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A new microsporidium, *Vairimorpha subcoccinellae* n. sp. (Microsporidia: Burenellidae), isolated from *Subcoccinella vigintiquatuorpunctata* L. (Coleoptera: Coccinellidae)

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ABSTRACT

A new microsporidium was isolated from *Subcoccinella vigintiquatuorpunctata* L. (Coleoptera: Coccinellidae), a pest of *Galega officinalis* L. in Turkey. Infection in larval and adult stages was systemic with mature spores produced in the midgut, gonads, Malpighian tubules and, most extensively, fat body tissues. The microsporidium was polymorphic with two sporulation sequences producing two types of spores, binucleate spores with 13–15 coils of the polar tube, and uninucleate spores with 7 coils of the polar tube that developed within a sporophorous vesicle (SPV) to form meiospores. The 16S small subunit rRNA (SSU rRNA) gene of the microsporidium was sequenced and compared with twenty-seven microsporidian sequences from GenBank. Based on the phylogenetic analysis of the SSU rRNA sequence, this microsporidium is unique within the *Vairimorpha* group. Morphological and genetic characters indicate that the described microsporidium is dissimilar to all known *Vairimorpha* species, and so is named here as *Vairimorpha subcoccinellae* n. sp.

1. Introduction

Subcoccinella vigintiquatuorpunctata L. is a member of the subfamily Epilachninae (Coleoptera: Coccinellidae). This family of phytophagous coccinellid species are pests of economically important plants (Riddick et al., 2009). *S. vigintiquatuorpunctata* causes damage on different plant species depending on the geographical location (Wheeler and Henry, 1981). In Turkey, larvae and adults of *S. vigintiquatuorpunctata* harm *Galega officinalis* (Fabaceae) by feeding on plant leaves. *G. officinalis* is mainly used medically and for animal nourishment throughout the world (Başaran et al., 2006; Duke, 1987; Lemus et al., 1999).

Microsporidia are eukaryotic obligate pathogenic organisms that infect many different Animalia taxa, especially Insecta (Bekircan et al., 2017a; Canning and Lom, 1986; Solter et al., 2012). This phylum has 200 genera and more than 1300 species (Becnel et al., 2014). They have nonlethal effects on their hosts, including reduced longevity or fecundity, and these tiny organisms can be used as natural regulators against certain insect pest species due to their detrimental effects on their hosts (Hajek and Delalibera, 2010). Because of these effects, microsporidians are also being studied as biological control agents. For instance, *Nosema algerae* (Vavra and Undeen, 1970) reduces the number of malarial oocysts formed in *Anopheles* mosquitoes (Schenker et al., 1992) and *Nosema whitei* (Weiser, 1953) is pathogenic to *Tribolium* (flour beetles) species (Bass and Armstrong, 1992). In addition, *Antonospora locustae* (Slamovits et al., 2004), previously known as *Nosema locustae* (Canning, 1953), is available as a commercial microbial pesticide against grasshoppers and allies (Roberts and Janovy, 2009). Therefore, studies have focused on characterization and description of new microsporidian species in recent years. In this study, a new microsporidian pathogen of *S. vigintiquatuorpunctata* is described based on morphological and molecular data.

2. Materials and methods

2.1. Light microscopy

Larvae and adults of *S. vigintiquatuorpunctata* were collected from April to August 2011–2016 in Ordu, Turkey. The samples were dissected in Ringer's solution and smeared on microscopic slides, then observed under a light microscope at different magnifications (Bekircan et al., 2017b). Infection positive smears were air-dried and fixed in methanol and stained with Giemsa stain (Undeen and Vávra, 1997). Microsporidian spores were photographed with a Nikon Eclipse Ci microscope combined with DS-Fi 2 digital camera. Spore measurements

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

Accession no	Organism name	Host	Order	Family
EU487251	*Vairimorpha* sp. CHW-2008a	*Ocinara lida*	Lepidoptera	Bombycidae
D85503	*Nosema bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
L39114	*Vairimorpha* sp.	*Bombyx mori*	Lepidoptera	Bombycidae
AY311592	*Vairimorpha* sp. C21	–	–	–
JQ083083	*Vairimorpha* sp. SB-2012	–	–	–
KT698948	*Vairimorpha* sp. Lake Erie	*Manayunkia speciosa*	Polychaeta	Sabellidae
KT698947	*Vairimorpha* sp. Klamath River	*Manayunkia speciosa*	Polychaeta	Sabellidae
Y00266	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
DQ996241	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
KP208681	*Vairimorpha* sp. GB-2014	*Bombyx mori*	Lepidoptera	Bombycidae
D85502	*Vairimorpha* sp. NIS-M12	*Bombyx mori*	Lepidoptera	Bombycidae
AF495379	*Oligosporidium occidentalis*	*Metaseiulus occidentalis*	Acari	Phytoseiidae
EU260046	*Endoreticulatus* sp. *CHW-2008 Austria*	*Thaumetopoea processionea*	Lepidoptera	Thaumetopoeidae
GQ337705	*Vairimorpha* sp. GKK-2009 clone 1	*Agrilus anxius*	Coleoptera	Buprestidae
GQ337707	*Vairimorpha* sp. GKK-2009 clone 3	*Agrilus anxius*	Coleoptera	Buprestidae
U11051	*Nosema necatrix* ATCC 30,460	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
AF141129	*Vairimorpha lymantriae*	*Lymantria dispar*	Lepidoptera	Erebidae
AF033315	*Vairimorpha lymantriae*	*Lymantria dispar*	Lepidoptera	Erebidae
AJ252955	*Pleistophora ovariae*	*Notemigonus crysoleucas*	Cypriniformes	Cyprinidae
AJ252953	*Pleistophora hippoglossoideos*	*Hippoglossoides platessoides*	Pleuronectiformes	Pleuronectidae
AJ252956	*Pleistophora typicalis*	*Myoxocephalus scorpius*	Scorpaeniformes	Cottidae
JQ082890	*Tubulinosema hippodamiae*	*Hippodamia convergens*	Coleoptera	Coccinellidae
KC412706	*Nosema adaliae*	*Adalia bipunctata*	Coleoptera	Coccinellidae
EF564602	*Ovavesicula popilliae*	*Popillia japonica*	Coleoptera	Scarabaeidae
AY009115	*Endoreticulatus bombycis*	*Bombyx mori*	Lepidoptera	Bombycidae
U26532	*Nosema furnacalis*	Ostrinia nubialis	Lepidoptera	Crambidae
U09282	*Nosema trichoplusiae*	Trichoplusia ni	Lepidoptera	Noctuidae
MF037236	*Vairimorpha subcoccinellae* n. sp.	*Subcoccinella vigintiquatuorpunctata*	Coleoptera	Coccinellidae

