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Tubulinosema loxostegi sp. n. (Microsporidia: Tubulinosematidae) from the Beet Webworm *Loxostege sticticalis* L. (Lepidoptera: Crambidae) in Western Siberia

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Abstract. Adults of beet webworm *Loxostege sticticalis* were collected in Western Siberia in 2009 and 2010. A microsporidium was found infecting 12 of 50 moths in 2010. The parasite develops in direct contact with host cell cytoplasm, sporogony is presumably disporoblastic. The spores are ovoid, diplokaryotic, 4.2 × 2.4 μm in size (fresh), without a sporophorous vesicle. Electron microscopy showed: (a) tubules on the surface of sporoblasts and immature spores; (b) slightly anisofilar polar tube with 10–14 coils, last 2–3 coils of lesser electron density; (c) bipartite polaroplast with anterior and posterior parts composed of thin and thick lamellae, respectively; (d) an indentation in the region of the anchoring disc; (e) an additional layer of electron-dense amorphous matter on the exospore surface. The spore ultrastructure is characteristic of the genus *Tubulinosema*. Sequencing of small subunit and large subunit ribosomal RNA genes showed 98–99.6% similarity of this parasite to the *Tubulinosema* species available on Genbank. A new species *Tubulinosema loxostegi* sp. n. is established.

Key words: Beet webworm, microsporidia, taxonomy, molecular phylogenetics, *Tubulinosema.*

INTRODUCTION

Biological mortality factors, namely the predators, parasitoids and pathogens, are of great concern in insect population ecology (Hawkins *et al.* 1997). Among pathogenic microorganisms affecting insect population density dynamics, an important role is played by Microsporidia, a unique group of unicellular parasitic eukaryotes with uncertain systematic placement, but phylogenetically related to the Fungi. Microsporidia are parasites of all the major taxa of animals, but most often reported from arthropods and fish. Dozens of microsporidial species have been reported from the Lepidoptera, the vast majority of which belong to the genera *Nosema* (Tsai *et al.* 2003, 2009; Hylis *et al*. 2006; Johny *et al.* 2006; Kyei-Poku *et al.* 2008; Zhu *et al.* 2010; Guan *et al.* 2012) and *Vairimorpha* (Canning *et al.* 1999, Vávra *et al.* 2006, Wang *et al.* 2009, Liu *et al.* 2012). Other genera that include species infecting

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Lepidoptera are *Cystosporogenes* (Canning *et al.* 1985, Kleespies *et al.* 2003, Solter *et al.* 2010), *Endoreticulatus* (Cali and Garhy 1991, Xu *et al.* 2012), *Orthosomella* (Andreadis *et al.* 1996) and *Vavraia* (Bourner *et al.* 1996). Microsporidial infections of Lepidoptera can affect the population density dynamics and limit the outbreak capacity in economically important species such as the cabbage white *Pieris brassicae* (Issi 1986), the gypsy moth *Limantria dispar* (Solter and Hajek 2009), the spruce budworm *Choristoneura fumiferanae* (Wilson 1973), the jack pine budworm *Choristoneura pinus* (van Frankenhuyzen *et al.* 2011), the green tortix *Tortrix viridana* (Lipa 1976, Franz and Huger 1971) and an undescribed microsporidium from the beet webworm *Loxostege sticticalis* (Frolov *et al.* 2008).

We describe here, based upon ultrastructural and molecular analysis, a new species of microsporidia from the beet webworm *L. sticticalis* sampled in Western Siberia. The beet webworm has a Holarctic distribution and the ability for long-distance migration resulting in regular, severe outbreaks in Eurasia (Frolov *et al.* 2008, Chen *et al.* 2008, Huang *et al.* 2008).

MATERIALS AND METHODS

Adults of *L. sticticalis* were hand neted during periods of mass flight in July 2009, September 2009 and July 2010 in the Novosibirsk region of Western Siberia, Karasuk district (53°42′N, 77°45′E). Dry cadavers were transported to the laboratory and stored frozen prior to analysis. Cadavers were homogenized in a drop of water and examined using conventional light microscopy. The spores were fixed and stained with diamidine phenylenindole (DAPI, Tokarev *et al.* 2007). For electron microscopy (EM), infected tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 4% sucrose (2 hr) and postfixed with 1% cacodylate–buffered osmium tetroxide (1 hr). The tissues were dehydrated in an ascending ethanol series followed by absolute acetone and embedded into epon–araldite resin. Ultrathin sections were cut using a Leica Ultracut or Ultratome–III (LKB) and stained with 2% uranylacetate in 50% ethanol followed by lead citrate for 10–20 min. The material was examined using an electron microscope LEO 910 (LEO Electron Microscopy Group, Germany) at an accelerating voltage of 80 kV.

For the molecular phylogenetic analysis, the individual specimens of infected adults were homogenized in 1.5 ml test tubes using an adapted Teflon pestle. The homogenates were filtered using a syringe plugged with cotton. DNA was extracted from the homogenates as described by Tokarev *et al.* (2010). Testing the microsporidial DNA sample quality was performed using PCR with primers 18f:530r. The partial small subunit (SSU), internal transcribed spacer (ITS) and partial large subunit (LSU) sequence of the ribosomal rRNA gene was amplified using two sets of primers: 18f:ss1492r and ss1061f:ls580r, spanning two overlapping regions, respectively (Weiss and Vossbrinck 1999). PCR was run using a Bio–Rad MyCycler in 20 µl volume containing 10 µl DNA template, 2 µl of 10 × PCR buffer, 1 µl of 10 µM dNTPs mixture, 1 µl of each forward and reverse 10 µM primers (Beagle, Russia) and 1 U of Colored Taq–polymerase (Sileks, Russia). A first cycle of denaturation was carried out at 95°C (5 min.), and a last cycle of extension was carried out at 72°C (10 min.). Samples were amplified for 30 cycles of denaturation at 95°C (60 s), annealing at 54°C (30 s) and elongation at 72°C (30 s with 18f:530r primers or 60 s with other primer sets). For amplification with primers 18f:1492r, the number of PCR cycles was increased from 30 to 50 to obtain visible bands. The PCR products were gel purified, cloned into pAL–TA vector (Evrogen, Russia) and sequenced in both directions. The alignment of the newly obtained sequences with those showing significant similarity (Table 1) was done automatically using CLUSTAL W algorithm and edited by eye in BioEdit v7.0.8.0 (Hall 1999). Regions containing gaps and ambiguous sites were removed, leaving an alignment of 1,087 nucleotides. Phylogenetic reconstructions were carried out with Bayesian Inference in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and Maximum Likelihood in PAUP* v4.0 β 10 (Swofford 2003).

