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A new microsporidian pathogen of the Mediterranean Flour Moth, *Ephestiae kuehniella* (Zeller) (Lepidoptera: Pyralidae)

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Abstract

In this study, a new *Vairimorpha* isolate was defined from *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) in Turkey (Malatya). Infection was determined in the host's hindgut, both larval and adult stages. Two types spore originated from the polymorphic sporulation was detected during the observations. While the binucleate ovocylindrical fresh spores were measured 4.38 ± 0.31 µm (5.09–3.77 µm, n = 100) in length and 2.80 ± 0.18 µm (3.21–2.43 µm, n=100) in width, the ovoid meiospores were measured as 3.50 ± 0.46 µm (4.98–2.51 µm, n = 100) in length and 2.02 ± 0.28 µm (2.94–1.50 µm, n=100) in width. The multilocus genetic analyses based on SSU rRNA and RPB1 genes were carried for determining the phylogenetic status of the current microsporidium. In ML and BI trees, the current microsporidium was separated into a different branch from *V. necatrix* strains within the *Vairimorpha* clade

Key words: *Ephestia kuehniella*, Lepidoptera, RPB1, SSU rRNA, *Vairimorpha*

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Akdeniz Un Güvesi, *Ephestiae kuehniella* (Zeller) (Lepidoptera: Pyralidae)'nin yeni bir mikrosporidian patojeni

Özet

Bu çalışmada, Türkiye'de (Malatya) *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) konağından yeni bir *Vairimorpha* izolatı tanımlanmıştır. Enfeksiyon, hem larva hem de yetişkin aşamalarında konağın arka bağırsağında belirlendi. Gözlemler sırasında polimorfik sporülasyon kaynaklı iki tip spor tespit edilmiştir. Çift çekirdekli ovosilindirik taze sporlar 4.38 ± 0.31 µm (5.09–3.77 µm, n = 100) uzunluğunda ve 2.80 ± 0.18 µm (3.21–2.43 µm, n=100) genişliğinde ölçülürken, oval meiosporlar 3.50 ± olarak ölçüldü. 0,46 µm (4,98–2,51 µm, n = 100) uzunluğunda ve 2,02 ± 0,28 µm (2,94–1,50 µm, n=100) genişliğinde. SSU rRNA ve RPB1 genlerine dayalı multilokus genetik analizler, mevcut mikrosporidyumun filogenetik durumunu belirlemek için gerçekleştirilmiştir. ML ve BI ağaçlarında, mevcut microsporidium, *Vairimorpha* dizisi içindeki *V. necatrix* suşlarından farklı bir dala ayrıldı.

Anahtar kelimeler: *Ephestia kuehniella*, Lepidoptera, RPB1, SSU rRNA, *Vairimorpha*

1. Introduction

Lepidoptera, which includes butterflies and moths, is one of the most significant Insecta order with about 180.000 species, 10 percent of the organisms' total known species [1, 2]. The larvae of many lepidopteran species are in the main category of pest insects, and most of these species are in families such as Tortricidae, Noctuidae, and Pyralidae [3]. The Pyralidae family is distinguished from others by the presence of species that cause damage to food processing facilities. *Ephestia kuehniella* Zeller, Mediterranean flour moth or mill moth, is one of these species' members, and this cosmopolitan moth attacks stored grains, seeds, dried fruits, and in general, on foodstuff [4, 5]. Since this moth's

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chemical control is quite problematic due to the residue problem, different methods are sought to manage this pest. In recent years, other combat techniques such as microbial control have attracted attention in pest control. Entomopathogenic organisms that cause infection in pests such as *E. kuehniella* frequently recorded in the literature, and there is increasing interest in isolating and identifying these organisms [6, 7, 8, 9, 10, 11].

Microsporidia, obligate intracellular pathogens, are tiny eukaryotic spore-forming organisms. They infect a broad range of hosts, from invertebrates to vertebrates [12, 13, 14]. Especially in recent years, microsporidial taxa have been preferred for biological control of agricultural and forest pest insects. The most successful and commercialized practice known has been the use of *Nosema locustae* Canning (1953) against grasshoppers in the United States, courtesy of USDA. This application is a milestone, and Microsporidia taxonomy moved to a different level in Turkey as well as all over the world. More than ten microsporidian species have been identified in Turkey from different insect taxa in recent years and made undefined records as much as defined species. Some of these records were made from lepidopteran hosts [10, 15]. These studies demonstrate the potential of Turkey in terms of microsporidium diversity. With this perspective, we present a new microsporidian isolate from *Ephestia kuehniella* (Pyralidae: Lepidoptera) through morphological and molecular characterization in the present study.

2. Materials and methods

2.1. Sample collection and Light microscopy

In 2019 and 2020, larvae and adult stages of the *E. kuehniella* were collected from dried apricot warehouses in Malatya (Yeşilyurt), Turkey. After the macroscopic observations, collected samples were put in the sterile plastic boxes and took the laboratory as soon as possible for further investigations. Care was taken not to break the cold chain during transportation. For light microscopy, larvae and adults were dissected with Ringer's solutions [16]. For signs of microsporidian infection, wet smears were observed with a light microscope (Nikon, Eclipse Ci) at different magnifications. To determine the life cycle stages, infection positive smears were stained with Giemsa stain as in the literature [17, 18]. Necessary photographs and measurements were taken with DS-Fi 2 digital camera attached Nikon Eclipse Ci microscope and Nikon NIS Elements imaging software. And some infected tissues were fixed in 75% alcohol for use in PCR amplification and stored in a refrigerator.

