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Original Research

Genetic diversity of Iranian honey bee (*Apis mellifera meda* Skorikow, 1829) populations based on ISSR markers

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Abstract: Honey bee is one of the most important insects considering its role in agriculture,ecology and economy as a whole. In this study, the genetic diversity of different Iranian honey bee populations was evaluated using inter simple sequence repeat (ISSR) markers. During May to September 2014, 108 young worker honey bees were collected from six different populations in 30 different geoclimatic locations from Golestan, Mazendaran, Guilan, West Azerbaijan, East Azerbaijan, Ardebil provinces of Iran. DNA was extracted from the worker honey bees. The quality and quantity of extracted DNA were measured. A set of ten primers were screened with the laboratory populations of honey bees. The number of fragments produced in the different honey bee populations varied from 3 to 10, varying within 150 to 1500 bp. The used ten ISSR primers generated 40 polymorphic fragments, and the average heterozygosity for each primer was 0.266. Maximum numbers of bands were recorded for primer A_1. A dendrogram based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method generated two sub-clusters. Honey bee populations of Golestan, Mazendaran, Guilan provinces were located in the first group. The second group included honey bee populations of Ardebil, West Azerbaijan, East Azerbaijan provinces, but this group showed a close relationship with other populations. The results showed obviously the ability of the ISSR marker technique to detect the genetic diversity among the honey bee populations.

Key words: Genetic diversity, Honey bee, ISSR marker, Polymorphism.

Introduction

Honey bee (*Apis mellifera* L.) is the most economically valuable pollinators of agricultural crops worldwide (1). One third of the total human diet is dependent on plants which are pollinated by insects; predominately honey bee (2). The Western honey bee, *Apis mellifera* L., is native to Africa, the Near and Middle East, and Europe. Based on morphometric analysis, Ruttner (1988) grouped honey bee subspecies into four geographic branches: a South and central African branch (A), a North African and West European branch (M), an East European and North Mediterranean branch (C), and a Near and Middle Eastern branch (O)(3). Subsequent studies via morphometry, along with mitochondrial DNA and microsatellite marker analysis, have confirmed or modified the subspecies distributions within these groups and have added a new Middle Eastern branch from Yemen (Y) (4-10). Based on morphometrics, the Near Eastern subspecies, Anatolian (*A. m. anatoliaca*), Caucasian (*A. m. caucasica*) and Iranian (*A. m. meda*), had been grouped within the O branch (3, 8, 11); however, mtDNA analysis showed that they in fact belonged to the C lineage (5, 6, 9, 10, 12-14). Ruttner (1988) using his morphometric analyses concluded that *Apis mellifera meda* all exist in Iran (3). Almost all of the geographical landmass of Iran is apparently occupied by *Apis mellifera meda*. To determine the status and resolution of honey bee populations in the region is used morphological methods, a variety of proteins and DNA fingerprinting (15). Ruttner *et al*., (1985) studied the dispersion of *Apis mellifera meda* in Iran and published its characteristics (16). These authors cited that this subspecies is native of Iran and has similarities with *Apis mellifera ligustica*, *Apis mellifera anatolica* and honey bees in the north of Iraq. A variety of molecular marker methods were used in honey bee, however PCR-based markers are more appropriate for fingerprinting studies such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) and the inter simple sequence repeat (ISSR). Nowadays, microsatellite markers play an important role in determining genetic diversity and phylogenetic relationships of animals, and especially insects (17). In plants as well as in insects the use of ISSR marker has increased in recent years. Between insects families of Noctuidae (Order Lepidoptera) and Bombycidae (Order Diptera) were used by different authors more frequently than other families of insects (18-20). Inter-simple sequence repeat (ISSR) markers were originally selected to differentiate between closely related plant cultivars but proved to be extremely useful for studies of populations of other organisms, between them, insects. The major advantage of this method is that it does not require prior knowledge of DNA sequence to design a primer. The markers are easily generated using minimal equipment and are hypervariable, yielding a reasonable amount of data for a reasonable amount of money to spend (21). Therefore, using molecular markers such as ISSR might be useful for identification of the location of target geno-

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mic region. The Northern provinces (the South coast of the Caspian Sea), West Azerbaijan, East Azerbaijan and Ardebil provinces of Iran which compose an important centre of honey production and produce the bulk amount of honey consumed in the country. This region is a well known region, to have modernized beekeeping area in Iran. North Iranian apiaries exist in the South coast of the Caspian Sea including Guilan, Mazendaran and Golestan provinces with 14711, 24000, and 8302 km^2 areas respectively, northwest Iranian honey bees exist in the northwest in the Iran including West Azerbaijan, East Azerbaijan, Ardebil provinces with 37437,17800, and 45650 km^2 areas respectively. These provinces have well defined ecological characteristics, and because of favorable environmental conditions, they are major destinations for seasonal colony displacement from other areas of the country. As these regions share the same favorable climate, so there are few displacements of bee colonies between them and it appears that these areas have their own unique groups of honey bees. Because of the fundamental role of this area in the country's honey production, we focused our study on these provinces.

