Genetic variation of local honeybees *Apis mellifera* in Basrah Governorate-Iraqi, using RFLP - PCR Technique

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Abstract

This study aimed to analyze the genetic diversity of honey bees *Apis mellifera* in Basrah Governorate, southern Iraq, using restriction fragment length polymorphism PCR (RFLP-PCR) of the intergenic COI-COII region in mitochondrial DNA (mtDNA). We collected 980 samples from 98 bee colonies distributed across six geographical regions. Results revealed moderate genetic diversity (75%), with three dominant bands (1200 bp, 800 bp, 400 bp) indicating polymorphism. UPGMA phylogenetic analysis clustered the samples into two main groups, reflecting genetic differences associated with geographical distribution. These findings underscore the significant role of geographic factors in shaping the genetic diversity of honey bees in the region.

Keywords: *Apis mellifera*, Mitochondrial DNA, Genetic polymorphism, RFLP-PCR, honeybees, Iraq.

Introduction:

Evolutionary history and genetic diversity of *Apis mellifera* flowering plants first appeared on earth approximately 100-150 million years ago. During this period, the genus Apis underwent a remarkable evolutionary transition from solitary to highly organized eusocial behavior over 30 million years, culminating in its modern form (Culliney, 1983). The honey bee was taxonomically classified as *Apis mellifera* by Linnaeus in 1758, with its initial distribution restricted to Europe, Africa, and the Middle East before achieving global dispersal. Currently, 29 recognized subspecies are distributed across five major geographic lineages: (M) northern and western Europe/northern Africa, (A) southern and central Africa, (C) northern Mediterranean/eastern Europe, (O) eastern Mediterranean, and (Y) eastern Africa to Ethiopia (Ruttner, 1988a; Garnery et al., 1992; Hall & Smith, 1991; Arias & Sheppard, 1996; Franck *et al*., 2000, 2001).

The honey bee populations of Iraq - geographically bounded by Turkey (north), Iran (east), and Syria (west) - belong to the Near Eastern subgroup of western honey bees (*Apis mellifera*). This group includes the following subspecies: *A. m. anatolica* (Maa), *A. m. adami* (Ruttner), *A. m. cypria* (Pollman), *A. m. syriaca* (Buttel-Reepen), *A. m. caucasica* (Gorbachev), *A. m. meda* (Skorikov), and *A. m. armeniaca* (Skorikov) (Ruttner, 1986, 1988b).

Traditional honey bee classification and phylogenetic reconstruction have primarily relied on morphological traits (Dechamp, 2003). However, genetic diversity and social evolution in organisms emerge through complex interactions between extrinsic and intrinsic factors. The Neutral Theory of Molecular Evolution (Kimura, 1983) provides a robust framework for understanding biodiversity, positing that much of the observed genetic variation in populations arises from random

accumulation of neutral mutations in non-coding or functionally insignificant genomic regions over evolutionary timescales.

The advent of polymerase chain reaction (PCR) technology by Kary Mullis in 1983 revolutionized genetic studies by enabling targeted amplification of specific DNA sequences, including individual genes or multiple loci (Saiki et al., 1988; Altshuler *et al.*, 2006). This breakthrough facilitated advanced analyses of genetic variation in honey bees. In the present study, we employed mitochondrial DNA (mtDNA) to investigate phylogenetic origins, evolutionary pathways, and gene flow dynamics in Iraqi honey bee populations. Restriction fragment length polymorphism (RFLP) analysis was applied to the intergenic COX2 region (COI-COII), a well-established marker for discriminating *Apis mellifera* subspecies (Cornuet *et al* ,1991; Hall & Smith, 1991; Franck *et al.*, 2000).

This study aims to analyze genetic variation in honey bees from Basra Governorate, Iraq using RFLP-PCR of mtDNA, and to determine the evolutionary relationships of Iraqi lineages within the global *A. mellifera* framework, thereby enhancing understanding of regional apian biodiversity.

Materials and Methods:

1. *Sampling:*

A total of 980 worker bees were collected from 98 honey bee colonies across six geographical regions in Basra Governorate (Al-Siba, Abi Al-Khasib, Shatt Al-Arab, City Center, Al-Zubair, and Al-Qurnah) Table (1) and Figure (1). Ten adult workers were sampled from each colony and preserved in 95% ethanol at - 20°C until analysis.