2.2. Electron microscopy

The fixed tissues with 2.5% glutaraldehyde for electron microscopy were washed with 0.1 M cacodylate buffer three times (15 min) before the post-fixation. The post-fixation was carried out with 1% osmium tetroxide (OsO4) for 2 h. The post-fixed tissues were again washed with 0.1 M cacodylate buffer and then dehydrated with alcohol series. Spurr's resin was used for embedding the samples [19]. The specimens were stained with saturated uranyl acetate and Reynolds' lead citrate after the sectioning with the ultramicrotome (Leica EM UC7) [20]. All observations were made using the JEOL JEM 1220 transmission electron microscope.

2.3. Genomic DNA extraction and Phylogenetic analysis

The ethanol-fixed infected tissues were washed three times with distilled water to eliminate the remnants. Then the tissues were put in the new Eppendorf tube and mechanically homogenized with micropestle. The genomic DNA was extracted from the prepared homogenate using the QIAGEN DNA Isolation Kit, No: 69504, following the manufacturer's instructions.

The phylogenetical analyzes were conducted based on the multilocus sequences in the present study. The partial sequences of the small subunit ribosomal RNA (SSU rRNA) and the largest subunit of RNA polymerase II (RPB1) genes were amplificated with this perspective. The primers, 18F and 1537R (18F/1537R: 5′-CACCA GGTTG ATTCT GCC-3′/5′-TTATG ATCCTGCTAA TGGTT C-3′), were used for amplification of the partial SSU rRNA gene [21]. And all reactants were proportioned for a 50 µl reaction system using the QIAGEN Multiplex PCR Kit (No. 206143). For the amplification, the thermocycler parameters were adjusted according to the kit's protocol. The primers used for amplification of the RPB1 gene were designed according to the SSU rRNA gene sequence. And the methodology used for the SSU rRNA gene was followed throughout the amplification process.

According to the BLAST search from the NCBI GenBank database and the literature, the sequences used in the phylogenetical analyses were chosen (Table 1). All sequences were edited and aligned with BioEdit software and the CLUSTAL_W algorithm. *Endoreticulatus bombycis* (AY009115) and *Ordospora colligata* (XM014708712) were used as outgroups. The Kimura-2 parameter model distance matrix was used for calculating the pairwise genetic distances/similarities. Phylogenetic analyzes were performed using the maximum likelihood (ML) method in MEGA X version 10.1.7. and Bayesian inference (BI) in MrBayes 3.2.6, respectively. According to the Akaike information criteria, the optimal evolutionary model was determined as GTR + I + G. Bootstrap confidence values were calculated with 1000 repetitions for ML analysis. The BI analysis was carried according to two independent runs with four chains

for one million generations. One sample was taken from phylogenetic trees every 100 generations. The first 25% of the samples were discarded from the cold chain (burninfrac = 0.25). The new combinations, re-assigned by Tokarev et al in 2020, were used in the phylogenetic analyses [22].

Table 1. Comparing current microsporidium and other related microsporidia based on the small subunit ribosomal RNA gene (SSU rRNA) and the largest subunit of RNA polymerase II (RPB1) gene by query cover, by nucleotide identity, by Pairwise distance analysis, and GC% content.

	MW740155	*Vairimorpha* sp. TME-2021	Query cover	Percent identity	Pairwise distances	GC content (36.7%)
SSU rRNA	KT020736	*Nosema fumiferanae*	91%	85.27%	0.2029	32.3%
	D85503	*Nosema bombycis*	91%	85.37%	0.2017	34.1%
	U09282	*Nosema trichoplusiae*	91%	85.38%	0.2015	34.1%
	MG907019	*Vairimorpha sp.WFH-2018b*	97%	99.64%	0.0036	37.1%
	EU219086	*Vairimorpha thomsoni*	99%	98.12%	0.0244	36.9%
	AY211392	*Nosema spodopterae*	91%	85.37%	0.2017	34.1%
	MN861969	*Nosema fumiferanae*	91%	85.27%	0.2008	33.5%
	U26532	*Nosema furnacalis*	91%	85.87%	0.1940	33.9%
	DQ996241	*Vairimorpha necatrix*	99%	97.42%	0.0273	37.1%
	AF033315	*Vairimorpha lymantriae*	99%	97.58%	0.0300	35.9%
	AJ011833	*Nosema granulosis*	95%	84.83%	0.1978	33.2%
	MT102274	*Vairimorpha oulemae*	98%	98.47%	0.0136	36.8%
	JX268035	*Vairimorpha pieriae*	99%	97.76%	0.0273	36.5%
	XR002966746	*Vairimorpha ceranae*	99%	96.46%	0.0347	36.2%
	AY009115	*Endoreticulatus bombycis*	27%	78.18%	0.4333	51.3%
	U11047	*Vairimorpha vespula*	99%	97.85%	0.0272	36.8%
	U11051	*Vairimorpha necatrix*	99%	97.50%	0.0272	37.0%
	MG907020	*Vairimorpha necatrix*	98%	96.31%	0.0280	37.5%
	MW773119	*Vairimorpha* sp. TME-2021	Query cover	Percent identity	Pairwise distances	GC content (34.7%)
RPB1	HQ457435	*Nosema fumiferanae*	90%	78.03%	0.1861	36.4%
	DQ996234	*Nosema trichoplusiae*	87%	78.38%	0.1882	36.7%
	DQ996231	*Nosema bombycis*	91%	77.09%	0.1934	36.6%
	HQ457438	*Nosema disstriae*	87%	77.23%	0.1948	35.6%
	AJ278948	*Nosema tyriae*	87%	77.70%	0.1880	36.7%
	DQ996233	*Nosema granulosis*	88%	74.67%	0.2110	42.9%
	DQ996232	*Nosema empoascae*	87%	73.54%	0.2280	43.6%
	XM 002995356	*Vairimorpha ceranae*	90%	83.08%	0.1307	31.4%
	AF060234	*Vairimorpha necatrix*	91%	81.67%	0.1462	32.5%
	DQ996230	*Vairimorpha apis*	92%	79.59%	0.1589	31.2%
	XM 014708712	*Ordospora colligata*	68%	72.08%	0.2579	43.3%
	DQ996236	*Vairimorpha necatrix*	91%	81.49%	0.1459	30.8%
	MG808087	*Vairimorpha necatrix*	30%	81.29%	0.1379	37.5%
	MG808086	*Vairimorpha necatrix*	30%	80.32%	0.1453	37.3%
	JX213749	*Vairimorpha lymantriae*	82%	78.85%	0.1652	36.0%
	JX239748	*Vairimorpha disparis*	82%	78.95%	0.1601	36.5%

