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GENETIC BIODIVERSITY OF THE TUNISIAN BEE *APIS MELLIFERA INTERMISSA* (BUTTEL REEPEN, 1906) (HYMENOPTERA: APIDAE)

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A B S T R A C T

The characterization of the Tunisian bee was performed using a cytoplasmic molecular marker: mitochondrial DNA (DNAm). Molecular analysis was carried out on 655 colonies from 7 populations. This study showed that the Tunisian bee *Apis mellifera intermissa* has a maternal origin belonging to the A line, which contradicts its belonging to the M line as demonstrated on the basis of the biometric study alone. There is a genetic polymorphism within the local breed by the presence of four haplotypes A1, A8 and A9 and A4. The distribution of haplotypes A4 and A9 depends on climatic conditions. The foreign haplotypes are present in the region of Ghardimaou, and represented by the haplotype C7.

Keywords: Tunisian bee, *Apis mellifera intermissa*, DNA^{t}_{m}, Genetic, polymorphism.

INTRODUCTION

Mitochondrial DNA ($DNA^{t)}$ is an excellent marker for the phylogenetic study (Mensel and Moritz, 1993) and the genetic structure of natural populations (Avise *et al*., 1987). In the case of *Apis mellifera,* all the members of a colony share the same type of mtDNA, since the queen transmits mitochondrial DNA to all of her offspring: male, female and new queen (Renaud, 2006). The DNA^{t}_{m} allows resolution between evolutionary lines (Brown *et al*., 1979). In addition, it allows precise detection of foreign haplotypes in the populations studied (Garnery, 1992). The physical map of mitochondrial DNA is established using 17 restriction enzymes and includes 46 sites (Cornuet *et al*., 1991). The mitochondrial DNA molecule of the honey bee has varying sizes ranging from 16500 to 17600 bp (Smith and Brown, 1990 and Smith *et al*., 1997). This diversity results from the size polymorphism of several regions of the molecule (Smith and Brown, 1990), particularly the region between the COI and COII genes (Mensel and Moritz, 1992). Two types of sequences can be recognized in the COI-COII region: P and Q (Garnery, 1992). Line A has a P_0 sequence (68pb) followed by one, two, or three tandem repeats of the Q sequence (192-196pb). In the M line, there is also a variable number of Q sequences, but the P_0 sequence is replaced by a shorter sequence (54bp) named P (Garnery, 1992). Line C does not have any of the P_0 and P sequences and only one copy of the Q sequence (Garnery *et al*., 1993). The Tunisian bee *Apis mellifera intermissa* is very active (Gould and Gould, 1993), but is characterized by its aggressiveness (Adam, 1985), its strong tendency to swarm (Ormel, 1987) and its variability in honey production. These characteristics are not appreciated by the modern beekeeper (CNEA, 2003). Such factors have prompted the professional Tunisian beekeeper to introduce alien subspecies, namely *Apis mellifera carnica, Apis mellifera Caucasia, Apis mellifera ligustica, Apis mellifera macedonica and Apis mellifera mellifera* due to their very interesting productivity in honey (Le Conte and Franck, 2005), and their least aggressiveness (FIDA, 1999). The introduction of subspecies of bees in Tunisia continues

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until now (INS, 2007). In this study we used the DraI restriction polymorphism of the COI-COII region of the mitochondrial DNA of the 7 populations studied in order to establish a balance sheet of the Tunisian apiary herd, to estimate the importance of the anthropic effects On the variability of the populations and to evaluate the level of differentiation of the Tunisian bee according to the bioclimatic stages.

MATERIALS AND METHODS

Location of apiaries and number of colonies: A population is a spatial unit characterized by particular climatic, morphological and topographical conditions that distinguish it from other populations. Tunisia is covered by ten natural areas (Souissi, 2000). In this study we selected 7 populations. The three Great Erg (GE) populations, Chotts (CH) and Dahars and Matmata (DM) are desert populations. 60% of the bee population is located in northern Tunisia rich in plants with a melliferous potential (OEP, 2009). Our sampling respects this distribution in addition it respects the proportionality of the number of apiaries by beekeeper. Bees are taken directly from the flight hole and immersed in pure ethanol to keep them for several months at room temperature. Each colony studied has been sampled by 5 workers, a single bee is sufficient for this study, the other bees are kept in case the first extraction is not good. The number of colonies collected from each population is shown in figure 1.

