Biodiversity Journal, 2020, 11 (1): 7–14 https://doi.org/10.31396/Biodiv.Jour.2020.11.1.7.14

Genetic diversity of the Honeybee *Apis mellifera* Linnaeus, 1758 (Hymenoptera Apidae) from Jijel (Northeast Algeria)

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ABSTRACT Traditionally, subspecific classification and phylogeographic inferences in *Apis mellifera* Linnaeus, 1758 (Hymenoptera Apidae) were first classified according to their morphological and behavioral traits along with their geographical distributions. Morphometrical analyses conducted on large datasets have established different evolutionary lineages of honeybees. In the last two decades of honeybee genetic analysis and conservation, subsequent studies based on variations in mitochondrial DNA largely confirmed Ruttner's phylogeographic structure of *Apis mellifera*. However, very little is known about Algerian honeybee diversity. Therefore, this study assessed the genetic diversity of honeybees (*Apis mellifera*) in Northeastern Algerian, in North Africa, using the mitochondrial DNA marker (mtDNA) COI-COII (Cytochrome Oxidase I and II). In total, thirty honeybee workers were sampled from three sites of Jijel province. A PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis of the mtDNA samples distinguished the honeybee evolutionary lineages from this region. Our study revealed that studied honeybee populations have A (African lineages) evolutionary group features. Notably, the northern Algerian honeybee populations had elevated diversity compared to the southern populations.

KEY WORDS Algerian honeybee populations; *Apis mellifera*; COI-COII intergenic region; Evolutionary lineage, Mitochondrial DNA marker.

Received 08.08.2019; accepted 17.12.2019; published online 30.01.2020

INTRODUCTION

Among insects, Apidae Linnaeus, 1758 (Hymenoptera) are the most important group of pollinators (Bénédicte, 2013) and *Apis mellifera* Linnaeus 1758 is the world's most widely distributed and well-known pollinator. It has naturally spread in Africa, Europe and Western Asia (Miguel et al., 2011), before they were spread around the world by many factors: natural migrations, adaptation to local conditions and some apicultural practices; such that the imports of queens, introduction of subspecies of foreign bees and the transhumance of colonies. These practices are therefore opposed to the forces exerted by natural selection that differentiates populations (Garnery et al., 1998a, b; Jensen et al., 2005). Based on morphometric characteristics, behavioral and biogeographical studies, 29 different honeybee subspecies have been identified (Ruttner et al., 1978; Ruttner, 1988; Sheppard et al., 1997; Sheppard & Meixner, 2003; Engel, 2004; Meixner et al., 2013) and are classified into

five evolutionary lineages: (M) from Northern Europe and Northern Africa, (A) from South and Central Africa, (C) from the Northern Mediterranean region and Eastern Europe, (O) from the Eastern Mediterranean and the Near and Middle East regions, and (Y) from the eastern African country of Ethiopia. Subsequent studies based on variation in mitochondrial DNA (mtDNA) have largely confirmed this classification (Hall & Smith, 1991; Garnery et al., 1993; Arias & Sheppard, 1996; Franck et al., 2000; Cánovas et al., 2008; Kekecoglu et al., 2009; Alburaki et al., 2011; Papachristoforou et al., 2013; Loucif-Ayad et al., 2014; Achou et al., 2015).

Mitochondrial DNA markers have been widely used to address population and evolutionary questions in the honeybee *A. mellifera*. The characterization of the mtDNA genome has been very useful for analyzing the genetic structure of *A. mellifera* subspecies, as it contains regions with variable evolutionary rates and it is, generally, maternally inherited without recombination (Meusel & Moritz, 1993). Therefore, molecular markers have facilitated more robust assessments of honeybee diversity, such as the reclassification of North African subspecies, *A. mellifera intermissa* (Buttel-Reepen, 1906) and *A. mellifera sahariensis* (Baldensperger, 1932), into the African branch instead of the West European branch (Garnery et al., 1992).

The honeybee subspecies that are native to North Africa belong to the African lineage A (Ruttner, 1988; Garnery et al., 1995; Franck et al., 2001). In Algeria, two honeybee subspecies have been identified: *A. mellifera intermissa*, described by Buttel-Reepen (1906), is a breed of North Africa, found in Tunisia, Algeria and Morocco along the Mediterranean coast (Cornuet et al., 1988; Grissa et al., 1990); *A. mellifera sahariensis*, described by Baldensperger (1932; see also Haccour, 1960) ranges from Djebel Amour and Ain Sefra in Algeria through the oases of the Sahara south of the Atlas Mountains to Figuig in the west of Morocco (Ruttner, 1968; De la Rúa et al., 2007).