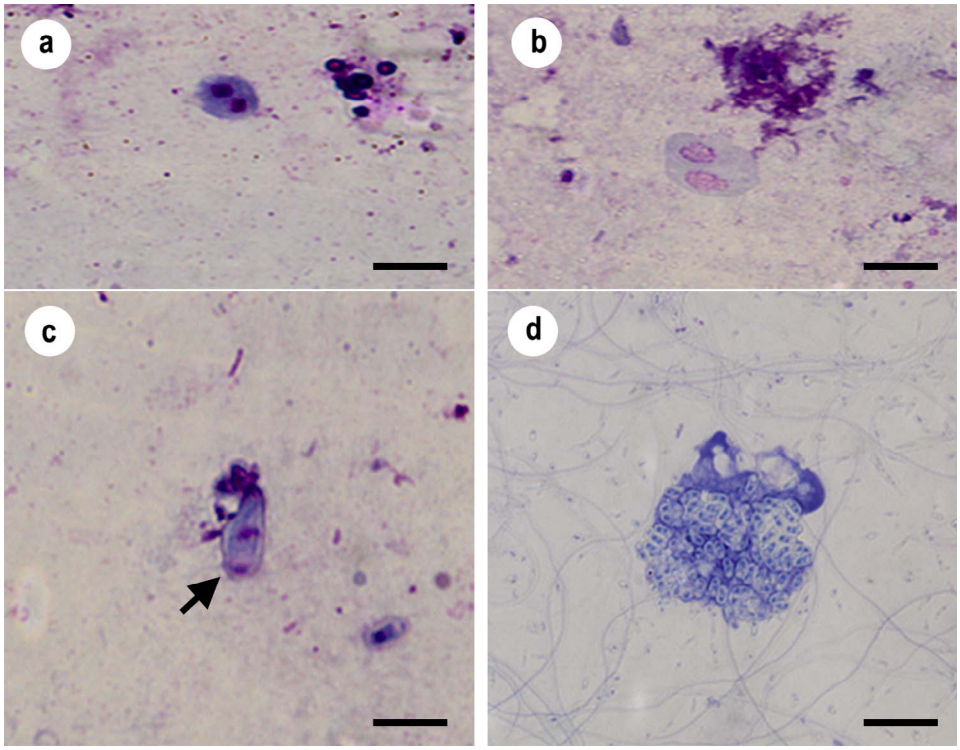

Fig. 1. Light microscopy of the Giemsa stained stages of *Vairimorpha subcoccinellae* n. sp life cycle. a: Spherical binucleate meront (Gut); b: Binucleate sporont (Gut); c: Sporoblast (Gut), (Unite bars = 1 μm); d: Mature spores (Malpighian tubules and gonads), (Unite bar = 2 μm).

were taken using Nikon NIS Elements imaging software.

2.2. Electron microscopy

Infected tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1–2 h, washed with cacodylate buffer and postfixed in 1% aqueous OsO_4 for 2 h. After postfixation, the tissues were washed with cacodylate buffer and dehydrated through an ascending alcohol series and acetone before embedding in Spurr's resin (Spurr, 1969). A Leica EM UC7 ultramicrotome was used to make thin sections, and these were mounted on Pioloform-coated copper grids, which were then stained with saturated uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). The samples were examined and photographed with a JEOL JEM 1010 transmission electron microscope.

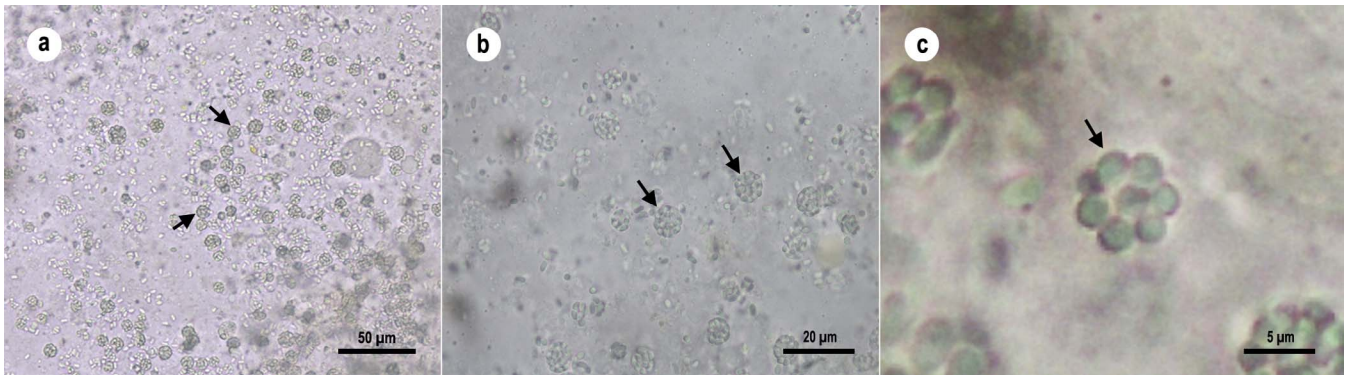

Fig. 2. Light micrograph of *Vairimorpha subcoccinellae* n. sp. in *S. vigintiquatuorpunctata* hemocoel which is heavily infected with binucleate spores and meiospores. Note that arrows show SPV structure containing meiospores.

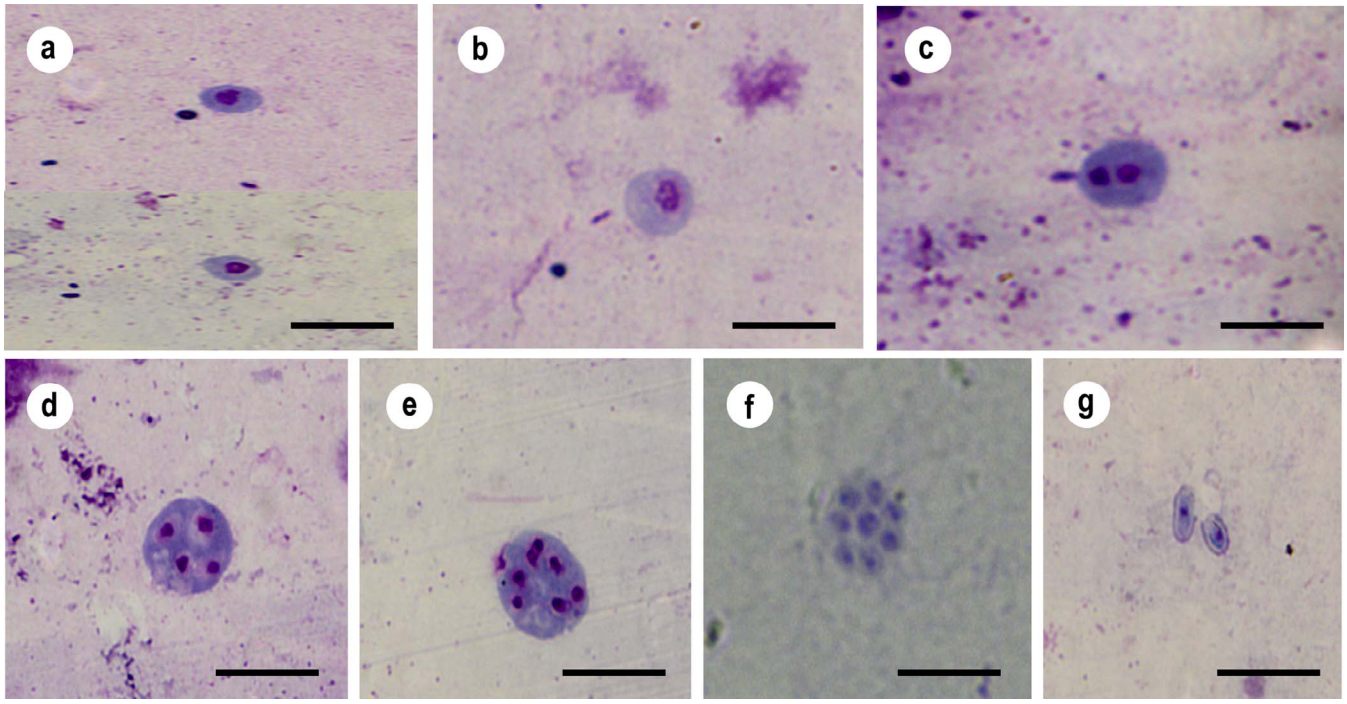

Fig. 3. Giemsa stained stages of *Vairimorpha subcoccinellae* n. sp. demonstrating the octosporous sporulation sequence. a: Uninucleate meront (Fat body), (Unite bar = 12 µm); b: Uninucleate meront in karyokinesis (Fat body), (Unite bar = 10 µm); c: Binucleate sporont (Fat body), (Unite bar = 1 µm); d: Tetranucleate sporont (Fat body), (Unite bar = 1 µm); e: Octanucleate sporont (Fat body), (Unite bar = 1 µm); f: Octospores within the SPV structure (Fat body), (Unite bar = 1 µm); g: Mature meiospores after treatment with 0.1 N HCl showing unikarya (Gut), (Unite bar = 4 µm).

