Journal of Invertebrate Pathology 124 (2015) 23–30

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Review of the genus *Endoreticulatus* (Microsporidia, Encephalitozoonidae) with description of a new species isolated from the grasshopper *Poecilimon thoracicus* (Orthoptera: Tettigoniidae) and transfer of *Microsporidium itiiti* Malone to the genus

Daniela K. Pilarska [a,e], Renate Radek [b], Wei-Fong Huang [c], Danail I. Takov [a], Andreas Linde [d], Leellen F. Solter [c,*]

[a] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1 Tzar Osvoboditel Blvd., Sofia, Bulgaria
[b] Freie Universität Berlin, Königin-Luise-Str. 1-3, 14195 Berlin, Germany
[c] Illinois Natural History Survey, University of Illinois, 1816 S. Oak St., Champaign, IL 61801, United States
[d] University of Applied Sciences, Eberswalde, Alfred-Möller-Straße 1, 16225 Eberswalde, Germany
[e] Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Kamycka 1176, 16521 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 16 May 2014
Accepted 22 September 2014
Available online 23 October 2014

Keywords:
Microsporidia
Phylogeny
Pleistophora
Endoreticulatus
Cystosporogenes

ABSTRACT

The historic genus *Pleistophora* (*Plistophora*) is a highly polyphyletic clade with invertebrate Microsporidia reassigned to several new genera since the 1980s. Two genera, *Endoreticulatus* and *Cystosporogenes*, clearly separate into distinct but closely related clades based on small subunit ribosomal RNA analysis but are included in different families that are each polyphyletic. A microsporidium with morphology resembling the *Endoreticulatus/Cystosporogenes* clade was isolated from the grasshopper *Poecilimon thoracicus* from a site in Northwest Bulgaria. It produced intense infections in the digestive tract of the host but no behavioral changes were noted in infected individuals. Prevalence of the microsporidium increased over the active feeding season yearly. Mature spores were oval and measured 2.58 ± 0.21 μm × 1.34 ± 0.24 μm, with 16 to approximately 32 spores in a parasitophorous vacuole. The spores were uninucleate and polar filament coils numbered 8–9 situated in a single row. The spore polaroplast consisted of an anterior lamellar section and a posterior vesicular section, and the posterior vacuole was reduced. Analyses of a 1221 bp partial SSU-rRNA sequence indicated that the isolate is more closely related to the *Endoreticulatus* clade than to *Cystosporogenes*, but shows earlier phylogenetic separation from species infecting Lepidoptera and represents a new species, *Endoreticulatus poecilimonae*. To compare sequences of *Endoreticulatus* spp. from Lepidoptera to those infecting other insect orders, an isolate, *Microsporidium itiiti* Malone (1985), described from the Argentine stem weevil, *Listronotus bonariensis*, was sequenced. Like the grasshopper isolate, the weevil isolate is closely related but basal to the lepidopteran *Endoreticulatus* clade. The original description combined with the new sequence data confirms species status and permits transfer of the isolate from *Microsporidium*, a genus erected for microsporidian species of uncertain taxonomic status, to *Endoreticulatus*.

1. Introduction

A clade of invertebrate microsporidian pathogens including the genera *Endoreticulatus* (Brooks et al., 1988; Cali and El Garhy, 1991), *Cystosporogenes* (Canning et al., 1985), and *Vavraia* (Weiser, 1977; Vavra and Becnel, 2007), were originally placed in the genus *Pleistophora* (also *Plistophora*) Gurley (1893) (Pleistophoridae), one of several genera recognized to be highly polyphyletic (Canning and Nicholas, 1980; Cali and El Garhy, 1991). The type species, *Pleistophora typicalis*, was described from the muscles of a fish, *Cottus scorpius* (Gurley, 1893) and it is unlikely that the insect clade is closely related (Canning and Nicholas, 1980; Bell et al., 2001). *Pleistophora* originally contained more than 36 described species and 5 additional isolates from vertebrate hosts, primarily fish, but also several species isolated from reptiles, amphibians and mammals (Sprague, 1977). Although many of these vertebrate pathogens have not been evaluated using molecular characters (but see Bell et al., 2001), many do appear to share generic morphological characters with the type species

* Corresponding author.
E-mail address: lsolter@illinois.edu (L.F. Solter).

http://dx.doi.org/10.1016/j.jip.2014.09.007
0022-2011/

(Canning and Nicholas, 1980; Cali and El Garhy, 1991). Only a few of the approximately 54 described species and 22 isolates from invertebrate hosts including insects ($n = 58$), crustaceans ($n = 12$), gastropods ($n = 2$) and 1 each gregarines, nematodes, trematodes and coelenterates (Sprague, 1977) have been reevaluated. Of the currently recognized genera, *Endoreticulatus* (primarily insect hosts) is assigned to the family Encephalitozoonidae and the sister clade *Cystosporogenes* (insect hosts) to Glugeidae; *Vavraia* (insect hosts) remains in Pleistophoridae and is also listed as Incertae sedis. Two related genera, *Euplotespora* (ciliate host) and *Mrazekia* (copepod host), are listed as Incertae sedis and Mrazekiidae, respectively (encyclopedia of life, http://eol.org).

Of the twenty microsporidian species in eight genera that have been reported or described from grasshoppers in the families Acrididae, Tettigonidae, Gryllidae, Pyrgomorphidae, Romaleidae and Tristiridae (reviewed by Sokolova et al., 2006) none are closely related to the former *Pleistophora* clade. One species, *Encephalitozoon romaleae*, is closely related to other *Encephalitozoon* species in the Encephalitozoonidae (Lange et al., 2009), the family to which *Endoreticulatus* is assigned, the latter probably erroneously based on small subunit ribosomal RNA (SSU rRNA) analysis (Vossbrinck and DeBrunner-Vossbrinck, 2005). Other isolates described from grasshoppers include eight *Nosema* species, three *Liebermannia* species, two *Paranosema* species, and one species each in the genera *Tubulinosema*, *Vairimorpha*, *Heterovesicula*, and *Johenrea*. Three species have not been identified to genus level (Sokolova et al., 2006). Most of the Microsporidia, including the well-known biological control agent *Paranosema* (*Nosema*) *locustae* (Canning, 1953; Sokolova et al., 2003), were isolated from grasshoppers collected in the Americas, Africa and Asia; however, one *Nosema* sp. was isolated in Spain from *Chorthippus albomarginatus*, a common European acridid grasshopper (Hernández-Crespo et al., 2001).

