# Mitogenomic Variation of Five *Apis mellifera* Subspecies Populations Using Mitochondrial Protein-Coding Genes

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## Abstract

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Honeybee, is essential to both the preservation of biodiversity and the security of the world's food supply. Identifying genetic variation is a crucial step in preserving diversity. The current study used thirteen mitochondrial coding protein genes to characterize molecular genetic variation among populations of five *Apis mellifera* subspecies. The results obtained showed that the populations of both subspecies, *Apis mellifera mellifera* and *Apis mellifera jemenitica*, had a higher mean in genetic diversity features such as nucleotide diversity, the number of pairwise differences, and polymorphic sites. While the *Apis mellifera ligustica* subspecies population had the lowest mean of the same parameters. The patterns of genetic differentiation and gene flow revealed that *Apis mellifera scutellate*, *Apis mellifera capensis*, and *Apis mellifera mellifera* populations were the most closely related in terms of their mitogenomic sequences, whereas *Apis mellifera jemenitica* and *Apis mellifera ligustica* populations were the most distant mitogenomically within and between populations. Phylogeny, PCA, and haplotype network analysis revealed that some individuals in different subspecies had the same haplotypes. These findings imply that the genetic integrity of native honeybees is threatened as individuals from several subspecies that share the same mitogenomics.

**Keywords:** Genetic diversity, honeybees, introgression, mitochondrial DNA.

## Introduction

In both natural and agricultural contexts, honeybees (genus *Apis*) are the most commercially useful pollinators and play an important role in maintaining ecosystem processes (Hung *et al.*, 2018). The *Apis* genus is thought to have originated in Asia, where it has since diverged into three distinct groups according to the morpho-behavioural taxonomy, including giant honeybees (*Apis dorsata*, *Apis laboriosa*, and *Apis breviligula*), dwarf honeybees, *Apis andreniformis* Smith, 1858 and *Apis forea* Fabricius, 1787, and cavity-nesting honeybees, *Apis mellifera* together with *Apis nigrocincta* Michener 1974, *Apis nuluensis* Engel, 1999, *Apis cerana*, and *Apis koschevnikov* Grigorii, 1866. They adapte to various ecological circumstances (Arias & Sheppard, 2005; Dogantzis *et al.*, 2021). Thirty-three different subspecies of honey bees are found in Africa (eleven subspecies), Western Asia and the Middle East (nine subspecies), and Europe (thirteen subspecies). These subspecies are further divided into five evolutionary lineages: lineage A (ten subspecies), lineage M (three subspecies), lineage C (10 subspecies), lineage O (three subspecies), lineage Y (one subspecies), and lineage C or O (3 subspecies) (Ilyasov *et al.*, 2020). *Apis mellifera* is the only species that is native to Africa and Europe, and it has over 30 nominal subspecies (Engel, 1999). Historically, these were classified into four continental categories known as ACMO (African, Continental, Mellifera, and Oriental) (Ruttner, 1988).

The geographical origin and evolutionary expansion of these categories, as well as their resolution into ancestor-descendant phylogenetic lineages, are still debated (Carr, 2023). Molecular research based on examinations of numerous nuclear genome elements generally concur that they should be rearranged as an MAOC backbone, rooted to provide two different origin theories: "Trice Out of Africa" (root within A) or "Trice Out of Asia" (root between A & O) (Dogantzis *et al.*, 2021; Whitfield *et al.*, 2006). Molecular approaches are effective instruments for addressing a wide range of insect ecology research concerns. For example, mitochondrial DNA (mtDNA) has been widely used as a useful molecular genetic marker in evolutionary biology, species identification, biodiversity assessment, ecology, and phylogenetic studies due to its technical ease-of-use and favorable biological properties such as maternal inheritance, near-neutrality, lack of recombination, and high mutation rate compared to nuclear DNA (Dong *et al.*, 2021; Freeland *et al.*, 2011).