The principal aim of the present study was to investigate the polymorphism and phylogenetic relationships between honey bee populations of the six provinces of Iran via the inter simple sequence repeats (ISSR) markers.

Materials and Methods

Honey bee samples

A total of 108 young worker honey bees from 54 colonies of *Apis mellifera meda* were sampled from 30 different localities distributed in Golestan, Mazendaran, Guilan, East Azerbaijan, Ardebil and West Azerbaijan provinces, north and northwest of Iran during May to September 2014.(Fig. 1 , Table 1). Samples were taken from honey bee colonies of most active apiaries, of five cities in each province, one apiary in each city (2-6 worker bees per city). Honey bees were collected directly from comb and were stored in 96% ethanol and kept at –20˚C until DNA extraction.

DNA extraction

Total DNA was extracted from the thorax and head sections of each young worker honey bee, using salting

Figure 1. The geographical locations of honey bee populations collected in this study.

Table 1. Sampling localities, geographical positions and number of honey bees used for ISSR analysis.

Locations		Geographical position			Honey bees analyzed for
Provinces	**cities**	**Latitude**	**Longitude**	**Altitude (m)**	**ISSR analysis**
	Kalaleh	37° 23′33.43 N	055° 32′00.82 E	225	2
	Galikesh	37° 17′07.30 N	055° 31′51.03 E	427	2
Golestan	Minodasht	37° 11′40.63 N	055° 23′38.21 E	150	2
	Gorgan	36° 50′47.46 N	054° 40′12.88 E	181	2
	Kurdkuy	36° 44′44.12 N	054° 10′24.55 E	268	2
	Neka	36° 34′14.44 N	053° 24′24.27 E	320	4
	Sari	36° 31′45.22 N	053° 11′46.15 E	223	4
Mazendaran	Amol	36° 25′22.19 N	052° 14′39.71 E	135	6
	Babol	36° 26′21.30 N	052° 42′20.19 E	35	2
	Chaloos	36° 38′46.72 N	052° 23′04.38 E	53	4
	Rasht	37° 14′30.22 N	040° 28′13.27 E	3	4
	Rodbar	36° 50′23.28 N	049° 25′57.83 E	329	6
Guilan	Lahijan	37° 09′23.83 N	049° 59′23.08 E	46	4
	Fouman	37° 14′02.60 N	049° 17′27.76 E	39	2
	Astara	38° 25′08.76 N	048° 48′02.79 E	84	4
	Bonab	37° 21′37.90 N	046° 05′07.08 E	1295	2
	Maragheh	37° 27′10.89 N	046° 10′09.56 E	1708	4
East Azerbaijan	Tabriz	38° 06′13.12 N	046° 11′10.99 E	1345	6
	Marand	38° 30′43.67 N	045° 43′43.04 E	1297	4
	Ahar	38° 27′19.12 N	047° 07′18.10 E	1290	4
	Bokan	36° 33′33.24 N	046° 21′35.98 E	1939	2
	Urmia	37° 34′04.14 N	044° 44′17.19 E	2138	6
West Azerbaijan	Salmas	38° 18′01.19 N	044° 31′40.64 E	2442	4
	Piranshahr	36° 41′22.54 N	045° 14′25.13 E	1900	4
	Khoy	38° 41′20.44 N	044° 59′47.94 E	1450	4
	Mashgin shahr	38°18′07.18 N	047° 36′48.22 E	2125	4
	Sarein	38° 08′19.24 N	048° 06′48.13 E	1570	4
Ardebil	Ardebil	38° 13′58.28 N	048° 10′05.22 E	1455	4
	Khalkhal	37° 39′27.87 N	048° 34′20.50 E	2212	4
	Germi	39° 01′42.00 N	048° 09′24.57 E	961	2

Table 2. Sequence and characteristics of the used ISSR primers.

Primer code	Core sequence 5'→3'	Tm (°C)
A_1	$(AGAC)_4GC$	51
A_2	$AC(GACA)_4$	51
A_3	$(GACA)_4GT$	50
A_4	$(GACA)_4CT$	53
A_5	$(ATG)_5GA$	50
A_6	$(TCC)_5GT$	54
A_7	$(CTC)_5GT$	50
A_8	$(AC)_8G$	53
A_9	$(CT)_8A$	50
A_{10}	$(AC)_8AT$	53

Tm: annealing temperature

out method described by Aljanabi and Martinez (1997) with minor modifications (22).

