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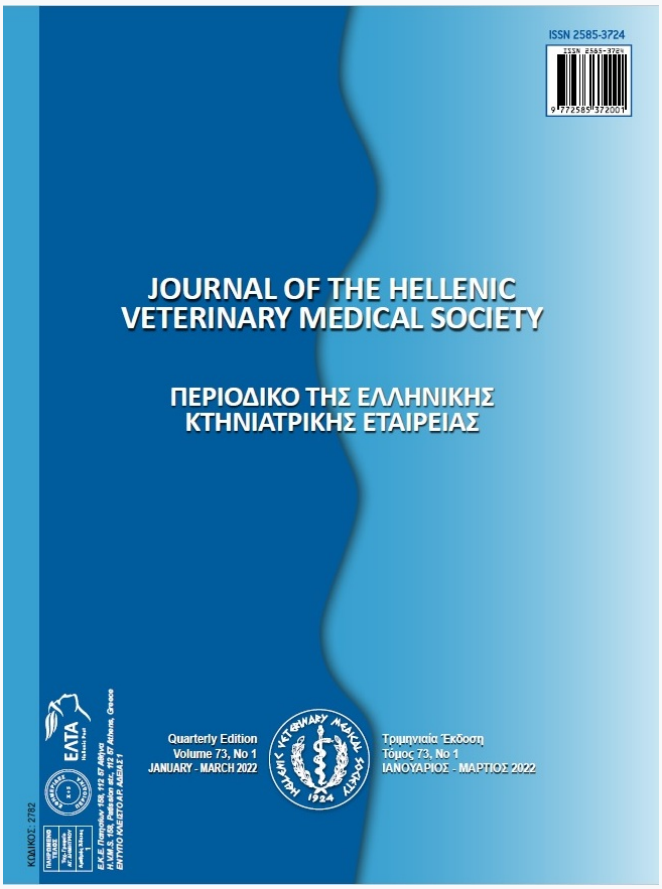

Identification of the Nosema spp., a microsporidian parasite isolated from the honey bees (Apis mellifera) and its association with honey bee colony losses in apiaries of Iran

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Research article
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Identification of the *Nosema* spp., a microsporidian parasite isolated from the honey bees (*Apis mellifera*) and its association with honey bee colony losses in apiaries of Iran

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ABSTRACT: The aim of this study was to determine the *Nosema* species by microscopic and molecular method and its association with honeybee colony losses (Colony Collapse Disorder) in apiaries of Urmia, Northwest of Iran. For this purpose, honeybee samples were collected from 840 colonies kept in 120 apiaries in five different location of Urmia. The specimens were examined for the presence of *Nosema* spores. After DNA isolation, the 16S rRNA gene was evaluated using multiplex PCR. Total infection prevalence with the microscopic evaluation was 32% while in PCR test was 58.2%. *Nosema* positive samples were evaluated by PCR sequencing. Based on the results of PCR, all identified cases were *N. ceranae*. The obtained sequences were transferred to GenBank/NCBI (samples accession numbers MT001887 and MT001893). The results showed the prevalence of Colony Collapse Disorder like symptoms in the studied honeybee colonies were 13.33%. *N. ceranae* was detected by PCR in 20.28% of honeybee colonies with Colony Collapse Disorder like signs. Our findings showed that there was a significant relation between Colony Collapse Disorder and presence of *N. ceranae*. The results of this study concluded that *N. ceranae* is the only specie that affects the honeybees which may have an important role in the occurrence of collapse of bee families and depopulation of hives in this area.