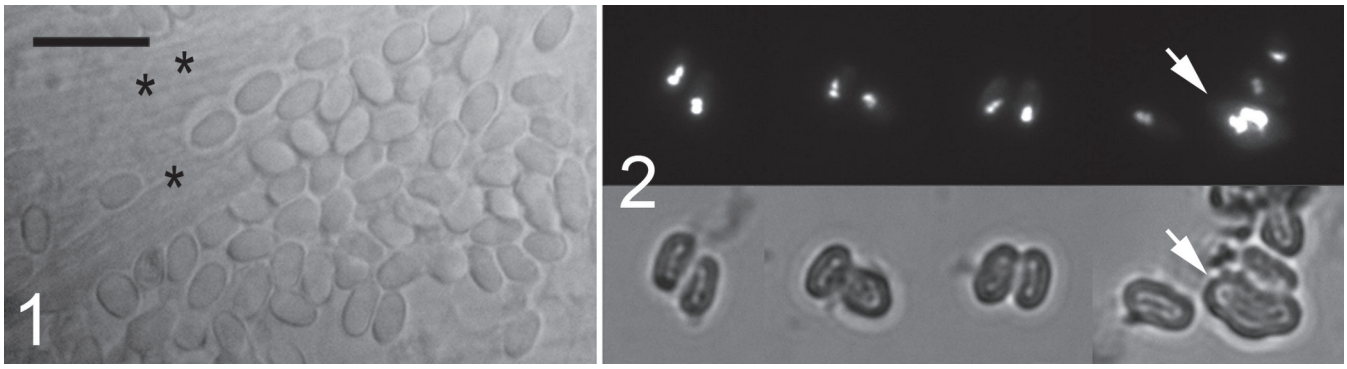
RESULTS

Light microscopy

No microsporidial infections were detected among moths sampled in July (number of moths examined, N = 15) or September (N = 15) 2009. However, in 2010, 12 of 50 examined moths were heavily infected with spores of similar shape and size suggesting a single species (Fig. 1). This corresponded to the 24.0 ± 6.0% prevalence rate. The condition of the samples (frozen dry cadavers) made it impossible to assess prespore developmental stages, which are delicate and prone to deterioration. As a result, the parasite's life cycle, precise tissue localization and infected tissue pathology could not be described. However, inner tissues of two types were found heavily loaded with the parasite's spores. Tissue of the first type contained lipid droplets and was indentified as the adipose tissue. Tissue of the second type possessed strands of filamentous material (Fig. 1). Fresh spores measured 4.20 ± 0.05 × 2.40 ± 0.02 µm in size, with a length to width ratio of 1.8 (n = 50). In DAPI-stained spores a diplokaryotic configuration of nuclei was evident (Fig. 2). The spores are often found in pairs, suggesting the disporoblastic sporogony. A small proportion of aberrant spores, "teratospores" of Tokarev *et al.* (2007), were also observed. They were enlarged, of irregular shape, and contained two diplokarya (Fig. 2). The prevalence of teratospores was 0.75 ± 0.38% (n = 400).

Table 1. Microsporidian species used for phylogenetic studies in the present work and their small subunit rRNA gene sequence similarity to the new microsporidium (in bold).

Microsporidia species	Host	Genbank Accession #	Maximal sequence similarity to *T. loxostegi*, %
Anncaliia algerae	*Homo sapiens* (Primates: Hominidae)	AY230191	76.1
Anncaliia meligethi	*Meligethes aeneus* (Coleoptera: Nitidulidae)	AY894423	75.4
Janacekia debaisieuxi	*Simulium* sp. (Diptera: Simulidae)	AJ252950	68.7
Kneallhazia carolinensae	*Solenopsis carolinensis* (Hymenoptera: Formicidae)	GU173849	78.0
Kneallhazia solenopsae	*Solenopsis invictae* (Hymenoptera: Formicidae)	AY312502	77.6
Tubulinosema acridophagus	*Schistocerca americana* (Orthoptera: Acrididae)	AF024658	99.6
Tubulinosema hippodamiae	*Hippodamia convergens* (Coleoptera: Coccinellidae)	JQ082890	99.3
Tubulinosema kingi	*Drosophila willistoni* (Diptera: Drosophilidae)	DQ019419	98.6
Tubulinosema loxostegi	*Loxostege sticticalis* (Lepidoptera: Crambidae)	JQ906779	ID
Tubulinosema ratisbonensis	*Drosophila melanogaster* (Diptera: Drosophilidae)	AY695845	99.4