PCR amplifications

Polymerase chain reaction was carried out to amplify in a final volume of 25 µl containing 30 ng of DNA, 2.5 µl of PCR buffer (10X), 1 mM of $MgCl_2$, 0.4 mM of dNTPs, 1 µM primer and 1 Unit of *Taq* DNA polymerase (SinaColon, Iran). Ten primers were used for PCR reaction (Table 2). The thermocycler parameters included of an initial denaturation step at 94°C for 7 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 50-54°C for 60s, extension step at 72°C for 2min, and then a final extension at 72°C for 7 min. PCR products were electrophoresed on a 1.5 % agarose gel in 1X TBE buffer and stained with ethidium bromide. The gel image was recorded using a Gel Documentation System.

Statistical analysis

ISSR markers derived from ten primers were scored with the 54 honey bee colonies under-study. Data were scored as (1) and (0) which stands for presence and absence of PCR product in all used primers. The POPGENE software version 1.31 (23) was used for estimating heterozygosity, and NTSYS package version 2.02 (24) used for drawing phylogenetic tree.

Results

One hundred and eight honey bee genotypes were used as materials for DNA genotyping. These samples were selected according to different geoclimatic conditions. All used primers showed polymorphism in amplified loci (Table 3). The total numbers of detected fragments were 70 with an average of 7 fragments per primer ranging from 3 to 10 fragments per primer (Table 3). ISSR-PCR profiles are represented in Figs. 2, 3 and 4. Forty loci were polymorphic and showed an average of 61.56 percent polymorphism. Primer A_1 showed the highest number of polymorphic fragments (7 fragments) (Fig. 2A). Primer No. A_9 showed the lowest number of polymorphic fragments (2 fragments) (Fig. 4A).

The total numbers of detected fragments per primer were expected because it depends on the number of ISSR loci in each colony. While, the total numbers of polymorphic fragments were 40 with an average of 4 per primer and the range was between 2 to 7 per primer. The percent of polymorphic fragments ranged between 45 to 85.71 with an average of 61.56 per primer. The range of fragment sizes was between 150 and 1500 bp. Variability for each locus was also measured. Each polymorphic fragment was scored as a locus with two allelic classes, so the maximum heterozygosity value of an ISSR locus was 0.5. Eventually, the average heterozygosity values for all detected loci for each primer were estimated. The average heterozygosity was 0.266 and the range was

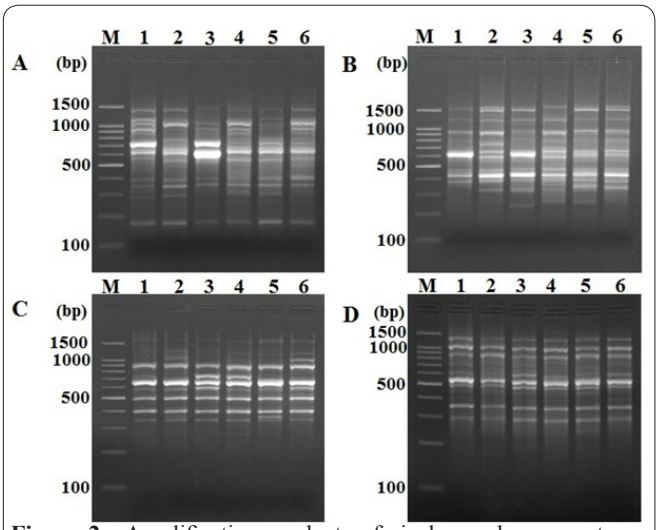

Figure 2. Amplification products of six honey bees genotypes obtained from ISSR primers namely; (A): $(AGAC)_4GC$, (B): $AC(GACA)_4$, (C): $(GACA)_4GT$ and (D): $(GACA)_4CT$. (M): 100 base-pair DNA ladder. 1: Ardebil, 2: West Azerbaijan, 3: East Azerbaijan, 4: Golestan, 5: Mazendaran and 6: Guilan.

Table 3. Levels of polymorphism detected by ten ISSR primers in 108 worker honey bees.

