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Characterisation of a Turkish Isolate of *Nosema ceranae* Fries et al., 1996 (Microsporidia) Recorded in Populations of *Apis mellifera* L. in Turkey

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Abstract: This study aimed to compare disease factors, in terms of morphological, molecular and ultrastructural characters, for nosemosis in honey bees in Turkey. *Nosema ceranae* Fries et al., 1996, a pathogen of *Apis mellifera* was detected with light microscopy and PCR-based methods. Then, its spore ultrastructure was studied with transmission electron microscopy (TEM) to compare with the known ultrastructure of isolates of *N. ceranae* from different countries. Mature fresh spores measured 4.5 x 2.5 μm (n=50) and Giemsa stained spores 4.4 x 2.6 μm (n=50). Polar filament had 22-23 coils, showed isofilar structure and measured 85-90 nm in diameter. Endospores measured 115-140 nm with low electron density, while exospore measured 15-30 nm. TEM studies confirmed that ultrastructure characters of the Turkish isolate of *N. ceranae* differed slightly from those of *N. ceranae* isolates from different countries. This is the first study of spore morphology and ultrastructure of *N. ceranae* recorded in honey bees in Turkey.

Key words: Nosemosis, ultrastructure, *Nosema ceranae*, *Apis mellifera*, Turkey

Introduction

Nosemosis is a serious and widespread disease in honeybees. It is known that the disease is caused by two species of microsporidian pathogens, *Nosema apis* (Zander, 1909) and *N. ceranae* Fries et al., 1996 (Ptaszynska et al. 2014). Microsporidia is a large group of obligate intracellular parasites, frequently infecting insects (Vavra & Larsson 1999). The genus *Nosema* Nägeli, 1857 generally infects many insect species. In honey bees, *N. apis* infects *Apis mellifera*, while *Nosema ceranae* infects both *A. ceranae* and *A. mellifera* (Fries 1997). Nosemosis is detected in infected honey bees by determining *Nosema* spores under light microscope but the differentiation between spores of *N. apis* and *N. ceranae* is very difficult (Ptaszynska et al. 2014) and this method is unable to characterise the disease agent at the species level as *N. ceranae* or *N. apis.* Furthermore, it is needed to compare different isolates of this microsporidium to further study its taxonomic characters and pathogenicity. In recent studies on microsporidia, ultrastructural characteristics of pathogen spores are always studied (Fries 1989, Larsson 1988, 1999, Yaman et al. 2011, 2016). However, only a few papers focus on the molecular characterisation of the disease agents at the species level, including *N. ceranae* and *N. apis* (Whitaker et al. 2011, Muz et al. 2010, Ütük et al. 2010).

In the present article, the spore ultrastructure of *N. ceranae* infecting honey bees in the Ordu Province, Turkey, is presented and compared with other *Nosema* spp. infecting honey bees from different countries.

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Materials and Methods

Insect samples and light microscopy

For this study, infected honey bee samples previously collected and stored -20ºC from Kabadüz, Gürgentepe, Gölköy and Mesudiye and field-collected honey bee samples from Gölköy, Gürgentepe, Fatsa, Perşembe, İkizce, Kabataş, Çatalpınar, Aybastı and Altınordu in the Ordu Province, Turkey were used. The bees were dissected in Ringer's solution and fresh smears of tissue samples were examined under a microscope for determination of nosemosis. When nosemosis was determined, a part of the insect gut or disturbed other tissues were used for preparation of Giemsa stained smears for confirming the microsporidian infection. Detected fresh and stained spores of microsporidian pathogens from seven localities in Ordu were measured and photographed using an Olympus BX51 microscope with a DP-25 digital camera and a DP2-BSW Soft Imaging System.