3. Results

3.1. Sample collection and Light microscopy

In the survey, 110 adults and 310 larvae of *E. kuehniella* were collected (420 samples). During the examination with the light microscope, the microsporidian infection was observed in 15 adults (13.6%) and 74 larvae (23.9%) (total infection rate 21.2%). The infection was restricted to the only hindgut epithelium and lumen of the host (Figure 1). During the observations, two types spores were detected. One of the spore types was larger than the other and

ovocylindrical in shape and direct contact with the host cytoplasm. Smaller spores were ovoid in shape. While the ovocylindrical fresh spores were measured 4.38 ± 0.31 µm (5.09–3.77 µm, n = 100) in length and 2.80 ± 0.18 µm (3.21–2.43 µm, n=100) in width, the ovoid smaller spores were measured as 3.50 ± 0.46 µm (4.98–2.51 µm, n = 100) in length and 2.02 ± 0.28 µm (2.94–1.50 µm, n=100) in width. Sporophorous vesicles (SPV) containing ovoid uninucleate spores (meiospores) were detected after the Giemsa staining (Figure 2a). The Giemsa stained ovoid uninucleate spores in SPV were 2.55 ± 0.27 µm (3.33-2.08) in length and 1.73 ± 0.20 µm (2.23-1.41) in width (n = 100), and SPV were 6.23–9.15 µm in diameter. Giemsa stained ovocylindrical spores were 3.51 ± 0.28 µm (3.99–2.88 µm, n = 100) in length and 2.03 ± 0.20 µm (2.51–1.61 µm, n=100) in width.

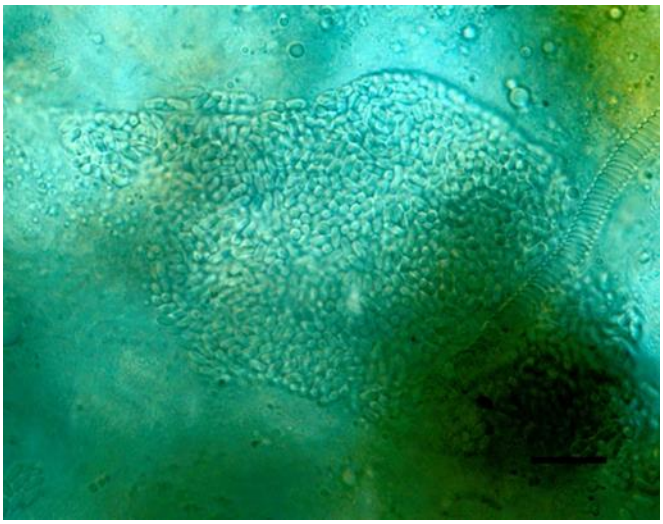

Figure 1. Light micrograph of *Vairimorpha* sp. TME-2021 in *E. kuehniella* hindgut (unit bar: 20 µm)

Nosema type and *Thelohania* type sporulations were also determined in Giemsa stained preparations (Figure 2b, g). In disporoblastic (*Nosema* type) life cycle, merogonial stage started with spherical binucleate meronts, and they were measured 4.78-6.23 µm in diameter (Figure 2b). The sporonts varied from spherical to ovoid, and they produced sporoblasts via binary fission (Figure 2c, d). In *Thelohania* type sporulations, the merogonial stage started with uninucleate meronts formed by germination of uninucleate meiospores (Figure 2e). The meronts usually spherical and measured 2.93-4.12 µm in diameter. In the sporogony stage, proliferation occurred in an SPV with bi-, tetra-, and octonucleate forms, and the meiospores developed from sporonts within an SPV (Figure 2f, g).