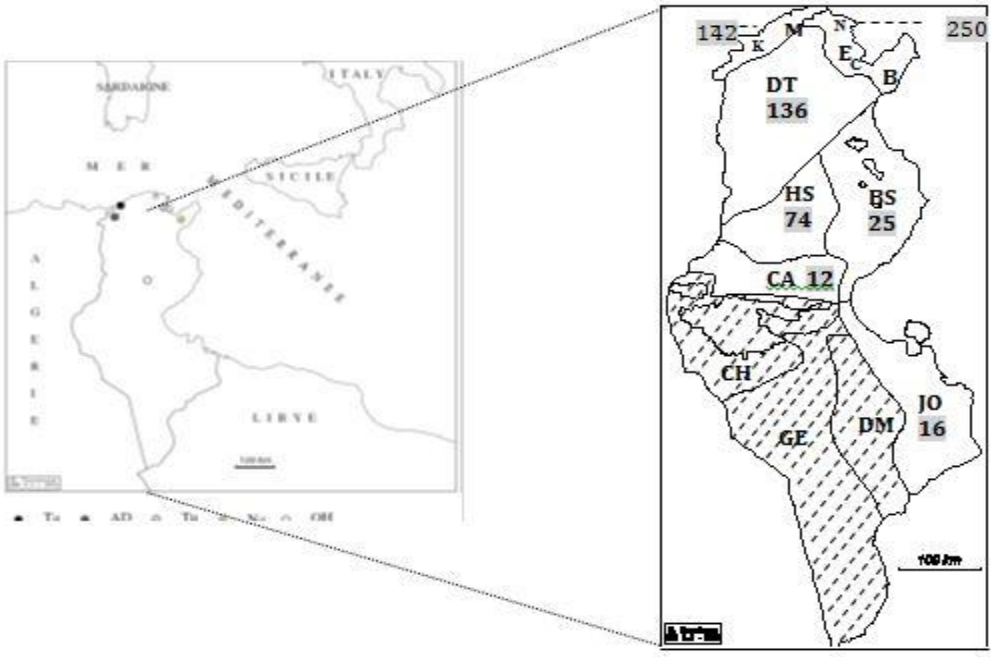

Figure 1. Number of colonies by population. (KM = Kroumirie Mogood, NECB = North East Cap, DT = Dorsal and Tell, BS = Low Steppe, HS = High Steppe, JO = Jeffara and Ouara and CA = Atlasic Chain, DM = Dahars and Matmata.

Extraction of total DNA: Total DNA was extracted from the thorax using the Chelex 10% method (Estoup *et al*., 1996). A solution of Chelex 10% previously heated to 60° C. with stirring is added at a rate of 600 µl per sample. The digestion of the proteins is initiated by the addition of 18 µl of proteinase K to each tube which will be stirred and placed in a water bath for 1 hour at 50° C. It is then incubated twice for 15 minutes at 95° C. with a pause Of 10 minutes at room temperature between the two incubations. Finally, the tubes are centrifuged for 10 minutes at 12000 rpm and are then stored with the Chelex beads at -20° C.

Evaluation of mitochondrial diversity: The study of mitochondrial DNA has focused on the inter-genic region which extends between the cytochrome oxidase I and II locus. This region is amplified by PCR using primers E2 (5'-GGCAGAATAAGTGCATTG-3') and H2 (5'-CAATATCATTGATGACC-3) (Garnery, 1992). The amplification is carried out from 1,2 µl of total DNA in a reaction volume of 25 µl (2,7 µl of buffer, 2,7 µl of $MgCl_2$ (1,5 10^{-9} pmoles), 2,7 µl of dNTP (0,25 pmoles), 2,7µl of E2 (25 pmoles), 2,7µl of H2 (25 pmoles), 0,4µl of BSA: Bovine Serum Albumin, 11,8µl of H_2O and 0,3µl of Taq polymerase (0.6 units). Each PCR was initially denatured