The insect mitogenome, like that of several animal species, is a circular, double-stranded molecule of 15-18 kb in length that contains 37 genes, including 13 mitochondrial protein coding genes (MPCGs), 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes, and a noncoding region (the control region, CR) that may regulate replication and transcription (Cameron, 2014). Several species and subspecies of the gene *Apis* have recently had their entire mitogenomes sequenced and used in phylogenetic relationships, including *Apis andreniformis* (Wang *et al.*, 2015), *Apis laboriosa* Michael, 1999 (Chhakchhuak *et al.*, 2016a), *Apis mellifera syriaca* Michael, 1999 (Haddad, 2016), *Apis cerena cerana* Johan Christian 1773 (Chhakchhuak *et al.*, 2016b), *Apis mellifera scutellate* Winston, 1992 and *Apis mellifera capensis* Hamilton,1964 (Eimanifar *et al.*, 2020), *Apis mellifera ligustica* subspecies Barrett, 1898, *Apis mellifera anatoliaca* Ruttner, 1988 and *Apis mellifera adansonii* Michael Adansonin, 1957 (Boardman *et al.*, 2020a; 2020b). These and previous studies,

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however, were limited to the complete sequencing, phylogeny, and characterization of individual mitogenomes. The mitogenomic variation of *Apis mellifera* subspecies at the population level has yet to be explored.

This study aims to investigate mitogenomic diversity indices, estimate genetic differentiation and gene flow, and establish haplotype networking in the populations of five *Apis mellifera* subspecies worldwide: *Apis mellifera scutellate* Winston 1992 (AMS), *Apis mellifera mellifera* Linnaeus, 1758 (AMM), *Apis mellifera capensis* Hamilton,1964 (AMC), *Apis mellifera jemenitica* Ruttner 1976 (AMJ), and *Apis mellifera ligustica* Barrett, 1898 (AML).

## Materials and Methods

**Complete protein coding genes**

Sixty seven complete mitogenomes of five subspecies of *Apis mellifera* worldwide: (AMS, N=6), (AMM, N=10), (AMC, N=15), (AMJ, N=15), and (AML, N=21) were downloaded from the GenBank of the NCBI database. The GenBank accession numbers for complete mitogenome for the five subspecies are presented in the phylogenetic tree (Figure 1). The complete mitogenomes of each subspecies were aligned using the Geneious Prime software (Kearse *et al.,* 2012). The same software was used to extract all 13 protein coding genes and concatenate them together. The total length of all 13 concatenated protein-coding genes was approximately 11029 bp.

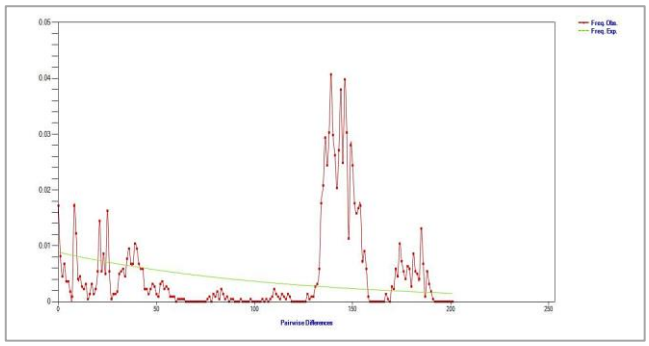

**Figure 1.** Mismatch distribution of populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML under the population expansion (growth) model. The x axis shows the number of nucleotide substitutions in a pairwise comparison of haplotypes, while the y axis represents the frequency (Freq.) of haplotypes. The dotted green line indicates the anticipated distribution (Exp.), while the solid red line reflects the observed distribution (Obs.).

**Phylogenetic analysis**

After alignment of mitogenome sequences of all 13 protein coding genes using the Geneious software, the software package MEGA version 11 (Molecular Evolutionary Genetics Analysis) (Tamura *et al.,* 2021) was used to discover the optimal substitution model. As a result, using the Geneious Prime software, the phylogenetic relationships between the 67 individuals of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ and AML based on the analysis of protein coding regions were built using the following parameters: genetic distance model; Tamura-Nei (TN93); tree build technique; Neighbour-Joining method; and bootstrapping 1000 number of iterations. The species (*Apis dorsata*) was used as an outgroup.

**Assessment of genetic variation**

Parameters of genetic diversity including number of polymorphic sites (PS), number of haplotypes detected (H), haplotype diversity (Hd±SD), nucleotide diversity (π±SD), average number of nucleotide differences (k), singleton variable sites (SVS), parsimony informative sites (PIS), Tajima's D and mismatch distribution of populations based on the analysis of protein coding regions of populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML were estimated using the DnaSP6 (DNA Sequence Polymorphism Analysis) software (Rozas *et al.,* 2017).