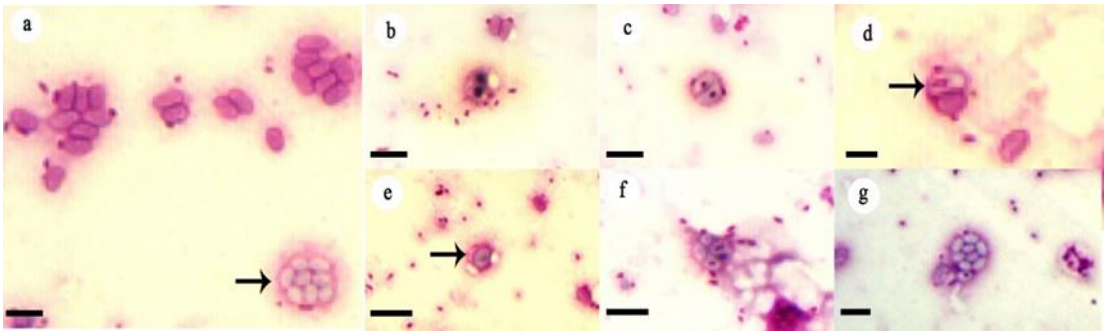

Figure 2. Giemsa stained life cycle stages of the *Vairimorpha* sp. TME-2021. a: Giemsa stained binucleate and meiospores. Note that the arrow shows SPV structure full with the meiospores (unit bar: 5 µm). b: Spherical binucleate meront (unit bar: 5 µm). c: Ovoid sporonts (unit bar: 5 µm). d: Elongated sporoblast (unit bar: 5 µm). e: Uninucleate meront (unit bar: 5 µm). f: Tetranucleate sporogony in SPV (unit bar: 5 µm). g: Eight-spore group (unit bar: 5 µm)

3.2. Electron microscopy

In transmission electron microscopy, mature spores and most life cycle stages, especially *Nosema* type sporulation, could not be observed. Unfortunately, this situation originated from different factors such a poor fixation, infiltration, and infection site. The *Thelohania* type sporulation, which started with uninucleate schizont, was observed (Figure 3a). The SPVs, which include bi- and tetraschizonts, were observed and measured 1.85 × 1.04 µm and 3.24 × 1.96 µm, respectively (Figure 3b, c).

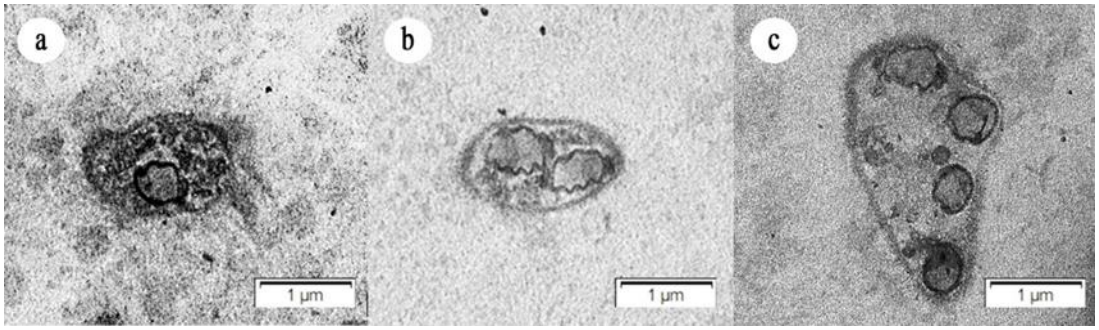

Figure. 3. Transmission electron micrographs of *Thelonia*-type developmental stages. a: Uninucleate schizont. b: Binucleate schizont. c: Tetranucleate schizont

3.3. Genomic DNA extraction and Phylogenetic analysis

The small subunit ribosomal RNA (SSU rRNA) and the largest subunit of RNA polymerase II (RPB1) genes partial sequences of the current microsporidium were deposited to the GenBank with MW740155 and MW773119 accession codes, respectively. The phylogeny of the current microsporidium that was isolated from *E. kuehniella* was based upon these sequences. The SSU rRNA gene sequence's GC content was 36.7%, and the per cent identities of the current microsporidium and other SSU rRNA sequences ranged between 78.1% and 99.6%. Pairwise phylogenetical distances varied from 0.0036 to 0.4333 (Table 1). The BLAST result indicated that the current microsporidium sequence was not identical to any microsporidian sequences listed in GenBank; however, most similar to *Vairimorpha* sp. WFH-2018b isolate Pi_CA_22 (MG907019; 99.6%). This unpublished microsporidian record was obtained from the *Plodia interpunctella* Hubner (Pyralidae: Lepidoptera) in California, USA.

The RPB1 gene sequence of the current microsporidium differentiates from all known RPB1 sequences. The pairwise distances/similarities between the current microsporidium and other microsporidian sequences ranged from 0.1307/83.1% to 0.2579/72.1% (Table 1). The current microsporidium was genetically distant from the other sequences obtained from lepidopteran hosts (below 82% sequence similarity). Surprisingly, it more similar to the honeybee pathogen, *Vairimorpha cerenae* record (XM_002995356) with 83.1%. And the GC content was 34.7%. Bayesian and maximum likelihood analyses of aligned genes sequences showed quite similar topologies (Figure 4, 5). Nosema and *Vairimorpha* species grouped in with their congeners in different branches in all tree topologies. The phylogenetical results robustly showed that the current microsporidium was a member of the *Vairimorpha* genus. Also, in the SSU rRNA gene trees, it formed sister grouped with *Vairimorpha* sp. WFH-2018b isolate Pi_CA_22 (MG907019).