at 92° C. for 3 minutes followed by 30 cyclic reactions and each of these cycles consisted of three parts: denaturation, hybridization and elongation. Finally, the reaction mixture was raised to 63° C. for 10 minutes. The determination of the variants was carried out on 2 µl of the amplified DNA of the COI-COII region mixed with 4 µl of bromophenol blue of each sample on a 1.4% agarose gel. The restriction fragment length polymorphism is generated by hydrolysis of the DNA with a DraI restriction endonuclease (4 units). The digestion takes place under incubation at 37° C. for 48 hours. The identification of precise haplotypes requires two migrations on acrylamide gel at 10% and 5% concentration. The 10% and 5% agarose and acrylamide gels are then immersed for 5 minutes in a solution of ethidium bromide (0.5 µg / ml), an ultraviolet (260 nm) fluorescent intercalating agent in order to visualize the length of the fragments.

Data Management: All data will be encoded and managed by Data Fauna Flora software (Barbier *et al*., 2002). The cartographic representation will be done using Carto Fauna Flora software (Barbier and Rasmont, 2000). The calculation of the frequency and the determination of the haplotypic diversity parameters (D, DNAtm) of the Tunisian bee were carried out using the EXCEL (Microsoft) software using the unbiased estimation formulas (Nei and Tajima, 1981):

$D= (n\ (1-\sum pi^2)/\ (n-1)$, where n is the number of individuals and pi the haplotype frequencies.

RESULTS

Determination of variants: The variant (P_0Q) is very predominant and accounts for about 94.66% of all variants of the Tunisian bee, in the populations of Lower Steppe, Atlas Chains and Jeffara and Ouara, this variant is dominant and represents 100%. The mean variant (P_0QQ) cohabitates with the short variant (P_0Q) in the populations of Haute steppe, Dorsale and Tell, North East Cap Bon and Kroumirie Mogood. The mean variant (P_0QQ) having a low percentage ranging from 2.2% to 8.5%. The distribution of the total variants P_0Q, P_0QQ and Q within the 7 populations is shown in figure 2.

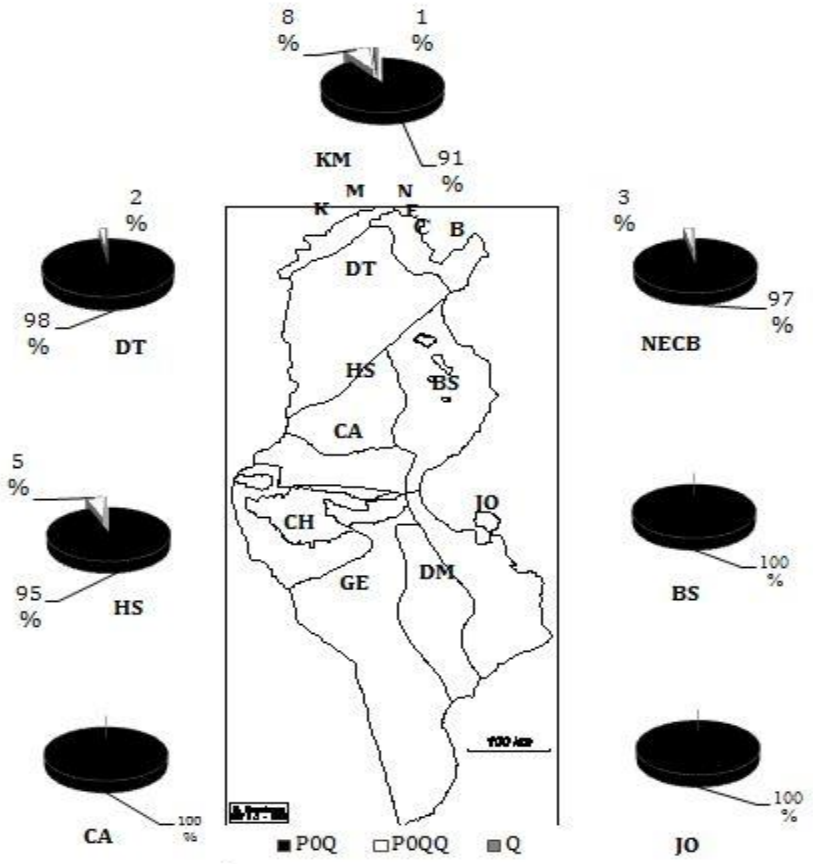

Figure 2. Percentages of variants in the 7 populations.