**Genetic differentian and gene flow**

The DnaSP6 software (Rozas *et al.,* 2017) was used to calculate genetic differentian (Fst) and gene flow (Nm) between the five populations of *Apis mellifera* subspecies. The ggplot2 package in Rstudio version 3.4.1 was used to create bar charts representing the values of genetic differentiation and gene flow of five subspecies of *Apis mellifera*. SPSS-IBM software was used to create the Principal Components Analysis (PCA) in order to visualize the genetic distance in the form of clusters between the five populations based on the pairwise Fst distances using factorial dimension reduction.

**Mitogenomic pairwise**

The mitogenomic differences between the 67 individuals of the five subspecies of the *Apis mellifera* in the form of the mitogenomic pairwise represented in the number of polymorphic sites were calculated using the Geneious software. TBTools (Chen *et al.,* 2020) was used to create Principal Components Analysis (PCA) in order to visualize genetic distance between 67 individuals of five subspecies of *Apis mellifera* based on the polymorphic sites of mitogenomic pairwise.

**Haplotype Network Analysis**

The PopART network analysis software (Population Analysis with Reticulate Trees) (Leigh & Bryant, 2015) was used to identify numbers, identities, networking and visualization of haplotypes of the 67 individuals of five subspecies of *Apis mellifera* through creating a median joining network.

## Results and Discussion

**Genetic variation patterns of inter-population**

Genetic variation is required for species adaptation and survival, and its assessment is critical for species management and the establishment of conservation measures, particularly for endangered species (Hedrick,

2004). Parameters of genetic diversity based on the analysis of thirteen mitochondrial protein coding genes of populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML were estimated using DnaSP v6 software. As a result, the populations of both subspecies AMM and AMJ had a higher mean in nucleotide diversity ($\pi$=0.0082, $\pi$=0.0077) and in the number of pairwise differences (k= 90.91, k= 85.44) than the populations of other rest subspecies (Table 1). Similarily, the same two populations AMM and AMJ had the highest number of polymorphic sites (PS= 283, PS=205) over the consensues of the sequence alignment of protein coding regions respectiveily. While, the population of AML subspecies had the lowest mean of nucleotide diversity ($\pi$=0.0011), lower number of pairwise differences (k=12.51) and polymorphic sites (PS=52) (Table 1). Regarding the haplotype diversity (Hd) mean, no changes were found in the three populations of subspecies AMS, AMM and AMC. However, the haplotype diversity mean was less than 1 in the populations of the two subspecies AMJ and AML (Hd=0.933±0.045, Hd= 0.852±0.055), respectively (Table 1). The five populations showed different numbers of single variable sites (SVS) and parsimony informative sites (PIS) in which, in total, the numbers of PIS were higher than the SVS numbers over the consensus of the alignment of 67 mitochondrial protein coding regions. Interestingly, genetic parameters like nucleotide diversity, haplotype diversity, haplotype number, and polymorphism site number were notably low in the population of the subspecies AML (Table 1). This indicates that these subspecies have low genetic variation between their individuals. Moreover, the analysis of mismatch distribution and neutrality tests revealed that all five populations (AMS, AMM, AMC, AMJ, and AML) were expanding demographically, owing to negative Tajima's D values in all populations except AMJ (Figure 1).

