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Natural occurrence of microsporidia infecting Lepidoptera in Bulgaria

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Abstract

We examined 34 lepidopteran species belonging to 12 families to determine presence and prevalence of microsporidian pathogens. The insects were collected from May 2009 to July 2012 from 44 sites in Bulgaria. *Nosema* species were isolated from *Archips xylosteana*, *Tortrix viridana*, *Operophtera brumata*, *Orthosia cerasi*, and *Orthosia cruda. Endoreticulatus* sp. was isolated from *Eilema complana.* The prevalence of all isolates in their hosts was low and ranged from 1.0% to 5.3%. Phylogenetic analyses of the new isolates based on SSU rDNA are presented.

Keywords

Biogeography, biological control, entomopathogens

Introduction

Microsporidia are obligate, intracellular pathogens of all major taxa of animals. Together with the Aphelida and Cryptomycota they form a sister group, Opisthosporida, of the true Fungi (Karpov *et al.* 2014). Insects are the most commonly reported hosts with 90 species listed as type hosts of various microsporidian genera (Solter *et al.* 2012). Although entomopathogenic microsporidia generally produce chronic effects leading to low or moderate mortality, they are primary pathogens that can reduce host reproduction and feeding, resulting in declining insect populations and, thus, reduced damage to host plants. Therefore, these pathogens may be candidates for use as classical biological control agents (Goertz *et al.* 2004). One species, *Paranosema* (*Nosema*) *locustae*, is commercially produced for control of grasshoppers and crickets, and other naturally occurring species have been implicated in regulation of their host populations (Bjørnson and Oi 2014, Ebert *et al.* 2000, Kohler and Holland 2001, Stentiford *et al.* 2014). In this respect, it is of interest to identify and investigate new microsporidian species infecting defoliating lepidopteran larvae. Understanding the pathogen complex, including species that produce chronic infections, is important for understanding the population dynamics of forest lepidopteran insects. In Bulgaria, insects of the genera *Lymantria*, *Orthosia*, *Archips*, *Agriopis*, *Erranis*, and others often outbreak in deciduous forests, leading to defoliation resulting in aesthetic and economic loss.

Since 1960, 11 microsporidian species have been recovered from Lepidoptera in the families Erebidae, Tortricidae and Noctuidae in Bulgaria (Pilarska *et al.* 2000; Solter *et al.* 2000; Hylis *et al.* 2006; Vavra *et al.* 2006), including five species isolated from Tortricidae and Erebidae (Panayotov *et al.* 1960; Atanasov 1982; Mirchev *et al.* 1987). More recently, research on microsporidian species isolated from different lepidopteran populations in Bulgaria included host specificity and prevalence studies (Pilarska *et al.* 2000; Solter *et al.* 2000; Pilarska 2000; Hylis *et al.* 2006; Vavra *et al.* 2006). Here we present new data on microsporidia isolated from the tortricids *Tortrix viridana*, *Operophtera brumata* and *Archips xylosteana*; the noctuids *Orthosia cerasi* and *Orthosia cruda*; and the erebiid *Eilema complana.*

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Materials and Methods

A total of 3,022 lepidopteran larvae representing 34 species in 12 families (Table I) were examined for microsporidian infections. The larvae were collected from foliage of small trees between early May 2009 to July 2012 from 44 sites in Bulgaria (Fig. 1). Collections were made by beating low hanging oak branches as previously described (Solter *et al.* 2010). The total number of larvae collected each year, 2009–2012, was 503, 571, 1,234 and 714, respectively. The larvae were transported to the Institute of Biodiversity of Ecosystem Research, Bulgarian Academy of Sciences in Sofia where they were identified and examined for infection.

The internal organs of each specimen were excised and examined for presence of microsporidia by light microscopy (400x). Tissues of infected insects were smeared on slides, fixed with methanol and stained with Giemsa (Sigma Diagnostic Accustain) (Becnel 2012; Solter *et al.* 2012). For transmission electron microscopy (TEM), infected tissues were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) and post-fixed for 2 hours in 2% OsO4. The tissues were

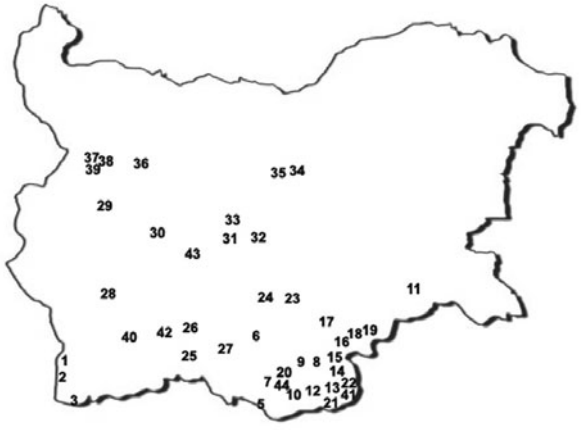


Fig. 1. Collection sites for Lepidoptera in Bulgaria.1 – Tsaparevo Malashevska Mt.; 2 – Nikudin Malashevska Mt.; 3 – Rupite; 4 – Karlanovo Pirin Mt.; 5 – Zlatograd East Rhodope Mt.; 6 – Komuniga East Rhodope Mt; 7 – Perperikon East Rhodope Mt; 8 – Perperek East Rhodope Mt; 9 – Gnyazdovo East Rhodope Mt; 10 – Momchilgrad, East Rhodope Mt; 11 – Kamenets, East Rhodope Mt; 12 – Zvezdel, East Rhodope Mt; 13 – Karamfil, East Rhodope Mt; 14 – Krumovgrad, East Rhodope Mt; 15 – Silen East Rhodope Mt; 16 – Stambolovo, East Rhodope Mt; 17 – Haskovo; 18 – Lyubimets, Sakar Mt.; 19 –Svilengrad Sakar Mt.; 20 – Ardino, East Rhodope Mt; 21 – Gugutka East Rhodope Mt; 22 – Ivaylovgrad, East Rhodope Mt; 23 – Parvomay; 24 – Stryama; 25 – Mihalkovo, West Rhodope Mt; 26 – Peshtera, West Rhodope Mt; 27 – Kuklen, West Rhodope Mt; 28 – Govedartsi, Rila Mt; 29 – Sofia; 30 – Pobit kamak, Sredna Gora Mt; 31 – Banya, Sredna Gora Mt; 32 – Gorni Domlyan, Sredna Gora Mt; 33 – Karlovo, Stara Planina Mt.; 34 – Plakovo, Stara Planina Mt; 35 – Tryavna, Stara Planina Mt; 36 – Skravena, Stara Planina Mt; 37 – Levishte, Stara Planina Mt.; 38 – Opletnya, Stara Planina Mt; 39 – Ochindol, Stara Planina Mt.; 40 – Alabak, West Rhodope Mt.; 41 – Huhla, East Rhodope Mt., 42 – Rakitovo, West Rhodope Mt., 43 – Hisarya, Sredna gora Mt; 44 – Kardzhali, East Rhodope Mt

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.