Spore purification, genomic DNA extraction and PCR amplification

Spore suspension, including heavily infected gut tissues, was used for DNA extraction. The semi-purified spore pellet was diluted with distilled water and an equal volume of spore suspension and glass beads (Sigma-G8772) were put in a 1.5 ml Eppendorf tube and was vigorously shaken for 1 min. at maximum speed using a vortex. After that, DNA extraction was performed with the QIAGEN DNA Isolation Kit (Cat. no. 69504) according to the manufacturer's guidelines. To amplify the specific genes of *Nosema* pathogen infecting honey bees, a pair of primers specific for *N. ceranae* (218MITOCFOR: 5'-CGGCGACGATGTGATATGAAAATATTAA-3' and 218MITOCREW: 5'-CCCGGTCATTCTCAAACAAAAAACCG-3') was used in the study as described previously (URL-1). Amplification was carried out in a thermocycler under the following conditions: initial denaturation step of 95˚C for 2 minutes, 35 cycles of 95˚C for 1 min., 50˚C for 1 min. and 72˚C for 1 min., with a final extension step of 72˚C for 5 minutes. The specificity of amplification was confirmed by cloning the purified PCR fragments from 1.5% agarose gel. Visualisation of the amplification products was made using standard procedures.

Transmission electron microscopy

Parts of the infected tissue could be used to document ultrastructural characters of the pathogen spore (Yaman et al. 2005). For this, portions (about 1 mm^3) of the infected tissues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1-2 h, postfixed in reduced OsO_4 for 1.5 h, and dehydrated in ethanol prior to embedding in resin. Thin sections were mounted on Pioloform-coated copper grids, which were then stained with saturated uranyl acetate and lead citrate. They were examined with a Philips 208 transmission electron microscope (TEM).

Results

In this study, *N. ceranae*, a pathogen of *Apis mellifera* from the Ordu Province, was detected firstly with light microscopy (Figs. 1, 2). Mature fresh and Giemsa stained spores were measured for each locality. Mature fresh spores measured 4.5 x 2.5 µm (n=50) and Giemsa stained spores - 4.4 x 2.6 µm (n=50). Light microscopic examinations of the parasitised individuals revealed the presence of the pathogen in the epithelial cells in the midgut,

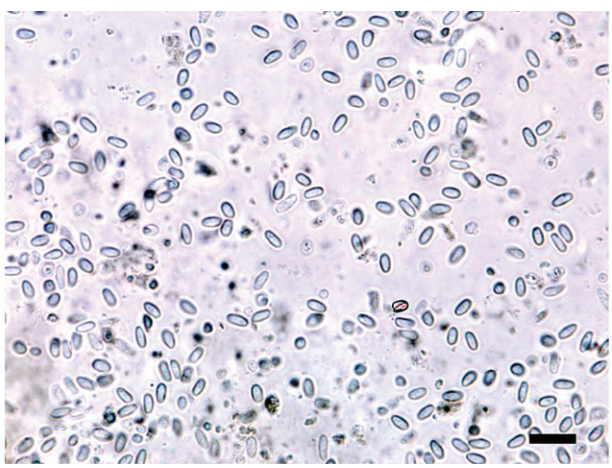

Fig. 1. Fresh spores of *N. ceranae* observed in *Apis mellifera* in Ordu. (Bar: 10 µm)

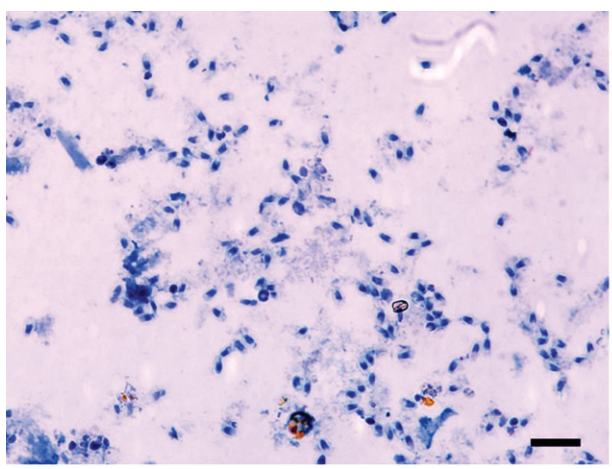

Fig. 2. Giemsa stained spores of *N. ceranae* observed in *Apis mellifera* in Ordu. (Bar: 20 µm)