***Keywords*:** Colony Collapse Disorder, Honeybee, *Nosema cerenae,* Iran.

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INTRODUCTION

Nosema species lead to a very serious disease in adult honeybees which is called nosemosis. The colonies are repeatedly infected, and all members of the colony are susceptible to this infection. The ingestion of spores through food or water is the main source of infection. Spores are germinated in the presence of physical and chemical features of the midgut and the vegetative stage of *Nosema* happens inside midgut cells (Chen et al., 2009). Just in two weeks after the infection, one can find 30-50 million of spores in a bee's midgut (Bailey and Ball, 1997). The spores also spread through the feces of the infected bees and create new source of infection (Chen et al., 2009). This parasite can cause digestive disorders and is a common reason for malnutrition, reduced longevity, and physiological aging and all these side-effects cause a reduction in the amount of honey produced by the bees (Hornitzky, 2008).

Nosema apis has long been known as a parasite which only affects Eurpean honeybees (*Apis mellifera*) (Matheson, 1996), but Asian honeybees, *A. ceranae* are mostly infected with *N. ceranae* parasite (Fries et al., 1996).The study conducted by Fries et al. (1996) reported *N. ceranae* which infects the adults of the eastern honey bee (*A.cerana*) in Beijing, China. In another report, Higes et al. (2006) noted that *N. ceranae* has infected *A. mellifera* in Europe. Other studies have reported *N. ceranae* from Asia and the Americas (Chauzat et al., 2007; Chen et al., 2008; Cox-Foster et al., 2007; Huang et al., 2007; Invernizzi et al., 2009; Teixeira et al., 2013; Emsen et al., 2016; Williams et al., 2008).

Since it is difficult to distinguish various *Nosema* species, various epidemiological and molecular investigations are conducted in order to identify the type of the species (Razmaraii et al., 2013). PCR is the most appropriate method used to detecting this microsporidian infection, since it can detect even the lowest levels of infection and it is also able to distinguish various stages of microsporidian life cycle (Aroee et al., 2017). The difference of *N. apis* and *N. ceranae* is related to their small subunit (16S) rRNA gene sequence (Fries et al., 2006), and this difference helps the researchers distinguish them (Paxton, 2010).

Due to evidence implicates the high colony losses or Colony Collapse Disorder (CCD) imposed by *N. ceranae* in the US and Europe, the occurrence of *N. ceranae* in *A. mellifera* is a one of the main points of interests of researchers working on honeybees all around the word (Chaimanee et al., 2010; Higes et al., 2008; Martín-Hernández et al., 2007; Prodanović et al., 2019).Rapid loss of adult bees from the colony is one of the main symptoms of CCD. There are no dead bees near or inside the colony. The final stages of CCD show that the queen is with a few recently grown up bees. The colonies which are collapsed often have some capped brood and food reserves. The first instance of CCD dates back to 2006, but there some initial reports regarding CCD in 2004. Some researchers hypothesize that CCD is the result of some unidentifiable infections (Coxfoster et al., 2007). In fact, the risk of colony depopulation is six times higher in colonies infected with *N. ceranae* than in uninfected ones (Martín-Hernández et al., 2007).

Previous studies used molecular techniques to investigate the presence of *N.apis* and *N. cerenae* in Iran (Aroee et al., 2017; Nabian et al., 2011; Razmaraii et al., 2013). The study by Nabian et al. (2011) was the first detection of *N. ceranae* in Iran. Previous reports regarding *Nosema* in Iran considered *N. apis* the only specie of *Nosema* which infected the honeybees (Lotfi et al., 2009; Razmaraii and Karimi, 2010; Tavassoli et al., 2009).The objective of this study was to identify *N. apis* and *N. ceranae* using light microscope examination and multiplex PCR in honey bee of Urmia, northwest of Iran.

MATERIALS AND METHODS

Study area

This investigation was done during spring and summer (March-July) of 2017 in the colonies of Urmia, West Azerbaijan province in Northwest of Iran. The study area is in a fertile agricultural region between 37º 32′ N and 45º 04′ E, with an area of 8000 km^2. The amount of rainfall in the area is 73.1 mm and the average relative humidity is 77% during different months of the year. The average temperature of the area varies from -3.8° C to +23.4° C in different seasons. The location is a four-seasoned area: cold (from January to March), spring (from March to June), summer (from July to September), and autumn (from October to December). The area shares border with Iraq and Turkey (Yakhchali and Hosseine, 2006) (Figure 1).The sampled apiaries were all the year in the same location.There was sufficient pollen and nectar in the studied area and the sampling beehives give a production of honey.