The used genetic marker in these studies was variation in the intergenic region between the cytochrome C oxidase I "COI" and II "COII" gene in mtDNA. It is unique to the genus *Apis* Latreille, 1802 (Cornuet & Garnery, 1991) as determined by restriction analysis "*DraI* restriction enzyme" (Garnery et al., 1993; Franck et al., 1998). This test has been widely used to analyze the evolutionary lineages of *A. mellifera* subspecies (Garnery et al., 1993; 1995; 1998 a, b; De la Rúa et al., 1998; Rortais et al., 2011). Using this method, different haplotypes can be distinguished and grouped into one of the five primary lineages described by Ruttner (1988) and reviewed by De la Rúa et al. (2009).

Studies were realized on the genetic biodiversity of *A. mellifera* in certain regions in Algeria (Chahbar et al., 2012; Loucif-Ayad et al., 2014; Achou et al., 2015; Hu et al., 2016).

The aim of this research was to analysis the genetic diversity of Algerian honeybee populations from three regions in the northeast (Jijel province) of the country. We used the COI-COII mtDNA marker (widely used in studies of honeybees) to identify the evolutionary lineages in our samples.

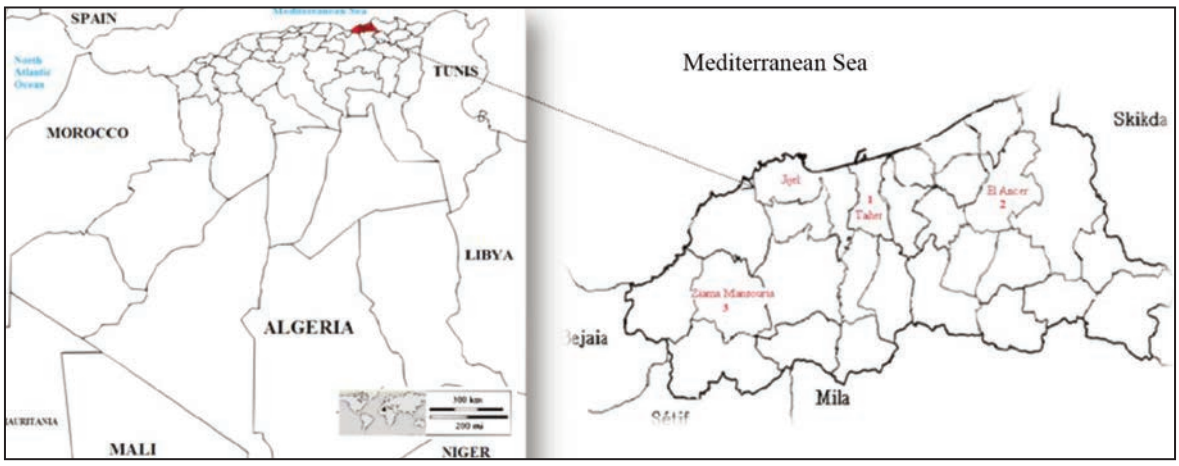

Figure 1. Sampling sites in the Jijel area (Algeria).

This work characterizes the mitochondrial genetic structure of the country's honeybee populations in Algeria.

MATERIAL AND METHODS

Honeybee samples

Thirty adult honeybees were sampled during autumn (October 2016) from three sites across Northeast Algeria (Jijel province). The geographical location and number of individuals sampled from each sites are shown in figure 1 and Table 1. This region spreading over an area of 2398,69 km^2, characterized by a Mediterranean climate (wet temperate) and per diverse vegetation (Greininger, 1982). Ten worker bees were sampled per site; preserved in absolute ethanol (90%) and stored at 4°C until DNA extraction.

DNA extraction

Genomic DNA was extracted from each bee's head according to the manufacturer's protocol (ZR Tissue & Insect DNA MiniPrep™Kit; Zymo Research, U.S.A). The head of the bees was crushed individually using mechanical homogenizer in a microcentrifuge tube then transferred to a ZR Bashing Bead™ Lysis Tubes. For optimal performance, β-mercaptoethanol supplemented the Genomic Lysis Buffer to a final dilution 0.5% (v/v). A Lysis Solution of 750 µl is added; with shaking by the vortex (10 min.); Lysis Tubes were centrifuged in a microcentrifuge (10000 x g/1min.). We transferred up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuged (7000 x g/1 min.); we added 1,200 µl of Genomic Lysis Buffer to the filtrate in the Collection Tube from the previous step and mixed; Then, 800 µl of the mixture from the previous step are transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge (10000 x g/1min.); the flow through from the Collection Tube was discarded and the previous step is repeated. We added 200µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge it (10000 xg/1 min.), then 500 µl g-DNA Wash Buffer are added to the Zymo-Spin™ IIC Column and centrifuged (10000 x g/1 min.). Finally, the Zymo-Spin™ IIC Column are transferred to a clean 1.5 ml microcentrifuge tube and we added 30 µl DNA Elution Buffer directly to the column matrix; then the tubes were centrifuged (10000 x g/30s) to elute the DNA.