In 2009 we isolated a microsporidium from a Bulgarian population of *Poecilimon thoracicus* (Tettigoniidae), a widely distributed grasshopper species in Europe. The pathogen infected the alimentary tract of the host and was recorded in one population of *P. thoracicus* from May to July each year from 2010 to 2012. Here we provide morphological and phylogenetic data for this new *Endoreticulatus* species infecting an orthopteran host. In addition, we provide sequence data for *Microsporidium itiiti* Malone (1985), a species isolated from the Argentine stem weevil, *Listronotus bonariensis* (Malone, 1985, 1987), permitting transfer of the weevil isolate to *Endoreticulatus* and phylogenetic comparison of lepidopteran *Endoreticulatus* species to the two species isolated from Coleoptera and Orthoptera.

2. Materials and methods

Nymphs and adults of *P. thoracicus* were collected individually by hand from various shrubs and perennial plants, primarily *Rubus fruticosus*, *Cotinus coggygria* and *Urtica dioica*, in two sites, the village of Gabrovnitsa (43°05.331′N:023°27.626′E; Northwest Bulgaria), and near Stryama (42°13.710′N:024°51.659′E; Central Bulgaria), in 2010, 2011 and 2012 (Table 1). The grasshoppers were transported to the laboratory and refrigerated to reduce activity and prevent transmission of the pathogen prior to dissection.

Spores of *M. itiiti* were obtained in 1985 from L. Malone, the New Zealand Institute for Plant & Food Research, Ltd., Auckland, New Zealand, and were held in liquid nitrogen storage at the Illinois Natural History Survey until sequenced in 2014.

2.1. Microscopic examination

Internal organs of *P. thoracicus* were excised and examined for presence of Microsporidia under light microscopy (400×). Tissues of infected insects were smeared on slides, fixed with methanol and stained with Giemsa (Sigma Diagnostic Accustain) (Becnel, 2012). For transmission electron microscopy (TEM), infected tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed for 2 h in 2% OsO_4. The tissues were then dehydrated through an ascending ethanol and acetone series and embedded in Epon–Araldite or in Poly/Bed 812/Araldite 502 (Becnel, 2012). Thick sections (1.0 μm), stained according to Richardson et al. (1960), were observed using light microscopy to locate infected cells. Thin sections were cut on an Ultracut E. Reichert microtome, stained with uranyl acetate and lead citrate, and examined with a Philips EM 208 electron microscope.

Table 1
Poecilimon thoracicus collection sites and prevalence of *Endoreticulatus poecilimonae* in one population.

Date of collection	Number collected	Prevalence (%) (N^a)
Gabrovnitsa		
06.26.2010	24	8.3 (2)
07.07.2010	35	37.1 (13)
07.23.2010	20	80.0 (16)
05.21.2011	41	7.3 (3)
05.29.2011	18	11.1 (2)
06.03.2011	35	5.7 (2)
06.18.2011	70	11.4 (8)
07.27.2011	17	88.2 (15)
07.15.2012	23	82.6 (19)
Stryama		
07.05.2010	32	–
07.21.2010	41	–
06.28.2011	27	–

[a] Number infected.

2.2. DNA isolation, SSU rRNA sequencing, and phylogenetic analysis

Microsporidian DNA was extracted from infected tissues of *P. thoracicus* and from thawed samples of *M. itiiti* using a modification of the Chelex method (Cordes et al., 2012). PCR was conducted using the microsporidian universal primer set 18f/1537r (Weiss and Vossbrinck, 1999) with an annealing temperature of 50 °C and Platinum Taq (Life Technologies) following the manufacturer's instructions. The PCR products were visualized using 1% agarose gel electrophoresis in TAE buffer, purified using alcohol precipitation and sequenced on an ABI 3730XL sequencer using the PCR primers. The sequence readings were reviewed using BioEdit (Hall, 1999) and unreliable sequence readings were manually trimmed. The assembled partial SSU rRNA sequences were analyzed using BLAST (nucleotide) services with nr database on NCBI. Related microsporidian species, including species in the genera *Endoreticulatus*, *Cystosporogenes* and other closely related genera, as well as Microsporidia isolated from orthopteran hosts, were included in the initial phylogenetic analysis. The sequences were aligned using ClustalW, and poorly aligned terminals were manually trimmed. Maximum likelihood analysis was performed using PhyML 3.0 (Guindon and Gascuel, 2003) with GTR nucleotide substitution model and optimized equilibrium frequencies. Support for the clade was calculated by 1000 times bootstrapping.

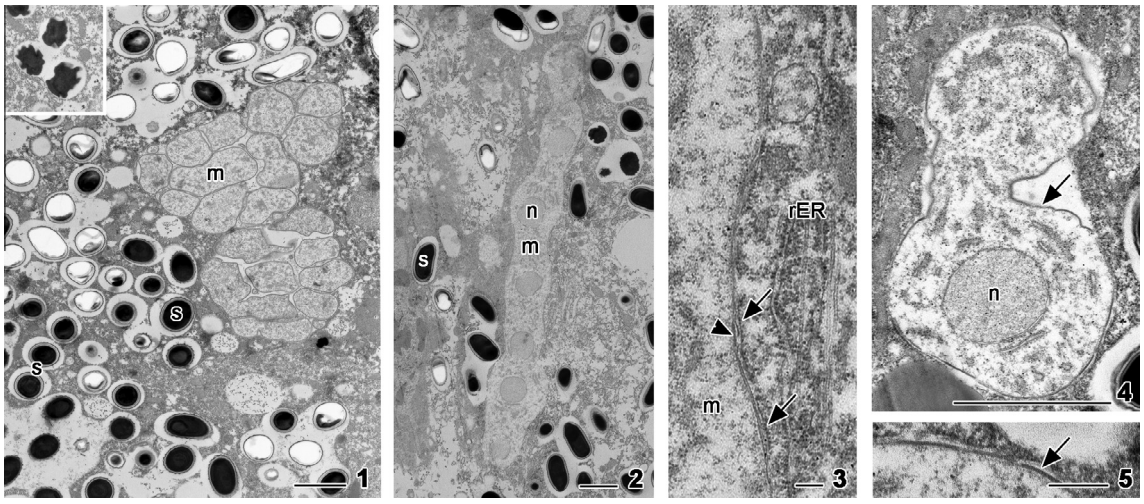
3. Results

The microsporidium isolated from *P. thoracicus* was found only in the Gabrovnitsa population. An increase in prevalence from less than 8% in May to over 80% in July was recorded yearly. The lowest prevalence, 5.7%, was documented in the beginning of June 2011 and the maximum, 88.2%, at the end of July of the same year (Table 1). The host was present in high numbers in the collection

site each year, approximately 15–30 nymphs and adults per host plant, suggesting that the pathogen was not driving population declines. The microsporidium was detected only in the midgut and gastric caeca cells of the hosts. The type of transmission is unknown, however, midgut infections suggest oral transmission.