Microsporidian DNA was extracted from infected tissues of individual larvae according to a slightly modified protocol of Andreadis *et al.* (2013). Each tissue sample was placed in a 0.5 ml microfuge tube with equal volumes of 0.5 mm and 0.1 mm glass beads (BioSpec Products) and 150 µl STE buffer (Fluka, BioUltra, pH 7.8). The tube was shaken in a Mini-Beadbeater (BioSspec Products) for 60–90 s at maximum speed. The mixture was immediately incubated at 95 °C for 5 min and centrifuged at 14,000 g for 5 min. The supernatant was removed and 1–3 µl were used for PCR. The primers 18f:1492r and 18f:1537r (Weiss and Vossbrinck, 1999) were used to amplify the SSU rDNA. The PCR reaction (95 °C for 2 min; 30 cycles of 94 °C for 1 min; 50 °C for 1 min; 72 °C for 2 min; and 72 °C for 10 min) was processed in a total volume of 25 µl, containing 25 pmol of each respective primer and GoTaq® Green Master Mix (Promega), according to the manufacturer´s instructions. The PCR product was separated using 1% agarose gel electrophoresis, extracted from the gel, purified using the DNeasy Tissue Kit® (QIAGEN) and prepared for automated sequencing with the primers 18f, 530f, 1047r, 1492r and 1537r (Weiss and Vossbrinck 1999) and the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an 3130XL Genetic Analyzer (Applied Biosystems).

Six newly obtained microsporidian SSU rDNA sequences (Gen Bank Acc.N. KY615712, KY615713, KY615714, KY615715, KY615716 and KY615717) were analysed in two separate datasets consisting of SSU rDNA sequences of selected microsporidian species. Five of the sequences aligned with 32 closely related species mostly from the genus *Nosema*. *Encephalitozoon cuniculi* and *Encephalitozoon hellem* were included as outgroup species. The remaining sequence aligned with 22 closely related microsporidian sequences from the genera *Endoreticulatus*, *Pleistophora* and *Cystosporogenes*. *Vittaforma corneae*, *Glugoides intestinalis* and *Microsporidium* sp. (KR303711) were included as outgroups. Datasets were aligned using MAFFT v6.626b (Katoh *et al.* 2005) using the E-INS-i multiple alignment method and following parameters: gap opening penalty: 1.0 and gap extension penalty 0.0. Alignments were cross-checked using SEAVIEW v3.2 (Galtier *et al.* 1996).

Phylogenetic trees were constructed from the datasets using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML analysis was done in RAxML v7.2.8 (Stamatakis 2006) under a GTR + Γ model. MP was done in PAUP* v4.0b10 (Swofford *et al.* 2001) with a heuristic search, random addition of taxa and Ts:Tv = 1:2.

Table I. Lepidopteran species collected, collection sites and numbers of larvae examined for microsporidian infections

Lepidoptera	Site №	No. larvae
Erebidae		
Eilema complana	28, 32	38
Lymantria dispar	5, 7, 8, 9, 11, 13, 17, 21, 22, 30, 37	528
Geometridae		
Agriopis aurantiaria	4, 39	8
Agriopis leucophaearia	4, 24, 28, 32, 36, 37, 38	34
Agriopis marginaria	37	7
Agriopis sp.	4	10
Alsophila aceraria	3, 29, 37, 38	17
Alsophila aescularia	32, 38, 39	15
Alsophila sp.	2	3
Biston strataria	37, 41	6
Colotois pennaria	3, 18, 32, 37	33
Erranis defoliaria	8, 9, 18, 29, 32, 37, 38	90
Operophtera brumata	4, 23, 24, 27, 29, 30, 32, 37, 38	151
Phigalia pilosaria	29, 37, 38	10
Lasiocampidae		
Eriogaster lanestris	1, 13	240
Malacosoma neustria	11, 24, 42	180
Noctuidae		
Anorthoa munda	37	1
Catocala nymphagoga	32, 36, 37	22
Cosmia trapezina	7, 29	2
Eupsilia transversa	36	2
Orthosia miniosa	7, 29	3
Orthosia cerasi	3, 4, 28, 29, 32, 33, 36, 37, 38	40
Orthosia cruda	3, 4, 7, 29, 32, 36, 37	61
Orthosia incerta	37	2
Orthosia sp.	15, 24, 29, 37, 38	39
Nolidae		
Bena bicolorana	3, 32, 37	4
Notodontidae		
Taumetopoea pityocampa	31	25
Nymphalidae		
Melitaea didyma	16	1
Pieridae		
Aporia crataegi	11	10
Pyralidae		
Acrobasis sp.	38	3
Sesiidae		
Paranthrene tabaniformis	10	19
Tortricidae		
Archips xylosteana	4, 14, 20, 16, 17, 19, 23, 32, 33, 35, 37, 42, 43	791
Tortrix viridana	3, 4, 6, 45, 9, 12, 13, 14, 17, 23, 30, 32, 33, 34, 35, 38, 42	586
Ypsolophidae		
Ypsolopha sp.	4, 38	4
Total collected	**2 985**	

Bootstrap support was calculated from 500 replicates in ML and 1000 replicates in MP analysis. BI was done using MrBayes v3.0 (Ronquist and Huelsenbeck 2003) with the GTR + Γ model of evolution (6 rates of substitution; gamma rate variation across sites; 4 categories used to approximate gamma distribution). MrBayes was run to estimate posterior probabilities over 1 million generations via 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Tracer v1.4.1 (Rambaut and Drummond 2007) was used to ascertain the sufficient length of burn-in period.

Results

Microsporidia in the genera *Nosema* and *Endoreticulatus* were isolated from 6 of 34 host species examined: *Archips xylosteana*, *Tortrix viridana*, *Operophtera brumata*, *Orthosia cerasi*, *Orthosia cruda* and *Eilema complana.* Because the amount of pathogen material was limited and some of the infected larvae died during transit from the field to the laboratory, not all isolates could be studied extensively by TEM.