DNA quality and concentration were measured using a spectrophotometer (NanoPhotometerTM-Pearl, Implen GmbH, Schatzbogen, Germany); DNA isolated from each honeybee sample was subsequently stored at -20 °C for further genetic analysis.

Mitochondrial DNA analysis

In this study, we amplified the mtDNA COI-COII intergenic region located between the tRNALeu gene and the second subunit of the cytochrome oxidase gene II (COII) using the primer pair (Table 2) (Hall & Smith, 1991; Garnery et al., 1991; Smith et al., 1991; Garnery et al., 1993; Alburaki et al., 2011; Meixner et al., 2013).

PCR amplification of the COI-COII intergenic region was performed in a volume of 25 µl via a Thermal cycler C1000TM "CFX96 Real-Detection System, Bio-Rad, U.S.A". Thermal cycling was as follows: 3 min. initial denaturing at 92 °C, 30 cycles of denaturation at 92 °C for 1 min., annealing at 48°C for 1 min., and extension at 62 °C for 2 min., and a final extension step at 62 °C for 7 min.

Honeybee evolutionary lineage

In order to identify the evolutionary lineage of each individual, 2 µl of each PCR product was loaded into 1.4% agarose gel and electrophoresed along with the M7123-100bp DNA ladder marker (Biomatik, Germany). The gel was then stained for 10 min with ethidium bromide (EtBrat 0.5µg/ml) and finally observed under UV illumination. The length of the amplified mtDNA COI-COII intergenic region was measured for each sample, using the M7123-100bp DNA ladder marker (Biomatik, Germany).

Haplotype identification

The remaining 23 µl volume of each PCR product was digested with four units of the *DraI* restriction enzyme (Takara, Japan) at 37°C for 48 hours.

DNA patterns of each sample were observed under UV illumination and the images were saved in jpeg format for storage.

RESULTS

Restriction enzyme digestion of the mitochondrial fragment containing the intergenic region with *DraI* resulted in two different patterns assignable to the A mitochondrial lineages (African evolutionary lineage) as described by Cornuet & Garnery, (1991).

In *A. mellifera*, the COI-COII intergenic region varies from 192 to 832bp according on the subspecies. This variability depends on presence or absence of the P or P0 patterns and number of the repetition of the Q pattern (Table 3). Compared to the marker we used (M7123-100bp DNA ladder), the short pattern P0Q or PQ (among 500-600 bp) is dominant and represents 99% of our samples (site

	Locations	Geographical position		Number of worker bees sampled
		Latitude	Longitude	
	Jijel province	36° 49'00″ N	5° 46'00″ E	
1	El-Taher	36° 46′ 19″ N	5° 53′ 54″ E	10
2	El-Ancer	36° 48′ 00″ N	6° 09′ 25″ E	10
3	Ziama mansouriah	36° 40′ 25″ N	5° 28′ 52″ E	10
Total	**3 sites**			**30**

Table 1. Location and number of honeybees sampled (ten worker bees/site) for Jijel province studied in Algeria.

Primer sequence (5'-3')	
Forward	**Reverse**
E2 : 5'-GGCAGAATAAGTGCATTG-3'	**H2** : 5'-CAATATCATTTGATGACC-3'

Table 2. Primers used for Molecular characterization of COI-COII intergenic region from honeybees sampled.

Variant type	**Interpretation**	**Lineages evolutionary**
Ultra short	Q	C
Short	PQ or P_0Q	M ou A
medium	PQQ or P_0QQ	M ou A
Long	PQQQ or P_0QQQ	M ou A

Table 3. Structural organization of COI-COII mitochondrial region from honeybees samples.

