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Ultrastructural and molecular characterization of *Nosema alticae* sp. nov. (Microsporidia: Nosematidae), pathogen of the flea beetle, *Altica hampei* Allard, 1867 (Coleoptera: Chrysomelidae)

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ABSTRACT

In this study, the first microsporidian pathogen from *Altica hampei* (Coleoptera: Chrysomelidae) is described based on light microscopy, ultrastructural characteristics and comparative 16S SSU rDNA analysis. All developmental stages of the microsporidium are diplokaryotic and in direct contact with the host cell cytoplasm. Giemsa-stained mature spores are oval in shape and measured 3.82 ± 0.35 µm in length and 2.54 ± 0.27 µm in width. The polar filament of the binucleate spores is isofilar with 12–14 coils. Coils are 140.28 ± 4.88 nm (135.59–147.06; n = 36) in diameter and consist of six concentric layers of different electron density and thickness. The spores have a relatively thick (161.72 ± 29.19 nm) trilaminar spore wall. Morphological, ultrastructural and molecular features indicate that the described microsporidium belongs to the genus *Nosema* and is named *Nosema alticae* sp. nov.

1. Introduction

The flea beetles (Alticinae) are members of a large and widespread subfamily of the Chrysomelidae. This subfamily includes over 1000 species in the Palearctic zone and is comprised of 21 genera and more than 200 species in Turkey (Aslan et al., 1999). Although different feeding behaviors are seen in this group, flea beetles are mostly oligophagous (Aslan et al., 1999), consuming foliage of the various plants including Asteraceae, Corylaceae, Ericaceae, Rosaceae, Salicaceae (Siede, 1998). While some species are direct agricultural pests and others cause economic damage indirectly by transmitting viruses that cause disease in plants (Jolivet et al., 1988), several species in the genera *Altica* and *Longitarsus* have the potential to be used as biological control agents against noxious weeds (Booth et al., 1990; Jolivet and Verma, 2002). In the northeast of Turkey, *Altica hampei* Allard 1867 adults and larvae cause considerable damage to *Cirsium echinus* (Asteraceae) by perforating the foliage.

Microsporidia are single-cell eukaryotic organisms that infect many animal taxa, especially insects (Bekircan et al., 2017; Solter et al., 2012). These obligate intracellular pathogens can acquire necessary ATP directly from the cytoplasm of their hosts (Heinz et al., 2012; Tsaousis et al., 2008) and produce detrimental effects on insects including reduced longevity and fecundity (Brooks, 1988; Solter et al. 2012). Microsporidiosis that impacts the host population dynamics is very common in Chrysomelidae (Zhu et al., 2011). Since the description of *Nosema phyllotretae* by Weiser in 1961, more than 30 species have been identified in chrysomelids and some of these species have significant potential to suppress populations of their insect hosts (Toguebaye et al., 1988; Hokkanen and Lipa, 1995). For these reasons, the Phylum Microsporidia has attracted attention in recent years that have focused on these pathogens for their potential as biological pest control agents. Here, we investigated and described a microsporidian pathogen of the chrysomelid *A. hampei*.

2. Materials and methods

2.1. Light microscopy

To describe the microsporidian pathogen of the *A. hampei*, 300 adult beetles were collected from Ordu, Turkey in 2017 and 2018. Wet smears, prepared with tissues from dissected beetles in Ringer's solution, were observed at different magnifications under a light microscope (Baki and Bekircan, 2018). Infection positive smears were stained with Giemsa stain according to a standard protocol (Undeen and Vavra, 1997). A Nikon Eclipse Ci microscope with a DS-Fi 2 digital camera was used to photographed fresh and stained microsporidian spores. Measurements were taken using Nikon NIS Elements imaging software.

2.2. Electron (TEM) microscopy

After light microscopy examinations, some infected tissues were selected and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer

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0022-2011/

Table 1
Small subunit (SSU) ribosomal RNA sequences used for phylogenetic analysis.