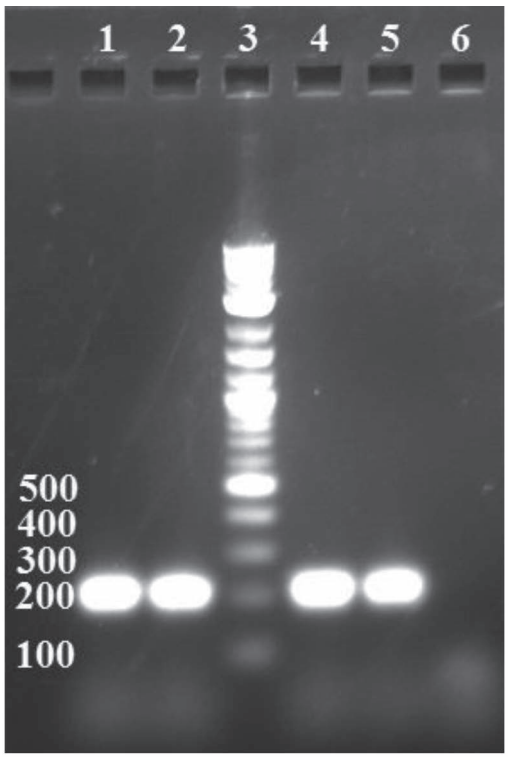

Fig. 3. Banding patterns on a 1.5% agarose gel after PCR with *N. ceranae* -specific primers (218MITOCFOR and 218MITOCREW). DNA ladder (Lane 3); negative control (Lane 6); DNAs prepared from *Nosema*-infected *A. mellifera* from Kabadüz (Lane 1), Gürgentepe (Lane 2), Gölköy (Lane 4) and Mesudiye (Lane 5).

Malpighian tubule systems, hypopharyngeal glands, salivary glands and venom sacs.

The microsporidium causing nosemosis in Gölköy, Kabadüz, Mesudiye and Gürgentepe was identified as *Nosema ceranae* by using a PCR-based method. The DNA sequence amplified by PCR using *N. ceranae* -specific primers was 218 bp (Fig. 3), thus it could be distinguished from *Nosema apis*.

The spore ultrastructure of the pathogen was studied with TEM to compare with the known isolates of *N. ceranae* that were previously studied from different countries. The ultrastructural studies showed that the free, oval spores contained two nuclei in diplokaryotic arrangement. The spore wall was thick, measured 135 to 160 nm and consisted of a clear endospore (115 to 140 nm) and an electron-dense, uniform, thin exospore (15–30 nm; Fig. 4). The polar filament was isofilar, relatively thin (85–90 nm) and had 22–23 coils (Fig. 5).

Additionally to morphological, molecular and ultrastructural characterisation of *N. ceranae,* we also studied its occurrence and distribution in the Ordu Province in the year 2015 to compare the results recorded previously. Totally 24 of the examined 731 bees from nine localities in the Ordu Province

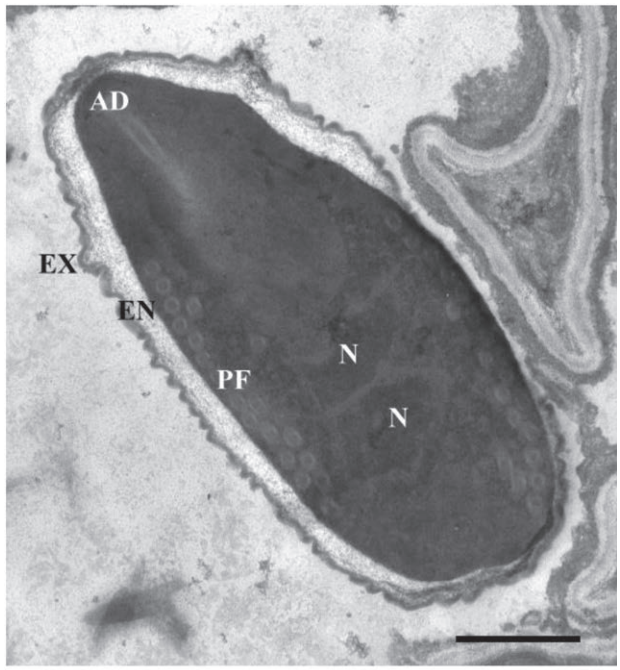

Fig. 4. Longitudinal section of *Nosema ceranae* spore from *Apis mellifera* in Ordu; EX: exospore, EN: endospore, PF: polar filament, AN: anchoring disc, N: nucleus. (Bar: 1 μm).