Collection of honeybee samples

This study was done in 840 colonies living in 120 apiaries in five different locations of Urmia. Samplings were done in accordance with the guidelines of the Office International des Epizooties (OIE, 2008). At least 60 adult forager bees were obtained from each of the colonies. Each sample was comprised of bees from the same hive. Three to six hives were sampled per apiary/location. Before the initiation of the sampling in each apiaries studies, for each sampled colony it was recorded if the characteristics of CCD (sudden disappearance of adult bees prior to colony death, unattended brood, colony weakness, no dead or trembling bees around the hives) were observed at the time of sampling and in the years afterwards (Stevanovic et al., 2010). Other data such as degree of infestation/apiary, in relation to bee losses, beekeeping manipulations or use of medicines for other diseases, such as *Varroa* and other symptoms like aggression, swarming were recorded for each sampled colony. The samples were immediately transferred to the parasitology laboratory of Urmia University.

Preparation of samples and microscopic assessment

The abdominal contents of twenty adult bees from the same colony were crushed in 4 ml of distilled water. The resulting solution was filtered via a two-layer muslin and then it was centrifuged for 20 minutes at 1000 g in order to remove the supernatant. precipitateof the separated spores were suspended again in 1.5 ml of distilled water and then it was transferred to a fresh tube (TUNCA et al., 2016). One drop of the sample was put under the microscope slide and it was examined for the spores of *Nosema* spp. under the magnification of 400X. Haemocytometer was used to count the number of spores (TUNCA et al., 2016). A software named Image Focus (V 2.0.0.0) was used to analyze spore size.

DNA isolation and PCR amplification

PCR of *N. apis* and *N. ceranae* was conducted on 16S rRNA locus. To this end, genomic DNA was extracted by a commercial kit (MBST, Iran), based on the manufacturer's instructions and isolated DNA was analyzed in order to confirm the *Nosema* species of the spores by Multiplex PCR as previously described using *N. apis* (321 Apis (FOR5'-GGGGGC ATG TCT TTG ACG TAC TATGTA 3', 321APIS REV 5'-GGG GGG CGT TTAAAA TGT GAA ACA ACT ATG-3') and 218 MITOC for *N. ceranae* (FOR 5'-CGGCGA CGA TGT GAT ATG AAA ATATTA A-3') 218MITOC: (REV 5'-CCC GGT CAT TCTCAA ACA AAA AAC CG-3') (Nabian et al. 2011).

It should be noted that multiplex PCR reaction was done in 20 µl of a solution with 10 ng of the template DNA, 10XPCR buffer (Fermentas, Germany), 2.5 mM $MgCl_2$, 0.4 µM of each of the primers, 0.3 mM dNTP mixture and 0.5U of *Taq* DNA polymerase (Fermentas, Germany) and high pure H_2O. The conditions for cycling was as follows: 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min with a final extension step of 72°C for 5 min. 1.5% agarose gel electrophoresis along with ethidium bromide staining and photography were used to investigate PCR products (Nabian et al., 2011). Positive controls were obtained from Tehran University and distilled water was chosen as the negative control for PCR amplification process.

DNA Sequencing

Two positive PCR products resulting from 16SrRNA gene were purified and subjected to sequencing with both forward and reverse primers by Sinaclon Co. (Tehran, Iran). Mega software (ver. 6) was used for the analysis of the sequencing results. The software was also used for comparison of the sequence with the reference sequence existing in the GenBank database which was the result of the BLAST analysis (refer to www.ncbi.nlm.nih.gov). Clustal O program was used to compare the partial nucleotide sequences with available sequences of *Nosema* species in other parts of the world (https://www.ebi.ac.uk/Tools/msa/clustalo).