Accession No	Organism name	Host	Order	Family
JX268035	*Nosema pieriae*	*Pieris brassicae*	Lepidoptera	Pieridae
D85503	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
U27359	*Nosema oulemae*	*Oulema melanopus*	Coleoptera	Chrysomelidae
KC412706	*Nosema adaliae*	*Adalia bipunctata*	Coleoptera	Coccinellidae
U11047	*Nosema vespula.*	*Vespula germanica*	Hymenoptera	Vespidae
KC596023	*Nosema thomsoni*	*Choristoneura conflictana*	Lepidoptera	Tortricidae
AF033316	*Nosema portugal*	*Lymantria dispar*	Lepidoptera	Erebidae
AF485270	*Nosema* sp.	*Pierris rapae*	Lepidoptera	Pieridae
DQ996241	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
L39114	*Vairimorpha* sp.	*Lymantria dispar*	Lepidoptera	Erebidae
AF426104	*Nosema carpocapsae*	*Cydia pomonella*	Lepidoptera	Tortricidae
DQ673615	*Nosema ceranae*	*Apis cerana*	Hymenoptera	Apidae
EU260046	*Endoreticulatus* sp. CHW-2008 Austria	*Thaumetopoea processionea*	Lepidoptera	Thaumetopoeidae
AF141129	*Vairimorpha lymantriae*	*Lymantria dispar*	Lepidoptera	Erebidae
AY009115	*Endoreticulatus bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
U26532	*Nosema furnacalis*	*Ostrinia nubialis*	Lepidoptera	Crambidae
U09282	*Nosema trichoplusiae*	*Trichoplusia ni*	Lepidoptera	Noctuidae
MG725375	***Nosema alticae* sp. nov.**	***Alticae hampei***	**Coleoptera**	**Chrysomelidae**

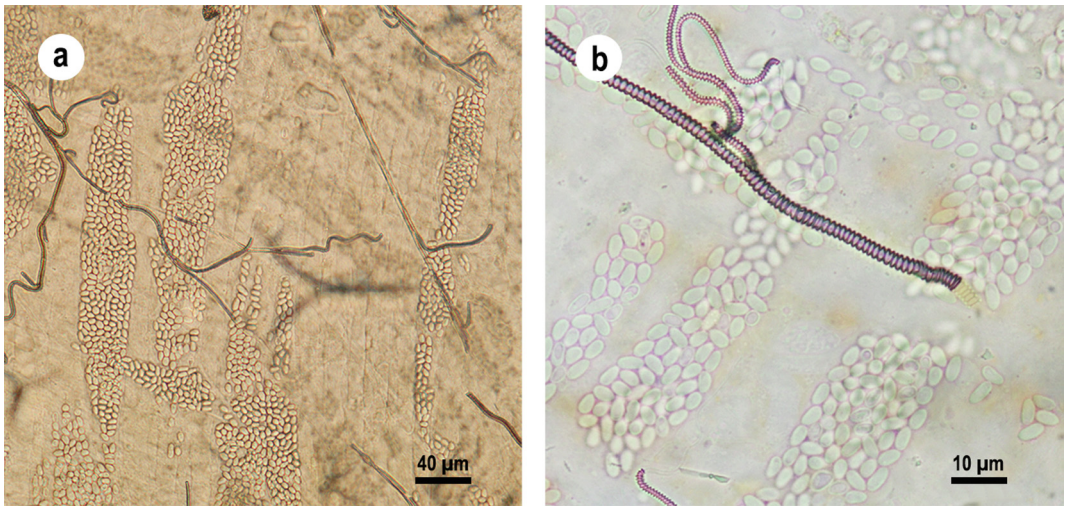

Fig. 1. Light micrograph of *Nosema alticae* nov. sp. in *A. hampei* midgut.

(pH 7.4) for 1–2 h for observation under the transmission electron microscope (TEM). Before post fixation (1% aqueous OsO_4 for 2 h) all samples were washed with cacodylate buffer, then dehydrated through an ascending alcohol series and embedded in Spurr's resin (Spurr, 1969). Thin sections were taken with a Leica EM UC7 ultramicrotome and mounted on Pioloform-coated copper grids which were then stained with saturated uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). A HITACHI HT7800 transmission electron microscope was used for observing and taking photographs from prepared samples.

