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Molecular Identification of *Nosema apis* and *Nosema ceranae* in Europian Honey Bee (*Apis mellifera*) in Northwestern of Iran

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Abstract: There is not enough information about the nosomiasis infection in Iran and also in other parts of the world. Moreover, the distinct genotype of each area in not fully determined, so, this study was aimed to detect microsporidians (*N. apis* and *N. ceranae*) of honeybee by and genotyping of the parasite using PCR-RFLP technique. During June to December, 2016, 180 adult honeybee samples were gathered from different parts of Northwest areas of Iran and nosomiasis was evaluated and genotyping by microscopic and PCR-RFLP technique and the results were confirmed by sequencing. About 75 samples out of 180 samples were microscopically positive for nosema spores. From 75 microscopically positive samples, 42 (56%) samples were positive by PCR amplifications and all were diagnosed as *N. ceranae*. Our results revealed that the *N. ceranae* opposite to the before believes is the main strain of Nosema in Northwest of Iran but future works with the larger sample size is recommended.

INTRODUCTION

The colonies of honey bee are in facing by numerous dangers. Microorganisms and especially, microsporidia are the main parasites of invertebrates[1]. *Nosema* spp. are the main microsporidian parasites for honey bees which decrease both the honey production and the residents of bee's colonies[2, 3]. European honey bee are the main species in northwest of Iran, that might be infected by species, *Nosema apis* (*N. apis*) and *Nosema ceranae* (*N. ceranae*) parasites[3, 4]. *N. apis* has a widely geographically distribution and have different degrees of pathogenicity in comparison to *N.ceranae*[5]. During last decades, *N. apis* was the only specie of nosema of European honey bee[6] but now, the rate of *N. ceranaeis* increasing[1, 7, 8]. *N. ceranae* is more pathogen than *N. apis* and is closely accompanying with honey reduction and increase mortality in cold seasons[7]. The spores of the *Nosema* species are morphologically identical but have genetic and functional differences and are not distinguishable by routine microscopic examinations[4]. So, it's necessary to practice a more sensitive procedure

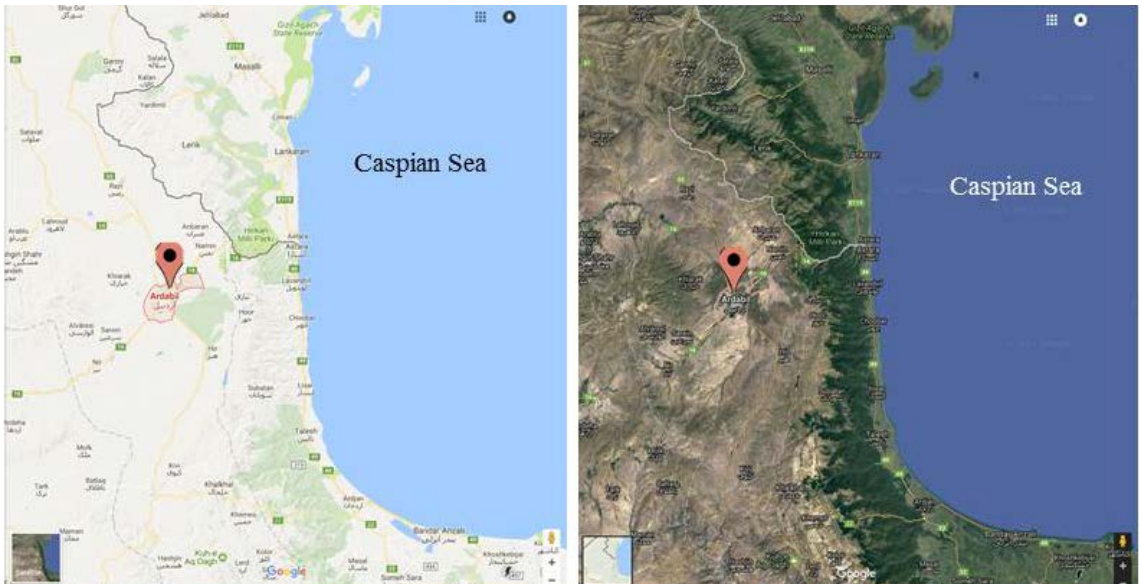

Fig. 1: Map indicating samples location in Northwest of Iran

Table 1: Primer name, sequence and size of PCR-amplified product for conventional PCR