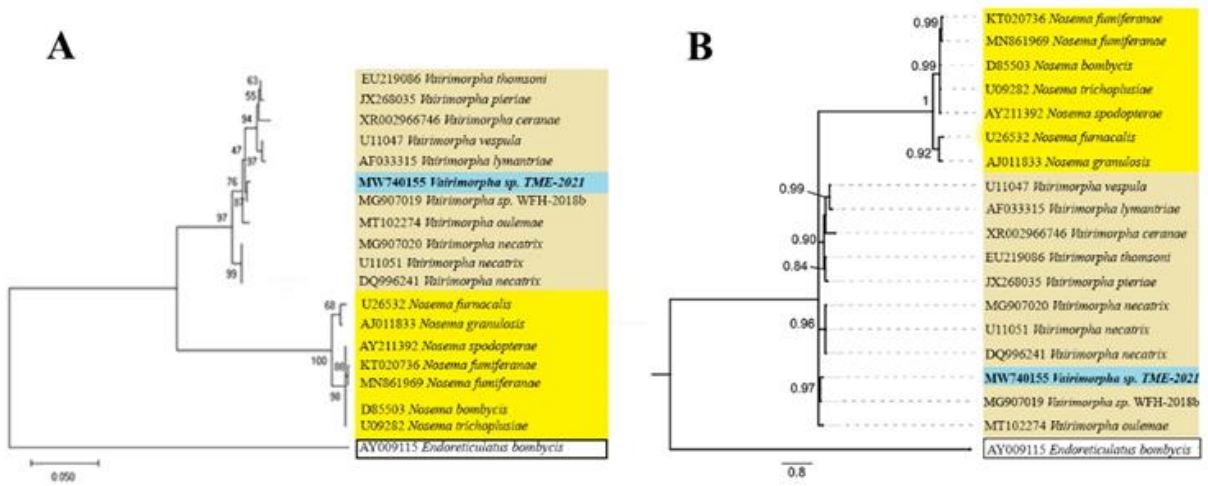

Figure. 4. Molecular phylogenetics of *Vairimorpha* sp. TME-2021 and related microsporidia, based on the SSU rRNA gene. A: The tree was constructed by the Maximum likelihood method. Numbers above the branches are bootstrap support values in percentage. Scale bar = 0.05 nucleotide changes per site. B: The tree obtained by Bayesian inference. Branch supports (Bayesian posterior probability) are indicated for clades with values below 1.0. Scale bar = 0.8 expected nucleotide changes per site

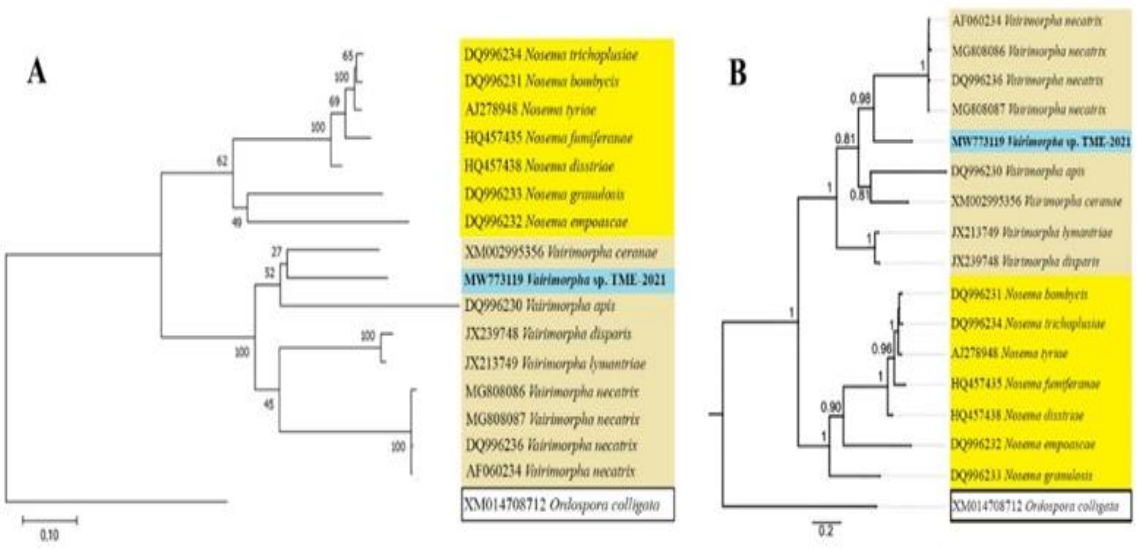

Figure 5. Molecular phylogenetics of *Vairimorpha* sp. TME-2021 and related microsporidia, based on the RPB1 gene. A: The tree was constructed by the Maximum likelihood method. Numbers above the branches are bootstrap support values in percentage. Scale bar = 0.1 nucleotide changes per site. B: The tree obtained by Bayesian inference. Branch supports (Bayesian posterior probability) are indicated for clades with values below 1.0. Scale bar = 0.2 expected nucleotide changes per site

4. Conclusions and discussion

Microsporidia are common pathogens in a wide range of insect taxa [6, 8, 14]. More than 100 microsporidia species have been identified in the Insecta until now, while almost 1 in 5 of them have been recorded from only lepidopteran [13]. These candidates of microbial control agents have detrimental effects on their hosts, such as reducing longevity or fecundity [23].