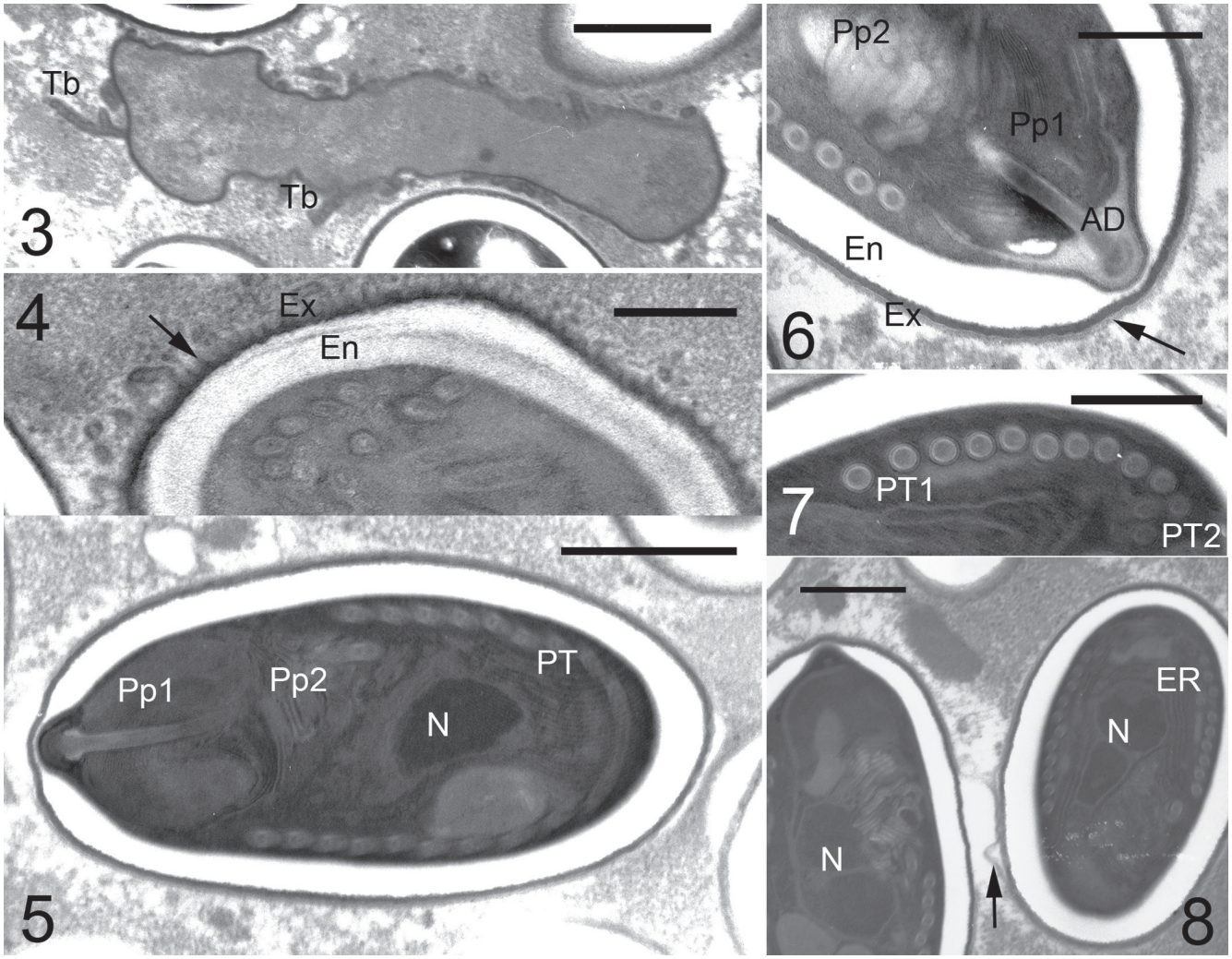


Figs 1–2. Light microscopy of *Tubulinosema loxostegi* spores. **1** – bright field image of a fresh smear, showing numerous spores and filamentous material of the host tissue (asterisks); **2** – fluorescent (upper) and bright field images (lower) of methanol-fixed and DAPI-stained smears, including a teratospore (arrow) with a double set of diplokaryotic nuclei. Scale bar: 10 μm.

Electron microscopy

On the ultrathin sections only the sporoblasts, immature and mature spores were observed, the latter being the most abundant developmental stage. The earlier developmental stages could not be visualized due to the limitations introduced by the specimen storage conditions. All parasite cells developed in direct contact with the host cell cytoplasm. The sporoblasts (Fig. 3) were rare, irregular in shape and averagely 2.8 ± 0.6 × 0.6 ± 0.1 μm in size (n = 3). Tubules about 30 nm in diameter were found adjacent or connected to the sporoblast surface. This could not be judged whether the sporoblast shape and extensions are not artifact due to poor tissue preservation. The immature exospore (Fig. 4) possessed short spiky extensions, some of which were connected to tubular structures similar to those observed in sporoblasts. The fixed mature spores were oval to elongated-oval, measuring 2.04 ± 0.04 × 2.1 ± 0.04 μm (n = 10) (Figs 5, 8). The nuclei were diplokaryotic in arrangement, 0.5–0.7 × 0.9–1.2 μm in diameter, and surrounded with 3–4 layers of polyribosome rich endoplasmatic reticulum (Figs 5, 8). The anterior nucleus of the diplokaryon was flattened toward the polaroplast and nearly conical shaped, while the posterior nucleus was spherical. The anchoring disc protruded out-

wards forming an indentation (Figs 5, 6) at the anterior pole of the spore. The polaroplast was bipartite, with the anterior part composed of tightly packed thin lamellae. The posterior part of the polaroplast consisted of thick lamellae (Fig. 5) though in some spores, they possessed the appearance of flattened vesicles of low electron density (Fig. 6). The polar tube was slightly anisofilar, possessing 10–14 coils with the posterior 3–4 coils being of smaller diameter and electron density (Fig. 7). The average diameter of anterior and posterior coils is 64 ± 1 (n = 41) and 49 ± 1 nm (n = 22), respectively. The endospore is 80 to 170 (average 120 ± 7, n = 13) nm thick, thinning over the anchoring disc. The exospore is 20 to 30 (average 25 ± 1, n = 13) nm thick. The exospore is covered with an outer layer of amorphous matter of moderate electron density, 13 to 28 (average 20 ± 1) nm thick (Fig. 8). A spore with the prominent pro-