2.3. DNA extraction, amplification, and phylogenetic analysis

Genomic DNA was extracted from microsporidia using QIAGEN DNA Isolation Kit, No: 69504 as previously described by Bekircan et al. (2017). For 16S SSU rRNA gene amplification, the QIAGEN Multiplex PCR Kit (No. 206143) and 18F/1537R primer set were used (18F/1537R: 5′-CACCA GGTTG ATTCT GCC-3′/5′-TTATG ATCCT GCTAA TGGTT C-3′) (Vossbrinck and Debrunner-Vossbrinck, 2005). All amplification processes were performed according to the kit's protocol, 16S SSU rRNA gene base sequences were determined by the Macrogen Inc. Company, The Netherlands.

The 16S SSU rRNA sequence of the microsporidium from *A. hampei* and other microsporidia sequences obtained from the NCBI GenBank database according to BLAST search and literature (Table 1), were edited and aligned using the BioEdit and CLUSTAL_W programs (Hall, 1999). The 16S SSU rRNA sequences of *Endoreticulatus bombycis* and *Endoreticulatus* sp. CHW 2008 Austria, were used as the outgroup. A phylogenetic tree based on 16S SSU rRNA gene sequences was constructed using the maximum parsimony method with PAUP* program (version 4.0a164 for 32-bit Microsoft Windows). Five thousand bootstrap replicates were used to test the robustness of the trees. The Fast PCR program was used for determining the GC content of the sequences.

3. Results

3.1. Light microscopy

Systemic microsporidiosis was observed in 132 of the 300 examined beetles (44%). In some samples, superinfections of eugregarines, neogregarines and microsporidia were observed. Microsporidian infection was localized mostly in the fat body, gonads, Malpighian tubules, gut and hemolymph of the host (Fig. 1). Mature spores and early developmental stages were observed during the examinations. Fresh spores, oval in shape, measured 4.27 ± 0.38 µm (3.35–5.47; n = 250) in length and 2.61 ± 0.20 µm (2.03–3.20; n = 250) in width. Giemsa stained binucleate spores were measured as 3.82 ± 0.35 µm