The pathogen, determined in the Malatya population of the Mediterranean flour moth (*E. kuehniella*), was a microsporidium without suspicion. In the literature, the first microsporidian infection of *E. kuehniella* was recorded in 1927 by Mattes, and later in 1928, he named this microsporidium as a *Thelohania ephestiae* [24, 25]. However, in 1985 this microsporidium assigned to the *Vairimorpha* genus and re-named as a *Vairimorpha ephestiae* by Weiser and Purrini [26]. And after this date, different studies have been done in the literature on *V. ephestiae* [27, 28]. With the study conducted based on multilocus sequence analysis in 2018, it is concluded that *V. ephestiae* is an isolate of *Vairimorpha necatrix*. Considering that *V. necatrix* able to infect different host species under natural or laboratory conditions, the validity of this study increases [29, 30].

Microsporidia taxonomy is shaped on certain taxonomic characters such as spore morphology and life cycle stages [31, 14]. The spore dimensions of the current microsporidium were 4.38 ± 0.31 µm × 2.80 ± 0.18 µm for ovocylindrical binucleate spores and 3.50 ± 0.46 µm × 2.02 ± 0.28 µm for ovoid uninucleate meiospores. The spore dimensions are range between *V. necatrix* strains based on the host species (Table 2). For instance, while the binucleate spore morphometrics was 4.33 ± 0.19 µm × 2.26 ± 0.18 µm for the isolate from *Spodoptera exempta* in 1976, it was 4.3 (3.9–5.0) µm × 2–3 (2.0–2.7) µm in *Heliothis* (*Helicoverpa*) *zea* and 4.7 ± 0.2 × 2.1 ± 0.2 µm in *Bombyx mori* [29, 30, 32]. Also, the spore dimensions in the strain of the V. necatrix (=syn *V. ephestiae*) isolated from *E. kuehniella* were measured as 4-4.5 µm × 2-2.5 µm for the binucleate spores and 2.5-3 µm × 1.5-2 µm for the meiospores [26]. The binucleate spore dimensions of the microsporidium isolated here are relatively similar to the *V. necatrix* strains. However, meiospores dimensions of the current microsporidium larger than all *V. necatrix* strains.

The ultrastructural characteristics of the mature spore are critical diagnostic characters for discriminating the microsporidian species [34]. Unfortunately, the mature spore of the current microsporidium could not be detected during the electron microscopical observations. This situation suggests that it was originated from the low intensity of the microsporidiosis and the microsporidiosis location (hindgut).

In the last quarter, molecular phylogeny has been an essential parameter for Microsporidia taxonomy. Although phylogenetic analyzes based on SSU rRNA are insufficient to reveal the current situation in some genera, it is still widely used in the microsporidian phylogeny [7, 21]. Phylogenetic comparison of the current microsporidium with *V. necatrix* strains based on SSU rRNA showed a significant genetic difference (2.72–2.79%). The current microsporidium was also settled the different branches from *V. necatrix* group in ML and BI trees. However, in the study conducted by Malysh et al. in 2018, the partial SSU rRNA sequence of the *V. necatrix* (=syn *V. ephestiae*) strain show 100% identity to that of the other *V. necatrix* strains (U11051 and DQ996241) [35]. On the other hand, the current microsporidium grouped on the same branch with the *Vairimorpha* sp. WFH-2018b (MG907019) isolated from *Plodia*

interpunctella Hübner both in ML and BI trees (Figure 4). This record, selected for high Query cover and Percent ident values (97% and 99.64%, relatively) in the BLAST analysis, was stored to NCBI GenBank as an unpublished record in 2018 from the USA. Therefore, it is impossible to evaluate this record according to other taxonomic parameters used in the microsporidian taxonomy.

Table 2. Comparison of spore morphologies of *Vairimorpha necatrix* strains and the current microsporidium, *Vairimorpha* sp. TME-2021

	Host	Spore shapes	Fresh spore dimensions	References
Vairimorpha necatrix	*Spodoptera exempta* Walker	Meiospores: ovoid Binucleate: elongate or cylindrical	Meiospores: 1.86 ± 0.10 µm × 1.08 ± 0.12 µm Binucleate: 4.33 ± 0.19 µm × 2.26 ± 0.18 µm	[30]
Vairimorpha necatrix	*Pseudaletia unipunctata* Haworth	Meiospores: ovocylindrical Binucleate: oblong	Meiospores: 3-4 µm × 2-2.5 µm Binucleate: 5-6 µm × 2-3 µm	[33]
Vairimorpha necatrix	*Heliothis (Helicoverpa) zea* Boddie	Binucleate: elongate or oval	Binucleate: 4.3 (3.9–5.0) µm × 2–3 (2.0–2.7) µm	[29]
Vairimorpha necatrix	*Helicoverpa armigera* Hübner	Binucleate: elongated oval	Binucleate: 4.88 ± 0.27 µm ×2.47 ± 0.11 µm	[32]
Vairimorpha necatrix	*Bombyx mori* L.	Binucleate:kidney-shaped or oval	Binucleate: 4.7 ± 0.2 × 2.1 ± 0.2 µm.	[32]
Vairimorpha necatrix (=*syn Vairimorpha ephestiae*)	*Ephestiae kuehniella* Zeller	Meiospores: oval Binucleate: elongated oval	Meiospores: 2.5-3 µm × 1.5-2 µm Binucleate: 4-4.5 µm × 2-2.5 µm	[26]
Vairimorpha sp. TME-2021	*Ephestiae kuehniella* Zeller	Meiospores: ovoid Binucleate: ovocylindrical	Meiospores: 3.50 ± 0.46 µm × 2.02 ± 0.28 µm Binucleate: 4.38 ± 0.31 µm × 2.80 ± 0.18 µm	Present study