2.3. Molecular studies

Microsporidian spores obtained from infected tissues together with 0.5 ml H_2O_2 (0.5%) were placed into a 1.5 ml Eppendorf tube and kept at room temperature for 1 h. An equal volume of ATL buffer (QIAGEN DNA Isolation Kit, No: 69504) and glass beads (0.425–0.600 µm) were added into the same tube and vigorously shaken for 2 min at maximum speed on the vortex (Hylis et al., 2005). DNA extraction was then performed with the QIAGEN DNA Isolation Kit, No. 69504 according to the manufacturer's guidelines. 16S SSU rRNA gene of microsporidian pathogen was amplified with an 18F/1537R primer set (18F/1537R: 5′-CACCA GGTTG ATTCT GCC-3′/5′-TTATG ATCCT GCTAA TGGTT C-3′) and the QIAGEN Multiplex PCR Kit (No. 206143) (Vossbrinck and Debrunner-Vossbrinck, 2005). The amplification was performed under the kit manufacturer's guidelines and then 16S SSU rRNA gene base sequences were determined in the Macrogen Inc. Company, The Netherlands.

In this study, twenty-eight microsporidia sequences were used for phylogenetic analyses. These sequences were selected from the NCBI Genbank database according to BLAST search and literature (Table 1). BioEdit and CLUSTAL_W programs were used for the sequence editing and multiple alignments (Hall, 1999). The maximum parsimony algorithm with MEGA 6.06 was used for analyzing the resultant alignment. The bootstrap analysis based on 5000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein, 1985). *Endoreticulatus bombycis* and *Endoreticulatus* sp. CHW 2008 Austria (Microsporida: Encephalitozoonidae), were used an outgroup in the analysis. The GC content of the SSU rRNA sequence of the current microsporidium and other sequences were analyzed with the FastPCR program.

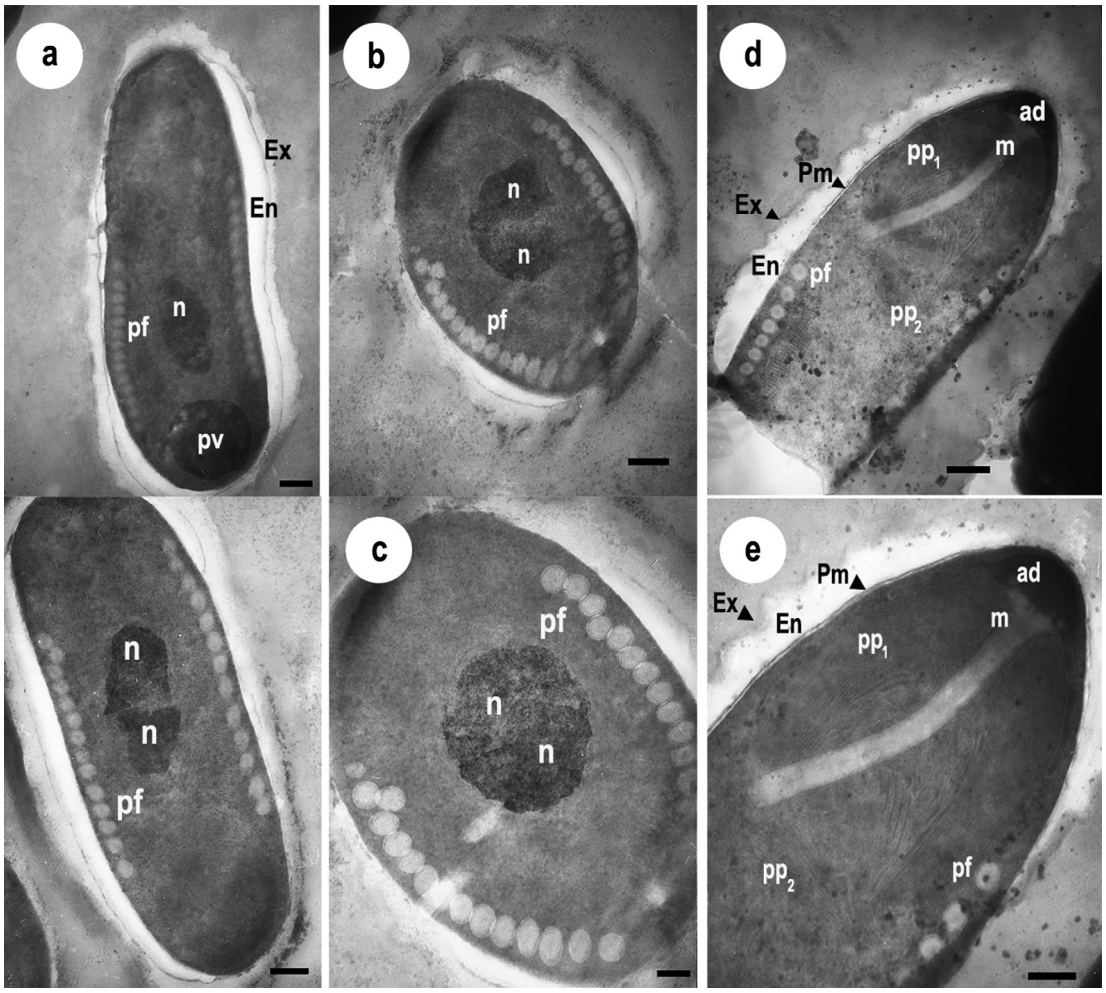

Fig. 4. Transmission electron micrographs of binucleate spores. a: Longitudinal section of mature spores with isofilar polar filaments, (Unite bar = 180 nm); b, c: Different magnifications of cross section of mature spores, (Unite bar for **b** = 150 nm, Unite bar for **c** = 105 nm); d, e: Longitudinal section through the anterior portion of a mature spore. Note the trilaminar structure of the spore wall and laminar and vesicular regions of the polaroplast. Anchoring disc (ad) attached to the manubroid portion of polar filament (m), (Unite bar for **d** = 270 nm, Unite bar for **e** = 180 nm). Abbreviations: ad Anchoring disc, En Endospore, Ex Exospore, m Manubrium, n Nucleus, pf Polar filament, pm Plasma membrane, pv Posterior vacuole, pp1 Lamellar polaroplast, pp2 Vesicular polaroplast.