Table (1): Coordinates of Regions in Basra Governorate, Iraq.

Region	Coordinates (Latitude, Longitude)	Notes
Al-Siba	30.5333° N, 47.8417° E	Agricultural area located west of Basra.
Abi Al-Khasib	30.4411° N, 47.9250° E	Rural area famous for date palm groves.
Shatt Al-Arab	30.5167° N, 47.8167° E	Approximate point where Tigris and Euphrates rivers meet.
City Center	30.5150° N, 47.8094° E	Administrative center of Basra city.
Al-Zubair	30.3892° N, 47.7078° E	Historic city rich in archaeological sites and oil fields.
Al-Qurnah	31.0333° N, 47.4333° E	Located at the confluence of the Tigris and Euphrates rivers.

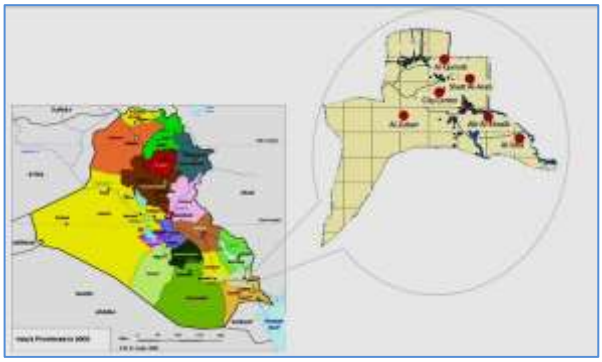

Figure (1): sampling location in Basrah, Iraq

2. DNA Extraction:

The following protocol was employed for DNA extraction from *Apis mellifera* worker bees:

Sample Preparation: Ten adult workers were processed by carefully removing legs and wings. The thorax region, rich in longitudinal and transverse flight muscles, was isolated to ensure optimal DNA yield (Winston, 1993).

DNA Extraction; 20 mg of muscle tissue was homogenized in a 1.5 mL Eppendorf tube containing 200 µL of 10% Chelex solution. 20 µL of Proteinase K (20 mg/mL concentration) was added for protein digestion. Samples were vortexed for 15 minutes followed by incubation at 56°C for 30 minutes.

DNA Purification: Samples were centrifuged at 12,000 rpm for 3 minutes, Supernatant was discarded and 200 µL of absolute ethanol (100%) was added to the pellet, Samples were vortexed again and dried at 37°C for 10-15 minutes, 20 µL of PureLink™ Genomic DNA solution was added, followed by incubation at 50°C for 24 hours.

Wash and Contaminant Removal: 200 µL PBS buffer with 20 µL Protease K, (10 mg/Ml), 20 µL RNase, Incubation at 37°C for 2 hours. Two wash steps using 500 µL Wash Buffer1, 500 µL Wash Buffer 2, Centrifugation at 10,000 rpm, Final pellet resuspended in 150-200 µL Elution Buffer, DNA stored at -80°C.

3. mtDNA Detection by Gel Electrophoresis:

A 1% agarose gel was prepared in 0.5× TBE buffer. Seven microliters of DNA were mixed with 2.5 µL loading dye (Loading Dye) and loaded into the gel wells. Electrophoresis was performed at 85 V and 48 mA for 30 minutes, with results visualized under a UV transilluminator.

4. Polymerase Chain Reaction (PCR):

The intergenic COI-COII region was amplified using the following specialized primers:

E_2 = 5′-GGCAGATAAGTGCATTG-3′

H_2= 5′-CAATATCATTGATGCC-3′

The PCR reaction was performed using primer pair E_2/H_2 under the following cycling conditions Table (2).

Table (2): Temperature gradient for primers.

Temp.	*Time/min*	*Cycle*	*Reference*
94°C	4 *min*	1	*Garnery et al. (1993)*
94°C	1 *min*	37	
37°C	1 *min*	1	
72 °C	2 *min*		
72 °C	10 *min*		
4	*storege*		

4. Restriction Digestion Protocol:

Enzyme Used: Dral (Thermo Scientific) at 10 units/µL concentration.