3.1. Microscopic examination

Parasitophorous vacuoles containing developmental stages filled the cytoplasm of host cells in infected tissues. Each vacuole contained 16 to more than 30 spores. Fresh spores were oval and



Figs. 1–5. Developmental stages, TEM. **Fig. 1** infected gut tissue with meronts (m) or sporonts (inset), or mature spores (s) in parasitophorous vacuoles. **Fig. 2** elongated meront (m) with several nuclei (n). **Fig. 3** plasma membrane (arrowhead) of meront (**Fig. 5**) and layer of rough endoplasmic reticulum (arrows) forming the border of the parasitophorous vacuole. rER = rough endoplasmic reticulum of the host cell. **Figs. 4 and 5** early stage sporont with electron dense deposits on the plasma membrane. Scale bars Figs. 1–4 = 2 μm; Fig. 5 – 0.02 μm.

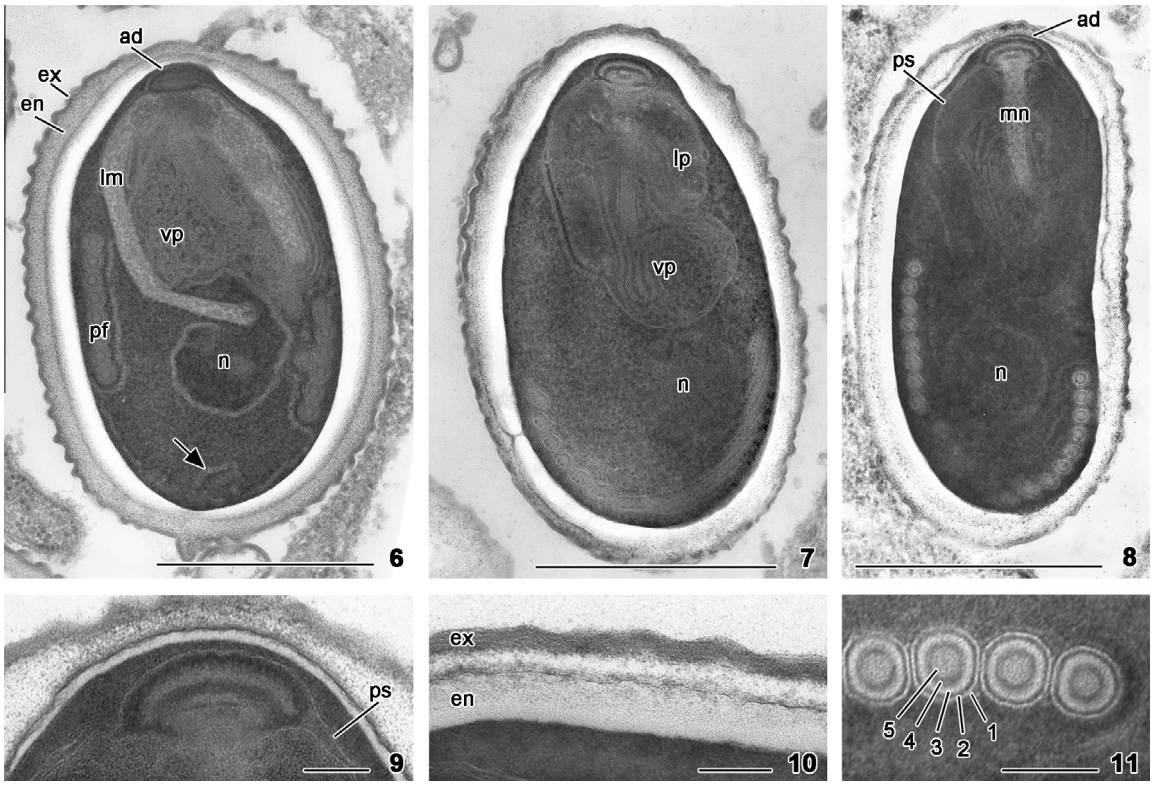


Fig. 6–11. Mature spores, TEM. **Figs. 6–8**. The spore wall consists of a thin, wavy (crenate) exospore (ex) and a thicker endospore (en). The anchoring disk (ad) with the bell-like polar sac (ps) lies terminally and gives rise to a straight manubrium (mn), which extends into 8 oblique coils of an isofilar polar filament (pf). The anterior (outer) part of the polaroplast is dense and probably lamellar while the posterior (inner) part is vesicular (vp). One nucleus (n), and reduced polar sac (arrow). **Fig. 9** multilayered, dome-shaped anchoring disk and polar sac. **Fig. 10** thin layered exospore (ex) and thick, slightly granular/fibrillar endospore (en). **Fig. 11** cross section of polar filament depicting three electron light and two electron dense layers (1–5). Scale bars: Figs. 6–8 = 1.0 μm; Fig. 9 = 0.1 μm; Figs. 10 and 11 = 0.02 μm.

small in size, averaging 2.58 ± 0.21 (2.08–3.14) μm × 1.34 ± 0.24 (0.93–2.88) μm (n = 70). Giemsa stained spores and spores stained for TEM and thick-sectioned were also examined. Ultrastructural micrographs revealed the presence of developing forms at the same stage within a parasitophorous vacuole, but different developmental stages in neighboring vacuoles including merogonial and sporogonial stages, sporoblasts, and spores (Fig. 1). Some merogonial stages were elongated (ribbon-like) and multinucleated (Fig. 2) while others appeared round in shape (Fig. 1), possibly a function of the plane of cut. The vacuole consisted of two membranes; the outer host-derived membrane was studded with ribosomes while the inner parasite plasmalemma was smooth, a principle characteristic of the genus (Brooks et al., 1988; Cali and El Garhy, 1991) (Fig. 3). Electron dense deposits were observed on the plasmalemma of early stage sporonts (Figs. 4 and 5).