Statistical Analysis

All data were analyzed by SPSS software, version 26.0 (SPSS Inc, Chicago, IL, USA). The prevalence of *Nosema* between colonies with CCD signs and colonies without CCD signs (or with and without disease) was analyzed by Chi-square. Difference were considered significant when $P < 0.05$.

RESULTS

The microscopic findings

Positive samples of the *Nosema* were found in all the five locations studied during the microscopic examination. The results of this diagnosis are shown in Table 1. *Nosema* spp. spores were found in 32.02% (269/840) hives of five location samples. The observations with light microscopy delineated that the

fresh spores of the *Nosema* were in the shape of a rod or had the oval shape and the average length of them were 4.2 ± 0.31 μm (mean ± SD) and the width were 2.1 ± 0.14 μm (mean ± SD).

The molecular findings

Molecular diagnosis of the honey bee samples were investigated using multiplex PCR for 16S rRNA gene of *Nosema* species. The PCR analysis showed that 488 of 840 beehives (58.09%) were positive for *N. ceranae* (Figure2), however, there were no positive apiaries for *N. apis*.

In this study, we used 16S rRNA gene to detect *Nosema* species successfully. The sequences of 16S rRNA gene were used to investigate diagnosis, and the PCR products were sequenced (Sinaclon Co., Tehran, Iran) and submitted to GenBank/NCBI (accession numbers MT001887, MT001893). The obtained sequences were compared with those of related species received from the GenBank and the diagnosis of *N. ceranae* were confirmed. The nucleotide sequences of the amplification products from the *Nosema* positive honeybee samples were 100% identical with the *N. ceranae* sequence deposited in the GenBank database. *N. ceranae* was reported in all the samples obtained from various locations of the study area.

The findings of Colony Collapse Disorder

The results of this showed that CCD prevalence in the studied honeybee colonies were 13.33% (112/840). The percentage of infected honeybee colonies detected by PCR which had CCD signs were 99/112 (20.28%). This study showed that there is a significant relation between *Nosema* infection and CCD ($p<0.05$) (Table 2).

DISCUSSION

The aim of performing this study was to determination prevalence of species of *Nosema* using microscopic examination and multiplex PCR based on small subunit rRNA (16SrRNA) and screening for significance of its association with CCD in apiaries of Urmia.

Previous studies used molecular techniques to investigate the presence of *N.apis* and *N. cerenae* in Iran (Aroee et al., 2017; Nabian et al., 2011; Razmaraii et al., 2013). The study by Nabian et al. (2011) was the first detection of *N. ceranae* in Iran. Previous reports regarding *Nosema* in Iran (Lotfi et al., 2009; Razmaraii and Karimi, 2010; Tavassoli et al., 2009) considered *N. apis* the only specie of *Nosema* which infected the honeybees. The replacement of *N. ceranae* with *N. apis* has been reported by various scientists all over the world (Martín-Hernández et al., 2007; Paxton, 2010). The difference in these two microsporidians is related to the speed by which *N. ceranae* causes colony death. *N. ceranae* weaken the colony and leads to its death. Furthermore, *N. ceranae* has more pathogenic features compared to *N. apis* and this leads to its expansion in various parts of the world (Paxton, 2010; Paxton et al., 2007; Whitaker et al., 2011).

The current study showed that *N. ceranae* is the only *Nosema* species found in northwest of Iran and no instance of *N. apis* have been found using molecular method from collected samples in years 2016 and 2017. The results are in line with that of Razmaraiiet al. (2013) and Nabian et al. (2011) which reported the species of *Nosema* in some other regions of Iran. The existence of different species is related to the pathogen features, because *N. ceranae* is highly pathogenic compared to *N. apis*. There is another potential reason for higher rate of *N. ceranae* infection. Since *N. ceranae* is mostly asymptomatic, many of the beekeepers do not pay enough attention to it and the infection rate increases in different colonies (TUNCA et al., 2016). The results of the study also suggest the colonization process of *N.ceranae* in this area and show that *N. cerenae* is not limited to its original host and has jumped from *A.ceranato A. mellifera* and hence it has been expanded in the area studied (Martín-Hernández et al., 2007). The same results have been reported in other regions of the world by various researchers (Chen et al., 2008; Klee et al., 2007; Razmaraii and Karimi, 2010).