Primer code	Primer	Total number of fragments	Number of polymorphic fragments	Percent of polymorphic fragments	Range of fragment sizes(bp)	Heterozygosity
A_1	$(AGAC)_4GC$	10	7	85.71	150-1400	0.224
A_2	$AC(GACA)_4$	8	5	66.66	220-1500	0.224
A_3	$(GACA)_4GT$	6	4	60	280-1100	0.266
A_4	$(GACA)_4CT$	5	3	54.55	300-1400	0.301
A_5	$(ATG)_5GA$	8	5	66.66	190-1400	0.205
A_6	$(TCC)_5GT$	7	4	62.55	180-1300	0.279
A_7	$(CTC)_5GT$	5	3	54.55	300-750	0.251
A_8	$(AC)_8G$	6	4	60	250-1000	0.302
A_9	$(CT)_8A$	3	2	45	300-600	0.287
A_{10}	$(AC)_8AT$	6	4	60	230-900	0.322
Total	-	70	40	-	-	-
Mean		7	4	61.56	240 - 1135	0.266

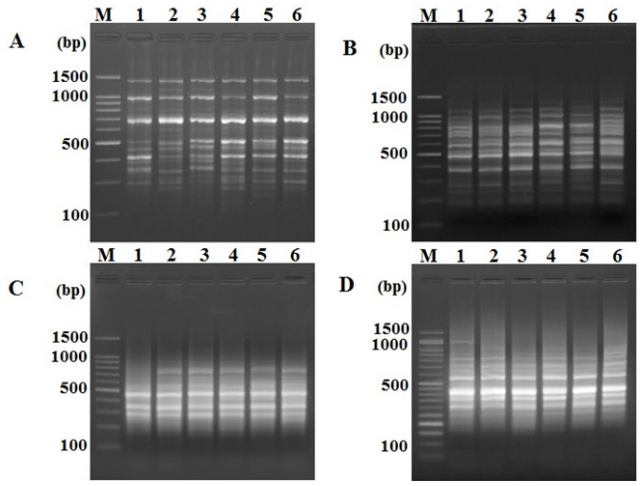

Figure 3. Amplification products of six honey bees genotypes obtained from ISSR primers namely; (A): $(ATG)_5GA$, (B): $(TCC)_5GT$, (C): $(CTC)_5GT$ and (D): $(AC)_8G$. (M): 100 base-pair DNA ladder. 1: Ardebil, 2: West Azerbaijan, 3: East Azerbaijan, 4: Golestan, 5: Mazendaran and 6: Guilan.

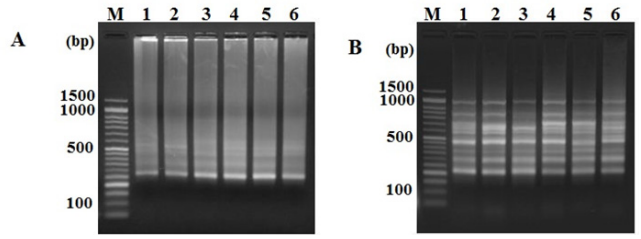

Figure 4. Amplification products of six honey bees genotypes obtained from ISSR primers namely; (A): $(CT)_8A$ and (B): $(AC)_8AT$. (M): 50 base-pair DNA ladder. 1: Ardebil, 2: West Azerbaijan, 3: East Azerbaijan, 4: Golestan, 5: Mazendaran and 6: Guilan.

between 0.205 and 0.322. These results indicated that the studied honey bees were relatively diverse.

Cluster analysis was implemented by UPGMA method to show the genetic relationships of the populations under-study and was presented in Fig. (5). The honey bee populations were divided into two groups based on the cluster analysis. The first group includes the group A (comprise honey bee populations from Ardebil, West Azerbaijan and East Azerbaijan provinces), and the second group consisted the group B (including honey bee populations from Golestan, Mazendaran and Guilan provines). Group A is divided into two subgroups. The first subgroup included honey bee populations of West Azerbaijan and East Azerbaijan provinces, and the second subgroup included honey bee populations from Ardebil province. Also, group B is divided into two subgroups. The first subgroup included honey bee populations from Golestan province, and the second subgroup included honey bee populations from Mazendaran and Guilan provinces.

