Molecular insights of mitochondrial 16S rDNA genes of the native honey bees subspecies *Apis mellifera carnica* and *Apis mellifera jementica* (Hymenoptera: Apidae) in Saudi Arabia

Reem Alajmi¹, Rewaida Abdel-Gaber¹²*, Loloa Alfozana¹

¹Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

²Faculty of Science, Department of Zoology, Cairo University, Cairo, Egypt

Corresponding author: Rewaida Abdel-Gaber

E-mail: rewaida@sci.cu.edu.eg

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ABSTRACT. The honey bee *Apis mellifera* is of major importance for the world’s agriculture and is also suitable for environmental monitoring. It includes several recognized subspecies distinguished by using morphological and morphometric variants. Here, 200 adult worker *Apis mellifera* honey bees were collected from Hail region, Saudi Arabia. Mitochondrial 16S rDNA was conducted to detect molecular polymorphism among honey bee *A. mellifera* subspecies. The amplified and sequenced gene regions of mtDNA revealed the presence of two different subspecies of *Apis mellifera carnica* (gb|MH939276.1) and *Apis mellifera jementica* (gb|MH939277.1). The sequences were compared with each other and with others retrieved from the GenBank demonstrating a high degree of similarity (up to 72%). The NJ tree indicated that all *Apis* species are clustered together in one clade in addition to the genetically origin of *Apis* species within family Apidae as a paraphyletic group within the African lineage.

Keywords: *Apis mellifera*; Genetic characterization
INTRODUCTION

Bees are the world’s most important pollinators in natural and agricultural ecosystems. They are very diverse with over 20,000 species (Engel, 2005, 2011a). Honey bees belong to the family Apidae with other social bees. The subfamily Apidae is comprised of a single genus, *Apis*. The genus *Apis* comprises *Apis florea* (the little bee), *Apis dorsata* (the giant bee), *Apis cerana* (the eastern bee) and *Apis mellifera* (the western bee). The honey bee, *Apis mellifera* Linnaeus, 1758, is naturally found throughout Europe, Africa and Western Asia (Miguel et al., 2011). Traditionally, the intraspecific taxonomy of the honey bee *Apis mellifera* L. has been based on morphology. Twenty-nine subspecies of *A. mellifera* are currently recognized on the basis of morphometric characteristics (Sheppard and Meixner, 2003; Miguel et al., 2011). These subspecies are also described as ‘geographic races’ because their distributions correspond to distinct geographic areas (Ruttner, 1992). Five evolutionary lineages have been characterized based on morphometric, molecular, ecological, ethological, and physiological traits (De la Rúa et al., 2005). The four primary lineages are found in the Mediterranean Basin, including the African lineage (A), west and north European lineage (M), south-east European lineage (C), and Near and Middle Eastern lineage (O) (Franck et al., 2000a, 2001; Miguel et al., 2007; Cánovas et al., 2008). The fifth proposed lineage is north-east African (Y) (Franck et al., 2001). 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 because they can be sensitive to environmental selection pressures (Franck et al., 2000b).

A number of molecular markers such as nuclear DNA (Hall, 1990; Tarès et al., 1993), mitochondrial DNA (mtDNA) (Moritz et al., 1986; Smith et al., 1989; Smith, 1991; Hunt and Page, 1992; Garnery et al., 1993; Oldroyd et al., 1995; Arias and Sheppard, 1996; Pedersen, 1996; De la Rúa et al., 2000), and microsatellites (Estoup et al., 1993; Garnery et al., 1998), are also used to study genetic variability in honey bees (Boore, 1999). The sequencing and characterization of the mtDNA genome have been very useful for analyzing the phylogeny and population genetic structure of the *Apis* species and of *A. mellifera* subspecies because it contains regions with variable evolutionary rates. In general, mtDNA contains genes for two ribosomal subunits (12S and 16S), 22 tRNA, and 13 proteins (three subunits of cytochrome c oxidase, cytochrome B, subunits 6 and 8 of ATP F0 synthase, and seven subunits of NADH dehydrogenase) (Shao et al., 2003; Silvestre and Arias, 2006). Genetic markers such as the mtDNA COXI–COXII intergenic region are unique to the genus *Apis* (Cornuet and Garnery 1991). Variations in the sequences of this region or the length of fragments produced using endonucleases are used extensively to differentiate the five honeybee lineages and to discriminate among *A. mellifera* subspecies (Garnery et al., 1992; Franck et al., 2000a; Sheppard and Smith, 2000). To date, no study has characterized Saudi honey bees. Therefore, the goal of this study was to determine the genetic diversity and phylogenetic relationships of *Apis mellifera* subspecies of Saudi Arabia via mitochondrial 16S rDNA genes.