Determination of haplotypes: The identification of restriction profiles of the COI-COII mitochondrial region (figure 3a) of 655 bees from 7 populations by the DraI enzyme revealed the existence of five haplotypes, four of which are African types belonging to line A And are A1, A4, A8 and A9; The fifth haplotype belonging to the C line is represented by the C7 haplotype. The mitochondrial COI-COII region of the haplotypes A1 and A8 is of the P0Q type, the haplotypes A4 and A9 are of the P0QQ type and the haplotype C7 is of type Q (figure 3b).

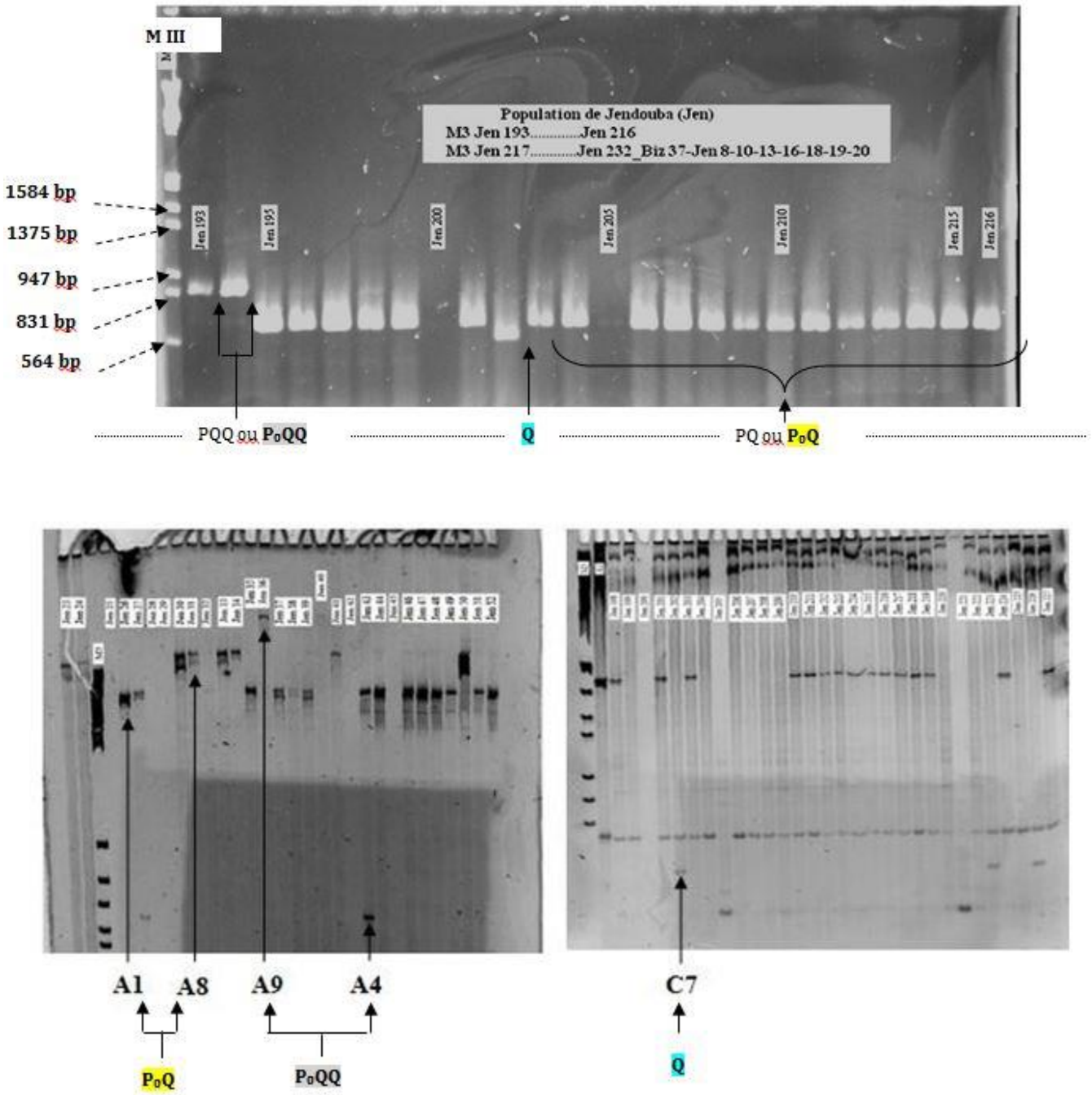

Figure 3. Migration of: a-variants on the agarose gel and b- haplotypes on acrylamide gel 5%.