3. Results

3.1. Light microscopy

Between 2011 and 2016, 1650 adults of *S. vigintiquatuorpunctata* were dissected and examined with the light microscope. In total, 406 infected beetles were detected during the observation (infection rate 24.60%). Examination by light microscopy revealed that the infection was mostly systemic and especially, the fat body, gut, gonads and Malpighian tubules were heavily infected. In addition, two different type spores (Type I and Type II) was detected during the examinations. Type I fresh spores were ovocylindrical in shape, and measured 3.50 ± 0.54 (2.71–4.83; n = 250) μm in length and 1.35 ± 0.18 (1.02–1.75; n = 250) μm in width. Type II fresh spores were ovoid, and measured 1.74 ± 0.28 (1.38–2.12; n = 250) μm in length and 1.43 ± 0.41 (0.92–1.96; n = 250) μm in width. After Giemsa staining, two sporulation sequences (*Nosema* type and *Thelohania* type) were determined (Figs. 1 and 2). A disporoblastic (*Nosema* type) life cycle was usually found to be in the intestine and gonads. In this cycle, the binucleate spores were in direct contact with the host cell cytoplasm. The merogonial stage started with spherical binucleate meronts measuring 6.48 ± 1.71 μm in diameter (n = 20) (Fig. 1a). The binucleate sporonts varied from spherical to ovoid and they produced binucleate sporoblast via binary fission (disporous) (Fig. 1c, d).

The *Thelohania* type sporulation sequence was mostly observed in the fat body of the host insect and this sporulation sequence was easily separated from the *Nosema* type life cycle by the presence of the sporophorous vesicle (SPV) structure that contain meiospores (octospores) (Fig. 2). The uninucleate spores (meiospores) (Type II) were formed in this sporulation sequence. In this type of sporulation, merogonial stages were structurally indistinguishable from the *Nosema* type cycle. On the other hand, unlike the *Nosema* type sporulation, the uninucleate meronts were formed by germination of meiospores formed by the continuation of the *Thelohania* type sporulation (Fig. 3a). The uninucleate meronts were usually spherical in shape and 5.02 ± 1.08 μm in diameter (n = 30). In this cycle, the binucleate meronts formed with the result of karyokinesis of the uninucleate meronts (Fig. 3b). The proliferation of sporogony took place in a SPV with bi-, tetra- and octonucleate forms (Fig. 3c–e). While the tetranucleate forms were 7.87 ± 0.89 μm in diameter (n = 5), the octonucleate form was 12.10 μm in diameter. The uninucleate (meiospores) spores developed from multinucleate sporonts within an SPV (Fig. 3f).

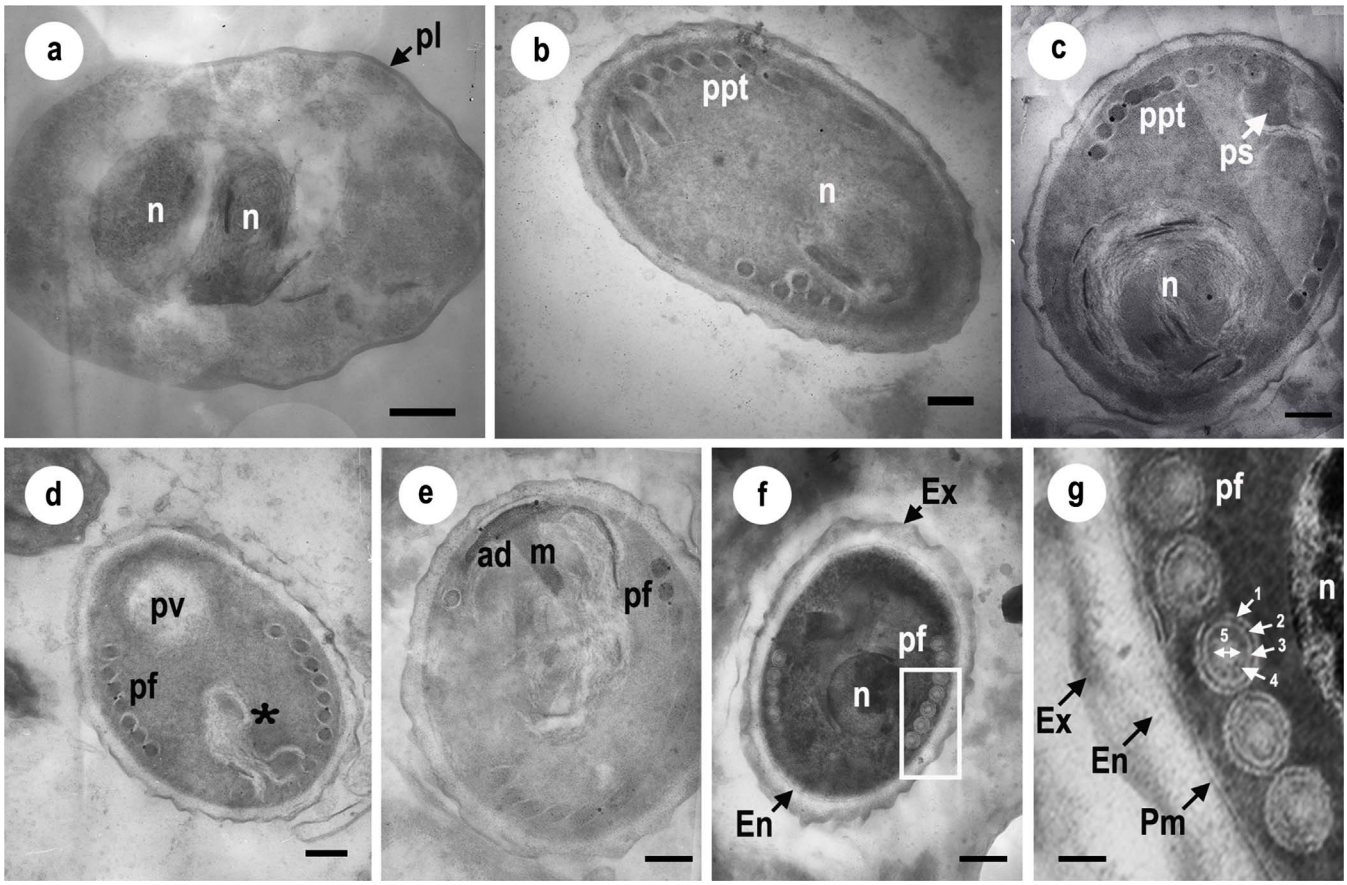

Fig. 5. Transmission electron micrographs of meiospores. a: Binucleate sporont, characterized by a thickened plasmalemma (pl) and less dense cytoplasm, (Unite bar = 150 nm); b: Sporoblast with primordium of the polar tube (ppt), (Unite bar = 130 nm); c: Polar sac formation (ps), (Unite bar = 150 nm); d: Maturing sporoblast with a single layer of the coils of the polar filaments; asterisk indicates the formation of the anchoring disc (ad), (Unite bar = 160 nm); e: Anchoring disc structure of meiospores, (Unite bar = 165 nm); f: Longitudinal section of mature meiospore with isofilar polar filaments, (Unite bar = 190 nm); g: Fine structure of the polar filaments (transversely sectioned); 1–5 indicate the most easily observed layers and trilaminar structure of spore wall, (Unite bar = 40 nm).