MATERIALS AND METHODS

Honey bee sample collection

A total of 100 samples were collected from adult worker bees of each breeder (200 samples) from honey bees in the Hail region of Saudi Arabia. Bee samples were collected during the month of September 2017 after approval from the beekeepers. Bees were collected inside the wooden cages containing food with passage of air and sunlight. The samples were transferred alive to an Entomology lab, frozen for 5 min to immobilize them, and then stored in 95% ethanol until processed. The two strains were identified according to the external characteristics with measurements of different body parts. Some samples were sent to a specialist for definition of the bee breeds with Dr. Yahya Al-Attal, Faculty of Agriculture, King Saud University, Saudi Arabia.
Molecular insights of mitochondrial 16S rDNA genes of the native honey bees subspecies *Apis mellifera carnica* and *Apis mellifera jementica* (Hymenoptera: Apidae) in Saudi Arabia

Molecular analysis

**DNA extraction, PCR amplification, and sequencing:** Mitochondrial genomic DNA was extracted from the thoracic legs of the ethanol-preserved samples. The tissue was homogenized using a glass pestle and a mortar. The genomic DNA in the homogenate was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The purity and concentration of extracted DNA was determined with a NanoDrop 2000 UV-VIS Spectrophotometer (Thermo Fisher Scientific Inc, USA) at 260/280 nm. The extracted DNA was stored at -20°C until use. Mitochondrial 16S rDNA gene fragments were amplified using GeneJETTM PCR Purification kit [Thermo (Fermentas)] following the manufacturer's protocol in a total volume of 50 µl including 5 µl 10 × buffer, 5 µl of each dNTP (10 mM), 10 µl of each primer (1 pmol/ µl), 0.3 µl of Taq polymerase (5 U/ml), 2.5 µl MgCl₂ (50 mM), and 2 µl of total genomic DNA. PCR amplification and subsequent DNA sequencing was carried out using the following mitochondrial 16S rDNA primers: 16SF (5’-CAC CTG TTT ATC AAA AAC AT-3’) and 16SR (5’-CGT CGA TTT GAA CTC AAA TC-3’), designed by Crozir and Crozier (1993).

The PCR cyclic conditions were processed under the following conditions: 95°C for 15 min (initial denaturation), 35 cycles of 15 s at 95°C (denaturation), 30 s at 62°C (annealing), and 1 min at 72°C (extension), and finally post-PCR extension for 5 min at 72°C. Each amplicons was examined by (1%) agarose gel electrophoresis in 1× Tris–acetate–EDTA (TAE) buffered gel stained with 1% Ethidium bromide. This was then visualized with a UV transilluminator, and bands with the predicted size were purified using QIAquick PCR Purification Kit (Qiagen) following the manufacturer’s instructions. Amplicons were sequenced (in both directions) using an ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) with an Applid Analyzer Biosystems (Thermo Fisher Scientific, USA) using the same primers for annealing.

Sequence alignment and phylogenetic analysis

BLAST search was done to identify related sequences on NCBI database. Sequences were aligned directly using CLUSTAL-X multiple sequence alignment (Thompson et al., 1997) and others available from GenBankTM selected to represent all available tapeworm lineages with an emphasis on taxa presumed to be related to the analyzed groups. The alignment was manually corrected using the alignment editor of software BIOEDIT 4.8.9 (Hall, 1999). Phylogenetic calculations were performed with the UPGMA method. The data were analyzed with the Maximum Composite Likelihood (MCL) approach based on the Kimura 2-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Phylogenetic and evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000).

