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Application of ISSR to study the genetic diversity of honeybee (*Apis mellifera* L.) populations in some areas of Iran

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Article info:

Received: 13 December 2013

Accepted: 9 March 2014

ABSTRACT

Inter-Simple Sequence Repeat (ISSR) molecular marker for the isolation of five races of honey bee *Apis mellifera* from Khuzestan, Kurdistan, Markazi, Fars and Isfahan provinces was studied. DNA was extracted from the worker bees. The quality and quantity of extracted DNA were measured. Values obtained from the bands on agarose gel (1.5%) were scored and analyzed. Results showed that the bands are in the range between 250 and 1000 bp. Maximum number of bands were recorded for primer 1. Cluster analysis of races placed them in two main groups. In the first group were Kurdistan and Khuzestan. The second group includes Markazi, Fars and Isfahan, but this group has shown no relationship between the populations. It seems that ISSR molecular marker could separate different races of honey bee.

Key words: molecular marker, honey bee, DNA, genetic diversity, ISSR

Introduction

Honey bee (*Apis mellifera* L.) is an important agricultural insect for honey production, for the pollination of more than 90 crops and for other products in world. The natural range of *A. mellifera* extends worldwide from northern Europe to southern Africa and from the British Isles to the Ural Mountains, western Iran, and the Arabian Peninsula (Ruttner, 1988). Iran is the most populated Asian country in terms of honey bee colonies. Current statistics (FAO, 2007) reported that there are more than 3 million colonies present in Iran. To determine the status and resolution of bee populations in the region is used morphological methods, a variety of proteins and DNA fingerprinting (Ruttner *et al.*, 1978). Today, microsatellites play an important role in determining genetic diversity and kinship ties of animals, and especially insects. Usually used ISSR markers for genetic diversity of plants and in the insects world now use these markers to assess the genetic diversity of Lepidopteran orders, especially from two families *Noctuidae* and *Bombycidae*, Order Diptera and

Hymenoptera have been the focus of the scientific community (Luque *et al.*, 2002; Hundsdoerfer *et al.*, 2005; Radjabi *et al.*, 2012). Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primers used can be either unanchored (Gupta *et al.*, 1994; Meyer *et al.*, 1993; Wu *et al.*, 1994) or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994). This study explores the usefulness of Inter Simple Sequence Repeat (ISSR) molecular marker to identify and discriminate several populations of honey bee *Apis mellifera* L. from Iran. This investigation was also conduct in order to

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analyze of phylogenetic relationships and determination of relationship among honey bee population of the five provinces of Iran based on ISSR molecular marker.

Materials and Methods

Sampling

A total of 45 colonies were sampled from 15 different localities distributed in Khuzestan, Kurdistan, Markazi, Isfahan and Fars during February 2011 and May 2012 (Figure 1). Samples were collected by aspirator. The bees were immediately kept at -20°C until laboratory processing.

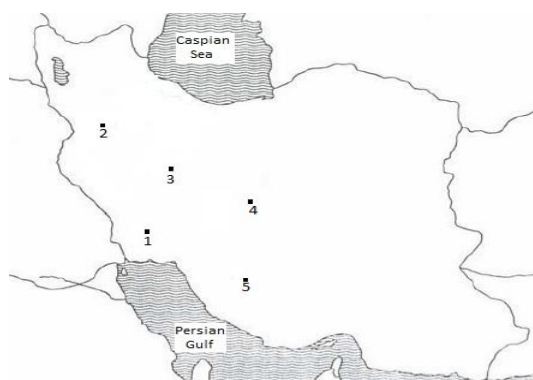


Figure 1. Sampling location of *Apis mellifera* from the Iran for the microsatellite analysis. 1-Khuzestan (Kh), 2-Kurdistan (Ku), 3-Markazi (Ma), 4-Isfahan (Is) and 5-Fars (Fa)

Molecular analysis

For genetic analysis of the honey bee races, DNA from adult body moths was isolated from 6 individual. After wings cutting, adult's body was smashed with a mechanical homogenizer in a microcentrifuge tube and suspended in DNA extraction buffer containing 100 $\mu\text{g}/\text{mL}$ proteinase K and 40 $\mu\text{g}/\text{mL}$ SDS. After digestion at 65°C for 2 h, to the samples were added 300 μl NaCl and centrifuged. The