3.2. Transmission electron microscopy (TEM)

Similar to the observations made by light microscopy, two different spore types of this novel microsporidium were detected with transmission electron microscopy (TEM). The binucleate mature spores were ovocylindrical (2.48 ± 0.11 μm × 1.09 ± 0.04 μm; n = 30) (Fig. 4a). The trilaminar structure of the spore wall was easily recognized and the spore wall was relatively thin (68–145 nm) (Fig. 4d, e). The exospore was 9–22 nm, and the endospore was 60–120 nm. In addition, the spore wall thicknesses of the anterior and posterior apex were very thin when compared to other parts of the spore wall (Fig. 4). The polar filament of Type I spores was isofilar and had 13–15 coils. Mature coils measured 70–88 nm in diameter (Fig. 4c). Both lamellar and vesicular type polaroplast were observed (Fig. 4d, e).

The sporogonic stage of the meiospores (Type II) started with binucleate sporonts, characterized by a thickened plasmalemma and less dense cytoplasm (Fig. 5a). During the observations, sporoblasts were characterized with the formation of primordial polar tubes, polar sacs and anchoring disc (Fig. 5b–e). Mature meiospores were ovoid in shape (1.37 ± 0.08 μm × 0.95 ± 0.04 μm; n = 4) and had relatively thick exospores when compared to Type I (Fig. 5). The thickness of the spore wall was homogenous throughout the spore structure (Fig. 5f). The trilaminar spore wall was 59–128 nm; the exospore thickness was 12–46 nm and the endospore thickness was 46–82 nm (Fig. 5f, g). The polar filament was isofilar and had 7 coils. Mature coils measured 65 nm in diameter and they consisted of 5 concentric layers of different electron density and thickness (Fig. 5g).

3.3. Molecular studies

The 16S SSU rRNA gene partial sequence of the current microsporidium consisted of 1173 bp, and the GC content was 36.4% (GenBank Accession code: MF037236). The identities of SSU rDNA sequences between current microsporidium and other species used in the phylogenetic analysis were 78–97% and the Pairwise phylogenetic distances varied from 0.02629 to 0.59392 (Table 2). Identities between the current microsporidium and the type species of the genera, *Vairimorpha necatrix* (Pilley, 1976) and *Nosema bombycis* (Nägeli, 1857), were 95–96.0% and 83.0%, respectively. According to maximum parsimony algorithm, the current microsporidium settled on a separate branch within the *Vairimorpha* clade (Fig. 6). Consequently, the phylogenetic status, light and electron microscopical observations showed that the current microsporidium was a new microsporidian pathogen of the *Subcoccinella vigintiquatuorpunctata*.

4. Discussion

In the literature, there are seven microsporidia that have been isolated and characterized from the Coccinellidae (Table 3). While six of these were *Nosema* spp., one species was found to be a *Tubulinosema*

Table 2
Comparison of current microsporidium and other twenty-seven related microsporidia based on the small subunit ribosomal RNA gene (SSU) by query cover, by nucleotide identity, by Pairwise distance analysis, and GC% content.

MF037236	*Vairimorpha subcoccinellae* n. sp	Query cover	Percent identity	Pairwise distances	GC content (36.4%)
EU487251	*Vairimorpha* sp. CHW-2008a	100%	97.0%	0.02801	36.8%
D85503	*Nosema bombycis*	93.0%	83.0%	0.18868	34.1%
L39114	*Vairimorpha* sp.	100%	97.0%	0.02629	36.9%
AY311592	*Vairimorpha* sp. C21	100%	97.0%	0.02985	36.6%
JQ083083	*Vairimorpha* sp. SB-2012	100%	97.0%	0.02896	36.8%
KT698948	*Vairimorpha* sp. Lake Erie	100%	96.0%	0.02718	35.0%
KT698947	*Vairimorpha* sp. Klamath River	100%	96.0%	0.02718	35.1%
Y00266	*Vairimorpha necatrix*	100%	95.0%	0.04001	37.4%
DQ996241	*Vairimorpha necatrix*	100%	96.0/%	0.03711	37.1%
KP208681	*Vairimorpha* sp. GB-2014	100%	95.0%	0.03900	37.1%
D85502	*Vairimorpha* sp. NIS-M12	100%	95.0%	0.04172	37.4%
AF495379	*Oligosporidium occidentalis*	99.0%	95.0%	0.04503	38.4%
EU260046	*Endoreticulatus* sp. *CHW-2008 Austria*	23.0%	78.0%	0.43506	51.3%
GQ337705	*Vairimorpha* sp. GKK-2009 clone 1	94.0%	96.0%	0.03192	37.4%
GQ337707	*Vairimorpha* sp. GKK-2009 clone 3	94.0%	95.0%	0.03959	37.3%
U11051	*Nosema necatrix* ATCC 30460	100%	96.0%	0.03710	37.0%
AF141129	*Vairimorpha lymantriae*	100%	97.0%	0.02718	35.4%
AF033315	*Vairimorpha lymantriae*	100%	97.0%	0.02717	35.9%
AJ252955	*Pleistophora ovariae*	3.0%	92.0%	0.51750	50.8%
AJ252953	*Pleistophora hippoglossoideos*	3.0%-	91.0%	0.51366	52.7%
AJ252956	*Pleistophora typicalis*	3.0%	92.0%	0.51536	52.7%
JQ082890	*Tubulinosema hippodamiae*	–	–	0.45199	42.3%
KC412706	*Nosema adaliae*	100%	95.0%	0.03697	37.4%
EF564602	*Ovavesicula popilliae*	3.0%	94.0%	0.59392	60.9%
AY009115	*Endoreticulatus bombycis*	24.0%	78.0%	0.43223	51.3%
U26532	*Nosema furnacalis*	93.0%	83.0%	0.18764	33.9%
U09282	*Nosema trichoplusiae*	93.0%	83.0%	0.18871	34.1%

"–" No significant similarity found.

species. In this study, a new microsporidian pathogen was described with light and electron microscopy observations in *S. vigintiquatuorpunctata* (Coleoptera: Coccinellidae). These observations showed that the current microsporidium was different in terms of many characters from all known species that infect other microsporidia isolated from Coccinellidae species.