**Genetic differentiation (Fst) and gene flow (Nm)**

In population genetics research, the fixation index (Fst) is one of the descriptive statistics that is most frequently used because it provides important insights into the evolutionary processes that affect the pattern of genetic variation both within and between populations (Holsinger & Weir, 2009). Additionally, gene flow is one of the factors that maintains genetic diversity within and between species, which is influenced by the behavior, geographical characteristics, migration, and natural selection (Garg & Chattopadhyay, 2021). The classification of *Apis mellifera* subspecies poses several challenges due to the complexity of their population structure and resolution of differentiation procedures (Ilyasov *et al.*, 2020). Therefore, in this study, and by using the DnaSP v6 program, the genetic distance based on the Fst index and gene flow between population pairs of five subspecies of *Apis mellifera* (Figure 2) was determined. Consequently, the patterns of genetic differentiation and gene flow showed that the populations that are most closely related to one another genetically were AMS with AMM, AMM with AMC, AMS with AMC, and AMM with AMJ, with Fst values ranging from 0.191 to 0.359 and gene flow ranging from 2.11 to 0.89, respectively (Table 2, Figure 2). The remaining population pairs, including AMS with AMJ, AMC with AMJ, AMM with AML, AML with AMJ, AMS with AML, and AML with AMC, were the most distant from one another mitogenomically. Their Fst values ranged from 0.621 to 0.875, whereas their gene flow values ranged from 0.3 to 0.07 (Figure 2). Due to the lack of natural separation mechanisms, most subspecies experienced continual gene flow, while some isolated populations remained in mountains and islands, such as the populations of dark honey bees in Russia's Ural Mountains and Denmark's Læsø Island (Ilyasov *et al.*, 2020). Additionally, using IBM SPSS's statistical software, the pairwise Fst distances at the population level from Figure 2 were plotted on the first two axes of a Principal Components.

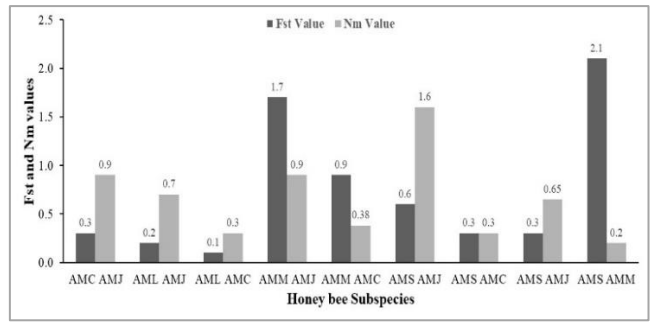


**Figure 2.** Pairwise Fst values and gene flow between populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of protein coding regions.

**Table 1.** Parameters of genetic diversity based on the analysis of protein coding regions of populations of five subspecies of *Apis mellifera*. worldwide: AMS, AMM, AMC, AMJ, and AML.

| | | Genetic diversity parameters | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| # | Population | N | PS | H | Hd±SD | Π | k | SVS | PIS | Tajima's D |
| 1 | AMS | 6 | 71 | 6 | 1.000±0.096 | 0.0024 | 26.66 | 56 | 15 | -0.9171 |
| 2 | AMM | 10 | 283 | 10 | 1.000±0.045 | 0.0082 | 90.91 | 118 | 165 | -0.5038 |
| 3 | AMC | 15 | 117 | 15 | 1.000±0.024 | 0.0021 | 23.26 | 77 | 40 | -1.5772 |
| 4 | AMJ | 15 | 205 | 10 | 0.933±0.045 | 0.0077 | 85.44 | 8 | 197 | 1.5141 |
| 5 | AML | 21 | 52 | 10 | 0.852±0.055 | 0.0011 | 12.51 | 25 | 27 | -0.5353 |
| | Overall | 67 | 559 | 51 | 0.983±0.008 | 0.0101 | 112.03 | 164 | 394 | -0.2406 |

The number of samples per population (N), number of polymorphic sites (PS), number of haplotypes detected (H), haplotype diversity (Hd±SD), nucleotide diversity ($\pi$±SD), standard deviation (SD), average number of nucleotide differences (k), singleton variable sites (SVS), parsimony informative sites (PIS), and Tajima's D.

Analysis (PCA) in order to better comprehend the genetic distances between all populations. Therefore, through the PCA feature, pairwise Fst values showed a number of specific categories (Figure 3). The subspecies AML population was concentrated on the left side of the plot, but the AMJ population was located on the top center side, showing that these two populations are genetically very distant from each other as well as from the other three subspecies AMM, AMS, and AMC populations (Figure 3). The populations of the subspecies AMM, AMS, and AMC were clustered on the right side of the plot, showing their close genetic similarity (Figure 3). The AMC subspecies from the center and east of Africa and the AMC populations from the Cape region of South Africa were combined in the PCA results, indicating high mitogenomic similarity between both subspecies (Figure 3). Both the AMC and AMS clusters shared a genetic relationship with the AMM population from northern Europe. Genetic differentiation between populations is higher in species with limited gene flow than in species with substantial gene flow (Hamrick *et al.*, 1991). It's interesting to note that populations of AML from Italy and AMJ from Saudi Arabia showed significant genetic separation from one another and from the other three population clusters of AMS, AMC, and AMM. The mitogenomic far distance of population of subspecies AMJ could be due to the ecological conditions. Only native AMJ can thrive in western and central Saudi Arabia, where temperature extremes typical of the Arabian Peninsula apply; other standard subspecies are unable to endure under these conditions (Alattal & Alghamdi, 2015). The analysis of genetic differentiation and gene flow at the population level of the five subspecies investigated herein revealed that while some subspecies are still significantly distinct, current management measures do not impede gene flow.