The spore structure was typical for Microsporidia in the genus *Endoreticulatus*. The electron-lucent endospore was 45–70 nm thick and a homogenous/fibrous structure was apparent in some micrographs. The exospore was 20–30 nm thick with a crenate surface and layered structure (Figs. 6–10). The mature spore possessed one nucleus, a bipartite cup-like lamellar anterior polaroplast and a posterior vesicular section. The polar filament was isofilar with 8–9 coils arranged in a single row (Fig. 8). The polar filament coils were not orthogonal to the longitudinal axis of the spore but oblique, with an inclination of 35–45°. Polar filament cross sections consisted of an electron-lucent center and four outer layers with alternating electron dense rings (Fig. 11). A mushroom-shaped anchoring disk was positioned at the anterior end of the spore (Figs. 6–8). A reduced posterior vacuole was observed at the posterior end of the spore (Fig. 6).

3.2. Phylogenetic analyses

The partial SSU rRNA (1221 bp) of the *P. thoracicus* microsporidian isolate has one polymorphic site (653rd bp) and is lacking approximately 61 bp at 3′ end compared to *E. schubergi* (GenBank Accession No. L39109), the most closely related described species. Lacking the 3′ end region did not cause difficulty for phylogenetic analysis because the terminal ends are usually poorly aligned and were trimmed before analysis. The isolate is genetically distinct from other Microsporidia described from orthopteran hosts (Fig. 12). The SSU rRNA sequence of the isolate shares 95.2% identity with *E. bombycis* and *E. schubergi*, and 87.7% with *Cystosporogenes legeri* (Figs. 12 and 13; Table 3). The topology of genetic structure as well as the morphological phenology support a closer phylogenetic relationship with *Endoreticulatus* spp. than with *Cystosporogenes* spp., and we assigned this isolate to the genus *Endoreticulatus* according to the current available data.

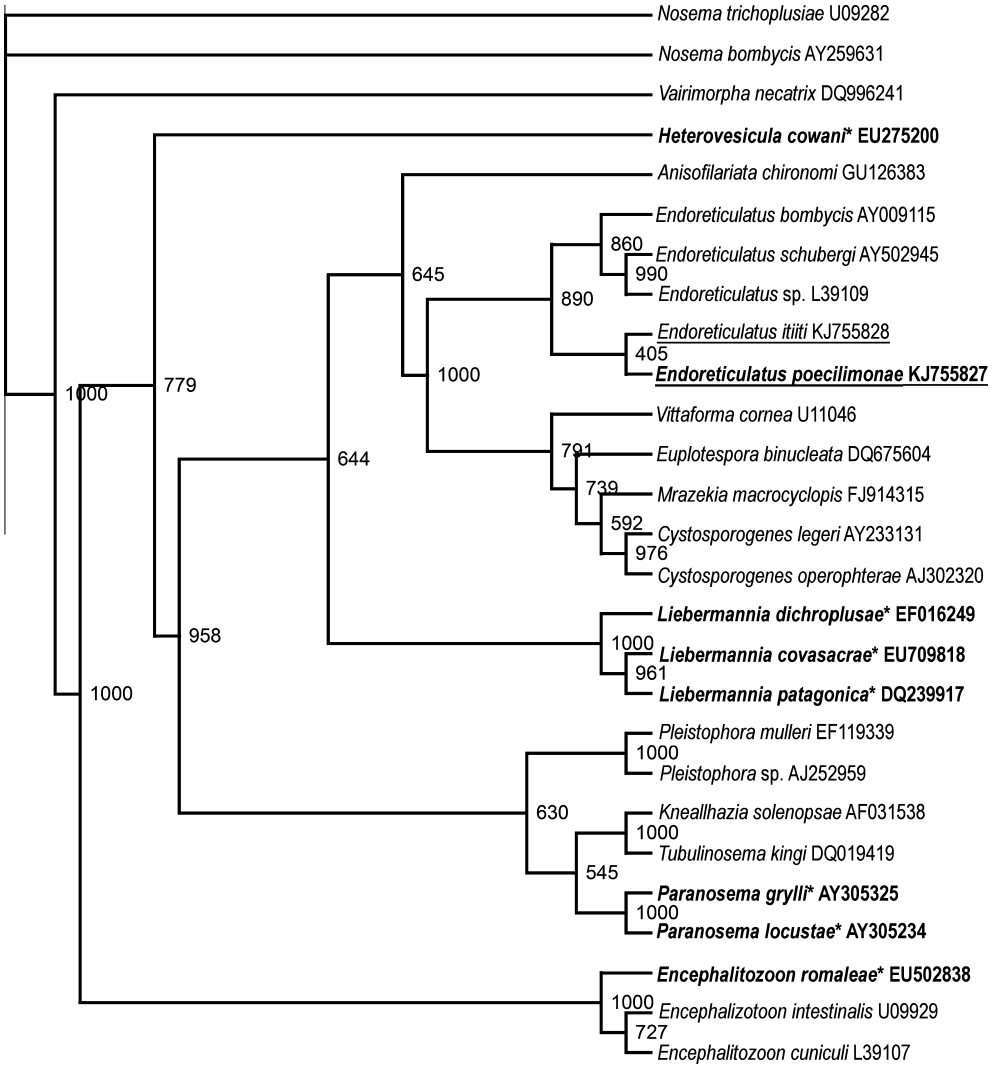


Fig. 12. Phylogenetic relationships among grasshopper Microsporidia and the *Endoreticulatus*/*Cystosporogenes* clade based on SSU rRNA. Microsporidian species in bold type were isolated from orthopteran hosts.