Reaction Mixture: 10 µL PCR product, 2 µL Dral restriction enzyme, 2 µL Tango Buffer (10X), 6 µL nuclease-free water.

Incubation Conditions: 37°C for 16 hours (overnight digestion) followed by final electrophoretic separation as described in the previous step.

Polymorphism was calculated using the Nei & Li (1979) formula, and a phylogenetic dendrogram was constructed using the UPGMA algorithm in MEGA-X software.

Results:

The RFLP-PCR analysis using DraI restriction enzyme Figure (2) revealed three dominant bands at 1200 bp, 800 bp, and 400 bp, indicating significant polymorphism in the mtDNA COI-COII intergenic region. The overall polymorphism rate across Basra samples reached 75%, demonstrating moderate genetic diversity.

Regional Variation: Al-Zubair samples showed the highest genetic diversity (all three bands present), Al-Siba samples exhibited the lowest heterogeneity (single band at 1200 bp).

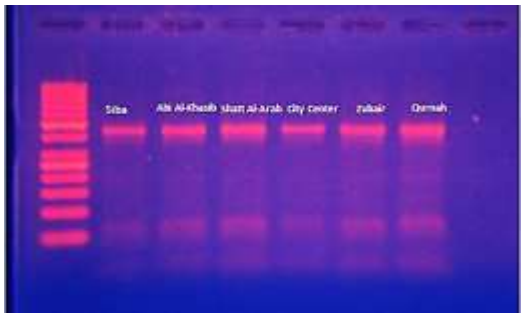

Figure (2): RFLP-PCR banding patterns of Apis mellifera mtDNA COI-COII region digested with DraI: Three polymorphic fragments (1200 bp, 800 bp, 400 bp) indicating regional genetic diversity in Basra populations.

The UPGMA analysis Figure (3) generated a genetic dendrogram revealing two distinct clades: Clade I: Al-Zubair and Abi Al-Khasib populations, Clade II: Al-Siba and Al-Qurnah populations. The Zubair samples showed the highest level of genetic diversity compared to other regions, with three gene bands (1200 bp, 800 bp, 400 bp) detected in RFLP analysis. This can be explained by several factors: Gene flow: Zubair is located near the Kuwaiti border, which may facilitate the mixing of local bee strains with those from neighboring regions through colony migration or beekeeper activities. In contrast, the sib showed low genetic diversity (one band at 1200 bp), which may reflect geographic isolation or dominance of a single dominant lineage due to harsh environmental conditions (e.g., high soil salinity).

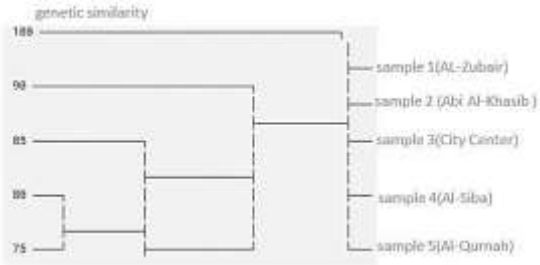

Figure (3): Genetic similarity matrix of Apis mellifera populations across Basra Governorate based on RFLP-PCR analysis of mtDNA COI-COII region.

Discussion :

The present study provides the first comprehensive assessment of mitochondrial genetic variation in honeybee populations from Basrah Province, southern Iraq, using the COI–COII intergenic region amplified by PCR-RFLP. The observed restriction patterns and the moderate level of genetic

diversity (75%) reveal a structured mitochondrial background consistent with populations belonging to the Near Eastern evolutionary lineage (O-lineage).

The COI–COII region has long been recognized as a robust molecular marker for distinguishing evolutionary lineages of *Apis mellifera*. Classical studies by (Garnery, *et al.* 1993) in Molecular Ecology demonstrated that DraI restriction profiles of this region effectively discriminate between African (A), West European (M), and Near Eastern (O) lineages. The restriction fragments obtained in the present study (1200, 800, and 400 bp) are consistent with the structural organization reported for eastern Mediterranean and Middle Eastern populations, supporting the hypothesis that Iraqi honeybees form part of the broader Near Eastern mitochondrial complex.

