

# Study of the genetic diversity of Iranian honey bee (*Apis mellifera meda* Skorikow, 1829) populations using the mtDNA COI–COII intergenic region

Ataollah Rahimi\*

Department of Plant Protection,  
College of Agriculture,  
Razi University, Kermanshah, Iran

Determining the genetic diversity amongst the populations of honey bee is one of the essential and important aims in race breeding of this useful insect. In the present study, the genetic diversity of the intergenic region COI–COII of mtDNA of the honey bee populations in the South of Iran has been studied. We used the PCR-RFLP technique, and 60 worker honey bees were collected from 6 provinces (Sistan and Baluchestan, Kerman, Fars, Hormozgan, Bushehr and Khuzestan) of Iran. Their DNA were extracted using a salting out method. To do PCR, 25 microliters of one pair of primers plus mtDNA were used. To ascertain the results of PCR, the PCR products were electrophoresed using 1.5% Agarose Gel. Thereafter, the *DraI* restriction enzyme was used for enzymatic digestion of the PCR product. The result of this digestion was to produce 4 diverse pieces (44, 55, 64 and 430 bp) of enzymatic restriction fragment patterns. The results obtained from this study showed that using *DraI* restriction enzyme digestion, the honey bee populations in the South of Iran belonged to the C mtDNA lineage (this honey bee race group consists of Central Mediterranean and South East European honey bee populations). Another result obtained from this study indicated the absence of genetic diversity between the samples collected from the South of Iran.

**Key words:** COI–COII intergenic region, *DraI* restriction enzyme, honey bee

## INTRODUCTION

The honey bee, *Apis mellifera* Linnaeus 1758, is naturally found throughout Europe, Africa and Western Asia (Miguel et al., 2011). From morphometric

and molecular studies, 29 subspecies of the honey bee, *Apis mellifera* L., are grouped into five evolutionary lineages: M from Northern and Western Europe and Northern Africa, A from Southern and Central Africa, C from the Northern Mediterranean region and Eastern Europe, O from the Eastern Mediterranean and the Near and Middle East

\* Corresponding author. E-mail: Rahimi.ata.1@gmail.com

regions, and Y from the east African country of Ethiopia (Ruttner, 1988; Hall, Smith, 1991; Garnery et al., 1992; Arias, Sheppard, 1996; Franck et al., 2000, 2001; Sheppard, Meixner, 2003; Meixner et al., 2011). Within Iran, wide ranges of climates and habitats are found, but only one honey bee subspecies has been described (Ruttner, 1988; Tahmasebi, 1996). Except for the East desert areas, nearly all of Iran is occupied by the Iranian honey bee (*Apis mellifera meda*). Recently, significant morphometric variations revealed 3 well-defined clusters of the native honey bee in Iran (Rahimi et al., 2014). Although morphological characteristics are still considered very important in the classification of honey bees, this approach is not well suited to characterize honey bee subspecies and analyze phylogenetic relationships, as they can be sensitive to environmental selection pressures (Franck et al., 2000b). Genetic markers such as the mtDNA COI–COII intergenic region are unique to the genus *Apis* (Cornuet, Garnery, 1991). Honey bee lineages can be distinguished by restriction and sequence analyses of the mitochondrial DNA (mtDNA) region between the cytochrome oxidase subunits I and II genes (CoxI–CoxII intergenic region) (Hall, Smith, 1991; Cornuet et al., 1991; Franck et al., 2000). *DraI* restriction has revealed more than 50 RFLP patterns in lineages A and M but only 5 fragment size patterns in lineage C (Franck et al., 2000, 2001; De la Rúa et al., 2004; Sušnik et al., 2004; Kandemir et al., 2006; Kozmus et al., 2007). Restriction fragment patterns of this region tend to be simpler in lineage C because of the absence of variable repeats found in other lineages (Hall, Smith, 1991; Garnery et al., 1992, 1993; Franck et al., 2000, 2001; De la Rúa et al., 2004). Variations in the sequences of this region or the length of fragments produced using endonucleases are used extensively to differentiate among five honey bee lineages and to discriminate among *A. mellifera* subspecies (Garnery et al., 1992; Franck et al., 2000a; Sheppard, Smith, 2000). Sequencing is more sensitive and may reveal new haplotypes that have not been previously described