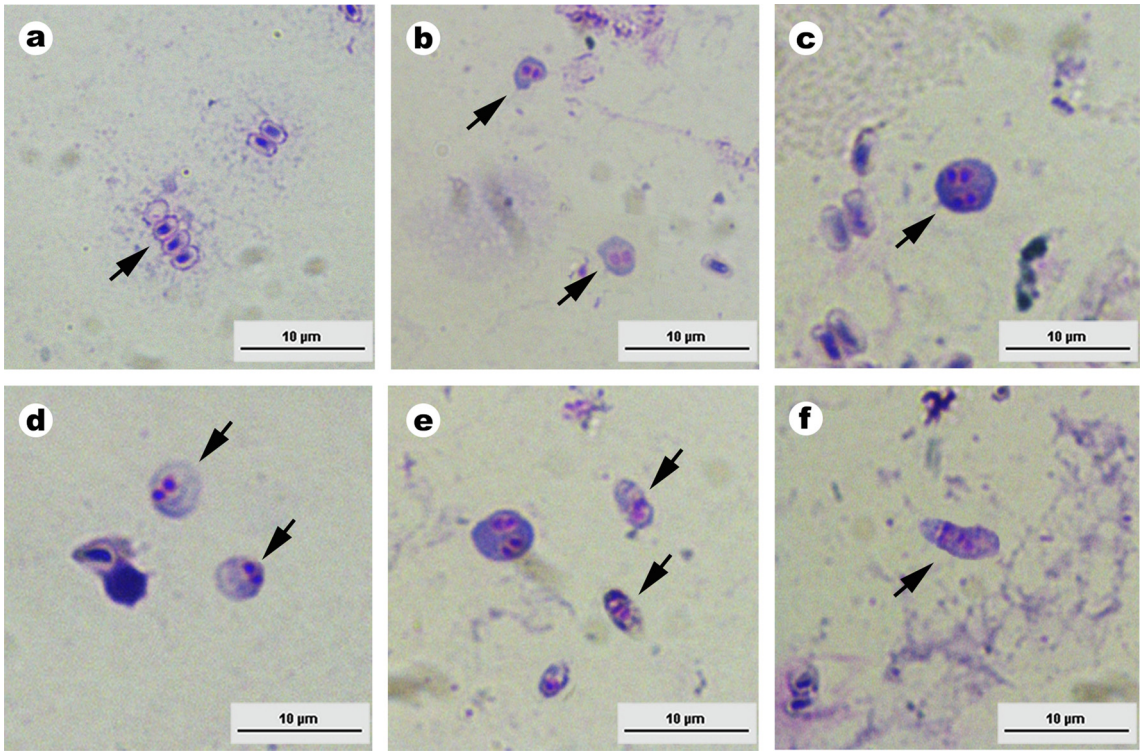

Fig. 2. Giemsa stained developmental stages of *Nosema alticae* nov. sp. (a) Mature spores, an arrow shows the spore chain specific to *Nosema* genus; (b) Binucleate spherical meronts; (c) Arrow shows tetranucleate meront; (d) Binucleate spherical sporonts; (e) Arrows show binucleate ovoid sporonts; (f) Elongated sporoblast.

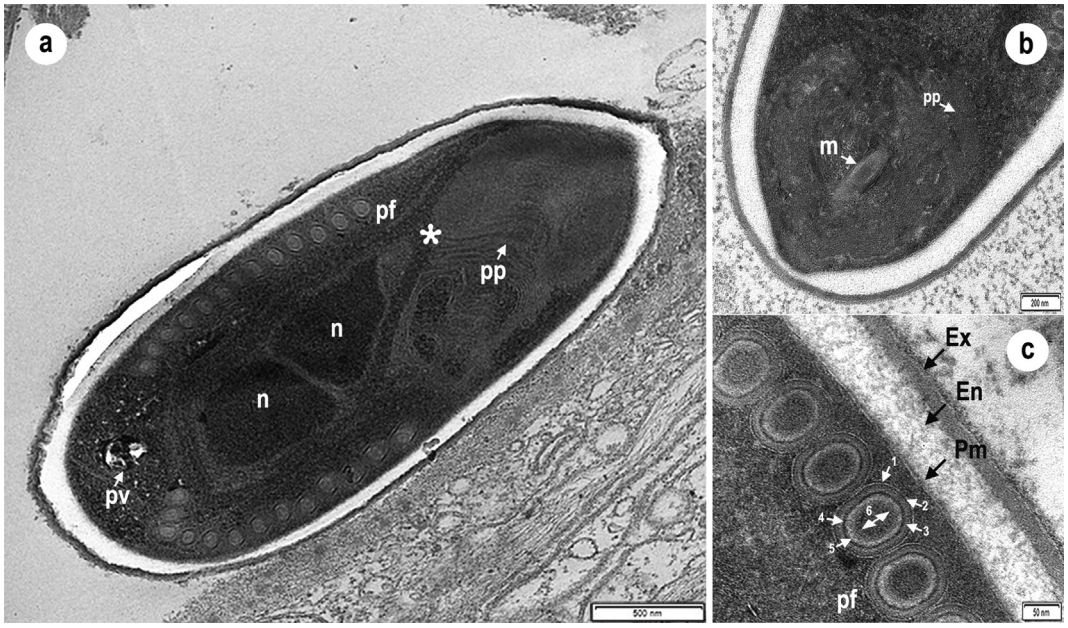

Fig. 3. Transmission electron micrograph of binucleate mature spore. (a) Longitudinal section of mature spores with isofilar polar filaments. Note that asterisk shows circularly arranged polyribosomes; (b) Longitudinal section through anterior part of the mature spores; (c) Cross section of mature spores with three laminar spore wall structure. Note the polar filaments that consist of 6 concentric layers. Abbreviations: En Endospore, Ex Exospore, m Manubrium, n Nucleus, pf Polar filament, pm Plasma membrane, pv Posterior vacuole, pp Lamellar polaroplast.