Host species and tissue specificity have historically been important taxonomic characteristics in microsporidia which infect Insecta (Sprague et al., 1992), and the microsporidium described herein is the first microsporidium isolated from *S. vigintiquatuorpunctata*. This novel microsporidium differs morphologically from the *Nosema* and *Tubulinosema* species. The former is polymorphic with one spore type contained within a sporophorous vesicle, whereas the latter are disporous and usually produce diplokaryotic spores in direct contact with the host cytoplasm. Furthermore, molecular studies revealed that the microsporidium is clearly different from the other species described in Coccinellidae. The presence of diplokaryotic spores together with octospores within a SPV places this novel microsporidium in the genus *Vairimorpha* as defined by Pilley (1976). In addition, phylogenetic analysis confirmed that the current microsporidium is most closely related to members of the *Vairimorpha* clade. When the microsporidium described here is compared with the type species, *Vairimorpha necatrix*, it differs from *V. necatrix* and other *Vairimorpha* species in several taxonomic features. *Vairimorpha necatrix* was described from a lepidopteran species (*Pseudaletia unipuncta* Franclemont, 1951) which is true for most *Vairimorpha* species (Pilley, 1976) and it was subsequently isolated in additional species of Lepidoptera (Solter et al., 2012). The spore dimension and structure are important species characteristics and these are widely used for differentiating microsporidian pathogens (Bekircan et al., 2017a; Becnel et al., 2002; Undeen and Vávra, 1997). The spore dimensions are very different between *V. necatrix* strains and the microsporidium described herein. The binucleate mature spores of *V. necatrix* measured 4.3 (3.9–5.0) μm × 2–3 (2.0–2.7) μm and the smaller meiospores spores measured 1.9 (1.5–2.0) μm × 1.1 (1.0–1.3) μm. (Mitchell and Cali, 1993; Pilley, 1976) (Table 4). Both binucleate mature spores and meiospores dimensions of the microsporidium described here are smaller than *V. necatrix* spore types (binucleate spores: 3.50 ± 0.54 μm × 1.35 ± 0.18 μm; meiospores: 1.74 ± 0.28 μm × 1.43 ± 0.41 μm). With respect to microsporidian taxonomy, the ultrastructural characteristics of spore structure, especially polar filament structure are important parameters for comparison of microsporidian species (Bekircan et al., 2017a; Becnel et al., 2002; Canning and Vávra, 2000; Ovcharenko et al., 2013). The binucleate mature spores and meiospores of the current microsporidium have an isofilar polar filament with 13–15 coils and 7 coils, respectively. However, in *V. necatrix* the number of polar filament coils can vary from strain to strain. For instance, while in 1978 Maddox and Sprenkel reported the 10–15 coils, Luo et al. (2014) reported 13–17 coils for their strain and they listed a different number of polar filament coils for different *V. necatrix* strains. Comparison of the current microsporidium with *V. necatrix* phylogenetically indicates there is a significant genetic difference (3.5–3.8%) between the two taxa and the novel microsporidium separated to a different branch within the *Vairimorpha* clade (Fig. 6). All phylogenetic analyses showed that the current microsporidium is a unique species and distinct from other species in the *Vairimorpha* complex. Similarly, when the current microsporidium is compared phylogenetically with *Nosema adaliae* (Steele and Bjornson, 2014) and *Tubulinosema hippodamiae* (Bjornson et al., 2011) which are microsporidian pathogen of Coccinellidae species, it differed from *Nosema adaliae* by 3.6% and 35% from *Tubulinosema hippodamiae*. These data show that the current microsporidium is phylogenetically and morphologically different from these species.

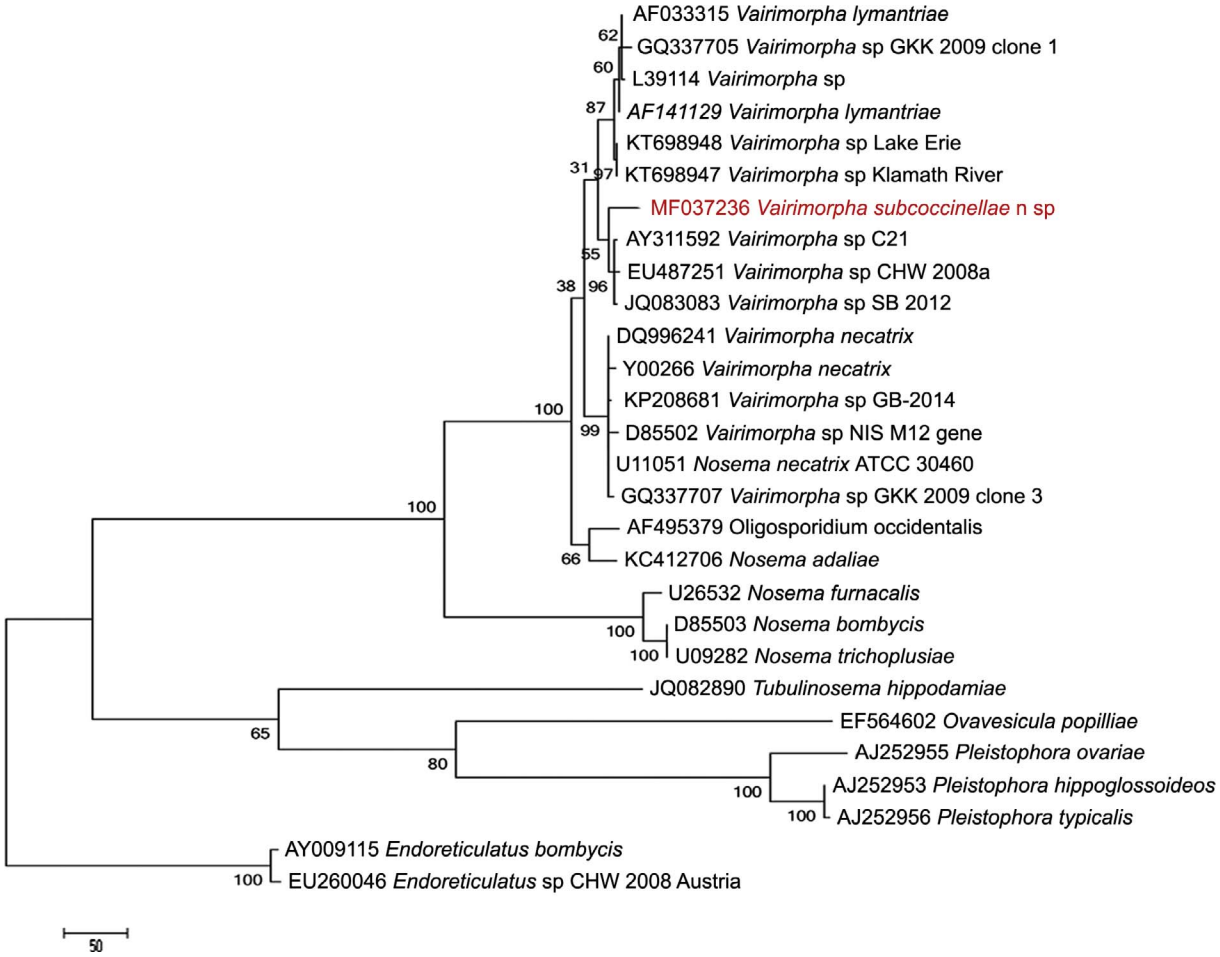

Fig. 6. Phylogenetic analysis of SSU rRNA gene showing the relationship of *Vairimorpha subcoccinellae* n. sp. with other microsporidia. *Endoreticulatus bombycis and Endoreticulatus* sp. CHW 2008 Austria (Microsporida: Encephalitozoonidae), were used an outgroup in the analysis. The phylogenetic tree was constructed by the maximum parsimony method.

4.1. Taxonomic description

***Vairimorpha subcoccinellae* n. sp.**

Phylum Microsporidia (Balbiani, 1882), Burenellidae (Jouvenaz and Hazard, 1978), *Vairimorpha* (Pilley, 1976).

Host: *Subcoccinella vigintiquatuorpunctata* L. (Coleoptera: Coccinellidae).

Site of infection: The infection is mostly systemic with the fat body, gut, gonads and Malpighian tubules heavily infected.

Spores: Two types of spores: (Type I) Binucleate mature spores are ovocylindrical with an average length of 2.48 ± 0.11 µm and a width of 1.09 ± 0.04 µm (fixed, n = 30). Fresh spores measured 3.50 ± 0.54 (2.71–4.83) µm in length and 1.35 ± 0.18 (1.02–1.75) µm in width. Spores contained an isofilar polar filament with 13–15 coils that measured 70–88 nm in diameter. The polaroplast was composed of 2 regions: an anterior lamellar portion and a vesicular posterior region. The posterior vacuole was moderately small. The spore wall has the typical trilaminar structure is relatively thin (68–145 nm). The spore wall thicknesses of the anterior and posterior apex are very thin compared to other parts.