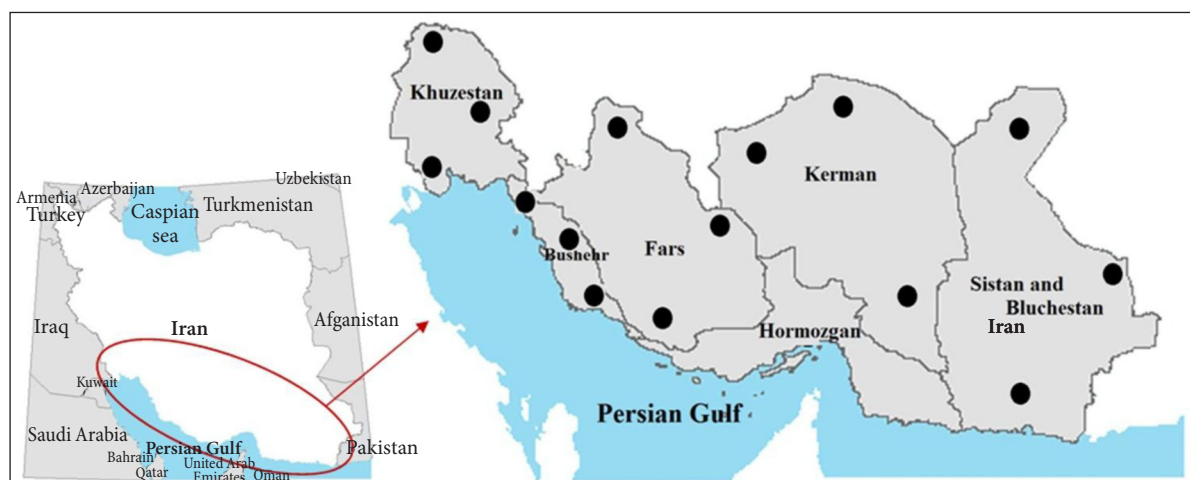
(Ozdil et al., 2009; Solorzano et al., 2009; Magnus, Szalanski, 2010; Magnus, Szalanski, 2010). Garnery et al. (1993) studied the areas between controller genes of COI and COII related to the honey bees mitochondria genome. This area was proliferated through PCR and cut by the *DraI* limited enzyme. Twenty one haplotypes were identified in the samples containing 302 populations of 12 honey bees subgroups. In part C, all the samples showed equal band population and were named C1, and part M showed 10 various haplotypes. Morits et al. (1994) studied 102 samples from 29 different areas of Southern Africa honeybees. In this study areas between controller genes of COI and COII were proliferated through PCR and cut by the *DraI* limited enzyme, and 4 parts of different sizes and 9 mitotypes were identified. Özdil (2007) carried out his study on 244 bee samples from 21 different areas of Turkey. Like previous researches, the area between controller genes of COI and COII of the mitochondria genome was cut by the *DraI* limited enzyme and 5 different haplotypes were identified. Length and restriction site polymorphisms within the CoxI–CoxII intergenic region of the honey bee mitochondrial genome have been particularly useful in differentiating evolutionary lineages and groups of subspecies (Cornuet, Garnery 1991; Hall, Smith, 1991; Garnery et al., 1992, 1993; Franck et al., 2000, 2001; Palmer et al., 2000). For example, *DraI* restriction of this region revealed more than 50 haplotypes in lineages A and M (Garnery et al., 1992, 1993; Franck et al., 2000, 2001) but only six haplotypes in lineage C (Franck et al., 2000; Kandemir et al., 2006; Ozdil et al., 2009), including one discovered recently (Ozdil et al., 2009).

