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PCR-based detection of *Acarapis* mites in Iranian honey bee *Apis mellifera meda* (Hymenoptera: Apidae)

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Abstract

Acarapis mites are one of the world's most important honey bee pests. A polymerase chain reaction (PCR) was used to amplify the mitochondrial cytochrome oxidase I gene to investigate the infestation of Iranian honey bee *Apis mellifera meda* populations with *Acarapis* species. From 2020 to 2022, bee workers were collected from apiaries across nine country provinces. The PCR assays were performed with four primer sets, and *Acarapis*-positive samples were sequenced to identify species. *Acarapis woodi* showed the highest infection rate, while *A. dorsalis* was less, and no infection with *A. externus* was observed. Different sets of primers were used in this investigation. Two primer sets showed different results: the prevalence of *A. woodi* ranged from 58.33 to 75% with primer set ACR2 and 25 to 50% with primer set ACR1. This is the first report on the existence of *Acarapis* mites in Iranian *A. mellifera* based on molecular techniques.

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Introduction

There are three species of *Acarapis* mites: *A. woodi* (Rennie, 1921), *A. externus* Morgenthaler, 1931 and *A. dorsalis* Morgenthaler, 1931 (Delfinado-Baker & Baker, 1982). These mites are known to parasitize specific locations on bees. *A. externus* is mainly found on the external surface of the head and thorax of bees. A. dorsalis is mostly found on the thorax especially in a groove between the mesoscutum and mesoscutellum of bees. Unlike these two species, *A. woodi* is an internal parasite that lives in the abdominal and thoracic air sacs of bees (Sammataro et al., 2000). All three species are prevalent all over many countries including UK, USA, Korea, Iran, Turkey, China, and Japan (Ahn et al., 2015).

Despite the death of colonies highly infested with *A. externus*, only *A. woodi* is considered to be of economic importance (Ibay, 1989; De Guzman et al., 2001). The most critical damage inflicted by tracheal mites on the honeybee colonies is colony death during winter (McMullan & Brown, 2009; Maeda, 2016). The traditional diagnostic methods for separating these species are very time consuming and they are based on direct visualization of *A. woodi* or its lesions in the tracheas. Other methods as enzyme-linked immunosorbent assays (Fichter, 1988; Grant et al., 1993; Ragsdale & Furgala, 1987; Ragsdale & Kjer, 1989) or methods based on the visualization of guanine under ultraviolet light, the main end product of nitrogen metabolism in mites (Mozes-Koch & Gerson, 1997), are little used in routine diagnostic. Nowadays, the preferred approach to detect *Acarapis* mites is PCR amplification of the mitochondrial cytochrome oxidase I (COI) subunit using different primer pairs (Evans et al., 2007; Kojima et al., 2011a, b; Garrido-Bailón et al., 2012; Ahn et al., 2015; Cepero et al., 1991). However, the limitation of this method is that most of the available primer sets lack specificity and may amplify any *Acarapis* mite found in honey bees (Evans et al., 2007;

Kojima et al., 2011a; Garrido-Bailón et al., 2012), therefore additional sequencing to avoid false positive identification and incorrect assessment of the real prevalence of *A. woodi* is required. Presence of *Acarapis* mites in 21 provinces of Iran from which *A. woodi* was found in 7 of the 21 provinces surveyed and *A. externus* and *A. dorsalis* were found in eight provinces (Mossadegh & Bahreini, 1994). In previous investigations: the average prevalence rate of Acariasis in the region in total population was 0.8, the highest prevalence was in Ardebil (2.3%), the lowest was in Fars (0.3%) and also negative total samples from West Azarbaijan, Isfahan, Lorestan, Kerman, and Yazd (Mossadegh & Bahreini, 1994). The infestation level and prevalence of bee colonies with *A. woodi* in the Alborz Province (Savojbolagh country) was 13% (Vaziritabar & Esmaeilzade, 2016). The prevalence of *A. woodi* infestation in apiaries of Kurdistan Province was 1%. Furthermore, *A. woodi* infestation rate of hives was 0.26% (Khezri & Moharami, 2017) based on old methods. The aim of this study is detecting *Acarapis* mites infection based on molecular technique.

Material and Methods

Sample collection

The study was carried out between 2020 to 2022. Sampling was carried out from Iranian apiaries including Kerman, Yazd, Fars, Hormozgan, Sistan-o-Baluchistan, Isfahan, Razavi Khorasan, Mazandaran, and East Azarbaijan Provinces. A total of 136 *Apis mellifera* colonies were sampled from 34 apiaries in 8 provinces. 30 adult bees were collected and pooled from 1-2 colonies in each apiary between July and November 2020. After collecting the bees, the samples were stored at -20˚C for DNA extraction.