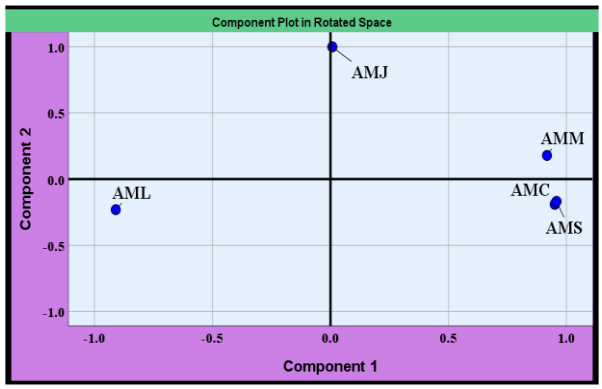


**Figure 3.** Principal Components Analysis (PCA) among populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of protein coding regions. PCA performed based on the pairwise Fst distances using factorial dimension reduction with SPSS software. Blue-circles represent populations.

**Phylogenetic relationships**

Phylogenetic studies based on mitogenomic lineages can be used to examine genetic diversity and track the origin and ancestry of distinct species by assessing nucleotide variations within mtDNA sequences (Al-Jumaili *et al.*, 2020; Mustafa, 2021; Mustafa *et al.*, 2018; 2022; Yang *et al.*, 2017; Yousif & Taha, 2023). Protein coding genes encoded by mitochondrial DNA in insects have been intensively exploited for maternal phylogeny, evolutionary relationships, population and conservation genetics, and genetic diversity (Dong *et al.*, 2021). The phylogenetic relationships between the 67 individuals of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of 13 mitochnodrial protein coding genes were studied (Figure 4). Results obtained showed that individuals of both subspecies AMS and AMC are highly related to each other (Figure 4). Whereas, individuals of both subspecies AML and AMJ showed two monophyletic clade with BP=100. However, individuals of subspecies AMM were distributed to different clades with individuals of other subspecies AMS, AMC and AMJ (Figure 4). Humans have carried *Apis mellifera* over the world as the primary commercial source of honey and beeswax, and it is also of great agricultural value as an efficient pollinator of some imported vegetable and fruit crops (Requier *et al.*, 2019). Individuals of both subspecies AMC and AMS are clustered within the same clade of phylogenetic tree and they are paraphyletic to other clades of subspecies AML and AMJ.

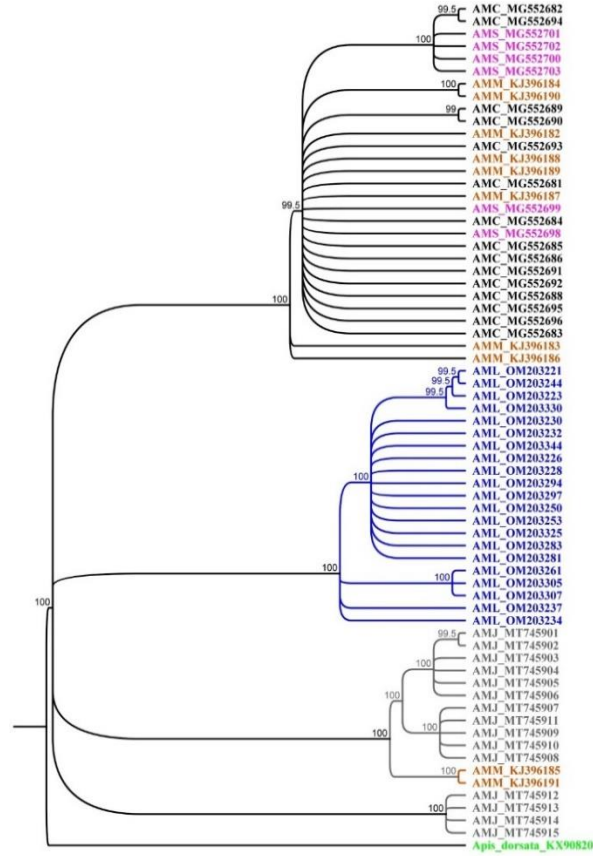


**Figure 4.** Phylogenetic relationships between populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of protein coding regions. The numbers beside the nodes or above the lines represent Bootstrap support (BP). GenBank accession numbers are shown before the scientific names, while their origin is shown in parentheses. The species (*Apis dorsata*) was used as an outgroup.