(2.58–4.91; n = 250) in length and 2.54 ± 0.27 µm (1.87–3.37; n = 250) in width (Fig. 2a). The binucleate spores were in direct contact with the host cell cytoplasm and showed a disporoblastic development. Spherical binucleate and tetranucleate meronts were observed and these measured 4.00 ± 0.77 µm (2.71–4.83; n = 6) × 4.04 ± 0.90 µm (3.13– 5.21; n = 6) and 5.15 ± 0.86 µm (4.29–7.08; n = 13) × 4.64 ± 0.80 µm (3.20– 5.79; n = 13), respectively (Fig. 2b, c). The binucleate sporonts varied from spherical to ovoid and produced a binucleate sporoblast via binary fission (disporous). Spherical sporonts measured 4.56 ± 0.40 µm (3.86–5.30; n = 15) × 4.21 ± 0.85 µm (3.07–6.08; n = 15); the ovoid sporonts were 4.66 ± 0.73 µm (3.84–5.87; n = 8) in length and 2.75 ± 0.34 µm (2.34–3.31; n = 8) in width (Fig. 2d, e). Sporoblasts were elongated and measured 8.48 µm in length and 3.15 µm in width

Table 2
Nosema species isolated in Turkey and their taxonomical characteristics.

Nosema species	Host	Spore size (µm)	Infected organs	Ultrastructural features			Spore wall thickness (nm)	Reference
				Polar filament	Polar filament diameter (nm)	Polaroplast		
Nosema pieriae	*Pieris brassicae*	4.21 × 1.91	Gut	6 coils	57–71	Lamellar	100–125	Yaman et al., 2014
Nosema phyllotretae	*Phyllotreta atra*	4.08 × 2.53	General infestation	13–15 coils	90–120	Lamellar	110–175	Yaman et al., 2005a
Nosema phyllotretae	*Phyllotreta nigripeens*	3.97 × 2.19	General infestation	14 coils	100–125	Lamellar	138–200	Yaman et al., 2005b
Nosema chaetocnemae	*Chaetocnema tibialis*	3.52 × 2.09	Gut, tracheae, muscles and Malpighian tubules	13 coils	115	Relatively vesicular	176.5–213	Yaman and Radek, 2003
Nosema leptinotarsae	*Leptinotarsa decemlineata*	4.69 × 2.43	General infestation	15–16 coils	125–160	Lamellar	180–250	Yaman et al., 2011
Nosema melasomae	*Cyrsomela populi*	4.86 × 1.64	Haemolymph, gut	6–8 coils	45–60	Lamellar	60–100	Yaman, 2018
Nosema tokati	*Chaetocnema tibialis*	3.82 × 1.3	Malpighian tubules	8–10 coils	90–100	Lamellar	85–100	Yaman et al., 2008
Nosema raphidiae	*Raphidia ophiopsis*	4.13 × 2.26	Gut	6–7 coils	140	Lamellar	200–280	Yaman et al., 2009
Nosema apis	*Apis mellifera*	~6.00 × 3.00	Gut	< 30 coils	–	–	–	Zander, 1909; Muz et al., 2010; Whitaker et al., 2011; Yaman et al., 2015
Nosema ceranae	*Apis mellifera*	4.4 × 2.2	Gut	20–23 coils	96–102	Lamellar	137–183	Ütük et al., 2010; Muz et al., 2010; Whitaker et al., 2011; Yaman et al., 2015
***Nosema aticae* sp. nov.**	*Altica hampei*	4.27 × 2.61	Fat tissue, gonads, Malpighian tubules, gut and hemolymph	12–14 coils	135–147	Lamellar	122–221	The present study

(Fig. 2f).

3.2. Transmission electron microscopy (TEM)

Similar to observations under light microscopy, electron microscopic observations confirmed that the oval mature spores of the novel microsporidium were diplokaryotic (Fig. 3a). A lamellar polaroplast occupied the anterior part of the mature spores (Fig. 3b). The trilaminar spore wall was relatively thick (mean: 161.72 ± 29.19 nm; range: 122.47–221.07; n = 100), the exospore was an average of 46.52 ± 7.26 nm thick (range: 35.00–60.77; n = 100), and the mean thickness endospore was 113.60 ± 21.54 nm (range: 82.35–141.44; n = 100) (Fig. 3c). The polar filament was isofilar with 12–14 coils. Mature coils measured an average of 140.28 ± 4.88 nm (range: 135.59–147.06; n = 36) in diameter and consisted of six concentric layers of different electron density and thickness (Fig. 3c).