DNA extraction and Polymerase Chain Reaction

Total DNA was extracted from the head and thorax of 24 adult bees (216 bees in total) using method described by Aljanabi & Martinez (1997) with some modifications. After quantification and qualification of isolated genomic DNA, four primer sets were used to identify *Acarapis* mite (Table 1) which were capable of amplifying mitochondrial cytochrome oxidase subunit I (COI) DNA fragments of *Acarapis* mites. The primer sets ACR1 and ACR2 are specific to *A. woodi* CO1 sequence and the primer sets KOJ and ACA amplify COI genes of three species of *Acarapis* mites. The PCR reactions were carried out in a total volume of 25 µl including 12.5 µl of PCR master mix Ampliqon Master Mix Red (Ampliqon, Copenhagen, Denmark), 1 µl of each 10 pM primers, 7.5-9.5 µl distilled water and finally 2-4 µl of the DNA template.

For the first two primers in the table, PCRs were performed using this program: initial denaturation at 94°C for 3 min, followed by cycling stages of denaturation at 94°C for 1 min, annealing at 47°C (ACR1) and 50°C (ACR2) for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. For primer ACA, PCR was performed using this program: initial denaturation at 95°C for 10 min, followed by cycling stages of denaturation at 95°C for 30 Sec, annealing at 56°C for 30 Sec, extension at 72°C for 30 sec and final extension at 72°C for 30 sec. For primer KOJ, PCR was performed using this program: initial denaturation at 94°C for 2 min, followed by cycling stages of denaturation at 94°C for 30 Sec, annealing at 55°C for 30 Sec, extension at 72°C for 30 sec and final extension at 72°C for 2 min. The PCR products were evaluated by 2% agarose gel electrophoresis.

Table 1. List of primers and their nucleotide sequences.

Primer name	Primer sequences (5'-3')	Amplicon Length (bp)	Annealing temperature (°C)	Reference
ACR1	F-TCTTCAATTTTAATTATACGT R1-CAAAAATCAGAATAAATGTTGAAATA	220	47	Kojima et al. (2011b)
ACR2	F-TCTTCAATTTTAATTATACGT R2-GTAATATTGTAATAGCACCTGCTAATACTGGTAAA	135	50	Kojima et al. (2011b)
KOJ	F- CAGTAGGGCTAGATATCGATACCCGAGCTT R- TGAGCTACAACATAATATCTGTCATGAAGA	247	55	Kojima et al. (2011a)
ACA	F- CGGGCCCGAGCTTATTTTACTGCTG R- GCGCCTGTCAATCCACCTACAGAAA	162	56	Garrido-Bailón et al. (2012)

Sequencing and phylogenetic analysis

All PCR products were purified using MG Gel extraction SV kit (Macrogen, Korea) and direct Sanger sequenced for both directions by Macrogen Inc. (South Korea). Obtained sequences were compared to sequences in the GenBank using the BLAST N software. The phylogenetic tree was generated by the

neighbor-joining method based on Nei-Gojobori model (Jukes-Cantor) (Nei & Gojobori, 1986) using software MEGA X 10.0.4 (Kumar et al., 2018). Bootstrap support was calculated using 3000 replicates. Rhinonyssus rhinolethrum (EU889353) was used as an outgroup in the phylogenetic analysis. Genetic distances between Acarapis sequences in GenBank calculated by MEGA 10.0.4 software using the Nei-Gojobori method with Jukes-Cantor.

Results

Quantification of isolated genomic DNA and PCR optimization

The purity and quantity of each DNA sample was determined using a Nanodrop ONE C (Thermo scientific). The integrity of genomic DNA was also determined by running the samples on 1% agarose gel. In temperature gradient PCR tests, the best amplification was obtained with an annealing temperature of 47, 50, 55 and 56°C. The amplified fragments of the isolated DNA of *A. mellifera* show a successful amplification at the correct size (220, 135, 247and 162 bp) (Fig. 1) and sequenced.

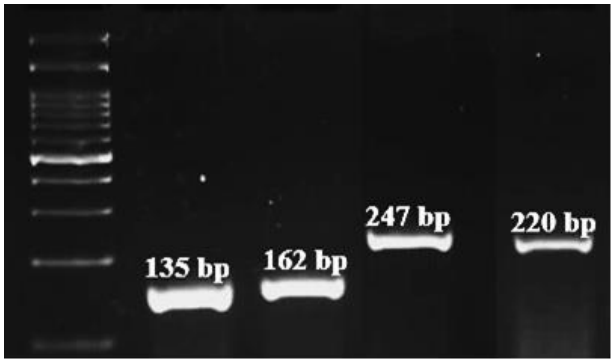

Fig. 1. PCR-amplified products obtained with four primer sets. M: 100 bp DNA ladder.