These findings are consistent with a previous study (Carr, 2023) that examined phylogenetic relationships among mtDNA genome sequences from more than 60 individual honey bees from 22 *A. mellifera* subspecies using parsimony analysis. They discovered that the Sub-Saharan clade included individuals from both subspecies AMS and AMC, and that these were paraphyletic to clades included individuals from additional subspecies AML, AMJ, and AMM (Carr, 2023). Furthermore, in the phylogenetic tree, the AMM individuals were dispersed over different clades of individuals of subspecies AMC, AMS, and AMJ. Similarly, hybridization between allopatric and sympatric populations of the AMM and A. m. carnica subspecies of honeybees has been observed through the use of microsatellite markers (Soland-Reckeweg *et al.*, 2009).

**Principal components analysis (PCA) and haplotype networking**

In order to visualize genetic distance between 67 individuals of five subspecies of *Apis mellifera*, the polymorphic sites of mitogenomic pairwise (Table 1), was used to create principal components analysis (PCA) using TBTools (Chen *et al.*, 2020). In order to examine the median-joining network analysis, the alignment of the 67 complete sequences of protein coding regions of five subspecies of *Apis mellifera* were compared with each other using PopART software (Figure 5). Overall, results of both PCA of polymorphic sites and the networking of the alignment of the 67 complete sequences of mitochondrial protein coding genes revealed three distinct clusters (Figure 6). Individuals of both AML and AMJ produced independent cluster of haplotypes, while the individuals of AMC and AMS shared their haplotypes. However, the AMM individuals were dispersed over the haplotypes of individuals of AMC, AMS and AMJ. Similarily, Muñoz & De la Rúa, 2021 provided evidence for introgression events from neighboring *Apis mellifera* subspecies based on the study of mtDNA and microsatellite analyses. The degree of introgression, the presence of foreign haplotypes in some of these groups of bees, and the formation of hybrid populations all indicated that non-native subspecies invasions pose a major danger to the genetic integrity of native honey bee populations (Muñoz & De la Rúa, 2021).The population-level data in this study revealed that the mean values of genetic diversity parameters such as nucleotide diversity, the number of pairwise differences, and polymorphic sites were higher in both AMM and AMJ subspecies. For the same features, the AML subspecies population had the lowest mean. According to patterns of genetic differentiation and gene flow, the AMS, AMC, and AMM populations are the most closely linked in terms of mitogenomic sequences, whereas the AMJ and AML populations are the most distant within and between populations. Individual-level results based on phylogenetic relationships, haplotype networking, and PCA analysis, on the other hand, revealed that AML and AMJ individuals are distinct subspecies, whereas AMC and AMS individuals, particularly AMM individuals, showed introgressive hybridization. These findings suggest that conservation strategies should be improved, which may entail greater mating control if introgression persists.