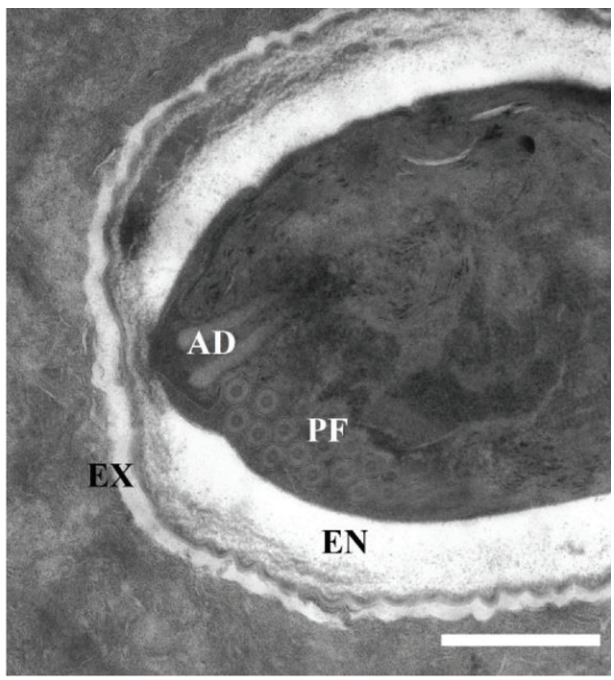

Fig. 5. Transmission electron micrograph of the anterior part of *Nosema ceranae* spore from *Apis mellifera* in Ordu; EX: exospore, EN: endospore, PF: polar filament, AN: anchoring disc (Bar: 750 nm).

were found to be infected (Table 1). The average infection rate was 3.28%. The disease was observed with the lowest rate (1.09%) in İkizce and with the highest rate (10.37%) in Aybastı. There was no infection in Gölköy and Fatsa.

Table 1. Occurrence and distribution of *Nosema ceranae* in Ordu province

Location	Years	Number of investigated sample	Number of infected sample	Infection rate (%)
Gölköy	2015	100	0	0
Gürgentepe	2015	56	1	1.78
Fatsa	2015	100	0	0
Perşembe	2015	40	2	5
Altınordu	2015	50	4	8
İkizce	2015	91	1	1.09
Kabataş	2015	88	1	1.13
Çatalpınar	2015	100	4	4
Aybastı	2015	106	11	10.37
Total		731	24	3.28

Table 2. Ultrastructural characters of different isolates of *N. apis* and *N. ceranae*

Nosema species	Host	Spore measurement (µm)	Ultrastructural features			References
			Polaroplast	Spore wall (nm)	Polar filament coils	
Nosema apis	*Apis mellifera*	---	Lamellar	---	26-32	(Fries 1989, Liu 1984)
		6.0 × 3.0	Lamellar	---	27-30	(Huang et al. 2012)
		---	Lamellar	---	30-35	(Shabanov 1973)
Nosema ceranae	*Apis ceranae*	4.7 × 2.7	Lamellar	137-183	20-23	(Fries et al. 1996)
Nosema ceranae	*Apis mellifera*	4.5 × 2.4	Lamellar	---	20-23	(Huang et al. 2007)
		4.4 × 2.2	Lamellar	48-53 (EX)	18-21	(Chen et al. 2009)
Nosema ceranae	*Apis mellifera* (Ordu)	4.5 × 2.5	Lamellar	135-160	22-23	In present study

Discussion

The nosemosis found in *Apis mellifera* was caused by a microsporidian pathogen, showing the typical characteristics of microsporidia, such as a polar filament with anchoring disc, polaroplast, posterior vacuole and lack of mitochondria (Larsson 1988, 1999, Yaman et al. 2005, 2011, 2016). The pathogen was firstly determined morphologically under light microscopy and then confirmed at species level using molecular and ultrastructural data. Morphological, ultrastructural and molecular data confirmed that this pathogen was *N. ceranae* (Figs. 1-5). The spore is accepted as the most important life cycle stage for taxonomical studies (Larsson 1999). We documented the spore ultrastructural characters of *N. ceranae* recorded from Turkey and compared them with other known isolates of this pathogen from different countries. The spore size of *N. ceranae* isolate in our study was different from that of all *N. apis* isolates (Table 3) and it showed slight similarities with *N. ceranae* isolates recorded by Fries et al. (1996) (4.7 x 2.7 µm) and Chen et al. (2009) (4.4 x 2.2 µm).