The haplotypes A8 and A9 are characterized by the existence of a single restriction site for the *DraI* enzyme, after migration onto the acrylamide gel, we detected 2 restriction fragments of different sizes (47 and 592bp) for the Haplotype A8 and (47 and 785bp) for haplotype A9. The haplotype A1 is characterized by the presence of two restriction sites for the enzyme *DraI,* on the acrylamide gel we detected 3 fragments of restrictions of different sizes (47,109-110 and 483-487 bp). The A4 haplotype

is characterized by the presence of 3 restriction sites for the enzyme *DraI*; on the acrylamide gel we detected 4 restriction fragments of respective sizes (592, 193, 109-110 and 47 bp). Of the 7 populations studied, haplotypes A1 and A8 are very predominant and account for about 94.65% of the honeybee rate studied. Of the five haplotypes, haplotype A1 is very dominant and represents 60.91% of all haplotypes of the Tunisian bee. The haplotypes A4, A8 and A9 represent respectively 1.37%, 33.74% and 3.51%. The distribution of haplotypes within the 7 populations is shown in figure 4.

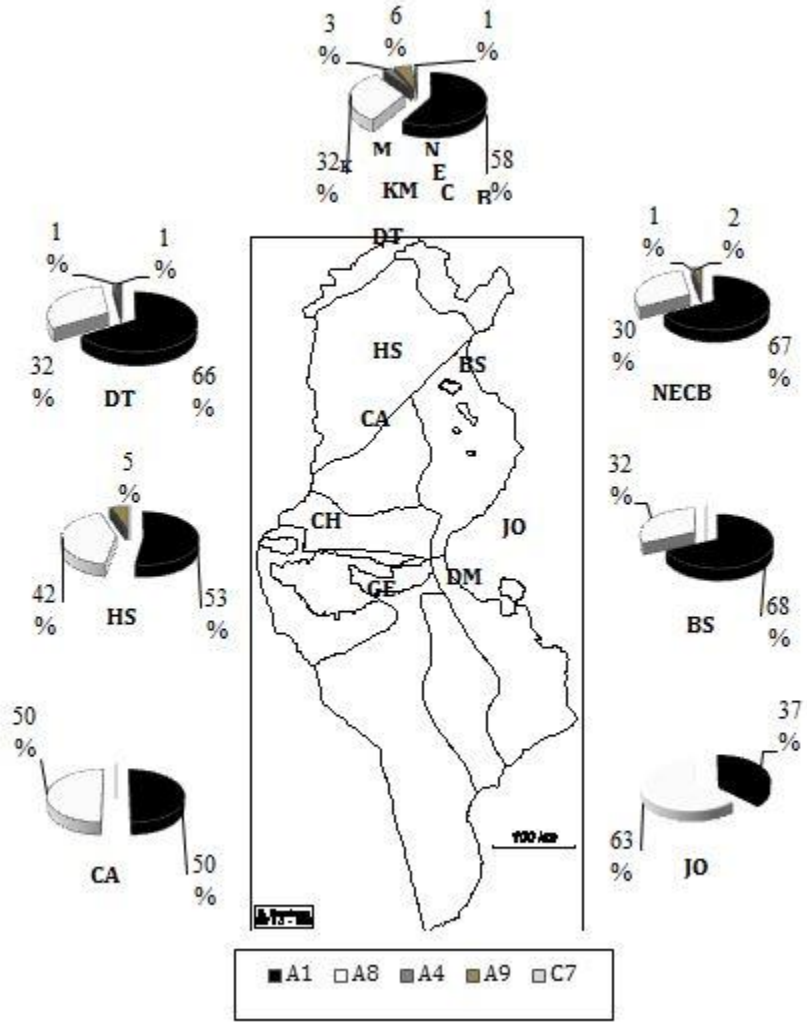

Figure 4. Percentages of haplotypes in the 7 populations.