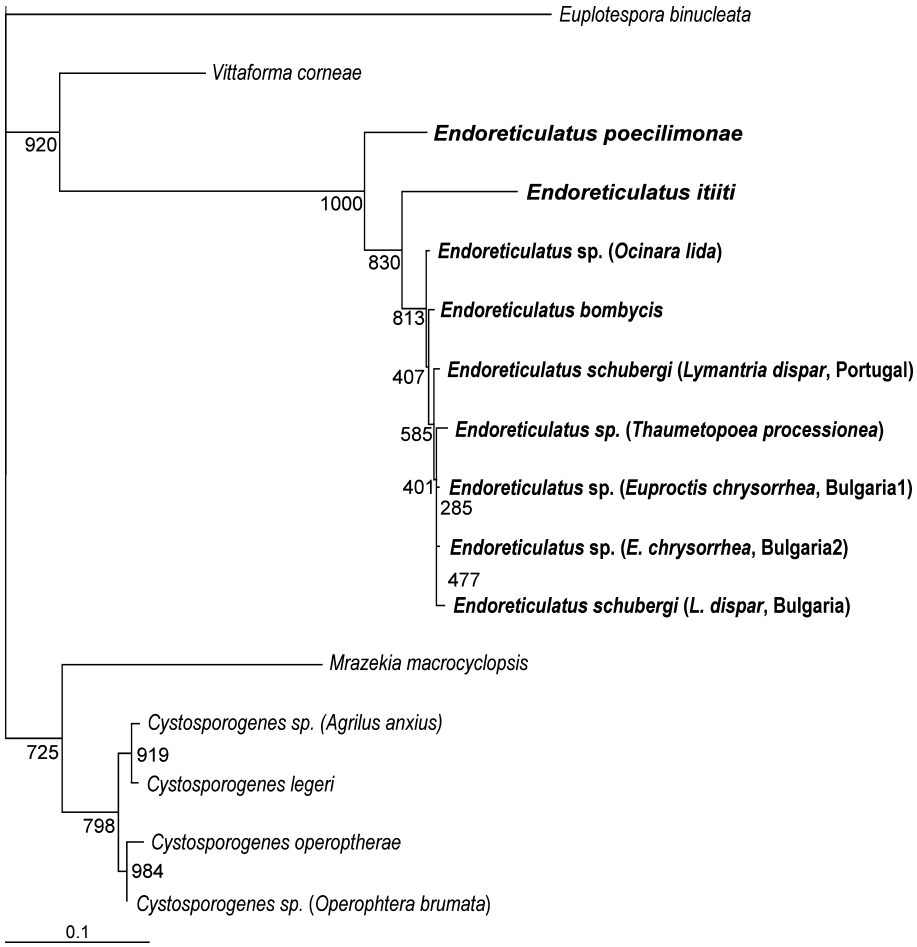


Fig. 13. Phylogenetic relationships among invertebrate Microsporidia in the *Endoreticulatus/Cystosporogenes* clade and closely related genera from invertebrate hosts.

M. itiiti SSU rRNA was included in our analysis to clarify the phylogenetic relationship between the *P. thoracicus Endoreticulatus* sp. and *Endoreticulatus* species isolated from Lepidoptera. *Endoreticulatus* spp. from different lepidopteran host species share high identities ranging from 98.7% to 99.8% (Fig. 13). The identity between *M. itiiti* and *E. schubergi* (95.1%) was similar to that between *P. thoracicus Endoreticulatus* and *E. schubergi* but *M. itiiti* and *P. thoracicus Endoreticulatus* shared only 93.1% identity. In the phylogenetic tree consisting of a larger spectrum of Microsporidia (Fig. 12), *M. itiiti* and *P. thoracicus Endoreticulatus* were grouped within the same clade, but the bootstrap value was only 405/1000. In the tree consisting primarily of *Endoreticulatus* species (Fig. 13), *M. itiiti* was distinct but grouped more closely than *P. thoracicus Endoreticulatus* with the lepidopteran *Endoreticulatus* species. The *P. thoracicus Endoreticulatus* was basal to all other sequenced *Endoreticulatus* species.

4. Discussion

Five species of Microsporidia pathogenic to invertebrate animals have been formally described and an additional three putative species were provisionally assigned to the genus *Endoreticulatus*. Five of these isolates were recovered from lepidopteran insects, one from a beetle and two from crustaceans (Table 2). Three insect isolates have been fully described, including the type species, *Endoreticulatus fidelis* (Brooks et al., 1988) isolated from the coleopteran *Leptinotarsa decemlineata* (no sequence available) and two species from Lepidoptera, *Endoreticulatus bombycis* isolated from *Bombyx mori* and several isolates of *Endoreticulatus schubergi* from *Choristoneura fumiferana*, *Lymantria dispar*, *Hyphantria cunea*, and *Euproctis chrysorrhoea*. Homology was greater than 99% for all sequenced lepidopteran isolates, with the exception of an isolate was recovered from *Thaumetopoea processionea* (Hoch et al., 2008), which was 98.7–99.3% similar to the other isolates. Various *Pleistophora* isolates from Lepidoptera were reviewed by Pilarska et al. (2002) and, although most of these historically reported isolates from different hosts were not sequenced to confirm species status, Microsporidia of similar morphotypes recently isolated and sequenced from the same host species suggest that they represent *Cystosporogenes* (van Frankenhuyzen et al., 2004) and *Endoreticulatus* species (Wang et al., 2005; Solter et al., 2010; WHF & LFS unpublished data).

In addition to the *Endoreticulatus* species isolated from insects, two species were described from other invertebrate taxa. *Endoreticulatus eriocheir* was described from the hepatopancreas of the mitten crab, *Eriocheir sinensis* (Wang and Chen, 2007). This isolate possesses some morphological characters that differ from those of the insect species (Table 2), including a smooth exospore, presence of only one developmental stage among parasitophorous vacuoles in one host cell, and much smaller spore size than other reported *Endoreticulatus* species. Unfortunately, the authors were not able to obtain a SSU-rRNA sequence from the fixed tissues and there is reason to question whether the isolate was correctly assigned to the genus. *Endoreticulatus durforti* was described from the crustacean *Artemia* sp. (Martinez et al., 1993) and, likewise, possesses significantly different morphology (Table 2) including smaller spores, up to 128 spores in the parasitophorous vacuole,

Table 2
Described and putative *Endoreticulatus* species.

Species	Host	Infected tissue	Spore size (μm)	# Coils	Exospore/endospore	References
Endoreticulatus bombycis	*Bombyx mori* (Lepidoptera)	Midgut epithelium	2.26 ± 0.21 × 1.19 ± 0.18	7–9	Exospore-crenate	Zhang et al. (1995), Wan (2005)
Endoreticulatus durforti[a]	*Artemia* sp. (Anostraca)	Intestinal epithelium	1.7 ± 0.15 × 0.98 ± 0.12	8–11	Exospore relatively smooth	Martinez et al. (1993)
Endoreticulatus eriocheir[a]	*Eriocheir sinensis* (Decapoda)	Hepato-pancreas	1.7 ± 0.2 × 1.0 ± 0.2	7	Exospore relatively smooth	Wang and Chen (2007)
Endoreticulatus fidelis[a]	*Leptinotarsa decemlineata* (Coleoptera)	Midgut epithelium	2.62 ± 0.03 × 1.51 ± 0.02	5–7	Exospore-crenate; endospore-electron transparent, thicker	Brooks et al. (1988)
Endoreticulatus poecilimonae	*Poecilimon thoracicus* (Orthoptera)	Midgut epithelium	2.58 ± 0.21 × 1.34 ± 0.24	8–9	Exospore – 20–30 nm crenate; endospore – 45–70 nm thick, sometimes with a homogenous/ fibrous structure	This study
Endoreticulatus schubergi	*Lymantria dispar*, *Hyphantria cunea*, *Choristoneura* fumiferana (Lepidoptera)	Midgut epithelium	2.4 ± 0.5 × 1.5 ± 0.5	7–9	Exospore –crenate; endospore-thinnest at the anterior part	Zwölfer (1927), Cali and El Garhy (1991)
Endoreticulatus sp.[a]	*Euproctis chrysorrhoea*, (Lepidoptera)	Systemic	2.48 ± 0.23 × 1.23 ± 0.17	8–9	Exospore– crenate	Pilarska et al., 2002
Endoreticulatus sp.	*Thaumetopoea processionea*	Midgut epithelium	2.5 × 1.4	–	–	Hoch et al. (2008)
Endoreticulatus sp.	*Ocinaria lida* (Lepidoptera)	Midgut epithelium	2.1 ± 0.2 × 0.9 ± 0.1	8–10	Exospore – 20 nm, crenate; endospore – 22.5–92.5 nm, some fibrous structure	Wang et al. (2005)
Endoreticulatus itiiti	*Listronotus bonariensis* (Coleoptera)	Systemic; primarily midgut epithelium	2.5 ± 0.04 × 1.4 ± 0.03	8–10	–	Malone (1985)