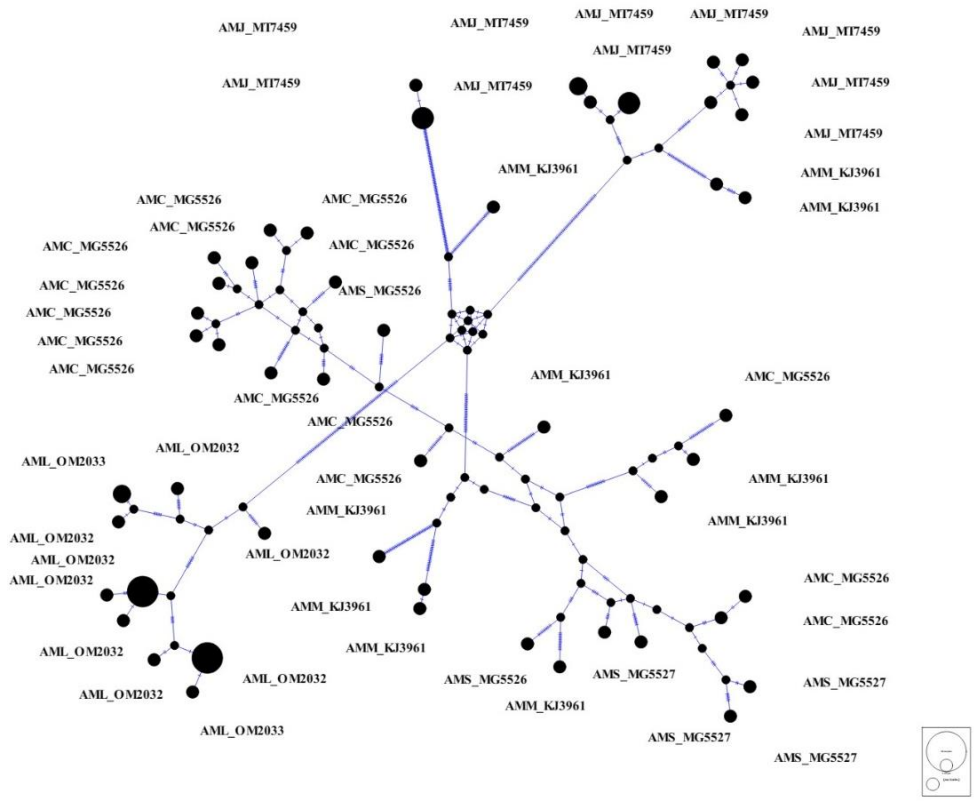


**Figure 5.** A median-joining network creates network structure and characterizes haplotype relationships between 67 samples of populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of protein coding regions. Each circle's size is proportional to the number of samples with a certain haplotype.