The analysis with light microscopy revealed that *N. cerenae* spores which are fresh have oval shape and they are rather uniform with respective length and width of 4.2 ± 0.31 μm and 2.1 ± 0.14 μm (mean ± SD). Studies conducted by Fries et al. (Fries et al., 2006; Fries et al., 1996), Higes et al. (2007) and Chen et al. (2009) have investigated the developmental and morphological qualities of *N. ceranae*. The study conducted by Chen et al. (2009) showed that *N. ceranae* spores are approximately 2.2-4.4 mm on fresh smears. The result of our study is in line with that of the Chen et al. (2009).

It should be noted that the prevalence of *Nosema*. spp. was 32% in microscopic examination, while it was measured as 58.2% in PCR. The potential reason

for this higher difference was due to high specificity and sensitivity of PCR amplification for detection and quantification of *Nosema*. spp (Chen et al., 2009). The prevalence of *Nosema* found in this research was lower than that in other previous studies in Iran. In a study conducted by Razmaraii et al. (2013) in East-Azerbaijan province or Iran, the prevalence of infection by PCR and microscopic examination was 67.1% and 58.1%, respectively.

In this study, *N. ceranae* isolate from Urmia region (Acc. No. MT001887 and MT001893) was showing maximum similarity (100%) with the sequence of *N. ceranae* isolate from Argentina (Acc. No. KX024757). Furthermore, in our study, the sequenced amplicons of *N. ceranae* (Acc. No. MT001887 and MT001893) were found to be 99.5% identical with the *N. ceranae* sequences previously published in Lithuania (Acc. No. JQ639308), Italy (Acc. No. HM859896) and North of Iran (Acc. No. JF431546) (Figure3). This was the first study of the type conducted in Urmia, northwest of Iran.

There is no definite answer to whether *N. ceranae* causes CCD per se or in combination with other factors like loss of the habitat or scarcity of floral resources (Paxton, 2010). Higes et al. (2008) were the first to note that *N. ceranae* can lead to collapse of bee colonies and they established a relationship between *N. ceranae* infection and death of honeybees. The weakness of the colony is mostly hidden until the queen loses its ability to replace the loss of the infected bees. Since the incubation period is asymptomatic, there are no signs before the collapse of the colony. Results of Three publications on Spanish honey bee colonies show that the Spanish honeybee colonies suffering from *N. ceranae* collapse in time period of 18 months after being infected (Higes et al., 2008, 2009; Martín-Hernández et al., 2007).

Our study shows that the prevalence of CCD in the studies area was 13.33% (112/840). Studies show that the occurrence of colony depopulation is different in various regions and this is indicated by different clinical and epidemiological patterns in Iran (Mohammadian et al., 2019).The study conducted by Mohammadian et al. (2019) showed that the occurrence of colony collapse in apiaries in various regions of Iran was 26.8%.

In our study, 99 out of 112 colonies affected with CCD were positive for *N. ceranae* (88.39%). Cox-Foster et al. (2007) were also unable to decide about the possibility of *N. ceranae* causing CCD alone or in combination with other factors. The results of our study does not agree with the results of the study conducted by Mohammadian et al. (2019) who believe that *N. ceranea* cannot be the cause of CCD. They concluded that presence or absence of *N. ceranae* does not affect the colony collapse.

CONCLUSION

Based on molecular data, our studies confirm that the microsporidia isolated from Urmia samples of honeybees are *N. ceranae*.The information obtained from this study and other similar studies help us improve management practices and they are also helpful in reducing the production cost. These results are also useful for beekeepers and forces them to use new hygiene policies to create an infection-free environment for bees.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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