Prevalence of *Acarapis* mite's infestations

All provinces with PCR test showed mite contamination with four sets of primers. *A. woodi* were detected in all surveyed provinces (Kerman, Yazd, Fars, Hormozgan, Sistan-o-Baluchistan, Isfahan, Razavi Khorasan, Mazandaran, and East Azerbaijan). *A. dorsalis* found in East Azerbaijan, Razavi Khorasan and Mazandaran. Among the species, *A. woodi* exhibited the highest infection rate, A. dorsalis showed a lower rate, and no infections by A. externus were detected. Since the ACR1 and ACR2 primer sets specifically identify *A. woodi*, the prevalence rate of this species was calculated according to the number of positive PCR samples in each province. The prevalence percentage of *A. woodi* achieved 70.83 in Kerman, 58.33 in Yazd, 66.66 in Fars, 62.5 in Hamedan, 66.66 in Sistan-o-Baluchistan, 58.33 in Isfahan, 62.5 in Razavi Khorasan, 75 in Mazandaran, 62.5 in East Azerbaijan with primer set ACR2 vs 25 in Kerman, 41.66 in Yazd, 25 in Fars and also in Hormozgan, Sistan-o-Baluchistan (25), 50 in Isfahan, 29.16 in Razavi Khorasan, 33.33 in Mazandaran, 37.5 in East Azerbaijan with primer set ACR1. Our results indicated that the ACA2 primer set has better performance than the ACR1 primer set.

DNA sequencing and Phylogenetic analysis

Acarapis-positive samples were sequenced and aligned. After alignment and trimming, the final length of the COI alignment for *A. woodi* was 220 and 135 bp, for A. dorsalis was 245 and 163 bp. These sequences have been deposited in GenBank for *A. woodi* with accession numbers MH919383 (Kerman), MW390964 (East Azerbaijan), MW390963 (Isfahan), MW390954 (Razavi Khorasan) and MW390962 (Mazandaran) and for A. dorsalis with accession numbers MW283291 (Razavi Khorasan), MW066758 (East Azarbaijan), OQ361650 (Mazandaran) and OQ361844 (Razavi Khorasan). Since the GenBank does not record sequences less than 150 bp, sequences of 135 bp couldn't be recorded.

The nucleotide sequence of COI of *A. woodi* with accession number MW390963, MW390954, MW390964 and MH919383 was most similar to sequences from AB634837, HQ162656, LC512730, KX790788 with identity of 99.09%; Accession number MW390962, showed 98.64% identity with AB634837, HQ162656 and 98.63% identity with LC512730, KX790788.

The COI sequence of A. dorsalis with accession number MW283291 showed 98.77% identity with HQ243439, HQ243434, HQ243433 and 98.16% identity with GQ916567; accession number MW066758 and OQ361844 showed 97.55% identity with HQ243439, HQ243434, HQ243433 and 97.14% identity with GQ916567; accession number OQ361650 showed 97.14% identity with HQ243439, HQ243434, HQ243433 and 96.73% identity with GQ916567.

Due to the use of different primer combinations in our study, the sequences differed in length and in position on the COI region, a central part of 110 bp was chosen for subsequent analysis. Each Iranian isolate *Acarapis* species was placed on a separate branch and closely related to the same *Acarapis* species (Fig. 2).