Nosema sp. was found in the silk glands and fat body tissues of *A. xylosteana* larvae collected in 2011 in 1 of 13 sites – Rakitovo (site no. 42), Rhodope Mountain. The prevalence in this site was 6.3% (total dissected larvae, n = 345). Total overall prevalence in all *A. xylosteana* individuals (n = 791) collected from all sites was 2.7%. The microsporidium was found in second instar larvae and the small amount of material was insufficient for ultrastructural studies. Phylogenetic studies placed this *Nosema* close to the *Nosema bombycis* clade (Fig. 2a).

A *Nosema* species was isolated from *Tortrix viridana* larvae in May 2010 (prevalence: 1.0% , n = 197, total prevalence in all 17 studied sites 0.3%, n = 586) collected from Karlanovo (site no. 4). The pathogen infected the fat body tissues. Because of insufficient infected material and the fact that most of the infected larvae were dead, no TEM data were obtained. Phylogenetic studies (Fig. 2a) placed this microsporidium close to the gypsy moth (*Lymantria dispar*) *Nosema lymantriae* and *Vairimorpha disparis* clade, and SSU-rDNA sequences differed from the previously described *Nosema tortricis*.

A *Nosema* species infected the fat body tissues of *Operophtera brumata* larvae collected in May 2010 in Levishte (Fig. 3, site no. 37). The prevalence was 6.8% (n = 44) in this site. No infections were found in *O. brumata* collected in eight other sites (overall prevalence 2.0%, n = 151). The spores measured 5.9 x 2.5 μm and the spore structure was typical for microsporidia in the genus *Nosema*. The polar filament was isofilar with 21–23 coils arranged in one or two rows (Fig. 4). Phylogenetic studies showed that this microsporidium is closely related to *Nosema thomsoni* described from *Choristoneura conflictana* (Fig. 2a).

A *Nosema* isolate also was recovered from the silk glands and fat body tissues of *Orthosia cruda* (Figs 5, 6) collected from Karlanovo (site no 4) in May, 2011. The prevalence in this site was 33.3% (n = 6) and the total prevalence in the seven sites we surveyed was 3.3% (n = 61). Phylogenetic studies showed a close relationship to *Nosema portugal* within the *Vairimorpha disparis* clade. The spore structure was typical for microsporidia in the genus *Nosema* (Figs 7–11). The exospore had a thickness of about 19 nm, possessed darkly contrasted lines at its borders and had a wavy contour (Figs 8, 8 inset, 10, 11). The electron-lucent endospore was about 50 nm thick when measured at cross-sectioned regions between the waves of the exospore. The mature spore possessed two closely apposed nuclei (Fig. 8), a polaroplast with many fine lamellae (Figs 10, 11) and a posterior vacuole (Fig. 8). The polar filament coils numbered 10–13, were sometimes situated in two rows (Fig. 8, 8 inset) and were tilted to the longitudinal axis (Fig. 8). The diameter of the isofilar polar filament was about 100 nm. At higher magnification six layers were seen in the cross-sectioned polar filament (Fig. 9). The polar filament was anchored apically but acentrically in a polar cap (Figs 10, 11). The manubrium took an oblique backwards course (Fig. 11). The spore size was 5.8 x 2.8 μm.

A *Nosema* isolate was identified in the fat body tissues and silk glands of *Orthosia cerasi*. The prevalence was 20.0% (n = 5) in larvae collected in May, 2010 from Gorni Domlyan (site no. 32), and the total prevalence in nine sites was 2.5% (n = 40). The only one infected larva was dead and no TEM data were obtained. Phylogenetic studies revealed that this microsporidium is closely related to *Nosema* sp. from *Orthosia cruda.*

A microsporidium in the genus *Endoreticulatus* was recorded in 7.4% (n = 27) of the *Eilema complana* larvae collected in Gorni Domlyan (site no. 32) in 2010 and 2011. The total prevalence in two sites, Gorni Domlyan and Govedartsi (site no. 32 and 28) was 5.3% (n = 38). The microsporidium infected the host gut epithelium (Figs 12, 13). The spore size was 2.8 × 1.2 μm. Spores numbering between 16 and approximately 32 were produced within a parasitophorous vacuole bordered by several layers (Fig. 14). The spores were uninucleate (nucleus 0.5–0.6 μm in diameter) with heavily undulant exospores (Fig. 15). The exospores were lamellate with 4 layers and were about 20 nm thick (Fig. 17). The endospore was bright with dark granules and measured 20–60 nm. The polar filament coils numbered 8–11 and were situated in a single row (Fig. 15). Cross-sections of the isofilar filament measured about 80 nm and revealed five layers (Fig. 16). The polaroplast of the spore had a compact, bright, and finely lamellar outer portion of about 60–70 nm in thickness and an inner, more posterior portion with wider spaced lamellae (Figs 18, 19). The wider spaced lamellae appeared to be more prominent on one side of the polaroplast. Phylogenetic analysis confirmed that this microsporidium is closely related to *Endoreticulatus*, however, it appears to be basal to known species and isolates of the genus (Fig. 2b).