[a] No sequence available.

different polar filament configuration and rosette-shaped fragmentation of sporogonal plasmodia. There is no published SSU-rRNA sequence for this species to confirm or reject placement in the genus *Endoreticulatus*.

The spore sizes of the *P. thoracicus Endoreticulatus* sp. and *M. itiiti* are very similar to *E. schubergi* and ultrastructural characteristics do not differ significantly from those of *E. fidelis* (Brooks et al., 1988), *E. schubergi* (Cali and El Garhy, 1991), *E. bombycis* (Wan, 2005) and *Endoreticulatus* sp. from *Ocinara lida* (Wang et al., 2005). Phylogenetic analyses indicated that these isolates are within the *Cystosporogenes/Endoreticulatus* clade and are both more closely related to *Endoreticulatus* species than to *Cystosporogenes* but are basal to the *Endoreticulatus* clade.

Endoreticulatus species have not been isolated previously from Orthoptera. The only other orthopteran microsporidium with spores in vesicles and parasitizing the midgut and gastric caeca tissues is *Encephalitozoon romaleae*, described from the lubber grasshopper *Romalea microptera* (Lange et al., 2009). *E. romaleae* spores were larger, 3.97 ± 0.23 by 1.95 ± 0.08 μm, no parasitophorous vacuole was present, and the sequence data, including a full genome, clearly placed the isolate with closest relatives *Encephalitozoon cuniculi* (89.1% homology) and *Encephalitozoon hellem* (95.6%

Table 3
Molecular relationships (SSU r-RNA) among *Endoreticulatus* species.

		1	2	3	4	5	6	7	8	9	10	11
1	*Endoreticulatus poecilimonae*	1033 bp	93.1%	95.2%	95.2%	95.1%	95.3%	95.3%	95.4%	94.5%	87.7%	86.7%
2	*Endoreticulatus itiiti*	93.1%	1025 bp	95.1%	95.1%	95.0%	95.2%	95.2%	95.2%	94.5%	87.3%	86.9%
3	*Endoreticulatus bombycis*	95.2%	95.1%	1028 bp	99.2%	99.2%	99.5%	99.5%	99.6%	98.7%	88.3%	87.4%
4	*Endoreticulatus schubergi* (*L. dispar*, Bulgaria AY502495)	95.2%	95.1%	99.2%	1028 bp	99.2%	99.6%	99.7%	99.2%	98.9%	88.5%	87.6%
5	*Endoreticulatus schubergi* (*L. dispar*, Portugal L39109)	95.1%	95.0%	99.2%	99.2%	1026 bp	99.5%	99.5%	99.2%	98.9%	88.6%	87.7%
6	*Endoreticulatus* sp. (*E. chrysorrhea*, Bulgaria1[a])	95.3%	95.2%	99.5%	99.6%	99.5%	1028 bp	99.9%	99.5%	99.1%	88.4%	87.5%
7	*Endoreticulatus* sp. (*E. chrysorrhea*, Bulgaria2[a])	95.3%	95.2%	99.5%	99.7%	99.5%	99.9%	1028 bp	99.5%	99.2%	88.4%	87.5%
8	*Endoreticulatus* sp. (*O. lida*, Taiwan)	95.4%	95.2%	99.6%	99.2%	99.2%	99.5%	99.5%	1028 bp	98.7%	88.3%	87.4%
9	*Endoreticulatus* sp. (*T. processionea*, Austria)	94.5%	94.5%	98.7%	98.9%	98.9%	99.1%	99.2%	98.7%	1028 bp	87.9%	87.0%
10	*Cystosporogenes legeri*	87.7%	87.3%	88.3%	88.5%	88.6%	88.4%	88.4%	88.3%	87.9%	1029 bp	98.2%
11	*Vittaforma cornea*	86.7%	86.9%	87.4%	87.6%	87.7%	87.5%	87.5%	87.4%	87.0%	98.2%	1032 bp

Listed sequence size was used in the alignment and to generate the phylogenetic tree (Fig. 13).
[*] Bulgaria1 = Sofia; Bulgaria2 = Melnik; distance between collection sites = 161 km.

homology), both vertebrate pathogens (Johny et al., 2008; Lange et al., 2009; Pombert et al., 2012). *E. romaleae* and the *P. thoracicus* microsporidium share only 59.7% identity.

The microsporidian SSU rRNA gene is highly conserved and only a few nucleotide differences (one to several) may exist among geographically isolated species with non-overlapping hosts (Canning et al., 1999; Tsai et al., 2005). Nevertheless, SSU rRNA remains the most commonly used marker for identifying a new microsporidian species. The sequences of the *P. thoracicus* isolate and the *M. itiiti* are the only *Endoreticulatus* SSU rRNA sequences reported from non-lepidopteran hosts. Identity among all sequenced lepidopteran isolates is approximately 99% (see also Wang et al., 2005). The *P. thoracicus* isolate and *M. itiiti* are distinct from one another and both isolates are separate from the lepidopteran clade. In addition, the *P. thoracicus* isolate is distinct from other orthopteran Microsporidia. While the morphological characters are nearly identical to those of the more closely related lepidopteran *Endoreticulatus* clade, the molecular data clearly demonstrate that the *P. thoracicus* isolate is a new species and we propose the name *Endoreticulatus poecilimonae*. Likewise, molecular data indicate that *M. itiiti* is a distinct species and is aligned with the *Endoreticulatus* clade.