supernatant was transferred to a new tube. Chloroform was added to the samples. After centrifugation and separation of the aqueous phase, cold absolute ethanol and 3 M sodium acetate was added to the samples for overnight at a temperature of -20°C . The DNA samples were centrifuged and washed with 70% alcohol. Then, 50 μl of distilled water was added to each sample. To ensure the quality of the extracted DNA and detected DNA fragments ethidium bromide stained 1% agarose gel was used. The quality of extracted DNA was measured by a spectrophotometer. PCR was carried out with a Thermo-Cycler using 15 μL of reaction mixture containing 1.5 μL of $10\times$ PCR buffer (15 mM MgCl_2), 1.5 μL of 2 mM dNTP, 0.6 μL of 2.0 mM MgCl_2 , 1 μL of 1.5 μM primer; 2 μL of DNA (10 $\text{ng}/\mu\text{L}$), 0.2 μL Taq DNA polymerase (5 U/ μL) and 8.2 μL double distilled water. Five suitable primers were used for PCR reaction (Table 2). The PCR schedule adopted was 1 cycle of 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 51°C for 1 min, 72°C for 2 min and a final extension of 10 min at 72°C . The PCR products were loaded on 2% agarose gel in Tris-boric acid/EDTA buffer (1X TBE) and electrophoresis was carried out with a constant voltage of 75 V for 4 h. The gel was stained with ethidium bromide (0.5 g/ml) and photographed with the gel documentation system for scoring as present (1) and absent (0) same other dominant molecular markers.

Results and Discussion

All used primers showed polymorphism in amplified loci (Table 1). These five primers produced 47 scorable bands with fragment size between 150-1000 bp (Figures 2 and 3). Twenty five loci were polymorphic and showed an average of 53.66 percent polymorphism. Primers 3 and 4 produced lowest number of loci and primer 1 produced the highest number of loci.

Table 1. Primers list, sequences and polymorphism.

Primer number	Sequence (5' _3')	Fragment size (bp)	Number of total bands	Number of polymorphic bands	Number of monomorphic bands	Percentage of polymorphism (%)
P1	(AG) ₈ T	200-1000	12	5	7	41.67
P2	(GA) ₈ T	250-800	10	6	4	60
P3	(AC) ₈ T	150-600	8	4	4	50
P4	(AC) ₈ YT	200-700	8	4	4	50
P5	(GA) ₈ A	150-800	9	6	3	66.66
Total	-	-	47	25	22	-
Mean	-	-	9.4	5	-	53.66

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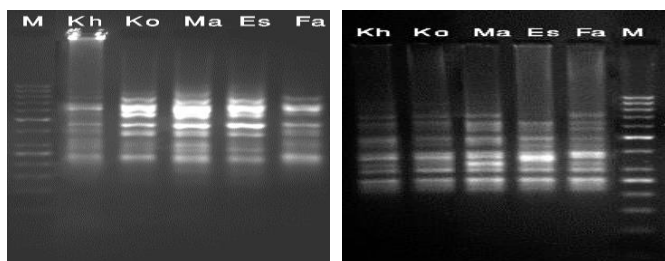


Figure 2. ISSR profile band of primer 1 (left) and primer 2 (right) on 2 % agarose gel stained by ethidium bromide, M = 50 bp Ladder

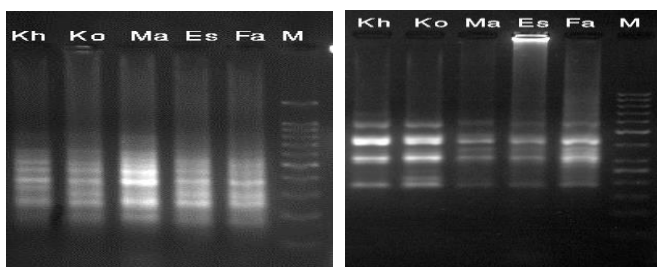


Figure 3. ISSR profile band of primer 3 (left) and primer 4 (right) on 2 % agarose gel stained by ethidium bromide, M = 50 bp Ladder

To determine the affinities and classification of genotypes based on molecular results UPGMA cluster analysis was used. Jaccard's similarity coefficient and a dendrogram was drawn. The bees were divided into two groups based on the dendrogram. The first group includes the Fars and the other group consists rest of the bees. Last group was additionally divided into two subgroups (Figure 4).

The number of bands produced in the study were between 4 and 12 bands. Compared with other studies these data showed some variations. Also, the number of amplified bands was from 2 to 10 bands (Paplauskienė et al., 2006; Al-Otaibi, 2008), 3 to 14 bands in the silkworm (Velu et al., 2008), in *Anthera mylitta* between 5 and 16 bands (Kar et al., 2005), 14 to 26 bands in flies *Simuliidae* (Dusinsky et al., 2006), 0 to 12 bands butterflies *Noctuidae* (Hundsdoerfer et al., 2005), 25 to 32 bands in *Homalodisca coagulata* (De León & Walker, 2004). Part of this difference is related to the use of different primers Dinucleotide, tri- and tetra-nucleotide and another part of the type used primers – normal or anchoring at both ends (Reddy et al., 1999). However, the results vary depending on the species studied. Tetra-nucleotide primers with sequences ACAG, GACA and ACTG showed better performance and these primers were used in a high percentage of polymorphism (Dusinsky et al., 2006). Di-

nucleotide primers in this study have produced more bands than other primers. Similar differences were seen in the results of other studies (Al-Otaibi, 2008; Paplauskienė et al., 2006).