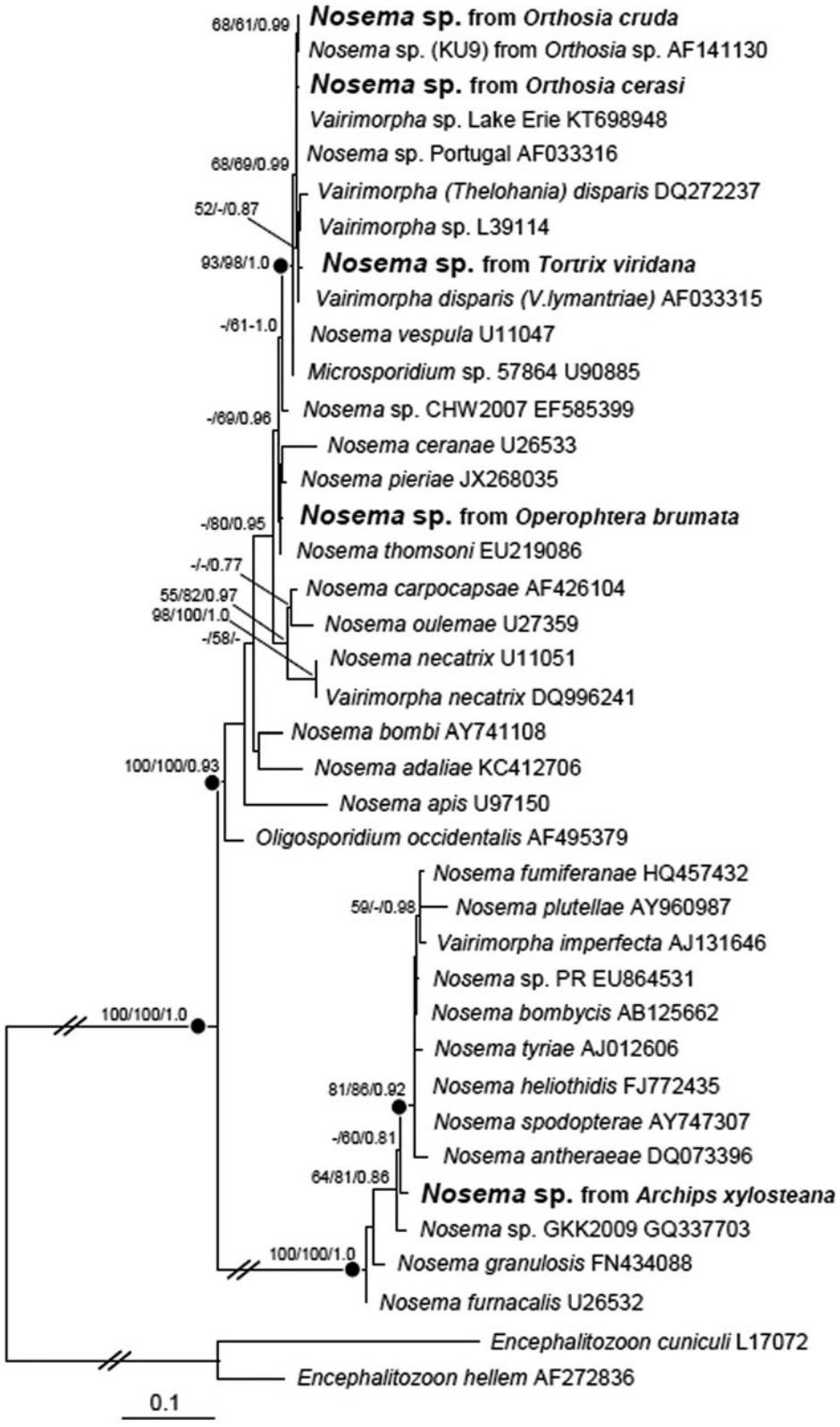


Fig. 2a. SSU rDNA maximum likelihood tree (–ln = –5930.399545, GTR + Γ model) constructed for the microsporidians closely related to *Nosema* spp. GenBank accession numbers are included with taxon names; newly sequenced taxa are in bold font. Numbers at nodes = maximum likelihood/maximum parsimony bootstrap support, and Bayesian posterior probabilities (shown for nodes gaining more than 50% bootstrap support and 0.5 posterior probability. Black full circle indicates well-supported nodes with bootstrap support (ML and MP) and BI posterior probability more than 80% and 0.9, respectively. Strikethrough branches indicate 50% of their original length. Scale bar is given under the tree

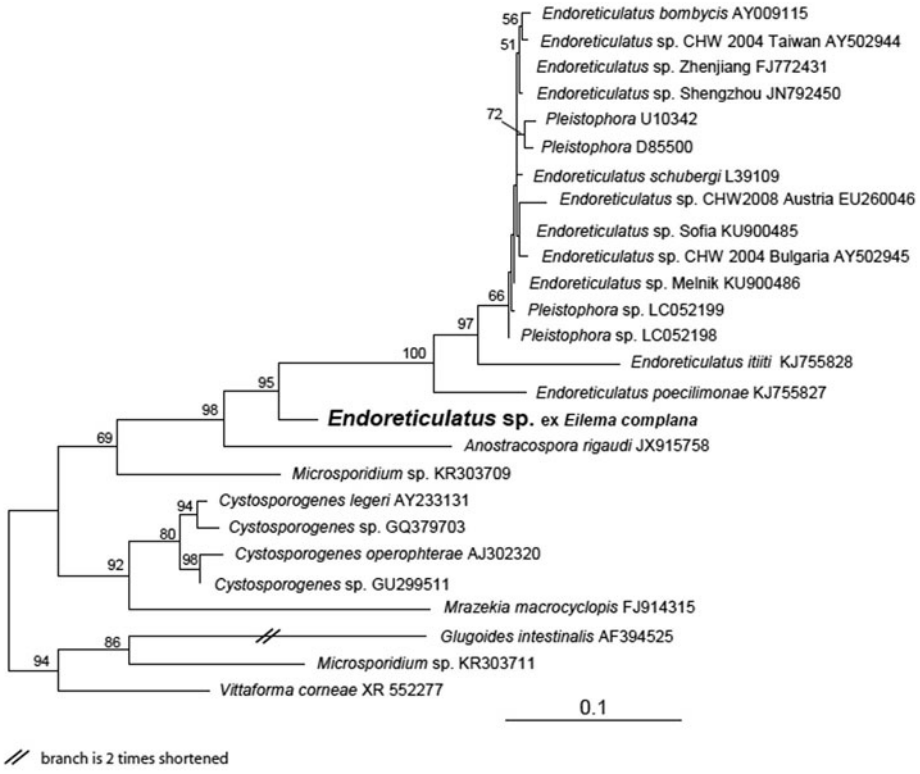
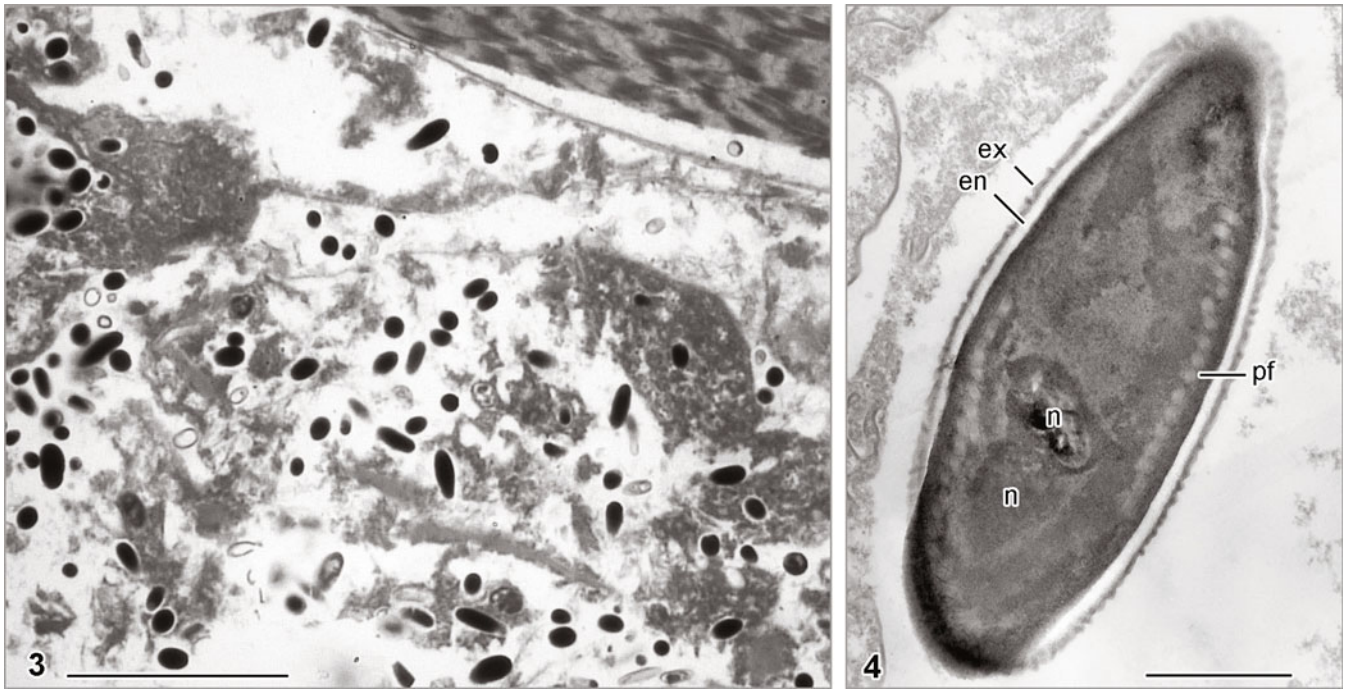
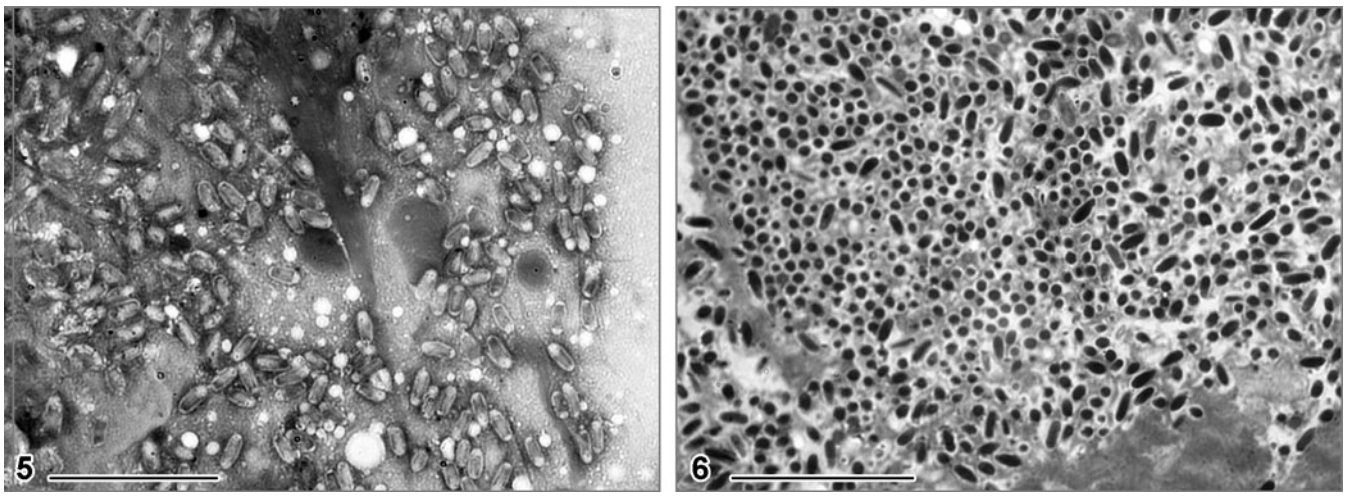