The ultrastructural results confirmed that the studied spores were of *N. ceranae* and possessed all of the ultrastructural characteristics of the genus *Nosema* (Larsson 1988). Mature spores were bounded by a thickened wall consisting of electron-dense exospore and electron-lucent endospore layers (Figs. 4, 5). It is known that the number of polar filament coils is an important taxonomic criterion that could be used to differentiate species or isolates (URL-1, Cheung & Wang 1995). The polar filament of *N. ceranae* spores in this study was isofilar and consisted of 22-23 coils. Electron microscopy confirmed that the number (22–23) of polar coils of *N. ceranae* described from Turkey was smaller from the number of coils of the seven *Nosema* isolates from *Apis mellifera* (Table 2); *N. apis* isolates, 26–32 coils (Fries 1989, Liu 1984) and 27-30 coils (Huang et al. 2012); *N. ceranae* isolates, 20-23 coils (Fries et al. 1996, Huang et al. 2007) and 18-21 coils (Chen et al. 2009).

The number of polar filaments of the pathogen described here also confirms the molecular data that the pathogen was clearly different from *N. apis*. However, Chen et al. (2009) have reported that the polar filament of *Nosema ceranae* spores have 18-21 coils. Our isolate has higher minimum number of polar filament coils than that of other *N. cera-*

nae isolates. Additionally, the diameter of the polar filaments of our isolates were 85-90 nm and much smaller than the ones recorded by Fries et al. (1996).

Similar differences were also seen in the thickness of the spore wall from other nosemosis infecting bees in different countries. We measured a maximum spore wall of 160 nm, which was thinner when compared with the spore wall thickness (183 nm) of *N. ceranae* recorded by Fries et al. (1996).

It used to be often difficult to compare nosemosis recorded in Turkey because the early descriptions of *Nosema* isolates from honey bees were based mainly on light microscopic spore morphology and ultrastructural details were not available. These restricted methods resulted in undescribed species. For example, it was believed that the nosemosis in honey bees in Turkey had been caused only by *N. apis* till the first detection of *N. ceranae* using molecular method (Ütük et al. 2010). However, Tosun (2012) described ultrastructural characteristics of one *Nosema* sp. without clear descriptions at the species level. Here, we present for the first time ultrastructural characters of *N. ceranae* infecting honey bees in Turkey. The description of the spore ultrastructure of *N. ceranae* provided here could be useful for the identification and comparison of other *Nosema* spp. infecting honey bees in Turkey.

We demonstrated that *Nosema ceranae* had dominated in Ordu for 5 years by confirming its first detection in the region by Tosun (2012) and Yaman et al. (2015).

The infection rate in honey bees in winter was generally high because of the climatic conditions, lack of nourishment and honey bee diseases (URL-1). Nevertheless, it was considerably lower as compared to previous studies. Sammataro & Yoder (2012) recorded that the mid-gut of honey bees with nosemosis may have intense infection and the body of the bee may become heavy. As a result, the bees cannot turn back the comb and can die.

Some episodes of high colony losses have happened in Turkey in autumn and winter. One of the characteristics of the infected apiaries is the depopulation of colonies with abandonment of the brood and the reserves of honey and pollen (Invernizzi et al. 2009). This resembles very much those described in colonies infected by *N. ceranae* in Europe (Higes et al. 2008) and America (Oldroyd 2007).

Nosemosis factors need to be explored in detail for both the microbiological quality of the honey (Dümen et al. 2013) and the bee hygiene (Yaman et al. 2015). Many biotic factors affect honey bee biology (Kirpik et al. 2010, Borum et al. 2015). Experimental infection of *Apis mellifera* with *N. ceranae* conducted by Higes et al. (2007) showed that the parasite was highly pathogenic and was a crucial threat to beekeeping industry. The morphological and molecular characterisation of *N. ceranae* in European honey bee (*Apis mellifera*) was conducted by Fries et al. (2006). However, characteristics of the different geographic strains of this pathogen, which would be more or less pathogenic as in some other bee pests (Aydin et al. 2007), remained to be demonstrated. Here, we present ultrastructural characters of *N. ceranae* from Turkey by comparing with different geographic strains for the first time.

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