3.3. Molecular studies

The 16S SSU rRNA partial gene sequence of the *A. hampei* microsporidium was deposited in GenBank with accession code MG725375 (amplicon length was 1151 bp; GC content was 36.8%). Pairwise phylogenetic distances between the *A. hampei* microsporidium and other species ranged from 0.0104 to 0.3274 (Table 2). According to the Maximum parsimony algorithm, the *A. hampei* microsporidium clustered with *Nosema oulemae*, a microsporidium isolated from the Coleopteran subtribe Criocerini (Fig. 4). The phylogenetic data, light and electron microscopical observations showed that the *A. hampei* microsporidium is new species.

4. Discussion

No microsporidian infections were previously reported to have been isolated from species in the *Altica* genus of chrysomelid beetles. We detected microsporidiosis in *A. hampei* via light microscopy and observed the life cycle using light and electron microscopy. The life cycle (disporoblastic development), spore structure (binucleate spore) and molecular results (16S SSU rRNA gene with 36.8% GC content) showed that current microsporidium belongs to the genus *Nosema* Nägeli, 1857. The genus *Nosema* is characterized with diplokaryotic merogony and sporogony. Disporoblastic sporogony follows proliferation by binary division (Weiss and Becnel, 2014). The *Nosema* genus includes more than 200 species, and these species mostly show chronic pathogenicity in the hosts (Cheung and Wang, 1995; Sprague, 1982).

Recently, numerous *Nosema* species, both those that were previously reported and recently described species, from different insect taxa in Turkey. The *A. hampei* microsporidium and other *Nosema* species isolated in Turkey were compared according to basic taxonomical characteristics. For microsporidia infecting insects, host species and tissue specificity traditionally have been important taxonomic characteristics (Canning and Vavra, 2000; Sprague et al., 1992). *Nosema* spp. are commonly isolated from the chrysomelid subfamily Alticinae in Turkey. Between 2003 and 2008, Yaman et al. identified three *Nosema* species (*Nosema chaetocnemae*, *Nosema phyllotretae*, *Nosema tokati*) from different Alticinae species (Yaman and Radek, 2003; Yaman et al., 2005a,b, 2008). Several taxonomic characters of the *A. hampei* microsporidium differ from those of these species. One of the most notable differences is the larger spore dimensions (4.27 ± 0.38 µm × 2.61 ± 123 0.20 µm). The ultrastructural characteristics of spore structure, especially polar filament structure, are important parameters for the comparison of microsporidian species (Becnel et al., 2002; Canning and Vavra, 2000; Ovcharenko et al., 2013). The *A. hampei* microsporidium polar filament number is 12–14 coils (135–147 nm diameter); 1 *N. phyllotretae* has 3–15 coils (90–120 nm diameter); *N. chaetocnemae* has 13 coils (115 nm diameter); and *N. tokati* has 8–10 coils (90–100 nm diameter) (Table 3). Unfortunately, the *A. hampei* microsporidium