Primer	Sequences	Size of PCR-amplified product (bp)
N.apis–Forward	5-GGGGGCATGTCTTTGACGTACTATGTA-3	312
N.apis-Reverse	5-GGGGGGCGTTTAAAATGGAAACAACTATG-3	
N. ceranae- Forward	5-CGGCGACGATGTGATATGAAAATATTAA-3	219
N. ceranae- Reverse	5-CCCGGTCATTCTCAAACAAAAAACCG-3	

with acceptable specificity. Molecular techniques are sensitive enough to distinguish the species of *Nosema* and could detect the very low copies of a parasite and moreover, can disclose all the steps of its life cycle[9, 10]. Geographical, climatic and natural situations are the vital condition for beekeeping and Northwest of Iran is completely suitable environment. This study was aimed to detect microsporidians (*N. apis* and *N. ceranae*) of honeybee by using PCR-RFLP technique (Fig. 1 and Table 1).

MATERIALS AND METHODS

Sample collection: During June to December 2016, 118 adult honeybee samples were gathered from Meshkin Shahr, Khalkhal, Garmi, Namin, Nayer and Ardabil cities, Northwest of Iran (Fig. 1). Sampling were done according the Office International des Epizooties (OIE) guidelines[11]. Species of the bee were identified by morphological characterations. Abdomen contains were collected, a direct smear was prepared for each sample and retain samples were stored in 99% ethanol and at -20°C.

DNA extraction: Total DNA was isolated from abdomen of bees by QIAamp DNA tissue mini kit (Qiagen, Germany) according to manufacturer instructions. The purified DNA was kept at -20°C until use for PCR amplification.

PCR amplification: Conventional PCR was performed to amplify 16 SSU rDNA locus from nosema species. Table 1 showed the specific primers to differentiate *N. apis* from *N. ceranae*.

PCR reactions were carried out using Little Genius the rmocycler (BIOER Technology Co., Ltd)., samples was heated to 95°C for 5 min, followed by 35 cycles of 94°C for 60 sec (denaturing), 62°C for 30 sec (annealing), 62°C for 30 sec (extension) and a final extension at 72°C for 10 min. PCR products of 219 bp for *N. ceranae* and 312 bp for *N. apis* were runned in electrophoresis in 1.5% agarose gels at 100 V for 35 min and cleared in a UV transilluminator after SYBR Safe staining (Invitrogen, USA). Amplicons size was determined using Gene-ruler ™ 50 bp Ladder Plus (Fermentas).

RESULTS AND DISCUSSION

A total of 180 samples were collected and 75 samples were microscopically positive for nosema spores (Table 1). From 75 microscopically positive

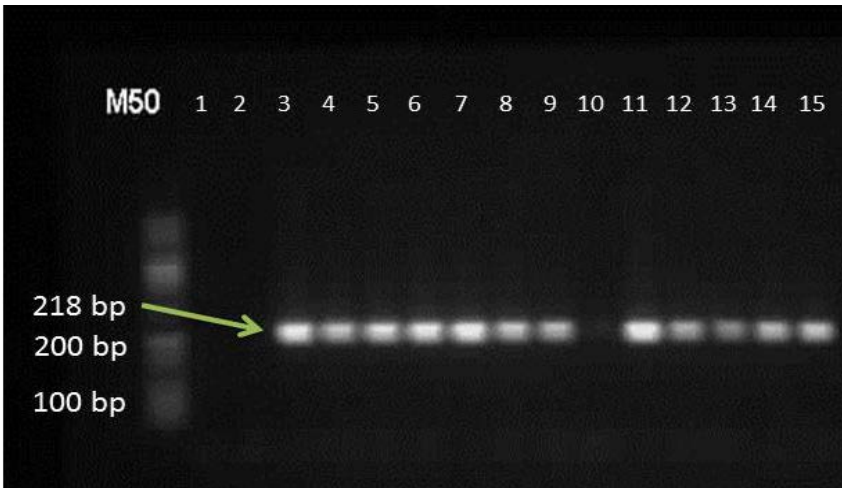

Fig. 2: Electrophoresis showing the results of Conventional PCR reaction. Line 1 and 15 are negative and positive control, respectively; Line 2 and 10 are negative (positive in microscopic methods); Line 3-9, 11-14 correspond to *N. ceranae*; M-Molecular marker 50 bp