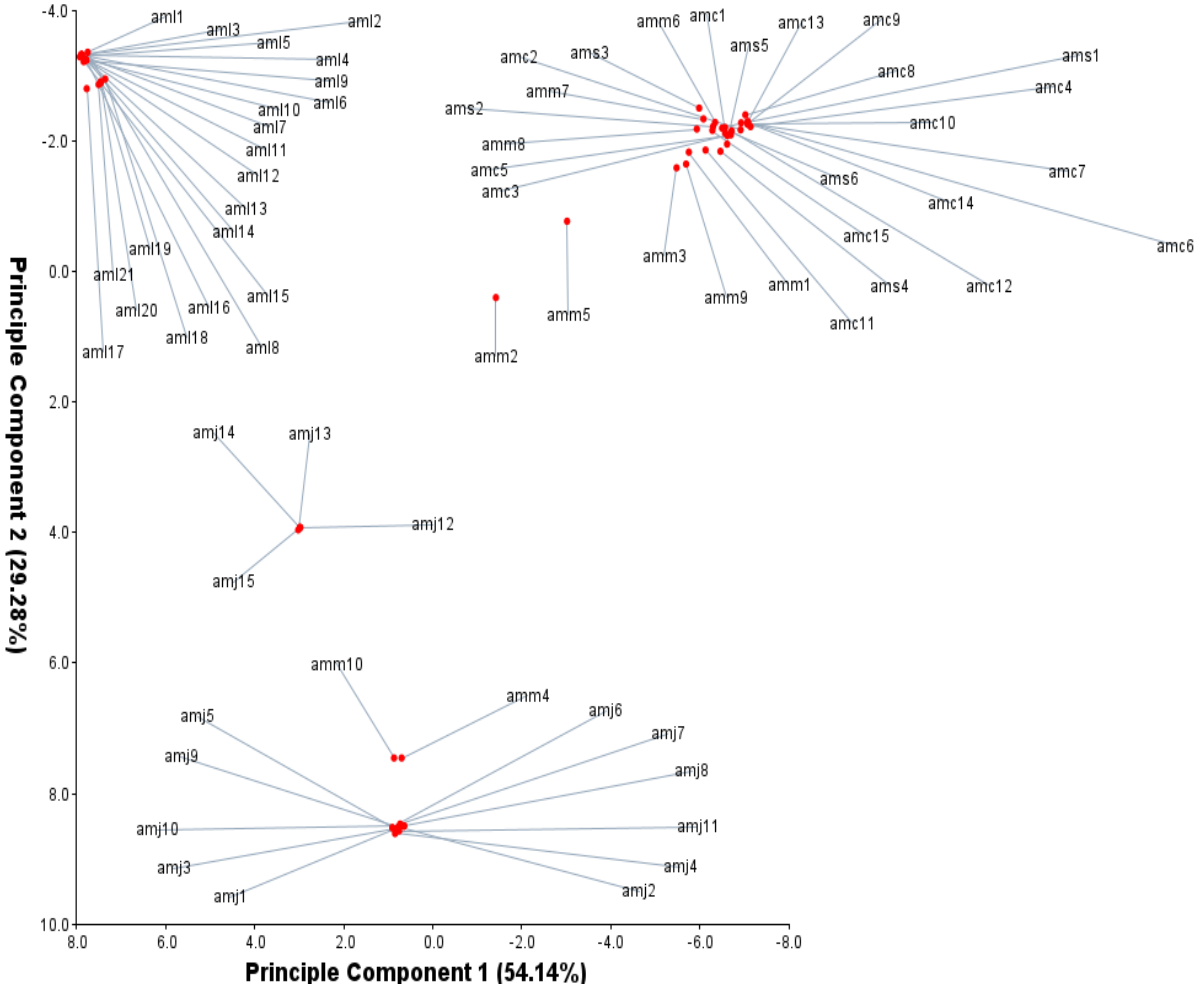


**Figure 6.** Principal Components Analysis (PCA) between 67 samples of populations of five subspecies of *Apis mellifera* worldwide: AMS, AMM, AMC, AMJ, and AML based on the analysis of protein coding regions. PCA performed based on the number of polymorphic sites using factorial dimension reduction with SPSS software. Grey-circles represent the individuals. See additional Table regarding polymorphic sites represented in matrix.


## الملخص

**بيبان، بشتيوان سعيد أمين. 2025. الاختلافات الجينية بين خمسة تحت أنواع لنحل العسل *Apis mellifera* باستخدام جينات الميتاكوندريا المشفرة للبروتينات. مجلة وقاية النبات العربية، 43(1):38-45.** https://doi.org/10.22268/AJPP-001294

إنّ نحل العسل ضروري لتعزيز الأمن الغذائي والتنوع الحيوي. ركزت هذه الدراسة على ثلاثة عشر جيناً في الميتاكوندريا لتوصيف الاختلافات الجينية بين خمسة تحت أنواع من نحل العسل (*Apis mellifera*). أظهرت النتائج بأن تحت النوعين *Apis mellifera mellifera* و *Apis mellifera jemenitica* كليهما ذي متوسط أعلى في سمات التنوع الجيني مثل تنوع النيوكليوتيدات، عدد الاختلافات الزوجية والمواقع متعددة الأشكال. في حين أن مجموعة سلالات *Apis mellifera ligustica* لديها أقل متوسط للمعايير نفسها. كشفت أنماط التمايز الوراثي وتدفق الجينات أن مجموعات تحت الأنواع *Apis mellifera scutellate*، *Apis mellifera capensis* و *Apis mellifera mellifera* كانت الأكثر ارتباطاً من حيث تسلسلاتها الجينية. في حين أن مجموعات *Apis mellifera jemenitica* و *Apis mellifera ligustica* كانت الأكثر بعداً من الناحية الميتوجينية داخل المجموعات وفيما بينها. كشف تطور السلالات وتحليل العناصر الأساسية (PCA) وشبكة النمط الإفرادي أن لبعض الأفراد من سلالات مختلفة نفس الأنماط الإفرادية. تشير هذه النتائج إلى أن السلامة الوراثية لنحل العسل الأصلي مهددة كأفراد من عدة سلالات فرعية تشترك في التركيب الوراثي نفسه للميتاكوندريا.



**كلمات مفتاحية:** التباين الوراثي، نحل العسل، الإضافات الجينية، الحمض النووي الميتوكوندري.



**عناوين الباحثين:** بشتيوان سعيد أمين بيبان، قسم علوم المختبر الطبية، كلية العلوم، جامعة شمرو، إقليم كردستان، العراق. البريد الإلكتروني للباحث المراسل: pshtiwan.saeed@charmouniversity.org

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