Genetic analyses clearly indicate that *Endoreticulatus* species are not closely related to other microsporidian species in the family Encephalitozoon, which are more closely aligned with the *Nosema/Vairimorpha* clade (van Frankenhuyzen et al., 2004; Vossbrinck and DeBrunner-Vossbrinck, 2005). It is, instead, a sister clade to the genera *Cystosporogenes*, *Euplotespora*, *Mrazekia*, and the human pathogen, *Vittaforma corneae* (Kleespies et al., 2003; van Frankenhuyzen et al., 2004; Fokin et al., 2008; Tokarev et al., 2010; this study).

5. Taxonomic summary

Endoreticulatus poecilimonae Pilarska, Radek, Huang, Takov, Linde, Solter 2014, n. sp.

- **Type host:** *Poecilimon thoracicus* (Orthoptera: Tettigonidae).
- **Transmission**: suggested per os as mature spores form in the midgut and gastric caeca.
- **Site of infection**: midgut and gastric caeca tissues.
- **Spores**: monokaryotic, broadly oval, $2.58 \pm 0.21 \times 1.34 \pm 0.24$ μm (fresh), enclosed in a semipersistent parasitophorous vacuole. Polar filament coils are of the isofilar type with 8–9 oblique coils in a single row.
- **Type locality**: the material for this description was isolated from *Poecilimon thoracicus* adults collected in 2011 from Gabrovnitsa in Northwest Bulgaria (43°05.331′N: 023°27.626′E).
- **Deposition of type specimens**: living spores of this isolate are maintained in the liquid nitrogen collections at the Illinois Natural History Survey. In addition, Giemsa-stained slides are held in the collections of D. Pilarska, Bulgarian Academy of Sciences. The nucleotide sequence of the SSU rRNA gene is deposited in NCBI GenBank, Accession No. KJ755827.

Endoreticulatus itiiti (Malone, 1985) n. comb.

- **Synonomy:** *Microsporidium itiiti* Malone (1985).
- **Biological data**: see Malone (1985).
- **Deposition of type specimens**: living spores of this isolate (Accession Number 1995-D) are maintained in the liquid nitrogen collection at the Illinois Natural History Survey. The nucleotide sequence of the SSU rRNA gene is deposited in NCBI GenBank, Accession No. KJ755828.

Acknowledgments

We thank D. Chobanov for identification of the grasshopper species and V. Djorova for technical assistance. The research was supported by the German Academic Exchange Service (DADD) Nos. A/13/03511 and A/13/03813, German Research Foundation (DFG) No. DFG GZ LI 551/10-1, National Science Fund of Bulgaria, Project DO-02-251/2008, and the Illinois Natural History Survey, University of Illinois.

References

Becnel, J.J., 2012. Complementary techniques: preparations of entomopathogens and diseased specimens for more detailed study using microscopy. In: Lacey, L.A. (Ed.), Manual of Techniques in Invertebrate Pathology. Elsevier, San Diego, pp. 451–470.

Bell, A.S., Aoki, T., Yokoyama, H., 2001. Phylogenetic relationships among microsporidia based on rDNA sequence data, with particular reference to fish-infecting Microsporidium Balbiani 1884 species. J. Eukaryot. Microbiol. 48, 258–265.

Brooks, W.M., Becnel, J.J., Kennedy, G.G., 1988. Establishment of *Endoreticulatus* n. g. for *Pleistophora fidelis* (Hostounsky & Weiser 1975) (Microsporidia: Pleistophoridae) based on the ultrastructure of a microsporidium in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). J. Protozool. 35, 481–488.

Cali, A., El Garhy, M., 1991. Ultrastructural study of the development of *Pleistophora schubergi* Zwölfer, 1927 (Protozoa, Microsporida) in larvae of the spruce budworm, *Choristoneura fumiferana* and its subsequent taxonomic change to the genus *Endoreticulatus*. J. Eukaryot. Microbiol. 38, 271–278.

Canning, E.U., 1953. A new microsporidian, *Nosema locustae* n. sp., from the fat body of the African migratory locust, *Locusta migratoria migratorioides* R. & F., Parasitology 43, 287–290.

Canning, E.U., Barker, R.J., Nicholas, J.P., Page, A.M., 1985. The ultrastructure of three microsporidia from winter moth, *Operophtera brumata* (L.), and the establishment of a new genus *Cystosporogenes* n. g. for *Pleistophora operophterae* (Canning, 1960). Syst. Parasitol. 7, 213–225.

Canning, E.U., Curry, A., Cheney, S., Lafranchi-Tristem, N.J., Haque, M.A., 1999. *Vairimorpha imperfecta* n. sp., a microsporidian exhibiting an abortive octosporous sporogony in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). Parasitology 119, 273–286.

Canning, E.U., Nicholas, J.P., 1980. Genus *Pleistophora* (Phylum Microspora): redescription of the type species, *Pleistophora typicalis* Gurley, 1893 and ultrastructural characterization of the genus. J. Fish Dis. 3, 317–338.

Cordes, N., Huang, W.-F., Strange, J.P., Cameron, S.A., Griswold, T.L., Solter, L.F., 2012. Interspecific geographic distribution and variation of two pathogens, *Nosema bombi* and *Crithidia bombi*, in United States bumble bee populations. J. Invertebr. Pathol. 109, 209–216.

Fokin, S.I., Di Giuseppe, G., Erra, F., Dini, F., 2008. *Euplotespora binucleata* n. gen., n. sp. (Protozoa: Microsporidia), a parasite infecting the hypotrichous ciliate *Euplotes woodruffi*, with observations on microsporidian infections in Ciliophora. J. Eukaryot. Microbiol. 55, 214–228.

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Gurley, R.R., 1893. On the classification of the Myxosporidia, a group of protozoan parasites infesting fishes. Bull. US Fish. Commun. 11, 407–420.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hernández-Crespo, P., Lipa, J.J., Santiago-Alvarez, C., 2001. Prevalence of a *Nosema* sp. (Microsporida: Nosematidae) in natural populations of *Chorthippus brunneus* (Orthoptera: Gomphocerinae) in Southern Spain. Biocontrol Sci. Technol. 11, 541–546.

Hoch, G., Verucci, S., Schopf, A., 2008. Microsporidian pathogens of the oak processionary moth *Thaumetopoea processionea* (L.) (Lep., Thaumetopoeidae), in eastern Austria. Mitt. Dtsch. Ges. Allg. Angew. Ent. 16, 225–228.