To date there is no information on the COI–COII DNA sequence variation of *Apis mellifera meda* in Iran, so the objective of our study was to determine the genetic diversity of honey bee populations from the Iran based on the area between controller genes of the cytochrome C oxidase I and II mitochondria genome identified by the indicator of RFLP on the bases of the PCR technique.

## MATERIALS AND METHODS

**Sampling.** A total of 120 adult worker honey bees each representing a different colony were collected from 6 provinces (Sistan and Baluchestan, Kerman, Fars, Hormozgan, Bushehr and Khuzestan) of Iran in 2012 (Fig. 1). In this way, I randomly chose three cities from each province, and also from each city I randomly selected one apiary. In addition, I randomly chose 5–8 colonies from each apiary, and I took one worker honey bee of each colony. Samples were preserved in 97% ethanol and stored at  $-20^{\circ}\text{C}$  until DNA extraction.

**DNA extraction and PCR.** Total DNA was extracted from each bee's thorax and head according to the salting out method with minor modification. In this study, all samples were amplified with using one pair primer (Table 1). PCR amplifications were performed in a  $25\ \mu\text{l}$  volume via a Thermocycler machine for amplified segments of the COI–COII intergenic region in accordance with the values and concentrations presented (Table 2). Thermal cycling was as follows: 4 min initial denaturing at  $94^{\circ}\text{C}$ , 37 cycles consisting of 30 s at  $94^{\circ}\text{C}$ , 30 s at the primer specific annealing temperature and 30 s at  $72^{\circ}\text{C}$  and 6 min extension step at  $72^{\circ}\text{C}$ .



**Fig. 1.** Sampling localities in the Sistan and Baluchestan, Kerman, Fars, Hormozgan, Bushehr and Khuzestan provinces

**Table 1.** Characteristics of COI–COII intergenic region primers used in this research

Primer sequence		T <sub>m</sub> (°)
Forward	Reverse	
5'GGCAGAATAAGTGCATTGGGC3'	5'CAATATCATTGATGACCTTA3'	55

**Table 2.** The amount of material used in each of the chain reaction polymerase

Materials	The amount of material used in each reaction ( $\mu\text{l}$ )	The final concentration
PCR Buffer	2.5	10X
Mgcl <sub>2</sub>	0.75	50 mM
dNTP mix	0.75	650 $\mu\text{M}$
Forward primer	1	10 $\mu\text{M}$
Reverse primer	1	10 $\mu\text{M}$
Taq DNA polymerase	0.4	1 U
Genomic DNA	3	40 ng
Sterile distilled water	15.6	–

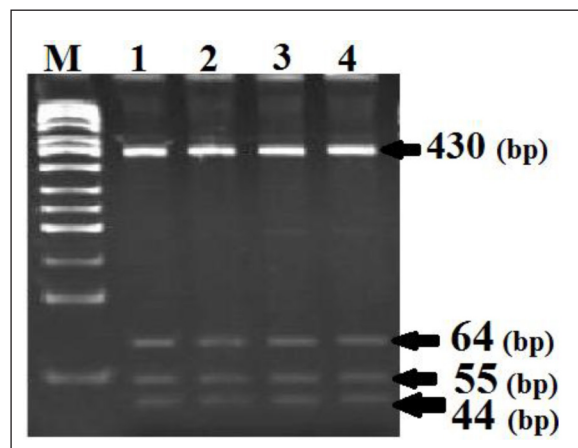
In order to ensure successful amplification and high quality PCR products, PCR products were electrophoresed using Agarose Gel 1.5% and then were stained with ethidium bromide. Thereafter, the PCR products were digested via *Dra*I enzymes. The reaction mix was incubated at 37 °C for 12 h. Afterwards, in order to separate the protected banding of the digested with *Dra*I restriction enzyme polyacrylamide gel 12% was used. After electrophoresis with polyacrylamide gel, ethidium bromide was used for stain of the polyacrylamide gel containing segments digested using the restriction enzyme. After the stain with ethidium bromide, all polyacrylamide gel was photographed via a gel documentation system and in later stages it was analyzed.