Haplotypic diversity within each population: The haplotypic diversity within the population depends on the number of haplotypes and especially on the percentage of each haplotype. Given the small percentage of haplotypes A9, A4 and C7, we can deduce that the bee population of the Tunisian bee consists essentially of two haplotypes A1 and A8 which represent 94.65%. When there is an equality between the two major haplotypes A1 and A8, the haplotypic diversity of the population is high; In the opposite case, ie the dominance of one or the other haplotype reduces the haplotypic diversity of the population. The haplotypic diversity of the 7 populations studied is variable on the Tunisian scale, haplotypic diversity is average (0.478). This is due to the dominance of haplotype A1, which alone accounts for about 2/3 of the bee population of Tunisia. The populations Cap Bon (0.346), Basse Steppe (0.383), Dorsal and Tell (0.481) and Jeffara and Ouara (0.458). The imbalance in the percentages of haplotypes has influenced the haplotypic diversity within these populations. The haplotype A1 is dominant in the northeastern Cape Bon, Basse Steppe, Dorsale and Tell populations. 66.9%, 68% and 64.17% of the bee population of these populations. In the Jeffara and Ouara population, haplotype A8 is dominant and accounts for 62.5% of the bee population in this population. The Kroumirie Mogod, Atlas and Upper Steppe populations have a high haplotypic diversity of 0.532, 0.545 and 0.604, respectively, due to the decrease in disproportion between the two major haplotypes of Tunisia A1 and A8.

Distribution of haplotypes according to the bioclimatic stages: The tolerance interval of the four haplotypes A1, A4, A8 and A9 with respect to temperature and precipitation is different. The haplotype A4 and A9 are present where the annual precipitation amount is greater than 300mm and the mean annual temperature is less than 19 ° c (table 1 and 2). Unlike haplotypes A4 and A9, haplotypes A1 and A8 are present in all temperatures and precipitation (table 1 and 2).

Table 1. Distribution of haplotypes according to annual mean temperatures (° C).

Temperature		14-14,34	15-15,325	16,81	17,55-17,85	18-18,7	19,73-20,5
P_0Q	A1	46	27	18	112	174	22
	A8	40	15	18	40	85	23
P_0QQ	A4	*	5	*	2	2	*
	A9	6	6	*	9	2	*
Q	C7	3	*	*	*	*	*

Table 2. Distribution of haplotypes according to annual precipitation (mm).

Precipitation		0-300	301-600	601-900	901-1200	1201 ET +
P_0Q	A1	48	247	27	53	24
	A8	42	111	8	50	10
P_0QQ	A4	*	3	*	1	5
	A9	*	13	1	7	2
Q	C7	*	*	*	3	*

The distribution of the haplotypes of the Tunisian bee according to the bioclimatic stages is not identical. Haplotypes A1 and A8 are present in all bioclimatic stages. The haplotypes A9 and A4 are present only in humid, subhumid and semi-arid to fresh winters bioclimatic stages. The size of the COI-COII inter-gene sequence appears to depend more on latitude and altitude, in fact in cold regions the mean Q sequence number is higher (Garnery, 1992). The distributions of the haplotypes A1, A4, A8 and A9 according to the bioclimatic stages are shown in the figure 5.