Discussion

The 10 primers used, produced 70 Bands ranging from 3 to 10 bands per primer, 40 out of 70 bands (61.56%) were polymorphic. Compared with other studies, the results of this study showed some differences. In addition, the numbers of amplified bands were from 2 to 5 bands (18, 25) and 4 to 12 bands (26). The part of this difference is related to the use of different primers dinucleotide,tri, and tetranucleotides and another part of the type used primers – normal or anchoring at both ends. Tetra-nucleotide primers with sequences AGAC and GACA showed better performance and these primers were used in a high percentage of polymorphism. Dušinsk *et al.,* (2006) and Reddy *et al.,* (1999) reported same results Tetra-nucleotide primers in this study have produced more bands than other primers (27, 28). The same differences were revealed in the results of other studies (18, 25, 26). It has been showed that honey bees 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 (29). 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. Also, 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 (30). According to our results 40 of bands were polymorphic. The average for each primer was about 4 bands. These results indicated that most of the areas studied were polymorphic. The results of this study is in accordance with the observations of other researchers (25, 29). Paplauskiene *et al.*(2006) observed fragments with range between 350-3000 and the fragments observed by Al-Otaibi (2008) were in range 100-850, and the fragments observed by Shouhani *et al.* (2014) were in range of 150-1500 nucleotides (25, 26, 31). Our results confirmed the findings of Shouhani *et al.* (2014) and we conclude that as we were successful to reveal polymorphism between populations of bees in spite of use of low number of primers, so ISSR markers should be considered as a powerful marker to reveal polymorphism in insect populations as a whole. Also, Al-Otaibi, (2008) and Shouhani *et al.* (2014) demonstrated that the ISSR marker was a good tool for discrimination and genetic structure analysis of *Apis mellifera* populations native to different geographical populations (25, 26). De Leon and Walker (2004) were able to reveal distinct banding patterns in *Gonatoerus* species (Hymenoptera: Mymaridae) three individuals emerging from different host tribes by using ISSR marker fingerprinting (32).

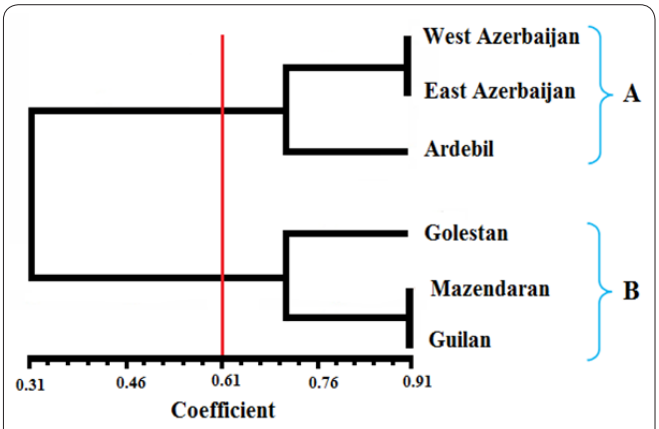

Figure 5. Dendrogram generated honey bee populations using UPGMA method and Jaccard similarity coefficient.

Dušinsk *et al.* (2006) used inter-simple sequence repeat (ISSR) markers successfully for discrimination among and within species of blackflies (Diptera, Simulidae). Meena *et al.* (2005) used ISSR analysis for molecular characterization of *Tospovirus* transmitting *Thrips* populations from India (33). De Leon *et al.* (2004) indicated that the ISSR markers were geographic-specific in *Homalodisca coagulata* (Homoptera: Cicadellidae) and can therefore be considered diagnostic since there was no band sharing between different populations. Philips *et al.* (2002) showed that amplification of inter simple sequence repeats regions of *Microctonus aethiopoides* (Hymenoptera: Braconidae) DNA showed clear genetic differences between French and New Zealand specimens of *Medicago aethiopoides* (34). Luque *et al.* (2002) have shown that some ISSR amplifications are possible and demonstrate their applicability in studying intra- and inter-specific variations in some Noctuid populations(19).

The cluster analysis based on UPGMA method divided the populations into two groups according to their origins. The first group included the North indigenous populations; the second group included the northwest indigenous populations. The dendrogram demonstrated clearly the ability of the ISSR markers to detect the genetic variability between and within the honey bee populations used in this study and to identify groups and subgroups with different levels of genetic distance. Besides, the results showed us that it is possible to distinguish the honey bee subspecies and based on phylogenetics inferences to select lines for highest genetic diversity between different populations of honey bees. Finally, ISSR markers can be used in molecular marker-assisted breeding programs. Khemakhem *et al.* (2005) and Shouhani *et al*. (2014) indicated that ISSR can be useful as DNA-based molecular markers for studying genetic diversity and phylogenetic relationships of *Mayetiola sp*. and honey bee haplotypes (26, 35). The results of present study showed that the levels of genetic similarity between honey bee populations in 6 different provinces of Iran was high. It could be due to the migration of bees to neighboring provinces. When we have made cluster analysis, we have found that two neighboring provinces of East and West Azerbaijan, formed a single group differentiated from the other groups, we consider this because of existing trade of queens as well as colonies of bees between beekeepers of these two provinces, so there are genetic similarities between honey bee populations of East and West Azerbaijan.

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