RESULTS

Molecular analyses based on the partial 16S rDNA gene sequence were performed to investigate the taxonomy and classification of the two recovered *Apis mellifera* subspecies. The amplified and sequenced gene regions and GC content in the samples from *Apis mellifera carnica* and *Apis mellifera jementica* were 1157 bp/29% and 682 bp/25.7%, respectively. The sequences were deposited in GenBank under the accession numbers, MH939276.1 and MH939277.1, respectively. The sequences were compared with each other and with other sequences available on GenBank from varied geographical regions (Table 1). The calculation of the percentage of identity between these novel sequences and a range of sequences for other arthropods of different host species demonstrated a high degree of similarity (up to 72%) with intra-specific differences that varied from 0.1% to 23.8% (Figure 1).
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<th>Parasite species</th>
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**Table 1.** Arthropoda species used in the phylogenetic analysis of the present sub-species *Apis mellifera*
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**Figure 1.** Estimates of evolutionary divergence between sequences. The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 168 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

The comparison of nucleotide sequences and divergence showed that 16S rDNA in the present species has the highest BLAST scores with lower divergence values than other apids previously sequenced; however, our sample was not identical to any prior specimen (Figure 2). A tree topology was automatically computed to estimate ML values (Figure 3). Two clades were clustered during the construction of the phylogenetic tree using the maximum likelihood and maximum parsimony of the present apids species. The major clade clustering all Pancrustacean species contained two classes: the first one was Crustacea represented here by *Daphnia pulex* (gb| DQ470571.1) within family Daphniidae; the second was Insecta with families Apidae (*A. mellifera scutellata* gb| MG552702.1, *A. mellifera capensis* gb| MG552697.1, *A. mellifera sahariensis* gb| MF351881.1, *A. mellifera Lamarckii* gb| KY464958.1, *A. mellifera syriaca* gb| KP162643.1, *A. mellifera intermissa* gb| KM458618.1, *A. mellifera monticola* gb| MF67858.1, *A. mellifera syriaca* gb| KY926882.1, *A. mellifera carnica* gb| JQ778284.1, *A. mellifera ligustica* gb| KP162643.1, *A. mellifera meda* gb| KY464957.1, *A. mellifera* gb| KX908209.1, *A. florea* gb| KU571744.1, *A. dorata breviligula* gb| JF393263.1, *A. laboriosa* gb| JQ317319.1, *A. mellifera mellifera* gb| KJ396191.1, *Trigona pallens* gb| L22902.1, *T. hypogea* gb| L22901.1, *Scaptotrigona luteipennis* gb| L22900.1, *Melipona compressipes* gb| L22899.1, *Xylocopa virginica* gb| L22905.1), Aphididae (*Acyrthosiphon pisum* gb| AK341909.1), Pteromalidae (*Nasonia vitripennis* gb| EU746617.1), Drosophilidae (*Drosophila melanogaster* gb| BT003592.1,
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The minor clade with the lowest blast scores and a high divergence value contained class Chelicerata represented by *Ixodes scapularis* sterol (gb|XM002408215.1). These results indicate that the recovered apid species are deeply embedded within the genus *Apis*.
Figure 2. Sequence alignment of mitochondrial 16S rDNA gene of the honey bee subspecies *Apis mellifera jemitéca* and *Apis mellifera carnica* with the most closely related Holometabola species within family Apidae. (Only variable sites are shown. Dots represent bases identical to those of the first sequences, and dashes indicate gaps).
Figure 3. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-2409.1376) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 168 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

Apidae is the largest family of bees, with over 5,700 described species (Ascher and Pickering, 2012). There are four different species of honey bees in the world: little honey bee (Apis florea) that is native to southeast Asia, the Eastern honey bee (Apis cerana) of eastern Asia including Korea and Japan; the Giant honey bee (Apis dorsata) native to southeast Asia; and the Western honey bee (Apis mellifera) native to Europe, Africa, and western Asia (Daly et al., 1982; Oldroyd and Wong, 2006; Rattanawannee et al., 2010). Apis mellifera is commonly known as the European, western, or common honeybee in different parts of the world and is extensively domesticated for honey, pollen, beeswax, propolis, royal jelly, and bee venom (Ratcliffe et al., 2011). It is a major agent of global ecology via pollination (Williams, 2002; Kajobe, 2006; Klein et al., 2007; Allsopp et al., 2008). It is a common model in scientific social behavioral studies (Breed et al., 2004; Solignac et al., 2004; Beye et al., 2006; The Honey Bee Genome Sequencing Consortium, 2006). The current study aimed to determine the genetic diversity and phylogenetic relationships of the A. mellifera subspecies of...
Saudi Arabia. The *Apis* species are often identified via molecular markers due to the lack of taxonomists skilled in apiculture in Saudi Arabia.