(Type II) The uninucleate spores (meiospores) are ovoid with an average length of 1.37 ± 0.08 µm and width of 0.95 ± 0.04 µm (fixed, n = 4). Fresh spores measured 1.74 ± 0.28 (1.38–2.12) µm in length and 1.43 ± 0.41 (0.92–1.96) µm in width. The isofilar polar filament was coiled 7 times around the centrally located nucleus. Mature coils measured 65 nm in diameter and they consisted of 5 concentric layers of different electron density and thickness. The meiospores develop from multinucleate sporonts within a SPV. The spore wall was trilaminar in structure and this spore type had a relatively thick, unlayered exospore compared to Type I. The thickness of the spore wall was homogenous throughout the spore structure (59–128 nm).

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

Transmission: Can occur either horizontally or vertically.

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. HÇVS-01. *V. subcoccinellae* SSU rRNA gene sequences from the samples were deposited to the GenBank with MF037236 accession code.

Etymology: The name of the species refers to the genus name of the host, *Subcoccinella vigintiquatuorpunctata*.

Table 3
Microsporidian records that isolated and characterized from the Coccinellidae members.

Microsporidium	Spore size (μm)	Infected organs	Host	Reference
Nosema hippodamiae	3.3–5.4 × 2.2–2.7 μm (fixed)	Midgut, fat body	*Hippodamia convergens*	Lipa and Steinhaus (1959)
Nosema tracheophila	3.1–4.4 × 1.9–3.2 μm (fixed) 4.0–5.3 × 2.2–3.1 μm (fresh)	Trachea, connective tissues	*Coccinella septempunctata*	Cali and Briggs (1967)
Nosema coccinellae	3.6–6.2 × 2.0–3.6 μm (fixed) 4.4–6.7 × 2.3–3.4 μm (fresh) 3.5–6.3 × 1.9–2.6 L μm (fixed)	Midgut, Malpighian tubules gonads, nerves, muscle	*Hippodamia tredecimpunctata* *Myrrha octodecimpunctata* *Adalia bipunctata* *Coccinella quinquepunctataExochromus quadripustulatus* *Coccinella. septempunctata*	Lipa (1968) Lipa et al. (1975) Lipa and Semjanov (1967)
Nosema epilachnae	5.3 ± 0.13 × 2.1 ± 0.03 μm (fresh)	Fat body, muscles, Malpighian tubules, gonads	*Epilachna varivestis*	Brooks et al. (1985)
Nosema varivestis	4.7 ± 0.06 × 2.6 ± 0.03 μm (fresh)	Malpighian tubules, gonads	*Epilachna varivestis*	Brooks et al. (1985)
Tubulinosema hippodamiae	3.58 ± 0.2 × 2.06 ± 0.2 μm (fixed)	Fat body, Malpighian tubules, gut, nerve, muscles, gonads,connective tissues	*Hippodamia convergens*	Bjornson et al. (2011)
Nosema adaliae	4.25 ± 0.09 × 1.82 ± 0.03 μm (fixed)	Fat body, flight muscles	*Adalia bipunctata*	Steele and Bjornson (2014)
Unidentified	7.4 × 3.1 μm (fresh) 4.6 × 2.2 μm (fixed)		*Hippodamia convergens*	Sluss (1968)
Vairimorpha subcoccinellae sp. n.	Meisospores: 1.37 ± 0.08 × 0.95 ± 0.04 μm Binucleate: 2.48 ± 0.11 × 1.09 ± 0.04 μm (fixed)	Fat body, Malpighian tubules, gut, gonads	*Subcoccinella vigintiquatuorpunctata* L.	Present study

Table 4
Comparison of morphological and ultrastructural features of, *Vairimorpha subcoccinellae* n. sp., and *Vairimorpha necatrix*.

	Vairimorpha necatrix	*Vairimorpha subcoccinellae* n. sp.
Host	*Pseudaletia unipunctata* (Haworth)	*Subcoccinella vigintiquatuorpunctata* L.
Spore shape	Meisospores: ovoid Binucleate: elongate, cylindrical	Meisospores: ovoid Binucleate: ovocylindrical
Spore dimensions	Meisospores: 1.9 (1.5–2.0) μm × 1.1 (1.0–1.3) μm Binucleate: 4.3 (3.9–5.0) μm × 2–3 (2.0–2.7) μm	Meisospores: 1.37 ± 0.08 μm × 0.95 ± 0.04 μmBinucleate: 2.48 ± 0.11 μm × 1.09 ± 0.04 μm
Polaroplast (anterior/posterior)	Lamellar/vesicular	Lamellar/vesicular
Polar filament type	Isofilar	Isofilar
Polar filament coils	13–14 in uninucleate spores; 14–15 in binucleate spores	7 in uninucleate spores; 13–15 in binucleate spores
Diplokaryotic free spores present	Yes	Yes
Octosporous vesicles with uninucleate spores	Yes	Yes