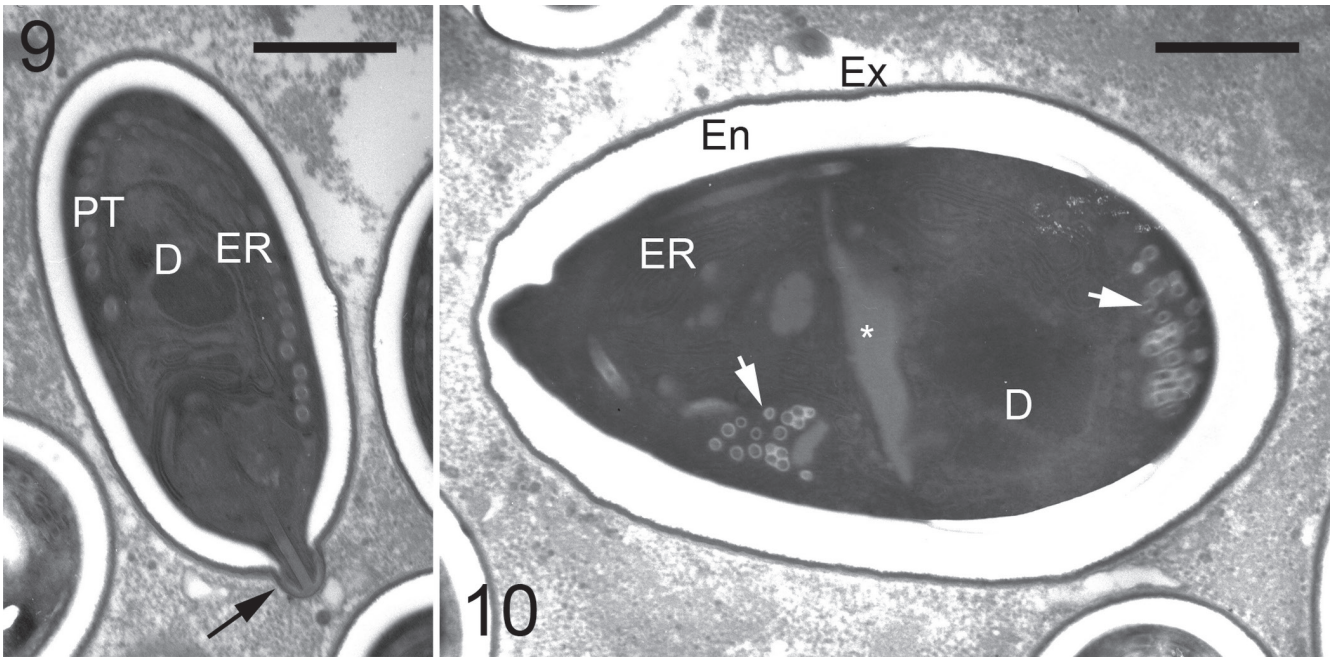
Figs 3–8. Electron microscopy of *Tubulinosema loxostegi*. **3** – the sporoblast with tubules on its surface; **4** – exospore of the immature spore with short spiky extensions (arrow); **5** – mature spore within the cytoplasm of the host cell showing the disposition of bipartite polaroplast, the polar tube and the nucleus; **6** – the anterior part of the spore demonstrating structure of the anchoring disc and the polaroplast, as well as the additional layer of the exospore (arrow); **7** – the polar tube coils, with posterior coils of lesser diameter; **8** – two spores showing the diplokaryotic arrangement of the nuclei and delamination of the outer layer of the exospore (arrow), possibly due to an artifact of poor tissue preservation. AD – anchoring disc, En – endospore, ER – endoplasmatic reticulum, Ex – exospore, N – nuclei, Pp1 – anterior part of the polaroplast, Pp2 – posterior part of the polaroplast, PT – polar tube, PT1 – anterior coils of PT, PT2 – posterior coils of PT, Tb – tubules. Scale bars: 1 μm (3, 5, 8), 0.5 μm (4, 6, 7).

trusion of the anchoring disc, characteristic of spore activation (perhaps due to the fixative) was found (Fig. 9). A teratoid spore (Fig. 10), approximately twice as large as typical spores, was also observed. Its inner content was remarkably different from that of normal spores, containing irregularly laid layers of amorphous matter, ER and tubules of varied diameter (compare Figs 9 and 10).

Molecular phylogenetics

The PCR products using 18f:530r primers showed two bands ca. 450 bp and ca. 600 bp respectively. The approximately 600 bp fragment (549 bp) (Genbank accession # JQ906778) is assumed, because of its close relationship to other lepidopteran species of the family Crambidae based on BLAST analysis (99.3% maximal sequence similarity to *Ostrinia furnacalis* 18S rRNA gene, Genbank accession # GU205787), to be the rRNA gene sequence of the insect host, *L. stricticalis*. Two amplicons ca. 1400 and 1000 bp were amplified using 18f:1492r and ss1061f:ls580r primers, respectively. The two sequences were identical in their expected region of overlap and the resulted concatenated sequence was 1876 bp long (Genbank accession # JQ906779). BLAST analysis showed the maximal similarity of this partial SSU-ITS-LSU sequence to the rRNA molecular haplotypes of *Tubulinosema ratisbonensis* and *Tubulinosema kingi* of 98.4 and 97.7%, respectively. The SSU sequence of the new microsporidium, 1399 bp long, showed high similarity to those of *T. acridophagus* (99.6%), *T. ratisbonensis* (99.4%), *T. hippodamiae* (99.3%) and *T. kingi* (98.6%). The ITS sequence, 41 bp long, showed similarity of 83.3 and 79.0% to *T. ratisbonensis* and *T. kingi*, respectively. The partial LSU rRNA gene sequence of the new microsporidium, 436 bp long, was similar by 96.7% to the respective sequences of *T. ratisbonensis* and *T. kingi*, the two latter being identical at the aligned region.

The close sequence similarity of the new microsporidium and the *Tubulinosema* species was reflected by their "tight" monophyletic grouping in the phylograms while the other members of the family Tubulinosematidae *sensu* Franzen *et al.* 2005 form a sister clade and include *Kneallhazia (Thelohania) solenopsae* and *Anncaliia meligethi* (Fig. 11).



Figs 9–10. Electron microscopy of abberant spores of *Tubulinosema loxostegi*. **9** – a spore with the prominent protrusion of the anchoring disc (arrow), characteristic of spore activation (perhaps due to the fixative); **10** – an oversized teratospore with irregularly laid layers of ER, amorphous matter (asterisk) and tubules (arrows). Abbreviations as in Figs 3–8. Scale bars: 1 μm.

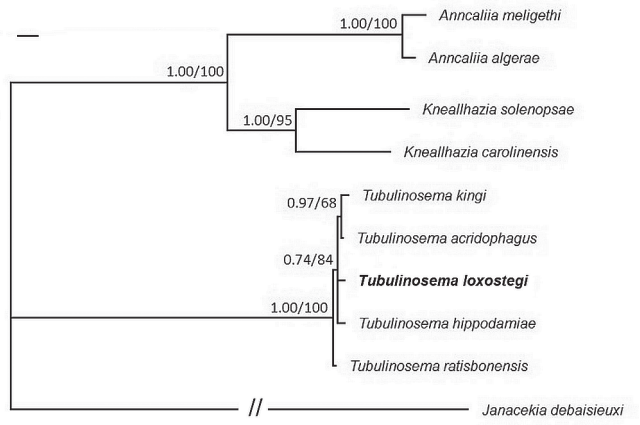


Fig. 11. Molecular phylogenetics of *Tubulinosema loxostegi* and related taxa, as obtained by Bayesian inference (BI) and Maximum likelihood (ML) from an alignment of respective ribosomal RNA gene sequences listed in Table 1. The branch support is given as posterior probability for BI and bootstrap value for ML. The branch length for the outgroup *Janacekia debaisieuxi* is reduced twofold (double slash). Scale bar: 0.01 expected changes per site.

