*. *Parasitology*, 135:903–929.
Sokolova, Y., Pelin, A., Hawke, J. & Corradi, N. 2015. Morphology and phylogeny of *Agmasoma penaei* (Microsporidia) from the type host, *Litopenaeus setiferus*, and the type locality, Louisiana, USA. *Int. J. Parasitol.*, 45:1–16.
Sokolova, Y. Y., Sokolov, I. M. & Carlton, C. E. 2010. New microsporidia parasitizing bark lice (Insecta: Psocoptera). *J. Invertebr. Pathol.*, 104:186–194.
Sprague, V. 1977. Annotated list of species of microsporidia. *In:* Bulla, L. A. & Cheng, T. C. (eds), Comparative Pathobiology. Plenum Press, New York. 2:p. 31–337.
Strunk, A. & Reavill, D. (2011) Intracellular parasite in the pelvic limb of a bearded dragon, *Pogona vitticeps*. 18th Annual Conference of the Association of Reptilian and Amphibian Veterinarians. Seatle: 152.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28:2731–2739.
Visvesvara, G. S., Moura, H., Leitch, G. J. & Schwartz, D. A. 1999. Culture and propagation of microsporidia. *In*: Wittner, M. (ed.), The Microsporidia and Microsporidiosis. American Society for Microbiology, Washington, DC. p. 363–392.
Vossbrinck, C. R., Andreadis, T. G., Vavra, J. & Becnel, J. J. 2004. Molecular phylogeny and evolution of mosquito parasitic microsporidia (Microsporidia: Amblyosporidae). *J. Eukaryot. Microbiol.*, 51:88–95.
Vossbrinck, C. R. & Debrunner-Vossbrinck, B. A. 2005. Molecular phylogeny of the microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitol.*, 52:131–142.
Weiss, L. M. & Vossbrinck, C. R. 1999. Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. *In:* Wittner, M. & Weiss, L. M. (eds), The Microsporidia and Microsporidiosis. American Society of Microbiology, Washington, D.C. p. 129–171.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Estimates of evolutionary divergence among 20 SSU-ITS rRNA sequences.