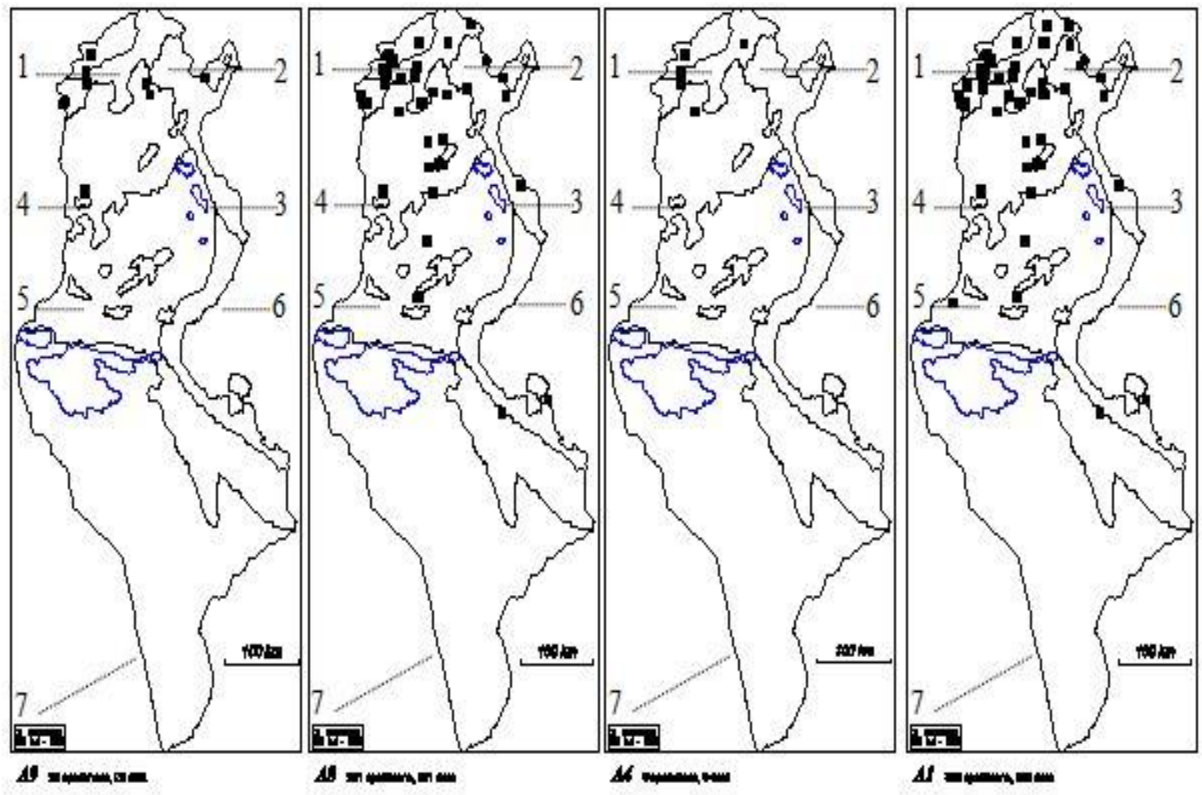

Figure 5. Distribution of haplotypes A1, A4, A8 and A9 according to the bioclimatic stages of Tunisia. (1-Wet, 2- Subhumid, 3- Semi-arid to mild winters, 4- Semi-arid to cool winters, 5- Arid to cool winters, 6- Arid to mild winters, 7- Saharan.

DISCUSSION

Evolutionary origin and haplotypic biodiversity of the Tunisian bee: The use of the *DraI* restriction polymorphism of the COI-COII region of DNAm showed the strong discriminating power of this molecular cytoplasmic marker in the analysis of the biodiversity of the Tunisian bee. The results for the analysis of 655 bees show that colonies with a maternal origin belonging to the very predominant line **A** in Tunisia represent about 99.54% of the bees sampled. This study made it possible to demonstrate the existence of the five haplotypes A1, A8, A4 and A9 and C7. The Tunisian bee has retained its originality as a bee belonging to line **A**. Based on our results and the results of Franck et al. (2001), De la Rua et al., (1998, 2001, 2002, 2003 and 2004a and b), Garnery et al., (1988), Sinacori et al., (1998) and Sheppard and Meixner (2000), haplotypes A1, A4, A8 and A9 are common in all African subspecies *Apis mellifera Unicolor, Apis mellifera Capensis, Apis mellifera Scutellata, Apis mellifera Monticola, Apis mellifera Adonsonii, Apis mellifera Sahariensis and Apis mellifera Intermissa*, the subspecies of several islands of the Mediterranean *Apis mellifera Sicula and Apis mellifera Ruthnerii* (Sheppard and Meixner 2000) and the Western European subspecies *Apis mellifera iberica* (De la Rua et al., 1998, 2001, 2002 and 2003 and Garnery et al., 1988).