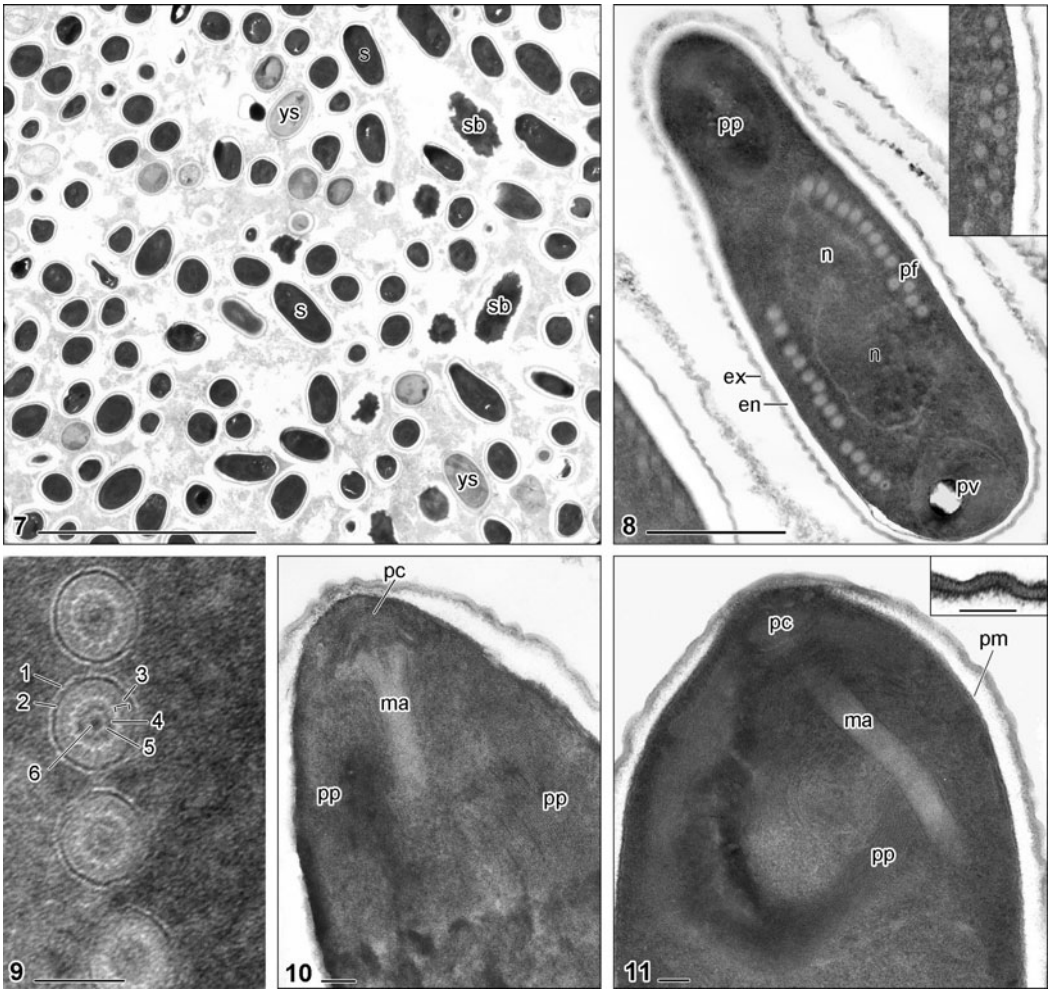
Fig. 2 b. SSU rDNA maximum likelihood tree (–ln = 5469.790579, GTR + Γ model) constructed for the microsporidians closely related to *Endoreticulatus* sp. from *Eilema complana*. GenBank accession numbers are included with taxon names. Numbers at nodes = maximum likelihood/maximum parsimony bootstrap support, and Bayesian posterior probabilities (shown for nodes gaining more than 50% bootstrap support and 0.5 posterior probability). Branch leading to *Glugoides intestinalis* is shortened to 50% of its original length. Scale bar is given under the tree