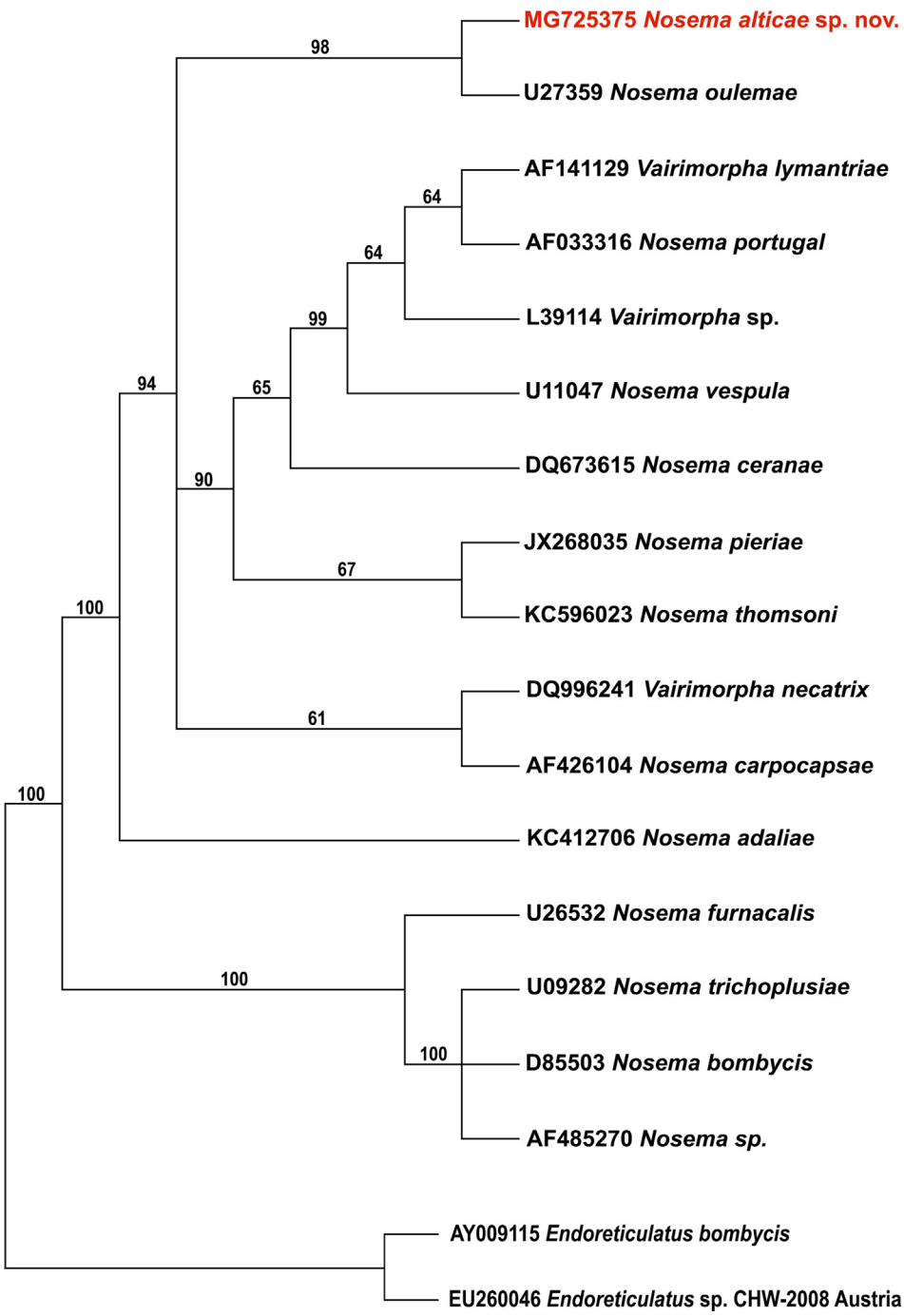

Fig. 4. Phylogeny inferred for *Nosema alticae* nov. sp. and related taxa with GenBank accession numbers. The tree is a bootstrap consensus based on maximum parsimony. *Endoreticulatus bombycis* and *Endoreticulatus* sp. CHW 2008 Austria (Microsporidia: Encephalitozoonidae), were used an outgroup in the analysis. Numbers above the branches are bootstrap support values in percentage.

could not be compared with these *Nosema* species phylogenetically because there is a lack of information on the 16S SSU rRNA gene sequences for these species.

According to our phylogenetic tree, the *A. hampei* microsporidium presented in this study grouped in the same branch with *Nosema oulemae* (U27359) recorded from *Oulema melanopus* L. 1758 (Coleoptera: Chrysomelidae). The base sequence of *N. oulemae* was selected via BLAST analysis, according to high Query cover and Percent ident values (100% and 99.0%, relatively) (Table 2). However, this record is stored in NCBI Genbank database as unpublished work and a study describing the taxonomical characteristics of *N. oulemae* is not available in the literature, so it is not possible to compare *N. oulemae* and the current microsporidium according to the taxonomic parameters used in Microsporidia taxonomy. In conclusion, the phylogenetic status, light, and electron microscopy observations suggest that the described *Nosema* species from *Altica hampei* is a novel species and is named as *Nosema alticae* sp. nov.