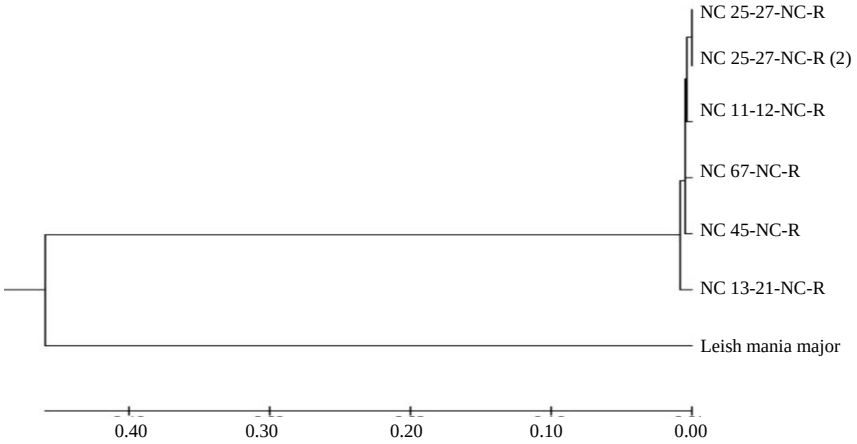

Fig. 3: Designing of phylogenic tree on 16 SSU rDNA locus from Nosemaspecies using Tamura3, Neighbor-Joining(NJ) by use of MEGA5 soft ware

Table 2: Differentiation of *Nosema* species in European honeybee in Northwest of Iran

Olecular results	Positive (%)	Negative (%)	Total
Nosema apis	0 (0)	75 (100)	75
N. ceranae	42 (56)	33 (44)	75

samples, 42(56%) samples were positive by PCR amplifications and all were diagnosed as *N. ceranae* (Table 2 and Fig. 2 and 3).

Because of the different degree of pathogenesis of *Nosema* spp. genotyping and determination of the main strain of each area is crucially important[12]. All species in current research are diagnosed as *N. ceranae*. In an experimental study conducted by Higes *et al*.[6] infections with *N. ceranae* cause mortality of 94.1% in laboratory model and had killed all infected bees after 8 days of infection. In a similar work, Paxton *et al*.[13] indicated a higher virulence in *N. ceranae* compared to *N. apis*. In 1909 for first time *N. apis* was isolated from *A. mellifera* (European honey bee) by Zander and after 87 years, Fries and his colleagues report *N. ceranae* in Asian honey bee (*Apis cerana*). But afterwards, revealed cross-infectivity in both parasites to host species in various reports[6]. Previous reports of nosemosis in Iran, supposed that *N. apis* is the only species that infected honey bees[10]. Recently, Nabian *et al*.[14] , reported *Nosema ceranae* in European honeybees (*Apis mellifera*) in Northern Iran. Northwest of Iran is an important site for beekeeping in middle East because of its environments, climate and geographical conditions[14]. Now a days the prevalence of *Nosema* and

its pathogenicity is increased in these areas and these phenomena caught the attention of beekeepers and researchers to genotyping the *Nosema*. Lotfi *et al*.[8] evaluate the prevalence of Nosemosis infection in honey bee colonies of Arasbaran Region (Northwest of Iran) and showed that 59.5% of honey bee were infected by *Nosema* spp.[8]. Based on our investigations, *N. ceranae* is a new species in Iran because in the former studies, *Nosemosis* infections were undoubtedly reported as *N. apis* only by light microscopic examinations. For instance, Branch and Marand[15] evaluated the prevalence of Nosemosis in 17 districts of East Azerbaijan (Northwest of Iran) and reported that 35.4, 59.8 and 44.5% of honeybees were infected by *N. apis* in April, May and June, respectively.

CONCLUSION

This study is conducted for first time that practice modern molecular methods to genotyping of the *Nosema* in Northwest of Iran. Our results recommended that it's crucially important to genotyping of *Nosema* species in different parts of Iran. Also, more research on the biology and transmission of *N. ceranae* are needed.

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