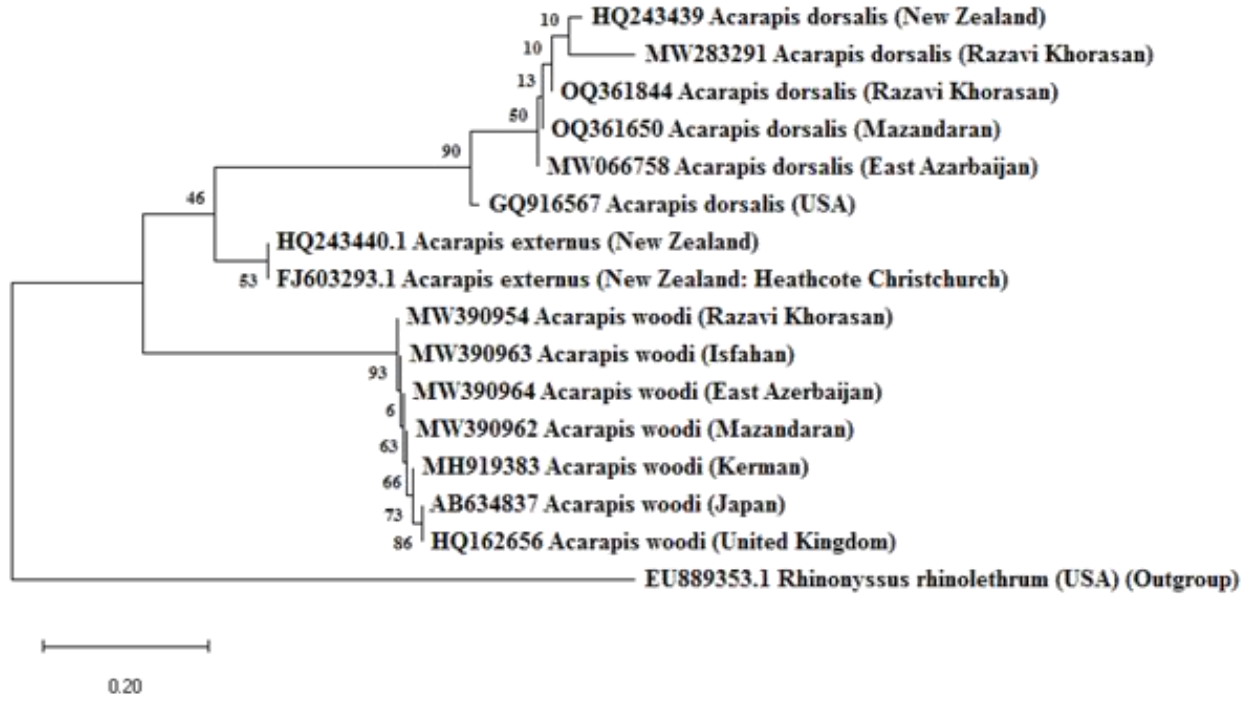

Fig. 2. Neighbor-joining tree (synonymous sites) of the nucleotide of mitochondrial cytochrome *c* oxidase subunit I (COI) DNA fragments from Iranian isolates of *Acarapis* mites and other references of *Acarapis* using Nei-Gojobori model (Jukes-Cantor). Scale bar represents number of nucleotide substitutions per site. Bootstrap was 3000 replicates. Numbers on nodes represent bootstrap values.

Discussion

This is the first extensive study of the prevalence of *Acarapis* mites in Iranian *A. mellifera* based on the molecular technique that uses a sensitive method and can be easily applied in the laboratory environment. In this study sequencing of all the amplified products confirmed the infection by *A. woodi* and *A. dorsalis*, and these products were identical to the COI sequences found in GenBank from this mite. Therefore, molecular analysis of bee DNA extracts using PCR and alignment indicate that two species of *Acarapis* mites are present in Iran from which *A. woodi* was found in nine provinces surveyed (Kerman, Yazd, Fars, Hormozgan, Sistan-o-Baluchistan, Isfahan, Razavi Khorasan, Mazandaran, East Azerbaijan) and *A. dorsalis* was found in three of the nine provinces (Razavi Khorasan, East Azerbaijan and Mazandaran), No other *A. externus* were detected in these samples. Although Mossadegh & Bahreini (1994) reported that *A. woodi* was found in 7 of the 21 provinces (Kerman, Khorasan, Isfahan, Mazandaran, Tehran, Gilan, Chaharmahal and Bakhtiari) and *A. externus* and *A. dorsalis* were found in five of the 21 provinces surveyed (Khorasan, East Azerbaijan, Chaharmahal and Bakhtiari, Khuzestan, Mazandaran) based on morphological investigations. Using four different primers, including ACR1, ACR2, KOJ, and ACA primer sets indicated that the ACA and KOJ primer sets was more sensitive than the ACR1 and ACR2 primer sets. Based on the Kojima et al. (2011b) and our results, the ACR1 and ACR2 primer set specifically identify *A. woodi*. However, each ACR1, ACR2, KOJ, and ACA primer sets require sequencing of amplicons to identify the species of the mite. Therefore, there is a need to design a primer which can amplify species-specific DNA fragments in future studies.

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Conflict of interest

The authors declare that there are no conflicts of interest present.

CRediT author statement

Z. Hajializadeh: Field and laboratory works & writing original draft preparation. **M. Asadi**: Supervision, methodology, writing, reviewing & editing. **M. Mansouri**: Supervision, methodology, writing, reviewing & editing.

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