The level of introgression of the Tunisian bee: The foreign lines are very poorly represented on all seven populations, 0% for the **M** line and 0.45% for the **C** line, which is represented by the C7 haplotype. These foreign bees are frequent in the population of Kroumirie Mogood, specifically in the region of Ghardimaou. Within this population, the percentage of this haplotype is of the order of 1.2%. These bees were sampled by the Breeding and Pasture Office in three different apiaries from three beekeepers. The presence of the same haplotype in the El Taref region of Algeria, which is a neighboring town of Ghardimaou, leads us to believe that the anthropogenic factor and the natural factor linked to the genetic character of the bee may explain the presence of these haplotypes foreign bees. These bees of line C may be the traces of the former introductions of foreign bees in Tunisia or in Algeria. Given the proximity of Ghardimaou and El Taref, by hunt swarms during the swarming, or by buying these bees, beekeepers Tunisians or Algerians make proliferate these foreign bees. The natural migration direction of *Apis mellifera Intermissa* is from south to north, that is from North Africa to East and West Europe. During the last glaciation, the Mediterranean was divided into two basins; among them a passage that encompassed southern Italy, Sicily and eastern Tunisia, the Tunisian bee migrated from south to north to meet *Apis mellifera Ligustica* giving rise to a subspecies ancestor of A*pis mellifera Ruttnerii* and *Apis mellifera Sicula* (Sheppard et al., 1997). The introduction of foreign subspecies in Tunisia is against the natural sense of migration. These long-term introductions can directly threaten the homogeneity and therefore the hybridization of the local breed, especially since all breeds of *Apis mellifera* are interfertile (Fresnaye, 1981) and indirectly influence the biodiversity of the honey flora that Results in the change of ecosystems. These introductions have led to the proliferation of enemies and infectious diseases: *Varroa destructor* which was introduced by bees imported from Romania (Fourgi, 1979).

Physiological adaptation of the four haplotypes of the Tunisian bee: In the evolutionary history of *Apis mellifera Intermissa*, it acquired in its gene pool the good adaptation to the climatic conditions of Tunisia: severe winter, hot summer (Grissa, 2000). Indeed, it can withstand temperatures that other sub-species cannot stand (Adam, 1955). In addition to adapting to climatic conditions, it is perfectly adapted to periods of local vegetation (Grissa, 2000). The haplotypes A4 and A9 have a tendency to fight against the cold (Chouchaine *et al*., 2014). They lower the temperature of the brood nest (Chouchaine *et al*., 2015). These haplotypes present a limited brood (Chouchaine, 2010). In winters, spawning stops, the temperature of the core of the cluster of these two haplotypes is 25 (± 3) ° C, the temperature of the periphery is 9 ° C and the cluster is formed around the queen. The outermost envelope of the cluster can never drop below 9 ° C. As soon as a situation threatens, the core of the cluster produces an excess of heat to restore adequate conditions to the periphery (Chouchaine *et al*., 2015). Haplotypes A1 and A8 are much more sensitive to low temperatures and cannot overwinter at extreme conditions. These two haplotypes tend to increase the mechanism of thermogenesis through thermolysis in order to keep the temperature of the periphery of the cluster higher than the triggering threshold of individual thermogenesis (Chouchaine *et al*., 2015). The phenomenon of overwintering is observed in *Apis mellifera mellifera Linnæus* (1758) (André 1990). In this case, it is attributed to proteins, fats and a substance called biopterin (Imdorf *et al*., 1996). The life expectancy of the bee is a key criterion in wintering (Imdorf et al., 1985). Indeed, genetic factors (Chouchaine *et al*., 2016) and environmental factors play a role (Chavalarias, 2007). The size of the COI-COII inter-gene sequence of mitochondrial DNA depends on latitude and altitude. In cold regions, the average number of Q sequences is higher (Garnery 1992). The oxygen consumption is proportional to the number of Q sequences (Chouchaine *et al*., 2016). Similarly, wintering depends on the oxygen consumption of haplotypes (Chouchaine *et al*., 2015).

CONCLUSION

The applications envisaged, such as the setting up of a conservatory for livestock management at the local and national level and impact studies are needed in order to better characterize the level of variability of local bees and to preserve genetic diversity before it is too late, and that local bees no longer have sufficient genetic resources to maintain them sustainably.

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