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References

Balbiani, G., 1882. Surles microsporidies ou psorospermies des articules. C.R. Acad. Sci. 95, 1168–1171.
Başaran, U., Acar, Z., Mut, H., Aşcı, Ö.Ö., 2006. Doğal Olarak Yetişen Bazı Baklagil Yembitkilerinin Bazı Morfolojik ve Tarımsal Özellikleri. J. Fac. Agric., OMU 21 (3), 314–317.
Bass, L.K., Armstrong, E., 1992. *Nosema whitei*: effects on oocyte development and maturation in *Tribolium castaneum*. J. Invertebr. Pathol. 59, 115–123.
Becnel, J.J., Jeyaprakash, A., Hoy, M.A., Shapiro, A., 2002. Morphological and molecular characterization of a new microsporidian species from the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Acari, Phytoseiidae). J. Invertebr. Pathol. 79, 163–172.
Becnel, J.J., Takvorian, P.M., Cali, A., 2014. Checklist of available generic names for microsporidia with type species and type hosts. In: Weiss, L.M., Becnel, J.J. (Eds.), Microsporidia: Pathogens of Opportunity. Wiley-Blackwell, USA, pp. 671–687.
Bekircan, Ç., Bülbül, B., Güler, H.İ., Becnel, J.J., 2017a. Description and phylogeny of a new microsporidium from the elm leaf beetle *Xanthogaleruca luteola* Muller 1766 (Coleoptera Chrysomelidae). Parasitol. Res. 116, 773–780.
Bekircan, Ç., Cüce, M., Baki, H., Tosun, O., 2017b. *Aranciocystis muskarensis* n. gen., n. sp., a neogregarine pathogen of the *Anisoplia segetum* Herbst (Coleoptera: Scarabaeidae). J. Invertebr. Pathol. 144, 58–64.
Bjornson, S., Le, J., Saito, T., Wang, H., 2011. Ultrastructure and molecular characterization of amicrosporidium, *Tubulinosema hippodamiae*, from the convergent lady beetle *Hippodamiaconvergens* Guerin-Meneville. J. Invertebr. Pathol. 106, 280–288.
Brooks, W.M., Hazard, E.I., Becnel, J.J., 1985. Two new species of *Nosema* (Microsporida: Nosematidae) from Mexican bean beetle *Epilachna varivestis* (Coleoptera: Coccinellidae). J. Protozool. 32 (3), 525–535.
Cali, A., Briggs, J.D., 1967. The biology and life history of *Nosema tracheophila* sp. n. (Protozoa: Cnidospora: Microsporidea) found in *Coccinella septempunctata* Linnaeus (Coleoptera: Coccinellidae). J. Invertebr. Pathol. 9, 515–522.
Canning, E.U., 1953. A newmicrosporidian, *Nosema locustae* n. sp., from the fat body of the African migratory locust, *Locusta migratoria migratorioides* (R. and F.). Parasitology 43, 287–290.
Canning, E.U., Lom, J., 1986. The Microsporidia of Vertebrates. Academic Press, London.
Canning, E.U., Vávra, J., 2000. Phylum microsporida. In: Lee, J.J., Leedale, G.F., Bradbury, P. (Eds.), The Illustrated Guide to the Protozoa. Allen Press Inc., Lawrence, pp. 39–126.
Duke, A.J., 1987. CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
Franclemont, J.G., 1951. The species of the Leucania unipunctagroup, with a discussion of the genericnames for the various segregates of Leucania in North America. Proc. ent. Soc. Wash. 53, 57–85.
Hajek, A.E., Delalibera, J.I., 2010. Fungal pathogens as classical biological control agents against arthropods. BioControl 55, 147–158.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.
Hylis, M., Weiser, J., Oborník, M., Vávra, J., 2005. DNA isolation from museum and type collection slides of microsporidia. J. Invertebr. Pathol. 88, 257–260.
Jouvenaz, D.P., Hazard, E.I., 1978. New family, genus, and species of microsporidia (Protozoa: Microsporida) fromthe tropical fire ant, *Solenopsis geminata* (Fabricius) (Insecta: Formicidae). J. Protozool. 25, 24–29.
Lemus, I., Garcia, R., Delvillar, E., Knop, G., 1999. Hypoglycaemic activity of four plants used in chilean popular medicine. Phytother. Res. 13, 91–94.
Lipa, J.J., 1968. *Nosema coccinellae* sp. n., a new microsporidian parasite of *Coccinella septempunctata*, *Hippodamia tredecimpunctata*, and *Myrrha octodecimguttata*. Acta Protozool. 5, 369–374.
Lipa, J.J., Pruszynski, S., Bartkowski, J., 1975. The parasites and survival of the lady bird beetles (Coccinellidae) during winter. Acta Parasitol. Pol. 23, 453–461.
Lipa, J.J., Semjanov, V.P., 1967. The parasites of the ladybirds (Coleoptera, Coccinellidae) in the Leningrad region. Entomologicheskoe Obozrenie 46, 75–80.
Lipa, J.J., Steinhaus, E.A., 1959. *Nosema hippodamiae* n. sp., a microsporidian parasite of *Hippodamia convergens* Guérin (Coleoptera, Coccinellidae). J. Invertebr. Pathol. 1, 304–308.
Luo, B., Liu, H., Pan, G., Li, T., Li, Z., Dang, X., Liu, T., Zhou, Z., 2014. Morphological and molecular studies of *Vairimorpha necatrix* BM, a new strain of the microsporidium *V. necatrix* (microsporidia, burenellidae) recorded in the silkworm, *Bombyx mori*. Exp. Parasitol. 143, 74–82.
Maddox, J.V., Sprenkel, R.K., 1978. Some enigmatic microsporidia of the genus Nosema. Misc. Publ. Entomol. Soc. Am. 11, 65–84.
Mitchell, M.J., Cali, A., 1993. Ultrastructural study of the development of *Vairimorpha necatrix* (Kramer, 1965) (Protozoa, Microsporida) in larvae of the corn earworm, *Heliothis zea* (Boddie) (Lepidoptera, Noctuidae) with emphasis on sporogony. J. Eukaryot. Microbiol. 40, 701–710.
Nägeli, C., 1857. Überdie neue Krankheit der Seidenraupe und verwandte Organismen. Bot. Zeitung. 15, 60–761.
Ovcharenko, M., Swiatek, P., Ironside, J., Skalski, T., 2013. *Orthosomella lipae* sp. n. (Microsporidia) a parasite of the weevil, *Liophloeus lentus* Germar, 1824 (Coleoptera: Curculionidae). J. Invertebr. Pathol. 112, 33–40.
Pilley, B.M., 1976. A new genus, *Vairimorpha* (Protozoa: Microsporida), for *Nosema* necatrix Kramer 1965: pathogenicity and life cycle in *Spodoptera exempta* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 28, 177–183.
Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208–212.
Riddick, E.W., Cottrell, T.E., Kidd, K.A., 2009. Natural enemies of the Coccinellidae: parasites, pathogens, and parasitoids. Biol. Control 51, 306–312.
Roberts, L.S., Janovy, J., 2009. Microsporidia and Myxozoa: parasites with polar filaments. In: Roberts, L.S., Janovy, J. (Eds.), Foundations of Parasitology, eighth ed. The McGraw-Hill Companies Inc, pp. 183–194.
Schenker, W., Maier, W.A., Seitz, H.M., 1992. The effects of *Nosema algerae* on the development of *Plasmodium yoelii nigeriensis* in *Anopheles stephensi*. Parasitol. Res. 78, 56–59.
Slamovits, C.H., Williams, B.A.P., Keeling, P.J., 2004. Transfer of *Nosema locustae* (Microsporidia) to *Antonospora locustae* n. comb. based on molecular andultrastructural data. J. Eukaryot. Microbiol. 51, 207–213.
Sluss, R., 1968. Behavioral and anatomical responses of the convergent lady beetle to parasitism by *Perilitus coccinellae* (Shrank) (Hymenoptera: Braconidae). J. Invertebr. Pathol. 10, 9–27.
Solter, L.F., Becnel, J.J., David, H.O., 2012. Microsporidian entomopathogens. In: Vega, F.E., Kaya, H.K. (Eds.), Insect Pathology. Elsevier Inc., London, pp. 221–263.
Sprague, V., Becnel, J.J., Hazard, E.I., 1992. Taxonomy of phylum microspora. Crit. Rev. Microbiol. 18, 285–395.
Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Clin. Microbiol. Res. 3, 197–218.
Steele, T., Bjornson, S., 2014. *Nosema adaliae* sp. nov., a new microsporidian pathogen from the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae) and its relationship to microsporidia that infect other coccinellids. J. Invertebr. Pathol. 115, 108–115.
Undeen, A.H., Vávra, J., 1997. Research methods for entomopathogenic protozoa. In: Lacey, L. (Ed.), Manual of Techniques in Insect Pathology. Biological Techniques Series. Academic Press, London, pp. 117–151.
Vavra, J., Undeen, A.H., 1970. *Nosema algerae* n. sp. (Cnidospora, Microsporida) a Pathogen in a Laboratory Colony of *Anopheles stephensi* Liston (Diptera, Culicidae). J. Protozool. 17, 240–249.
Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol. 52, 131–142.
Weiser, J., 1953. Schizogregarinen aus Mehlschadlingen. Acta Societatis Zoologica Bohemoslovenicae. 17, 199–211.
Wheeler, A.G., Henry, T.J., 1981. Seasonal history and habits of the European alfalfa beetle, *Subcoccinella vigintiquatuorpunctata* (L.) (Coleoptera: Coccinellidae). Coleopterists Bull. 35, 197–203.