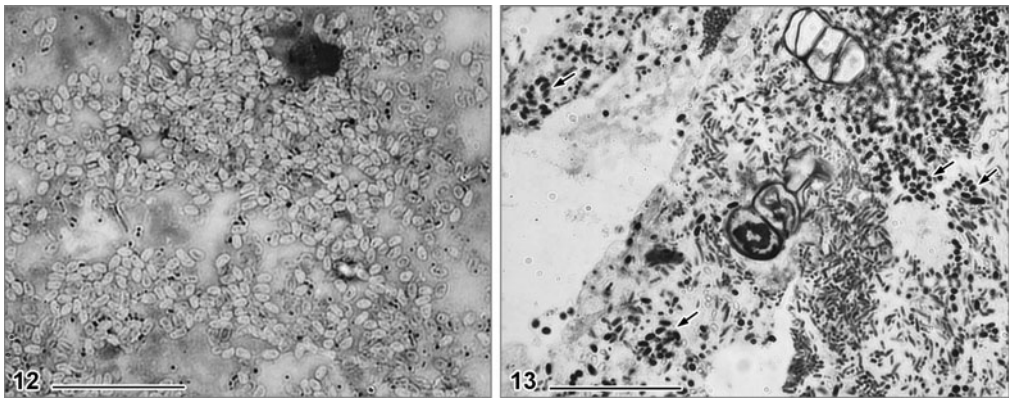
Figs 3–**4.** *Nosema* sp. from *Operophtera brumata*. **Fig. 3.** Spores in the fat body, semi-thin section. **Fig. 4.** Mature spore. Transmission electron microscopy (TEM). Bar **Fig. 3** = 20 μm, **Fig. 4** = 1 μm



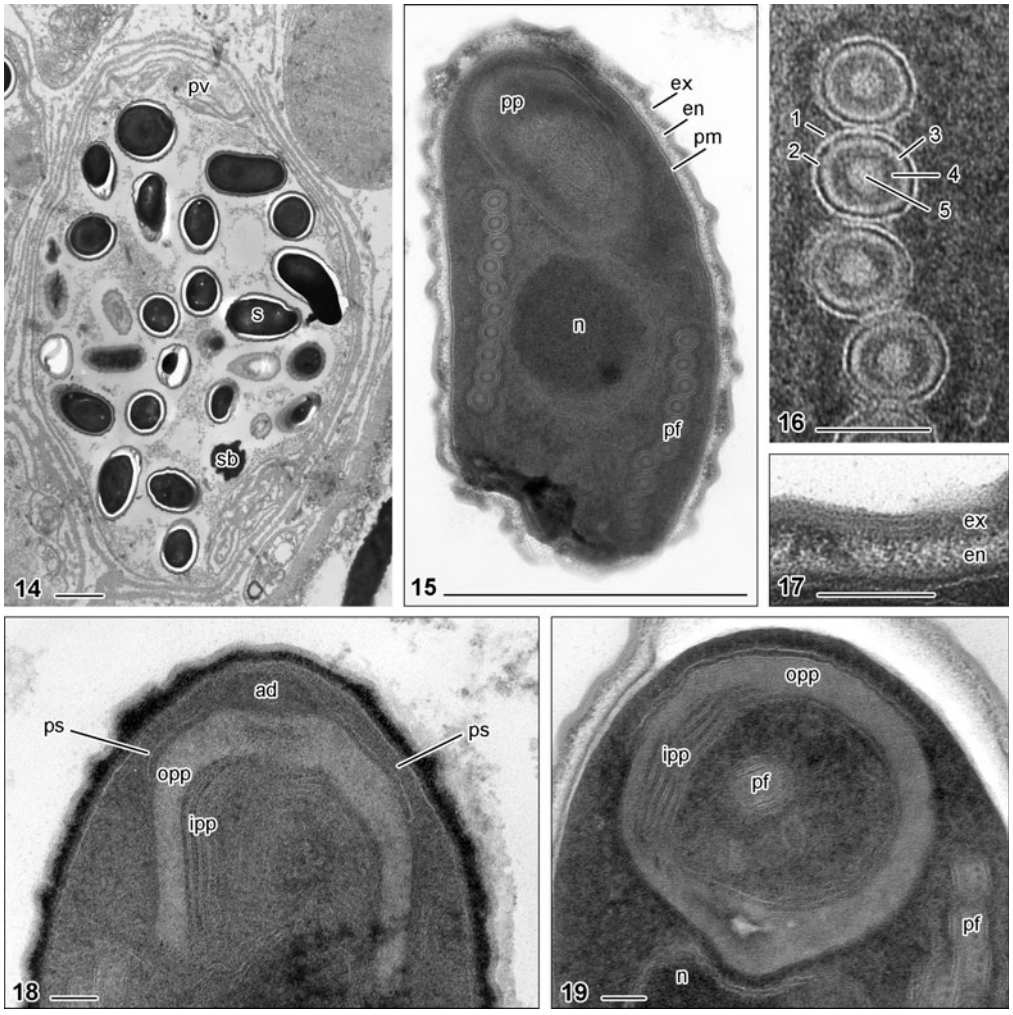
Figs 5–**6.** *Nosema* sp. from *Orthosia cruda*, light microscopy**. Fig. 5.** Giemsa stained spores. **Fig. 6.** Spores in silk glands, semi-thin section stained by Richardson. Bars = 20 μm