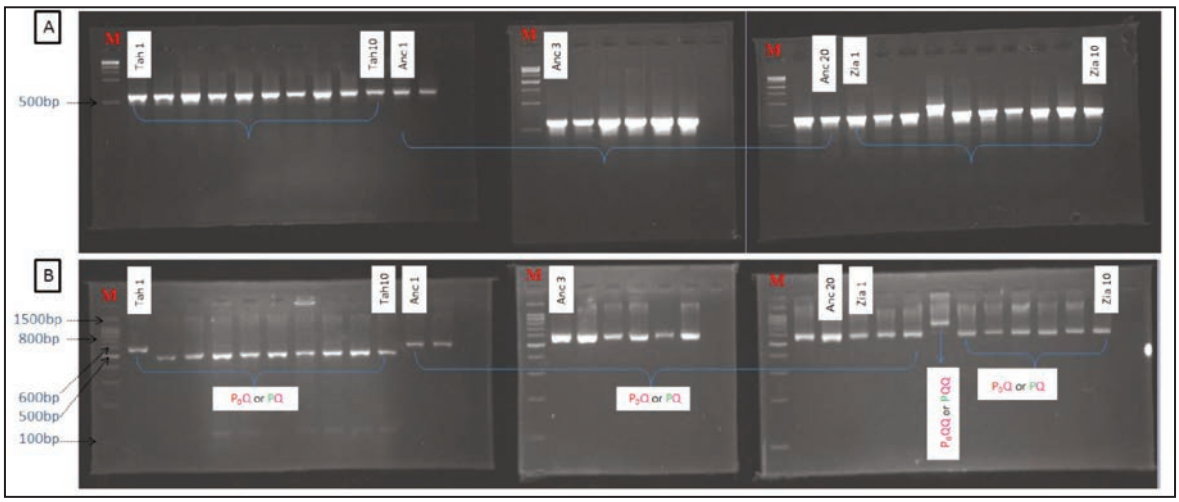

Figure 2. Visualization of different sizes of the COI-COII fragment on the agarose gel (M: M7123-100bp DNA ladder marker), lane Tah1-Tah10: Site 1 (El-Taher); lane Anc1-Anc10: Site 2 (El- Ancer); lane Zia1-Zia10: Site 3 (Ziama mansouriah); (A): Before digestion with *DraI* restriction enzyme; (B): After digestion with DraI restriction enzyme.

1 and 2), the samples from Ziama mansouriah were characterized by medium pattern P0QQ or PQQ, having a low percentage 1%, which coexists with the short pattern P0Q or PQ (Fig. 2).

DISCUSSION

Our study was based mainly on the COI-COII mtDNA marker in order to characterize the genetic variability of the Algerian honeybee populations on a large geographical scale. The COI-COII intergenic mtDNA region had different lengths in the studied populations. Among our samples, the size range was detected between 500bp and 800bp on agarose gel (1.4%) (Fig. 2) compared to the marker we used (M7123-100bp DNA ladder).

As expected from previous results (Cornuet et al., 1991; Garnery et al., 1993, 1995; Franck et al., 2001), this study has shown that Algerian honeybee populations correspond to the African evolutionary lineage. A comparison of the genetic variability between the Algerian populations and Tunisian honeybee populations (Chouchaine, 2010) revealed the presence of three types of restriction fragment P0Q or PQ, P0QQ or PQQ and Q. But, P0Q or PQ variant is a majority and represents about 94.66% of all variants of the Tunisian bees, identification of this profiles by DraI restriction enzyme revealed existence of four African haplotypes A1, A4, A8 and A9; based on this polymorphism, Tunisian honey bees belong to African lineages.

Previous studies have demonstrated similar results in Algerian honeybee populations (Chahbar et al., 2012) in which African haplotypes were strongly dominant despite past importations of European honeybees. These results are highly consistent with those based on prior microsatellite analysis of the honeybee samples (Loucif-Ayad et al., 2014); the data of Achou et al. (2015) revealed the presence of three different honeybee lineages among the studied populations, comprising the African (A) lineages, North Mediterranean (C) and West Mediterranean (M) whose eight different mtDNA haplotypes (A1, A2, A8, A9, A10, A13, C7 and M4).

CONCLUSIONS

In conclusion, our data demonstrated that Algerian honeybee populations belong to the African (A) lineage. According to our research, there is no introgressions (importation of foreign honeybees) among these populations were recorded, which can cause an impact on the genetic diversity of local honey bees. Expanding the sampling to the Algerian honeybee populations is of major importance, as it

will allow a better understanding of the diversity. From a conservation point of view, limiting foreign queen importations to preserve the local genetic diversity of the Algerian honeybee populations is needed.

ACKNOWLEDGEMENTS

We would like to thank Algerian beekeepers for their contribution to sampling. We are especially grateful to Mr. Abed El-khalak for his intensive collaboration in honeybee sampling. This work was supported by the Ministry of High Education and Scientific Research of Algeria to Pr. Noureddine Soltani (Laboratory of Applied Animal Biology).

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