DNA sequence-based identification techniques have revealed the morphological and ecological traits of many species during larval stages (Foltan et al., 2005; Smith et al., 2006; Hayashi and Sota, 2010). Mitochondrial DNA (mtDNA) markers have been widely used to address population and evolutionary questions from the *A. mellifera* honey bee. The sequencing and characterization of the mitochondrial DNA genome has been very useful for analyzing the phylogeny and population genetic structure of the *Apis* species and of the *A. mellifera* subspecies. It contains regions with variable evolutionary rates. In principle, the general pattern of subspecies distribution has been supported by various genetic studies using molecular tools (Garnery et al., 1993; Franck et al., 2000a; 2001; Whitfield et al., 2006; Cánovas et al., 2008). Mitochondrial introgressions can be assessed using the “DraI” test—a molecular test that highlights the sequence variability between subunits I and II of the cytochrome oxidase gene (Garnery et al., 1993). This test has been widely used to analyze the biogeography of *A. mellifera* L. subspecies and races (Rortais et al., 2011) as well as for other *Apis* species (Smith and Hagen, 1997).

We used this method to distinguish different haplotypes and group them into one of the four primary lineages (Ruttner, 1988; De la Rúa et al., 2009). The mitochondrial *16S rDNA* was studied here for the first time to detect the molecular polymorphism between two different subspecies of *Apis mellifera* worker honey bee. Previously, the mitochondrial CoxI–CoxII intergenic region was utilized to distinguish honey bee lineages. Schiff and Sheppard (1995) used mitochondrial DNA and allozyme variations to characterize 142 breeder queen colonies from 22 apiaries in the southeastern United States. In addition, Franck et al. (2000a) described the different haplotypes of the CoxI-CoxII intergenic region found in major honey bee lineages. Kozmus et al. (2007) analyzed the mtDNA of honeybee ectotypes as the initial step of molecular characterization of the indigenous honeybee populations from Serbia. Papachristoforou et al. (2013) combined analysis of mtDNA and microsatellite data from *A. m. cypria* to describe the genetic structure of the Cyprian honey bee population. Techer et al. (2015) analyzed the genetic diversity of the honeybee population in Rodrigues using the mitochondrial CoxI-CoxII intergenic region. Božič et al. (2016) analyzed mtDNA for the characterization of carniolan honey bee *A. M. carnica*.

This study indicated that closely related *Apis* species have 90% similarity in the standardized DNA sequence and distantly related species have less than 90% similarity in the same genes sequence. This agrees with Gurney et al. (2000). The NJ tree was constructed here based on the multiple aligned sequence data for different *Apis* species and other arthropods. The tree separates the genomes into two main clades. All *A. mellifera* species were included in one clade; Chelicerata represented by *Ixodes scapularis* sterol were in another clade. Our results verify the conclusions of Prentice (1998), Melo (1999), Michener (2007), Cardinal et al. (2010), and Haddad et al. (2017) who found that all *Apis* species are clustered together in one clade in addition to the genetic origin of *Apis* species within family Apidae as a paraphyletic group.

Franck et al. (2001) and Cánovas et al. (2008) demonstrated that there are five honey bee lineages found in the Mediterranean Basin. These results indicated that the recovered apid species are deeply embedded within the genus *Apis* within the African lineage. A recent similar study by Haddad et al. (2017) analyzed the mt genome haplotypes and indicated that the north African Sahara Honey bee, *A. m. sahariensis* in the African lineage was genetically distinct from the northern African lineage exemplified by *A. m. intermissa*. The branch length of *A. m. carnica* (gb |MH939276.1) was lower than *A. m. jementica* (gb |MH939277.1) indicating less divergence from its ancestor. This result was confirmed by data from Rukhsana et al. (2014) who indicated that the branch length of the NJ tree is a degree to indicate the divergence from the common ancestor.

**CONCLUSION**

A recent field study provides tools for the rapid identification and phylogenetic analysis of honey bees inhabiting Saudi Arabia via mitochondrial *16S rDNA* gene from the recovered *Apis mellifera* subspecies. This yielded a unique genetic sequence that confirms their taxonomic position within the family Apidae.
COMPETING INTERESTS

The authors have declared that they have no conflict of interest regarding the content of this article.

ACKNOWLEDGEMENTS

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COMPLIANCE WITH ETHICAL STANDARDS

All procedures contributing to this work comply with the ethical standards of the relevant national guides on the are and use of laboratory animals and have been approved and authorized by the Institutional Animal Care and Use Committee (IACUC) at King Saud University, Riyadh, Saudi Arabia.

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