DISCUSSION

Our limited sampling data did not allow us to evaluate any potential correlation between the abundance of *L. sticticalis* and microsporidial infection rates in Western Siberia. We note, however, that when the insect population density was at its peak and the infested area in Siberian Federal District (including Western and Eastern Siberia) equaled to 2344 insects ha^{-1} in 2009, no microsporidian infections were detected. However, in 2010, when the insect population collapsed the infested region in Siberia was reduced to 629 insects ha^{-1}, the microsporidian infection rate was 24%. This decrease of the pest population continued in 2011 and the infested region in Siberia comprised 372 ha (Govorov *et al.* 2012), but populations were not sampled in the vicinities of Novosibirsk.

Unfortunately, as the insect samples were transported to our laboratory as dry moth cadavers, and larval populations were not sampled in Novosibirsk region during the pest outbreak, we did not obtain ultrastructural information on the prespore developmental stages (due to limitations of the tissue preservation) nor did we obtain incidence rates data concerning parasite-host interactions (due to arbitrary "snaphot" sampling of a limited number of moths). However, we feel that the information presented here, including the spore ultrastructure and the ribosomal RNA gene sequence, represents the most critical factors for the description of a new species of microsporidia (Tokarev *et al.* 2010).

The ultrastructural and molecular characters indicate a close affinity of the newly discovered microsporidium to the genus *Tubulinosema* (Franzen *et al.* 2005). The sequence similarity between the species of this genus is high, ranging from 98.5% (between *T. hippodamiae* and *T. kingi*) to 99.7% (between *T. acridophagus* and *T. ratisbonensis*). The sequence similarity of 99.6% between the new microsporidium and its closest relative, *T. acridophagus,* confirms that both species belong to the same genus, *Tubulinosema*. This relatedness corresponds well to the ultrastructural similarity in *Tubulinosema* spp., sometimes making it problematic to differentiate among these species at the ultrastructural level. For example, *Tubulinosema kingi* (Armstrong *et al.* 1986, Franzen *et al.* 2006) and *Tubulinosema acridophagus* (Streett and Henry 1993) share such characteristics as the spore dimensions, lamellar polaroplast, 10 to 14 polar tube coils with 3–4 posterior coils of lesser diameter, and a layer of tubular elements, 20–40 nm in dimater, on the surface of meront cells. The main difference between these two species, apart from their rRNA gene sequence divergence, is their natural host range (Henry 1967, Burnett and King 1962, Kramer 1964, see Table 2). Actually, all known *Tubulinosema* species possess diplokaryotic nuclei, a lamellar (or bipartite) polaroplast, a slightly anisofilar polar tube with 10–15 rows, an indentation at the anterior pole of the spore and tubular structures on the surface of the prespore developmental stages; the differences are only slight (Table 2). The only ultrastructural characteristic found unique in this new microsporidium is the uniform layer of moderate electron density covering the exospore. It also possesses bipartite lamellar polaroplast, as opposed to bipartite polaroplast in *Tubulinosema maroccanus* (Issi *et al*. 2008) with lamellar and tubular parts and uniform lamellar polaroplast in other species of *Tubulinosema* (Table 2). An additional feature is due to spiky extensions seen on the exospore of a young spore (all other species of *Tubulinosema* possess only tubular or vesicular structures on their cell surface, see Table 2) but this character is presented by a single sample only (Fig. 4). *T. maroccanus* from the orthopteran host (*Dociostaurus maroccanus*) and *T. hippodamiae* from the coleopteran host (*Hippodamia convergens*) are clearly different in their spore sizes from this new microsporidium from the beet webworm. This new species, described here, is

Table 2. Microsporidia of the genus *Tubulinosema*.

Microsporidia species	**Type host** and host range	Infected tissue	Spore shape and size (μm), spore ratio[a]	Polar tube coils[d]	Polaroplast	Endospore thickness (nm)	Surface ornamentation	References
Tubulinosema acridophagus (Henry 1967) Franzen *et al*. 2005	***Schistocerca americana***, *Melanoplus* spp. (Orthoptera, Acrididae), *Helicoverpa zea* (Lepidoptera, Noctuidae)	Mg, G, P, N, FB	ovoid with a narrowed anterior pole 4.1 × 2.6[b]; 3.9 × 2.5[c] 1.6	10–12 (3–4)	lamellar	no data	plasma membrane covered by a layer of tubular elements, ∅ 35 nm (in M)	Henry 1967, Henry and Oma 1974, Streett and Henry 1993
Tubulinosema hippodamiae Bjørnson *et al*. 2011	***Hippodamia convergens***, *Adalia bipunctata*, *Coccinella septempunctata*, *Coccinella trifasciata perplexa*, *Harmonia axyridis* (Coleoptera, Coccinellidae)	FB, MT, O, Pve, HE, Mu, N, CT	slightly pyriform 3.9 × 2.5[c] 1.6	10–14 (?)	lamellar	ca. 300	not found	Bjørnson *et al.* 2008, 2011; Saito and Bjørnson 2006, 2008
Tubulinosema kingi (Kramer 1964) Franzen *et al*. 2005	***Drosophila willistoni*** (Diptera, Drosophilidae)	Mg, G, MT, FB	oval 4.3 × 2.6[b]; 3.7 × 2.1[c] 1.7–1.8	10–14 (3–4)	lamellar	180–200	surface coat of tubular elements, ∅ 20–40 nm (in M)	Burnett and King 1962, Kramer 1964, Armstrong *et al.* 1986, Franzen *et al.* 2006
Tubulinosema loxostegi	***Loxostege sticticalis*** (Lepidoptera, Crambidae)	FB, CT, Mu	wide-oval 4.2 × 2.4[c] 1.8	10–14 (2–3)	bipartite: thin and thick lamellae	80–170	tubular structures, ∅ 30 nm (in SS and young S); continuous layer of amorphous matter (in S)	this paper
Tubulinosema maroccanus (Krilova and Nurzhanov 1987)	***Dociostaurus maroccanus*** (Orthoptera: Acrididae), *Calliptamus italicus* (Orthoptera, Catantopidae), Lepidoptera	GI	ellipsoidal 4.6 × 3.2[b]; 3.9 × 2.7[c] 1.4	12–15 (3–4)	bipartite: lammelar and tubular parts	up to 150	additional layer, formed by microtubules, ∅ ca. 20 nm (in M, S); small vesicles, ∅ up to 50 nm with electron-dense cores (in SS, S)	Krilova and Nurzhanov 1987, Krylova and Nurzhanov 1989, Issi *et al.* 2008
Tubulinosema ratisbonensis Franzen *et al*. 2005	***Drosophila melanogaster*** (Diptera, Drosophilidae)	FB, Mg, G, MT, Mu, N, SG	slightly pyriform 4.2 × 2.5[b]; 3.9 × 2.4[c] 1.6–1.7	9–14 (3–4)	no data	no data	additional layer of membrane-bound small tubular elements (in M)	Franzen *et al.* 2005