Figs 7–11 *Nosema* sp. spores from *Orthosia cruda.* TEM. **Fig. 7.** Overview showing mature spores (s), young spores (ys), and sporoblasts (sb). **Fig. 8.** Mature spore in longitudinal section with wavy exospore (ex), endospore (en), paired nuclei (n), polar filament (pf), polaroplast (pp), and posterior vacuole (pv). Inset: polar filament in two rows. **Fig. 9.** Cross-sections of polar filament (pf) with six internal layers (1–6). **Figs 10 and 11**. Anterior poles of spores with polar cap (pc), manubrium (ma) of polar filament, and lamellar polaroplast (pp). pm = plasma membrane. Bars: **Fig. 7** = 10 μm, **Fig. 8** and inset in **Fig. 8** = 1 μm, **Figs 9–11** and inset in **Fig. 11** = 0.1 μm



Figs 12–13. Spores of *Endoreticulatus* sp. from the gut epithelia of *Eilema complana*. **Fig. 12.** Giemsa-stained smear. **Fig. 13.** Semi-thin section stained by Richardson. Bars = 20 μm



Figs 14–19. *Endoreticulatus* sp. isolated from *Eilema complana*, TEM. **Fig. 14.** Spores (s) and sporoblasts (sb) of *Endoreticulatus* sp. in a parasitophorous vacuole (pv) bordered by several layers. **Fig. 15.** Longitudinal section of spore depicting wavy exospore (ex), endospore (en), plasma membrane (pm), anterior polaroplast (pp), single nucleus (n), and polar filament coils (pf). Posteriorly collapsed. **Fig. 16.** Five layers in cross-section of the polar filament (1–5). **Fig. 17.** Exospore (ex) with four fine, dark layers, granular endospore (en)**. Figs 18 and 19.** Anterior poles of spores. Outer finely lamellate part of polaroplast (opp) surrounds the inner, wider spaced lamella of the polaroplast (ipp) like a bell. An anchoring disc (ad) lies inside the polar sac. (ps). n = nucleus, pf = polar filament. Bars **Figs 14 and 15** = 1 μm, **Figs 16–19** = 0.1 μm

Discussion

Microsporidia are frequently described from Lepidoptera, including many agricultural and forest pests. Most of these pathogens are in the genera *Nosema* and *Vairimorpha*, but other commonly reported microsporidian genera isolated from Lepidoptera include *Endoreticulatus*, *Cystosporogenes*, *Orthosomella*, *Vavraia*, and *Tubulinosema* (Malysh *et al.* 2013). Congeneric microsporidian species are also found in other insect orders, including some pest species, albeit less frequently.

In Bulgaria, microsporidia belonging to *Nosema*, *Vairimorpha* and *Endoreticulatus* were previously recorded in Lepidoptera (Table II). *Nosema* spp. were reported from *Carpocapsa pomonella* (Atanasov 1982; Pilarska 1993), *Cydia molesta* (Pilarska 1995), *Lymantria dispar* (Mirchev *et al.* 1987; Pilarska and Vavra 1991; Pilarska *et al.* 1998), *Orthosia* sp. (Solter *et al.* 2000), *Archips xylosteana* and several unidentified tortricids (Solter *et al.* 2000), and *Euproctis chrysorrhoea* (Hylis *et al.* 2006). *Vairimorpha disparis* was described from *L. dispar* (Vavra *et al.* 2006) and another *Vairimorpha* sp. was isolated from *Archips xylosetana* (Solter *et al.* 2000). Microsporidia belonging to *Endoreticulatus* were found only in two hosts, *L. dispar* (Pilarska *et al.* 1998) and *E. chrysorrhoea* (Solter *et al.* 2000; Pilarska *et al.* 2001). The prevalence of previously reported microsporidia varied widely, from 0.8% (in *V. disparis*) to 42.8% (*N. carpocapsae*) (Table II).

Nosema spp. isolated from *O. brumata* and *O. cruda* are quite similar in size and general morphology, however, molecular and ultrastructural data show that these two isolates are different species. Additionally, *Nosema* sp. from *O. brumata* infects only the fat body tissues. while *Nosema* sp. from *O. cruda* infects the silk glands of its host as well.

The phylogenetic data also revealed that the *O. brumata* isolate is not closely related to the other microsporidia isolated from *O. brumata*, including *N. wistmansii*, *Orthosomella*, and *Cystosporogenes*, but the SSU rDNA sequences show a close relationship to *Nosema thomsoni. Nosema* sp. from *O. brumata* differs in SSU rDNA sequence from *Nosema thomsoni* by one nucleotide. *N. thomsoni* should be regarded as a Holarctic species.

The SSU rDNA sequences of the *Nosema* spp. isolated from *T. viridana* and *A. xylosteana*, both tortricid hosts, show that they are not phylogenetically related. The *T. viridana* isolate aligns most closely with the *Vairimorpha disparis* clade, and the *A. xylosteana* isolates align with the *Nosema bombycis* clade. Both clades exhibit the highest bootstrap support (black full circle in Fig. 2a) but closer examination reveals other molecular differences among species forming these clades.

Nosema sp. from *A. xylosteana* is distinct from *Nosema bombycis* clade and is more closely related to the beetle microsporidium *Nosema* sp. GKK2009 isolated from the mountain pine beetle *Dendroctonus ponderosae* (99.09 % / 12 nucleotide substitutions) than to the nearest lepidopteran microsporidia *N. bombycis*, *N. heliothidis*, and *N. spodopterae* (all 98.62% / 17 nucleotide substitutions). *Nosema* sp. from *A. xylosteana* may represent a new species, although it is possible that it is conspecific with the *D. ponderosae Nosema* sp.

A similar situation occurs in the *V. disparis* clade (top clade represented in Fig. 2a) where sequence differences among described species and isolates from a variety of hosts are very small. For example, the range of sequence similarity (nucleotide substitutions) of *Nosema* sp. isolated from *T. viridana* to other members of the *V. disparis* clade is 99.6–99.84% (2–6 nucl. subst.). Likewise, microsporidia isolated from two *Orthosia* species align within *V. disparis* clade (>99%) and are closest to KU-9 (100% similarity), which was also isolated from an *Orthosia* species, possibly *O. gothica* (Solter *et al.* 2000). This situation suggests that microsporidia currently described in multiple genera may actually represent several genotypes of one species that parasitize a broad range of Lepidoptera (*Tortrix*, *Orthosia*, *Lymantria*), and may even include an isolate from a hymenopteran host (*N. vespula*, GenBank acc. No. U11047) and an isolate from the polychaete, *Manayunkia speciosa* (Annelida) (GenBank acc. No. KT698948). The apparently broad host range of some species with unexpected hosts (fresh water annelids) might be explained by broad host specificity and host switches or narrow host specificity and an alternative explanation of phylogeny.