## RESULTS AND DISCUSSION

In this study, genetic diversity of the COI–COII intergenic region of mtDNA in 120 worker honey bees of 6 provinces of Iran using the PCR-RFLP technique and the *Dra*I restriction enzyme was analyzed. The genetic area between the controller genes of cytochrome C oxidase I and II samples were amplified using the polymerase chain reaction and then the digested *Dra*I restriction enzyme. In all samples, this enzyme produced cutting in 3 sites, and the result of this digestion was to produce 4 di-

verse pieces (44, 55, 64 and 430 bp) on polyacrylamide gel 12% (Fig. 2). The data obtained in this study indicated that honey bee populations in the South of Iran have C evolutionary group features. Similar results on the African honey bee populations (Franck et al., 2001), on the samples collected in 1993 from Australia (Garnery et al., 1993) and on the populations of honey bees in the North and North West of Iran in 2011 (Pish Jang et al., 2011) have been reported. Frank et al. (2000a, 2001) in 3 different researches on the area between controller genes of the C cytochrome oxidase I and II mitochondria genome of honey bees related to the subspecies of *A. m. cypria*, *A. m. armeniaca*, *A. m. caucasica* and *A. m. anatolica* observed that similar bands were created in the mentioned genetic area by the *Dra*I restriction enzyme cutting and these honey bees were placed in the C genetic place. On the other side, *A. m. syriaca* were on the O place according to the mitochondria genome.

All polyacrylamide gel images for the survey of existence and the level of genetic diversity in the samples collected in this study were analyzed. The results of this study showed that the samples of honey bees collected from 6 provinces were similar to the diversity of the area between the genes COI–COII, using the PCR-RFLP technique and the restriction enzyme *Dra*I, and the absence of genetic diversity between samples collected from these provinces via these methods.



**Fig. 2.** The fragment produced from digested PCR products using *Dra* I restriction enzyme (M: size marker)

## CONCLUSIONS

The genetic diversity of the COI–COII intergenic region of mtDNA in 120 worker honey bees of 6 provinces of Iran using the PCR-RFLP technique and the *Dra*I restriction enzyme was analyzed in this study. The results obtained from this study showed that using *Dra*I restriction enzyme digestion, the honey bee populations of the South of Iran belonged to the C mtDNA lineage. In addition, the results obtained from our study indicated the absence of genetic diversity between the samples collected from the South of Iran.

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#### Ataollah Rahimi

#### IRANO NAMINIŲ BIČIŲ (*Apis Mellifera Meda* SKORIKOW, 1829) POPULIACIJŲ GENETINĖS ĮVAIROVĖS TYRIMAI mtDNA COI–COII INTERGENINIAME REGIONE

#### Santrauka

Kryžminant skirtingų rasių naminės bitės yra svarbu įvertinti genetinę šių bičių populiacijų įvairovę. Tyrime buvo nustatyta medaus bičių mtDNA COI–COII intergeninio regiono genetinė įvairovė tarp skirtingų Irano pietuose esančių populiacijų. Panaudojus PCR-RFLP DNR tyrimų metodą ištirta 60 bičių darbininkių iš 6 Irano provincijų (Sistano ir Baluchestano, Kermano, Farso, Hormozgano, Bushehro ir Khuzestano). DNR išskirta druskini-mo metodu, PGR rezultatai įvertinti elektroforezės 1,5 % agarozės gelyje metu. Panaudojus *DraI* restrikcijos fermentus, gauti keturi skirtingo dydžio (44, 55, 64 ir 430 bp) fragmentai. Rezultatai atskleidė, kad Irano pietinės dalies populiacijų naminės bitės yra artimos centrinės Viduržemio jūros ir pietrytinės Europos bičių populiacijoms. Taip pat nustatyta, kad šio regiono populiacijų naminėms bitėms būdinga nedidelė genetinė įvairovė.

**Raktažodžiai:** naminės bitės, COI–COII intergeninis regionas, genetinė įvairovė