CT – connective tissues, FB – fat body, G – gonads, GC – gastric caeca, GI – generalized infection, H – haemocytes, HE – hindgut epithelium, Mu – muscles, Mg – midgut, MT – Malpighian tubules, N – nervous tissue, O – ovaries, P – pericardium, SG – salivary glands, T – trachea, M – meronts, SS – sporogonial stages, S – spores, M/St – transitional meronts/sporont stages; [a] spore length to width ratio, [b] fresh spores, [c] fixed and stained spores, [d] slightly anisofilar polar tube with number of posterior coils of lesser diameter in parentheses; ∅ – diameter.

unique from all other microsporidial species including all *Tubulinosema* species in its rRNA gene sequence. Basing upon these findings, a new species, *Tubulinosema loxostegi* sp. nov., is erected.

The spores of abberant type, referred to as "teratospores", were found both on light and electron microscopy levels. They apparently resulted from spore wall maturation at the stage of undivided sporoblasts as they contained two diplokarya. A small proportion of abberant spores is often found in microsporidia, and their number might be enhanced by various stress factors (Ditrich *et al.* 1994, Solter *et al.* 1997, Ovcharenko *et al.* 1998, Tokarev *et al.* 2007). In *T. hippodamiae*, abberant spores of another type are observed, with multilayered concentric rings of ER in early spores and unidentified vesicular masses in mature ones (Bjørnson *et al.* 2011).

As stated above, the precise tissue tropism of *T. loxostegi* could not be established. However, the spore masses were found in the fat body (identified by presence of lipid droplets) and a tissue possessing strands of filamentous material which could be either connective or muscular one. This is typical of the *Tubulinosema* parasites to infect multiple tissues or cause generalized invasion of their insect hosts (Table 2). A wide host range is observed for the closely related group of the *Tubulinosema* species (collectively as a genus), including orthopteran (*T. acridophagus*, Henry 1967; *T. maroccanus*, Krilova and Nurzhanov 1987), coleopteran (*T. hippodamiae*, Bjørnson *et al.* 2008, 2011; Saito and Bjørnson 2006, 2008), lepidopteran (*T. loxostegi*, this paper) and dipteran insects (*T. kingi*, Burnett and King 1962, Kramer 1964; *T. ratisbonensis*, Franzen *et al.* 2005). Their generalist nature is further confirmed by high infectivity rates and successful transmission of *T. maroccanus* (Krylova and Nurzhanov 1989) and *T. acridophagus* (Henry and Oma 1974) from their orthopteran hosts to lepidopteran larvae as well as by two cases of recent isolation of *T. acridophagus* from immunosupressed patients with disseminated microsporidiosis (Choudhary *et al.* 2011, Meissner *et al.* 2012). The members of the family Tubulinosematidae demonstrate therefore evolutionary expansion into rather diverse taxa of invertebrate and vertebrate hosts.

Diagnosis of *Tubulinosema loxostegi* sp. nov.

Type host: *Loxostege sticticalis* (Lepidoptera, Pyraloidea, Crambidae).

Type locality and collection date: Karasuk district, Novosibirsk region, Russian Federation (53°42′N 77°45′E); July 2010.

Site of infection: Adipose tissue; infection of other tissues (connective or muscular) is presumed.

Merogony: Process unknown.

Sporogony: Presumably disporoblastic.

Interface: Development in direct contact with host cell cytoplasm.

Morphology of the life cycle stages: The microsporidium is monomorphic, diplokaryotic, sporogony is presumably disporoblastic. The surface of the sporoblasts is covered with tubular structures, 30 nm in diameter. Mature spores are ovoid, 4.2 × 2.4 μm in size (alive), with an indentation in the region of the anchoring disc. The slightly anisofilar polar tube possesses 10–14 coils with the last 2–3 coils of lesser diameter and electron density. The lamellar polaroplast is bipartite with anterior and posterior parts composed of thin and thick lamellae, respectively. The nuclei are surrounded by several layers of polyribosomes. The endospore and exospore are 120 and 25 nm thick, respectively. The exospore surface is covered with an additional layer of electron-dense amorphous matter 20 nm thick.

NCBI GenBank nucleotide accession number: *T. loxostegi (L. sticticalis)* – JQ906779

Deposition of type specimens: The slides with fixed smears, epon-araldite embeddings, ethanol-fixed insects and frozen DNA extracts are deposited at the State Collection of Entomopathogenic and Phytopathogenic Microorganisms and their Metabolites affiliated to the All-Russian Institute of Plant Protection RAAS (Podbelsky sh. 3, 196608 St. Petersburg, Pushkin, Russian Federation). Deposition number TL-LS-Kar-2010.

Etymology: Specific name after the host genus.