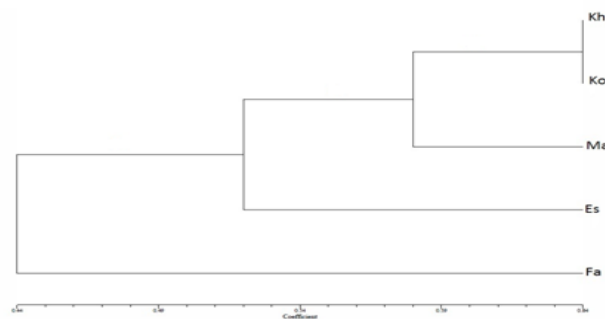


Figure 4. Cluster analysis of honey bee according to the grouping from UPGMA method for Jaccard standard genetic distance.

It has been showed that honey bee genome sequences are A+G and A+C. AC and AG sequences contain abundant and common sequences of animal groups, including insects such as fruit flies and other insects (Schug et al., 1998a, 1998b). Extending the primer (anchoring) with 1 to 4 degenerate nucleotides at the 3' end or 5' end assures annealing only to the ends of a microsatellite in template DNA thus obviating internal priming and smear formation. Secondly, the anchor allows only a subset of the microsatellites to serve as priming sites. When 5' anchored primers are used, the amplified products include the microsatellite sequences and their length variations across a genome and therefore give more number of bands and a higher degree of polymorphism (Reddy et al., 2002).

According to the results 25 of bands were polymorphic. The average for each primer was about 5 bands. These data indicate that most of the areas studied were polymorphic. Results are consistent with the observations of other researchers. Paplauskienė et al. observed fragments with range between 350-3000 and the fragments observed by Al-Otaibi were in range 100-850 (Paplauskienė et al., 2006; Al-Otaibi, 2008). Studied strains were compared by calculating the percentage of polymorphic positions, based on the results obtained in the study of polymorphism in races. For Khuzestan it was 53.33%, for Isfahan – 42.42%, for Fars – 37.14%, for Central and Kurdistan – 32.25%, Percentage of polymorphic positions in the native bees is 88.57%, bee hybrids – 51.61% and the rate for the race “*carnica*” – 34.78%, respectively (Paplauskienė et al., 2006; Al-Otaibi,

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2008). Our data showed that the ISSR technique is a suitable method for the detection of polymorphism in the honey bee, and ISSR bands in numerous iterations on various breeds are highly repeatable in comparison with other molecular techniques even in close races. Results indicated low levels of genetic similarity between races in 5 different provinces. This could be because bees across the successive migrations are adjacent to the provinces although sampling performed from bee keeper that never has moved out of the province. The bee colonies traded popular among bee keepers in every city and county together. In this study, different similarity coefficients (Jaccard, Sorenson and Dice) and simple matching lead to the conclusion that there is no difference between the coefficients of similarity in the cluster analysis. However, it has been reported that the similarity coefficient of Jaccard, Dice and Sorenson compared with other coefficients indicate better performance cluster analysis of RAPD and ISSR, especially compared to the group with distant taxa (Landry & Lapointe, 1996). Hallden *et al.* (1994) believe that the choice should be based on similarity of DNA of different taxa (Kosman & Leonard, 2005). Jaccard and Dice similarity coefficients results in better performance in general and in our studies we used the Jaccard similarity coefficient. Therefore, cluster analysis of the AFLP marker is comparable. In other words, differences in the genetic diversity of strains by ISSR and AFLP marker do not exist and given the low cost and quick work of ISSR markers are used. Although ISSR markers such as RAPD markers are dominant, the big difference and repeatability of the results are closely consistent pattern of AFLP bands and could be regarded as a suitable and effective marker, which can be used with confidence (Dalirsafat *et al.*, 2009; Radjabi *et al.*, 2012).

Interestingly, in a recent study Kurdistan and Khuzestan bees were inserted in a group, indicating the similarity of the two provinces bee populations. It is demonstrated that the two provinces heavily traded bee keepers and colonies have migrated to the province, which lead to similarities between two mentioned provinces bee populations. The geographical distance has better performance. For species in close geographic areas or similar geographical conditions cannot have a good performance and application should be considered on other aspects such as the relationship between some morphological traits or physiological characteristics.

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