4.1. Taxonomic description

***Nosema alticae* sp. nov.**

Phylum Microsporidia (Balbiani, 1882), Nosematidae (Labbe, 1899), *Nosema* (Nägeli, 1857).

Host: *Altica hampei* Allard, 1867 (Coleoptera: Chrysomelidae).

Table 3
Comparison of current microsporidium and other seventeen related microsporidia based on the small subunit ribosomal RNA gene (SSU) by query cover, by nucleotide identity, by Pairwise distance analysis, and GC% content.

MG725375	*Nosema alticae* sp. nov.	Query cover	Percent identity	Pairwise distances	GC content (36.8%)
JX268035	*Nosema pieriae*	100%	97.0%	0.02799	36.5%
D85503	*Nosema bombycis*	86.0%	85.0%	0.17610	34.1%
U27359	*Nosema oulemae*	100%	99.0%	0.01042	36.9%
AF485270	*Nosema* sp.	88.0%	84.6%	0.16474	33.3%
U11047	*Nosema vespula*.	100%	97.0%	0.02622	36.7%
KC596023	*Nosema thomsoni*	100%	97.0%	0.02797	36.9%
AF033316	*Nosema portugal*	100%	96.0%	0.02798	35.7%
AF426104	*Nosema carpocapsae*	100%	97.1%	0.02313	35.3%
DQ996241	*Vairimorpha necatrix*	97.0%	97.0/%	0.03151	37.0%
L39114	*Vairimorpha* sp.	100%	96.0%	0.02799	36.9%
KC412706	*Nosema adaliae*	100%	94.9%	0.03685	37.3%
DQ673615	*Nosema ceranae*	100%	95.0%	0.03674	35.8%
EU260046	*Endoreticulatus* sp. *CHW-2008 Austria*	26.0%	78.0%	0.32744	51.3%
AF141129	*Vairimorpha lymantriae*	100%	96.0%	0.02798	35.4%
AY009115	*Endoreticulatus bombycis*	26.0%	79.0%	0.32534	51.3%
U26532	*Nosema furnacalis*	89.0%	85.0%	0.17182	33.9%
U09282	*Nosema trichoplusiae*	86.0%	85.0%	0.17593	34.1%

Site of infection: Fat body, gonads, Malpighian tubules, gut, and hemolymph.

Spores: All life cycle stages of the current microsporidium are diplokaryotic. Binucleate mature spores are oval in shape with an average length of a 3.82 ± 0.35 (2.58–4.91) μm and a width of 2.54 ± 0.27 (1.87–3.37) μm (fixed, n = 250). Fresh spores measured 4.27 ± 0.38 (3.35–5.47) μm in length and 2.61 ± 0.20 (2.03–3.20) μm in width (n = 250). Polar filament isofilar; 12–14 coils. Mature coils measured 140.28 ± 4.88 nm (135.59–147.06; n = 36) in diameter and consisted of six concentric layers of different electron density and thickness. The polaroplast is the lamellar type. The spore wall has the typical trilaminar structure is relatively thick 161.72 ± 29.19 nm (122.47–22107; n = 100). The spore wall thickness of the anterior apex is very thin compared to other parts.

Locality: Specimens described here were collected from Ordu, Turkey.

Deposition of specimens: The samples for light and electron microscopy are preserved in Research Laboratory, Department of Plant and Animal Production, Espiye Vocational School Giresun University, Giresun – Turkey, with the Catalog No. HÇNH-01. *N. alticae* 16S SSU rRNA gene partial sequences from the samples were deposited to the GenBank with MG725375 accession code.

Etymology: The name of the species refers to the genus name of the host, *Altica hampei* Allard, 1867 (Coleoptera: Chrysomelidae).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2019.107302.

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