Microsporidian phylogeny has gained a different dimension with the widespread use of multilocus sequence analysis in recent years [10, 22]. With this perspective, the partial sequence of the RPB1 gene of the current microsporidium was analyzed in this study. The distance analysis, based on the current microsporidium's RPB1 gene, demonstrated a genetic difference from all *V. necatrix* strains between 13.79 to 14.62% values. In ML and BI trees, the current microsporidium was separated into a different branch from *V. necatrix* strains within the *Vairimorpha clade* (Figure 5). All phylogenetic analyses demonstrated that the current microsporidium is distinct from other species in the *Vairimorpha* complex.

Although the results of the light microscopy and molecular analysis of the current microsporidium were enough to assign a novel species, the lack of ultrastructural characteristics of the current microsporidium weakened this claim. Therefore, in this study, it was considered appropriate to temporarily define the current microsporidium as *Vairimorpha* sp TME-2021 to avoid creating an incomplete or incorrect registration of a new species.

In conclusion, a new *Vairimorpha* isolate was defined non-*Vairimorpha necatrix* strains from *Ephestia kuehniella* in Turkey (Malatya) with this study.

References

[1] Capinera, J. L. (2008). Butterflies and moths. In Encyclopedia of Entomology (Vol. 4, pp. 626-672): Springer.

[2] Powell, J. A. (2009). Lepidoptera. In V. H. Resh & R. T. Carde (Eds.), Encyclopedia of Insects (Vol. 2, pp. 557-587): Academic Press.

[3] Scoble, M. J. (1995). The Lepidoptera: Form, Function and Diversity (1 ed.). Oxford University: Oxford University Press.

[4] Benson, J. F. (1973). The biology of Lepidoptera infesting stored products, with special reference to population dynamics. *Biological Reviews*, 48, 1-26.

[5] Carter, D. J. (1984). Pest Lepidoptera of Europe with special reference to the British Isles. Netherlands: Dr. W. Junk Dordrecht.

[6] Baki, H., & Bekircan, Ç. (2018). A new microsporidium, Vairimorpha subcoccinellae n. sp. (Microsporidia: Burenellidae), isolated from Subcoccinella vigintiquatuorpunctata L. (Coleoptera: Coccinellidae). *Journal of Invertebrate Pathology*, 151, 182-190. doi:10.1016/j.jip.2017.12.004

[7] Bekircan, Ç. (2020). Assignment of Vairimorpha leptinotarsae comb. nov. on the basis of molecular characterization of Nosema leptinotarsae Lipa, 1968 (Microsporidia: Nosematidae). *Parasitology*, 147(9), 1019-1025. doi:10.1017/s0031182020000669

[8] Bekircan, Ç., Bülbül, U., Güler, H. İ., & Becnel, J. J. (2016). Description and phylogeny of a new microsporidium from the elm leaf beetle, Xanthogaleruca luteola Muller, 1766 (Coleoptera: Chrysomelidae). *Parasitology Research*, 116(2), 773-780. doi:10.1007/s00436-016-5349-y

[9] Bekircan, Ç., Cüce, M., Baki, H., & Tosun, O. (2017). Aranciocystis muskarensis n. gen., n. sp., a neogregarine pathogen of the Anisoplia segetum Herbst (Coleoptera: Scarabaeidae). *Journal of Invertebrate Pathology*, 144, 58-64. doi:10.1016/j.jip.2017.01.014

[10] Tosun, O. (2020). A new isolate of Nosema fumiferanae (Microsporidia: Nosematidae) from the date moth Apomyelois (Ectomyelois) ceratoniae, Zeller, 1839 (Lepidoptera: Pyralidae). *Parasitology*, 147(13), 1461-1468. doi:10.1017/s0031182020001481

[11] Yıldırım, H., & Bekircan, Ç. (2020). Ultrastructural and molecular characterization of Nosema alticae sp. nov. (Microsporidia: Nosematidae), pathogen of the flea beetle, Altica hampei Allard, 1867 (Coleoptera: Chrysomelidae). *Journal of Invertebrate Pathology*, 170, 107302. doi:10.1016/j.jip.2019.107302

[12] Lacey, L. A. (2012). Manual of Techniques in Invertebrate Pathology (Second ed.). USA: Academic Press.

[13] Vega, F. E., & Kaya, H. K. (2012). Insect Pathology (Second ed.). UK: Elsevier.

[14] Weiss, L. M., & Becnel, J. J. (2014). Microsporidia Pathogens of Opportunity. USA: John Wiley & Sons, Inc.

[15] Yaman, M., Bekircan, Ç., Radek, R., & Linde, A. (2014). Nosema pieriae sp. n. (Microsporida, Nosematidae): A New Microsporidian Pathogen of the Cabbage Butterfly Pieris brassicae L. (Lepidoptera: Pieridae). *Acta Protozoologica*, 53, 223-232. doi:10.4467/16890027AP.14.019.1600

[16] Bekircan, Ç., Tokarev, Y. S., Tosun, O., & Baki, H. (2016). Detection of Neogregarine and Eugregarine (Apicomplexa) Infections from Chrysolina herbacea (Duftschmid 1825) (Coleoptera: Chrysomelidae) in Turkey. *Turkish Journal of Life Sciences*, 1(2), 59-64.