Our findings are also in agreement with results published in (Alburaki, *et al.* 2013), which reported clear mitochondrial structuring in transitional geographic zones of the Middle East. That study emphasized the role of the region as a contact zone between evolutionary lineages, where historical gene flow and secondary introgression events have shaped present-day diversity. Iraq occupies a central biogeographic position between Anatolia, the Levant, and the Iranian Plateau; therefore, the moderate mitochondrial diversity observed in Basrah populations likely reflects both historical dispersal routes and contemporary anthropogenic movement of colonies.

Recent genomic investigations published in (Lukić, *et al.* 2024) further highlight the importance of fine-scale population structuring in honeybees, demonstrating that environmental gradients and commercial queen trade strongly influence mitochondrial and nuclear genetic composition. The differentiation detected between Al-Zubair and Al-Siba populations in our cluster analysis (UPGMA) may thus be interpreted as the combined result of local ecological pressures—such as extreme temperature regimes and habitat fragmentation—and human-mediated colony exchange. The higher diversity detected in Al-Zubair could plausibly be linked to cross-border beekeeping activities and importation of queens, given its geographic proximity to Kuwait and regional trade routes.

The clustering pattern obtained in this study is consistent with previously described phylogeographic structures in Near Eastern populations, where genetic grouping often correlates more strongly with ecological and historical factors than with simple geographic distance. Similar patterns have been documented in populations from Syria, Turkey, and Iran, where mitochondrial haplotypes reflect both ancient colonization processes and recent introgression events.

From a biogeographical perspective, the Iraqi honeybee populations examined here appear to represent a southern extension of the O-lineage distribution described by Garnery and subsequent authors. However, the detected polymorphism suggests that these populations are not genetically

homogeneous. Instead, they display a moderate level of internal differentiation, likely shaped by climatic extremity, salinity gradients in southern Iraq, and the long-standing tradition of migratory beekeeping.

Importantly, the genetic diversity observed in this study falls within the range reported for neighboring Middle Eastern populations, reinforcing the idea that Iraqi honeybees are integrated into a broader regional gene pool rather than being isolated. This finding has practical implications for conservation and breeding programs. Preservation of locally adapted mitochondrial haplotypes is crucial, particularly under the current challenges of climate change and increasing colony trade (Franck, *et al*.2000).

Conclusions:

The present results corroborate previous regional and international findings on the phylogeographic structure of *Apis mellifera* in the Near East. By providing the first mitochondrial characterization of honeybee populations in Basrah Province, this study fills an important knowledge gap and establishes a molecular baseline for future integrative studies combining mitochondrial, nuclear, and genomic approaches. Further investigations using high-resolution markers (e.g., SNP panels or whole-genome sequencing) are recommended to clarify the extent of introgression and to support evidence-based management strategies for Iraqi honeybee populations.

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التنوع الوراثي لنحل العسل الغربي Apis mellifera في محافظة البصرة –العراق، باستخدام تقنية RFLP-PCR

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الملخّص

هدفت هذه الدراسة إلى تحليل التنوع الوراثي لنحل العسل *Apis mellifera* في محافظة البصرة جنوب العراق باستخدام تقنية تعدد أشكال طول قطع التقييد (RFLP-PCR) لمنطقة COI-COII في الحمض النووي للميتوكوندري (mtDNA). تم جمع 980 عينة من 98 خلية نحل موزعة على ست مناطق جغرافية. أظهرت النتائج تنوعاً وراثياً متوسطاً (75%)، مع ظهور ثلاث نطاقات سائدة (1200 زوج قاعدي و800 زوج قاعدي و400 زوج قاعدي) تشير إلى تعدد الأشكال. صنّف التحليل التطوري UPGMA العينات إلى مجموعتين رئيسيتين، مما يعكس اختلافات وراثية مرتبطة بالتوزيع الجغرافي. تؤكد هذه النتائج التاثير الهام والمعنوي للعوامل الجغرافية في تشكيل التنوع الوراثي لنحل العسل في المنطقة المدروسة.

الكلمات المفتاحية: *Apis mellifera*، الحمض النووي للميتوكوندري، تعدد الأشكال الوراثية، RFLP-PCR، نحل العسل الغربي، العراق.