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REFERENCES

Andreadis T., Maier C. T., Lemmon C. R. (1996) *Orthosomella lambdinae* n. sp. from the spring hemlock looper, *Lambdina atharasia* (Lepidoptera: Geometridae). *J. Invertebr. Pathol.* **67:** 169–177

Armstrong E., Bass L., Staker K., Harrel L. (1986) A comparison of the biology of *Nosema* in *Drosophila melanogaster* to *Nosema kingi* in *Drosophila willinstoni*. *J. Invertebr. Pathol.* **48:** 124–126

Bjørnson S. (2008) Natural enemies of the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville, used for augmentative biological control: their inadvertent importation and potential significance. *Biol. Control.* **44:** 305–311

Bjørnson S., Le J., Saito T., Wang H. (2011) Ultrastructure and molecular characterization of a microsporidium, *Tubulinosema hippodamiae*, from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville. *J. Invertebr. Pathol.* **206:** 280–288

Bourner T. C., Glare T. R., O'Callaghan M., Jackson T. A. (1996) Towards greener pastures – pathogens and pasture pests. *New Zealand J. Ecol.* **20**: 101–107

Burnett R. G., King R. C. (1962) Observations on a microsporidium parasite of *Drosophila willistoni* Sturtevant. *J. Insect Pathol.* **4:** 104–112

Cali A., Garhy M. E. (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. Protozool.* **38:** 271–278

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

Chen X., Zhai B., Gong R., Yin M., Zhang Y., Zhao K. (2008) Source area of spring population of meadow moth, *Loxostege sticticalis* L. (Lepidoptera: Pyralidae), in Northeast China. *Acta Ecol. Sin.* **28(4):** 1521–1535

Choudhary M. M., Metcalfe M. G., Arrambide K., Bern C., Visvesvara G. S., Pieniazek N. J., Bandea R. D., DeLeon-Carnes M., Adem P., Choudhary M. M., Zaki S. R., Saeed M. U. (2011) *Tubulinosema* sp. microsporidian myositis in immunosuppressed patient. *Emerg. Infect. Dis.* **17:** 1727–1730

Ditrich O., Lom J., Dykova I., Vavra J. (1994) First case of *Enterocytozoon bieneusi* infection in the Czech-Republic – comments on the ultrastructure and teratoid sporogenesis of the parasite. *J. Eukaryot. Microbiol.* **41:** S35–S36

Franz J. M., Huger A. M. (1971) Microsporidia causing the collapse of an outbreak of the green tortrix *Tortrix viridana* L. in Germany. *Proc. Int. Colloq. Insect Pathol. 4th College Park, MD.* 48–53

Franzen C., Fischer S., Schroeder J., Schölmerich J., Schneuwly S. (2005) Morphological and molecular investigations of *Tubulionsema ratisbonensis* gen. nov., sp. nov. (Microsporidia: Tubulinosematidae fam nov.), a parasite infecting a laboratory colony of *Drosophila melanogaster* (Diptera: Drosophilidae). *J. Eukaryot. Microbiol.* **52:** 141–152

Franzen C., Futerman P. H., Schroeder J., Salzberger B., Kraaijeveld A. R. (2006) An ultrastructural and molecular study of *Tubulinosema kingi* Kramer (Microsporidia: Tubulinosematidae) from *Drosophila melanogaster* (Diptera: Drosophilidae) and its parasitoid *Asobara tabida* (Hymenoptera: Braconidae). *J. Invertebr. Pathol.* **91:** 158–167

Frolov A. N., Malysh Yu. M., Tokarev Yu. S. (2008) Biological features and population density forecasts of the beet webworm *Loxostege sticticalis* L. (Lepidoptera, Pyraustidae) in the period of low population density of the pest in Krasnodar Territory. *Entomol. Rev.* **88:** 666–675 (in Russian)

Govorov D. N., Zhivykh A. V., Ipatova N. V., Ibryamova A. S., Chetvertin S. N., Novoselov E. S., Proskuryakova M. Yu., Matveeva O. G. (2012) Review of the phytosanitary situation of agricultural crops in Russian Federation in 2011 and forecast of harmful objects development in 2012. *Moscow, Russian Agricultural Center*, 31–40 (in Russian)

Guan R., Shen Z., Zhu F., Chen D., Zhang J., Hou J., Dong S., Tang X., Xu L. (2012) Phylogenetic characterization of a microsporidium (*Nosema* sp.) isolated from the mulberry pest, *Hemerophila atrilineata*. *Folia Parasitol. (Praha)*. **59:** 87–92

Johny S., Kanginakudru S., Muralirangan M. C., Nagaraju J. (2006) Morphological and molecular characterization of a new microsporidian (Protozoa: Microsporidia) isolated from *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). *Parasitology.* **132:** 803–814

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

Hawkins B. A., Cornell H. V., Hochberg M. E. (1997) Predators, parasitoids and pathogens as mortality agents in phytophagous insect populations. *Ecology* **78:** 2145–2152

Henry J. E. (1967) *Nosema acridophagus* sp. n. a microsporidian isolated from grasshoppers. *J. Invertebr. Pathol.* **9:** 331–341

Henry J. E., Oma E. A. (1974) Effects of infections by *Nosema locustae* Canning, *Nosema acridophagus* Henry, and *Nosema cuneatum* Henry (Microsporida, Nosematidae) in *Melanoplus bivittatus* (Say) (Orthoptera: Acrididae). *Acrida* **3:** 223–231

Huang S. Z., Jiang X. F., Lei C. L., Luo L. Z. (2008) Correlation analysis between periodic outbreaks of *Loxostege sticticalis* (Lepidoptera: Pyralidae) and solar activity. *Acta Ecol. Sin.* **28:** 4824–4829

Hylis M., Pilarska D. K., Oborník M., Vávra J., Solter L. F., Weiser J., Linde A., McManus M. L. (2006) *Nosema chrysorrhoeae* n. sp. (Microsporidia), isolated from browntail moth (*Euproctis chrysorrhoea* L.) (Lepidoptera, Lymantriidae) in Bulgaria: characterization and phylogenetic relationships. *J. Invertebr. Pathol.* **91:** 105–114

Issi I. V. (1986) Microsporidia as a phylum of parasitic protozoa. *Protozoology. Leningrad, Nauka* **10:** 1–136 (in Russian)

Issi I. V., Tokarev Y. S., Seliverstova E. V., Nassonova E. S. (2008) Specified ultrastructural data on *Tubulinosema maroccanus* comb. nov. (*Nosema maroccanus* Krilova et Nurzhanov 1987) (Microsporidia) from the Moroccan locust *Dociostaurus maroccanus* (Orthoptera). *Acta Protozool.* **47:** 125–133

Kleespies R. G., Vossbrinck C. R., Lange 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

Kramer J. (1964) *Nosema kingi* sp. n., a microsporidium from *Drosophila willistoni* Sturtevant and its infectivity for other Muscoides. *J. Insect Pathol.* **6:** 491–499

Krilova S. V., Nurzhanov A. A. (1987) *Nosema* sp. n. (Nosematidae) – microsporidium from Marokkan locust *Dociostaurus maroccanus* Thunb. (Orthoptera). *Bull. All-Union Inst. Plant Protection, St. Petersburg.* **68:** 10–14 (in Russian)