[17] Bekircan, Ç., Tosun, O., & Baki, H. (2018). Influence of climatic conditions on microsporidiosis that originated from Rugispora istanbulensis in Elm Leaf Beetle, Xanthogaleruca luteola Muller (Coleoptera: Chrysomelidae). *Kastamonu Üniversitesi Orman Fakültesi Dergisi*, 18(2), 230-235. doi:10.17475/kastorman.344845

[18] Bekircan, Ç., Tosun, O., & Yıldırım, H. (2020). Survey of Entomopathogenic Organisms of the Cantharis livida Linnaeus, 1758 (Coleoptera: Cantharidae) in Turkey. *Biological Diversity and Conservation*, 13(3), 187-193. doi:10.46309/biodicon.2020.736577

[19] Spurr, A. R. (1969). A Low-Viscosity Epoxy Resin Embedding Medium for Electron Microscopy. *Journal of Ultrastructure Research*, 26, 31-43.

[20] Reynolds, E. S. (1963). The Use of Lead Citrate at High pH as an Electron-Opaque Stain in Electron Microscopy. *The Journal of Cell Biology*, 17(1), 208-2012.

[21] Vossbrinck, C. R., & Debrunner-Vossbrinck, B. A. (2005). Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitologica*, 52, 131-142.

[22] Tokarev, Y. S., Huang, W. F., Solter, L. F., Malysh, J. M., Becnel, J. J., & Vossbrinck, C. R. (2020). A formal redefinition of the genera Nosema and Vairimorpha (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics. *Journal of Invertebrate Pathology*, 169, 107279. doi:10.1016/j.jip.2019.107279

[23] Hajek, A. E., & Delalibera, I. J. (2010). Fungal pathogens as classical biological control agents against arthropods. *BioControl*, 55, 147-158. doi:10.1007/s10526-009-9253-6

[24] Mattes, O. (1927). Parasitiire Krankheiten der Mehlmottenlarven und Versuche tiber ihre Verwendbarkeit als biologisches Bekiimpfungsmittel. Zugleich ein Beitrag zur Zytologie der Baktericn. . Retrieved from Sitzungsber. Ges. zur Bef. Ges.

[25] Mattes, O. (1928). Uber den Entwicklungsgang der Mikrosporidie Thelohania ephestiae und die von ihr hervorgerufcncn Krankheitserscheinungen. Z. wiss, Zool., 132, 526-582.

[26] Weiser, J., & Purrini, K. (1985). Light- and electron-microscopic studies on the microsporidian Vairimorpha ephestiae (Mattes) (Protozoa, Microsporidia) in the meal moth Ephestia kühniella. Archiv für Protistenkunde, 130(3), 179-189. doi:10.1016/s0003-9365(85)80060-9

[27] Moawed, S. M., Marei, S. S., Saleh, M. R., & Matter, M. M. (1997). Impact of Vairimorpha ephestiae (Microsporidia: Nosematidae) on Bracon hebetor (Hymenoptera: Braconidae), an external parasite of the American bollworm, Heliothis armigera (Lepidoptera: Noctuidae). *European Journal of Entomology*, 94, 561-565.

[28] Vorontsova, Y. L., Tokarev, Y. S., Sokolova, Y., & Glupov, V. V. (2004). Microsporidiosis in the wax moth Galleria mellonella (Lepidoptera: Pyralidae) caused by Vairimorpha ephestiae (Microsporidia: Burenellidae). *Parazitologiya*, 38(3), 239-250.

[29] 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. *Journal of Eukaryotic Microbiology*, 40(6), 701-710.

[30] Pilley, B. M. (1976). A new genus, Vairimorpha (Protozoa: Microsporida), for Nosema necatrix Kramer 1965: Pathogenicity and life cycle in Spodoptera exempta (Lepidoptera: Noctuidae). *Journal of Invertebrate Pathology*, 28(2), 177-183. doi:10.1016/0022-2011(76)90119-1

[31] Sprague, V., Becnel, J. J., & Hazard, E. I. (1992). Taxonomy of Phylum Microspora. *Critical Reviews in Microbiology*, 18(5/6), 285-395.

[32] Luo, B., Liu, H., Pan, G., Li, T., Li, Z., Dang, X., . . . 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. *Experimental Parasitology*, 143, 74-82. doi:http://dx.doi.org/10.1016/j.exppara.2014.05.001

[33] Kramer, J. P. (1965). Nosema necatrix sp. n. and Thelohania diazoma sp. n., microsporidians from the armyworm Pseudaletia unipuncta (Haworth) *Journal of Invertebrate Pathology* 7(2), 117-121.

[34] Canning, E. U., & Vávra, J. (2000). Phylum microsporida. In J. J. Lee, G. F. L. le, & P. Bradburry (Eds.), The Illustrated Guide to the Protozoa (Vol. 1, pp. 39-126). Lawrence: Allen Press Inc.

[35] Malysh, J. M., Vorontsova, Y. L., Glupov, V. V., Tsarev, A. A., & Tokarev, Y. S. (2018). Vairimorpha ephestiae is a synonym of Vairimorpha necatrix (Opisthosporidia: Microsporidia) based on multilocus sequence analysis. *European Journal of Protistology*, 66, 63-67. doi:10.1016/j.ejop.2018.08.004