Host specificity of microsporidia in the *V. disparis* clade was previously reported. Solter *et al.* (1997) documented different but relatively high levels of host specificity among closely related isolates (*Vairimorpha*/*Nosema* spp.) collected from the European gypsy moth *Lymantria dispar* to non-target North American lepidopteran hosts. Low susceptibility of sympatric non-target Lepidoptera to *Nosema lymantriae* and *Vairimorpha disparis* isolated from *L. dispar* were reported in field studies (Solter *et al.*, 2010). Both species appear to have a very narrow host range in the field. It is clear from the observations that host specificity of microsporidia within *V. disparis* clade is limited. How can microsporidian species that infect only a limited range of lepidopteran hosts infect annelids, or *Nosema* spp., usually isolated from Lepidoptera infect amphipods and crayfish? Apparently, even small nucleotide changes in this subclade represent distinct and often different species and not genotypes of one species.

Taxonomic classification of *Endoreticulatus* sp. isolated from *E. complana* appears to be less complex. Phylogenetic analysis revealed that *Endoreticulatus* sp. isolated from *E. complana* is not closely related to other existing *Endoreticulatus* clades and species. This microsporidium forms a separate basal line to all other *Endoreticulatus* species (Fig. 2b) including those isolated from other Lepidoptera and other related species, including *Endoreticulatus poecilimonae* from a grasshopper, *Poecilimon thoracicus*, and *Endoreticulatus itiiti* from a weevil, *Listronotus bonariensis*) (Pilarska *et al.*, 2015). *S*equence similarity is as close to *Anostracospora rigaudi* (92.25%) isolated from brine shrimp *Artemia* as to *E. poecilimonae* (92.136 %) and *E. itiiti* (90.91%). Because we do not have enough data for an in-depth comparison of

Table II. Microsporidia reported from Lepidoptera in Bulgaria

Species	Host	Infected tissue	Spore size (µm)	Prevalence (%)	Ultrastructure data	References
Nosema carpocapsae	*Carpocapsa pomonella*	gut midgut epithelium, gut muscle, silk glands, fat body, tracheae, haemolymph, somatic muscles and gonads	3.48 (2.97–3.48) 3.48 (2.97–3.48) × 1.79 (1.54–2.10) fresh 2.8 (2.27–3.34) × 1.6 (1.2–1.97) fixed	42.8	11–12 polar filament coils arranged in a single row; exospore – 12 nm, endospore – 98 nm;	Atanosov (1982), Pilarska (1987, 1993)
	Laspeyresia molesa		3.55 (3.25–4.34) × 1.89 (1.60–2.40) fresh 2.88(2.39–3.38) x1.62 (1.25–1.86) fixed	17.5	No data	Pilarska (1994)
Nosema serbica	*Lymantria dispar*	Midgut, silk glands, fat body, somatic muscles, Malphigian tubules	5.24 (4.31–5.84) × 2.4 (2.0–2.82) fresh 5 (3.99 – 5.60) × 2.27 (2.00 – 2.65) fixed	15.0	8–9 polar filament coils arranged in a single row;	Pilarska and Vavra (1991)
Nosema chrysorrhoeae	*Euproctis chrysorrhoea*	Silk glands	6.12 (5.52–6.67 X 2.21 (1.99–2.38) fresh	7.5	10–12 isofilar polar filament coils, exospore – 46–58 nm, endospore – 81–93 nm	Hylis *et al*. (2006)
Nosema lymantriae	*L. dispar*	Silk glands, fat body	4.98 × 2.21	9.9	No data published	Panajotov *et al*. (1960) Mirchev (1987) Pilarska *et al*. (1998) Pilarska *et al*. (2000)
Nosema sp.	*Orthosia* sp. (possibly *O. gothica*)	Silk glands, fat body	No data	8.3	No data	Solter *et al*. (2000)
Nosema sp.	*Orthosia cerasi*	Silk glands, fat body	4.95 × 2.15	2.5	No data	This study
Nosema sp.	*Orthosia cruda*	Silk glands, fat body	5.8 x 2.8 fixed	3.3	10–12 polar filament coils arranged sometimes in two rows	This study
Nosema sp	*Archips xylosteana*	Silk glands, fat body	No data	No data	No data	Solter *et al*. (2000)
Nosema sp.	*A. xylosteana*	Silk glands, fat body	No data	2.7	No data	This study
Nosema sp.	*Tortrix viridana*	Fat body	No data	0.3	No data	This study
Nosema sp.	*Operophtera brumata*	Fat body	5,9 x 2,5	2.0	21–23 isofilar polar filament coils arranged in one or two rows	This study
Endoretuculatus schubergi	*L. dispar*	Midgut epithelium	2.32x1.3	5.8	No data published	Pilarska *et al*. (1998) Pilarska *et al*. (2000)
*Endoreticulatis schubergi**	*E. chrysorrhoea*	Midgut epithelium, silk glands, tracheal matrix and fat body of larvae	2.48 (±0.23) × 1,23(±0.17) unfixed	No data	8–9 polar isofilar filament coils arranged in single row;	Pilarska *et al*. (2002)
Endoreticulatus sp.	*Eilema complana*	Midgut epithelium	2.8 × 1.2 µm fresh This study	13.5	7–8 polar filament coils arranged in a single row; lamellar spore polaroplast	This study

Cystosporogenes (Microsporidium) legeri	*Lobesia botrana*	Midgut epithelium, silk glands, fat body and somatic muscles	2.03 (1.84–2.26) × 1.20 (1.12 – 1.43) fixed	8.3	No data	Pilarska (1987, 1995)
Vairimorpha disparis	*L. dispar*	Fat body	Diplokaryotic secondary spores – 5.1 × 2.6; monokaryotic octospores – 4.6 × 2.8 fresh	5.5	Secondary diplokaryotic spores – 11–13 coils arranged in a single row; octospores – 30 coils arranged in two rows	Vavra *et al*. (2006)
Vairimorpha sp.	*A. xylosteana*	Fat body	No data	No data	No data	Solter *et al*. (2000) Pilarska *et al*. (2000)

*Most probably *Cystosporogenes* sp.

Anostracospora (Rode *et al.* 2013) and *Endoreticulatus* at the structural level, and structural data for our isolate from *E. complana* support a relationship with *Endoreticulatus*, we suggest that this isolate is a new *Endoreticulatus* species.

Our studies show that a considerable number of species/biotypes of microsporidia occur in different lepidopteran populations as primary pathogens. Five new microsporidian isolates were recovered from six lepidopteran species, elucidating diversity and possible host specificity within common microsporidian clades. Microsporidia are known to be major factors driving population cycles of some insect species, particularly Lepidoptera. Surveys provide information about the importance of these natural enemies in targeted insect populations including prediction of pest species outbreaks and identification of potential biological control agents.

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