Johny, S., Larson, T.M., Solter, L.F., Edwards, K.A., Whitman, D.W., 2008. Phylogenetic characterization of *Encephalitozoon romaleae* (Microsporidia) from a grasshopper host: relationship to *Encephalitozoon* spp. infecting humans. Infect. Genet. Evol. 9, 189–195.

Kleespies, R.G., Vossbrinck, C.R., Land, M., Jehle, J.A., 2003. Morphological and molecular investigations of a microsporidium infecting the European grape vine moth, *Lobesia botrana* Den. et Schiff., and its taxonomic determination as *Cystosporogenes legeri* nov. comb. J. Invertebr. Pathol. 83, 240–248.

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.

Malone, L.A., 1985. A new pathogen, *Microsporidium itiiti* n. sp. (Microsporidia) from the Argentine stem weevil, *Listronotis bonariensis* (Coleoptera: Curculionidae). J. Protozool. 32, 531–535.

Malone, L.A., 1987. Longevity and fecundity of Argentine stem weevils, *Listronotis bonariensis* (Coleoptera: Curculionidae) infected with *Microsporidium itiiti* (Protozoa: Microspora). J. Invertebr. Pathol. 50, 113–117.

Martinez, M.A., Vivares, C.P., Bouix, G., 1993. Ultrastructural study of *Endoreticulatus durforti* n. sp., a new microsporidian parasite of the intestinal epithelium of *Artemia* (Crustacea, Anostraca). J. Eukaryot. Microbiol. 40, 677–687.

Pilarska, D., Linde, A., Solter, L., McManus, M., Takov, D., 2002. New data on the biology of the microsporidium *Endoreticulatus schubergi* infecting the browntail moth *Euproctis chrysorhoea* (Lepidopter: Lymantriidae). Acta Zool. Bulg. 54, 55–62.

Pombert, J.F., Selman, M., Burki, F., Floyd, B.P., Farinelli, K., Solter, L.F., Whitman, D.W., Weiss, L.M., Corradi, N., Keeling, P.J., 2012. Gain and loss of multiple functionally-related horizontally transferred genes in the reduced genomes of two microsporidian parasites. Proc. Natl. Acad. Sci. 109, 12638–12643. http://dx.doi.org/10.1073/pnas.1205020109.

Richardson, K.C., Jarrett, L., Finke, E.H., 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technol. 35, 313.

Sokolova, Y.Y., Dolgikh, V.V., Morzhina, E.V., Nassonova, E.S., Issi, I.V., Terry, R.S., Ironside, J.E., Smith, J.E., Vossbrinck, C.R., 2003. Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* Gen. Nov., Comb. Nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus* Deg. J. Invertebr. Pathol. 84, 159–172.

Sokolova, Y., Lange, C., Fuxa, J., 2006. Development, ultrastructure, natural occurrence, and molecular characterization of *Liebermannia patagonica* n. g., n. sp., a microsporidian parasite of the grasshopper *Tristira magellanica* (Orthoptera: Tristiridae). J. Invertebr. Pathol. 91, 168–182.

Solter, L.F., Pilarska, D.K., McManus, M.L., Zubrik, M., Patočka, J., Huang, W.-H., Novotny, J., 2010. Host specificity of microsporidia pathogenic to the gypsy moth, *Lymantria dispar* (L.): field studies in Slovakia. J. Invertebr. Pathol. 105, 1–10.

Sprague, V., 1977. Systematics of the microsporidia. In: Bulla, L.A., Cheng, T.C. (Eds.), Comparative Pathobiology, vol. 2. Plenum Press, New York, pp. 1–510.

Tokarev, Y.S., Voronin, V.N., Seliverstova, E., Dolgikh, V., Pavlova, O.A., Ignatieva, A.N., Issi, I.V., 2010. Ultrastructure and molecular phylogeny of *Anisofilariata chironomi* g.n. sp.n. (Microsporidia: Terresporidia) from *Chironomus plumosus* L. (Diptera: Chironomidae). Parasitol. Res. 107, 39–46.

Tsai, S.J., Huang, W.F., Wang, C.H., 2005. Complete sequence and gene organization of the *Nosema spodopterae* rRNA gene. J. Eukaryot. Microbiol. 52, 52–54.

van Frankenhuyzen, K., Ebling, P., McCron, B., Ladd, T., Gautheir, D., Vossbrinck, C., 2004. Occurrence of *Cystosporogenes* sp. (Protozoa, Microsporidia) in a multi-species insect production facility and its elimination from a colony of the eastern spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). J. Invertebr. Pathol. 87, 16–28.

Vavra, J., Becnel, J.J., 2007. *Vavraia culicis* (Weiser, 1947) Weiser, 1977 revisited: cytological characterisation of a *Vavraia culicis*-like microsporidium isolated from mosquitoes in Florida and the establishment of *Vavraia culicis floridensis* subsp. n. Folia Parasitol. 54, 259–271.

Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol. 52, 131–142.

Wan, Y.J., 2005. Study and control on several population of pathogenic microsporidian in the silkworm, *Bombyx mori* (Bombycidae, Lepidoptera). Ph.D. Thesis. Agriculture and Biotechnology College, China Agricultural University, Beijing, China. 91 pp.

Wang, C.Y., Solter, L.F., T'sui, W.H., Wang, C.H., 2005. An *Endoreticulatus* species from *Ocinara lida* (Lepidoptera: Bombycidae) in Taiwan. J. Invertebr. Pathol. 89, 123–135.

Wang, W., Chen, J., 2007. Ultrastructural study on a novel microsporidian, *Endoreticulatus eriocheir* sp. nov. (Microsporidia, Encephalitozoonidae), parasite of Chinese mitten crab, *Eriocheir sinensis* (Crustacea, Decapoda). J. Invertebr. Pathol. 94, 77–83.

Weiser, J., 1977. Contribution to the classification of microsporidia. Věstn. Čs. Spol. Zool. 41, 308–320.

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.), Microsporidia and Microsporidiosis. ASM Press, Washington D.C., pp. 129–171.

Zhang, L., Du, Y., Wang, Y.J., 1995. A new species of *Endoreticulatus* (Protozoa, Microspore) from the larva of silkworm, *Bombyx mori* L. J. Southwest Agric. Univ. 17, 363–367 (In Chinese with English abstract).

Zwölfer, W., 1927. Die Pebrie des Schwammspinner und Goldafters, eine neue wirtschaftlich bedeutungsvolle Infektionskrankheit. Z. Angew. Entomol. 12, 498–500.