Krylova S. V., Nurzhanov A. A. (1989) Specificity of microsporidium *Nosema maroccanus* from Marokkan locust for the other

insects. *Bull. All-Union Inst. Plant Protection, St. Petersburg* **74:** 7–11 (in Russian)

Kyei-Poku G., Gauthier D., van Frankenhuyzen K. (2008) Molecular data and phylogeny of *Nosema* infecting lepidopteran forest defoliators in the genera *Choristoneura* and *Malacosoma*. *J. Eukaryot. Microbiol.* **55:** 51–58

Lipa J. J. (1976) Microsporidians parasitizing the green tortrix in Poland and their role in the collapse of the tortrix outbreak in Puszcza Niepolomicka during 1970–1974. *Acta Protozool*. **15:** 529–536

Liu H., Pan G., Li T., Huang W., Luo B., Zhou Z. (2012) Ultrastructure, chromosomal karyotype, and molecular phylogeny of a new isolate of microsporidian *Vairimorpha* sp. BM (Microsporidia, Nosematidae) from *Bombyx mori* in China. *Parasitol. Res.* **110:** 205–210

Meissner E. G., Bennett J. E., Qvarnstrom Y., da Silva A., Chu E. Y., Tsokos M., Gea-Banacloche J. (2012) Disseminated microsporidiosis in an immunosuppressed patient. *Emerg. Infect. Dis.* **18:** 1155–1158

Ovcharenko M., Molloy D., Wita I. (1998) Unusual polar filament structure in two microsporidia from water reservoirs with radionuclide and organic pollution. *Bull. Polish Acad. Sci. Biol.* **46:** 47–50

Ronquist F., Huelsenbeck J. P. (2003) MrBayes version 3.0: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. **19(12):** 1572–1574

Saito T., Bjørnson S. (2006) Horizontal transmission of a microsporidium from convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), to three coccinellid species of Nova Scotia. *Biol. Control.* **39:** 427–433

Saito T., Bjørnson S. (2008) Effects of a microsporidium from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), on three non-target coccinellids. *J. Invertebr. Pathol.* **99:** 294–301

Solter L. F., Hajek A. E. (2009) Control of gypsy moth, *Lymantria dispar*, in North America since 1878. Use of microbes for control and eradication of invasive arthropods. New York, Springer

Solter L. F., Maddox J. V., McManus M. L. (1997) Host specificity of microsporidia (Protista: Microspora) from European populations of *Lymantria dispar* (Lepidoptera: Lymantriidae) to indigenous North American Lepidoptera. *J. Invertebr. Pathol.* **69:** 135–150

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

Streett D. A., Henry J. E. (1993) Ultrastructural study of *Nosema acridophagus* Henry (Microspora: Nosematidae) from a grasshopper. *Parasitol. Res.* **79:** 173–177

Swofford D. L. (2003) PAUP*. Phylogenetic Analysis using Parsimony (*and other Methods), v40b10 Sinauer Associates, Sunderland, MA, USA

Tokarev Y. S., Sokolova Y. Y., Entzeroth R. (2007) Microsporidia-insect host interactions: teratoid sporogony at the sites of host tissue melanization. *J. Invertebr. Pathol.* **94:** 70–73

Tokarev Y. S., Voronin V. N., Seliverstova E. V., Dolgikh V. V., Pavlova O. A., Ignatieva A. N., Issi I. V. (2010) Ultrastructure and molecular phylogeny of *Anisofilariata chironomi* sp. n. g. n. (Microsporidia: Terresporidia), a microsporidian parasite of *Chironomus plumosus* L. (Diptera: Chironomidae). *Parasitol. Res.* **106:** 1381–1389

Tsai S. J., Lo C. F., Soichi Y., Wang C. H. (2003) The characterization of microsporidian isolates (Nosematidae: *Nosema*) from five important lepidopteran pests in Taiwan. *J. Invertebr. Pathol.* **83:** 51–59

Tsai Y. C., Solter L. F., Wang C. Y., Fan H. S., Chang C. C., Wang C. H. (2009) Morphological and molecular studies of a microsporidium (*Nosema* sp.) isolated from the thee spot grass yellow butterfly, *Eurema blanda arsakia* (Lepidoptera: Pieridae). *J. Invertebr. Pathol.* **100:** 85–93

van Frankenhuyzen K., Ryall K., Liu Y., Meating J., Bolan P., Scarr T. (2011) Prevalence of *Nosema* sp. (Microsporidia: Nosematidae) during an outbreak of the jack pine budworm in Ontario. *J. Invertebr. Pathol.* **108:** 201–208

Vávra J., Hylis M., Vossbrinck C. R., Pilarska D. K., Linde A., Weiser J., McManus M. L., Hoch G., Solter L. F. (2006) *Vairimorpha disparis* n. comb. (Microsporidia: Burenellidae): a redescription and taxonomic revision of *Thelohania disparis* Timofejeva 1956, a microsporidian parasite of the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae). *J. Eukaryot. Microbiol.* **53:** 292–304

Wang C. Y., Solter L. F., Huang W. F., Tsai Y. C., Lo C. F., Wang C. H. (2009) A new microsporidian species, *Vairimorpha ocinarae* n. sp., isolated from *Ocinara lida* Moore (Lepidoptera: Bombycidae) in Taiwan. *J. Invertebr. Pathol.* **100:** 68–78

Weiss L. M., Vossbrinck C. R. (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. The microsporidia and microsporidiosis. Washington, ASM. 129–171

Wilson G. G. (1973) Incidence of microsporidia in a field population of a spruce budworm. *Can. For. Serv. Bi-monthly. Res. Notes* **29:** 35–60

Xu X., Shen Z., Zhu F., Tao H., Tang X., Xu L. (2012) Phylogenetic characterization of a microsporidium (*Endoreticulatus* sp. Zhenjiang) isolated from the silkworm, *Bombyx mori*. *Parasitol. Res.* **110:** 815–819

Zhu F., Shen Z., Xu X., Tao H., Dong S., Tang X., Xu L. (2010) Phylogenetic analysis of complete rRNA gene sequence of *Nosema philosamiae* isolated from the lepidopteran *Philosamia cynthia ricini*. *J. Eukaryot. Microbiol.* **57:** 294–296

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