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Characterization of the native honey bee subspecies in Saudi Arabia using the *mt*DNA COI–COII intergenic region and morphometric characteristics

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Abstract

Morphometric and genetic markers are very common and powerful tools used to characterize honey bee subspecies. In Saudi Arabia, morphometric analysis using 24 characteristics separated the Saudi honey bee between two reference subspecies. Most Saudi honey bee samples clustered with *Apis mellifera jemenitica* reference group, but others were more similar to *Apis mellifera litorea* reference group. Based on sequence analysis of the *mt*DNA COI–COII region, 18 new and clearly separated haplotypes were characterized for the first time. Sequence analyses showed that most haplotypes belonged to the O lineage and are very close to the Syrian haplotypes found in the gene databank (NCBI). However, two other haplotypes belonged to the A lineage and are clearly different from the Ethiopian haplotypes previously described as *A.m. jemenitica*. However, no previous genetic data for the *A.m. jemenitica* from Saudi Arabia, Yemen or Oman are available in the databank for comparison purposes. Additionally, this high genetic diversity in the *mt*DNA COI–COII region and the clear morphometric variation suggest that the honey bee of Saudi Arabia represents more than one distinct honey bee subspecies.

Key words: Yemeni honey bee, morphometric characterization, *mt*DNA, Saudi Arabia, O lineage.

Introduction

The honey bee, *Apis mellifera* L. 1758, is naturally found throughout Europe, Africa and Western Asia (Miguel *et al.*, 2011). In recent years, based on morphometry, 26 subspecies have been identified and clustered into four evolutionary lineages (Ruttner, 1988; Sheppard *et al.*, 1997; Engel, 1999; Sheppard and Meixner, 2003; Miguel *et al.*, 2011). The indigenous honey bee of Saudi Arabia, which is the focus of this publication, was characterized morphometrically by Ruttner in 1975 as *Apis mellifera jemenitica*, a honey bee subspecies that has evolved and adapted to hot and adverse climatic conditions. Recently, significant morphometric variations revealed three well-defined clusters of the native honey bee in Saudi Arabia (AlGhamdi *et al.*, 2012). 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, as they can be sensitive to environmental selection pressures (Franck et al., 2000b). Genetic markers such as the *mt*DNA COI–COII 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 among five honey bee lineages and to discriminate among *A. mellifera* subspecies (Garnery *et al.*, 1992; Franck *et al.*, 2000a; Sheppard and Smith, 2000). Sequencing is more sensitive and may reveal new haplotypes that have not been previously described (Ozdil *et al.*, 2009; Solorzano *et al.*, 2009; Magnus and Szalanski, 2010; Szalanski and Magnus, 2010). To date, a characterization of the Saudi honey bee is lacking. In the present study, morphological characteristics and *mt*DNA COI-COII sequence data were used to characterize the honey bees within Saudi Arabia.

Materials and methods

Morphometric study

Samples of 10 honey bee workers each were collected from local honey bee colonies, covering seven regions within Saudi Arabia (figure 1). Samples were preserved in 70% ethanol and then dissected according to Ruttner

Figure 1. An explanatory outline map of Saudi Arabia that shows sampling locations (Albaha 20°16'15"N 41°26'25"E; Almadinah 24°33'28"N 39°43'42"E; Altaif 21°41'3"N 40°27'21"E; Asir 18°59'41"N 42°51'6"E; Jazan 17°22'49"N 42°44'29"E; Najran 17°28'12"N 44°35'5"E; Alqaseem 26°20'27"N 43°57'44"E).

Table 1. List of morphometric characteristics used in this analysis and their Ruttner's No.

No.	Characteristic	No.	Characteristic
13+14	Body size	*19:20*	Index of slenderness
5	Length of proboscis	*6*	Length of femur
7	Length of tibia	*8*	Length of metatarsus
9	Width of metatarsus	*10*	Pigmentation of tergite 2
29:30	Cubital index	*9:8*	Metatarsus index
11	Pigmentation of tergite 3	*12*	Pigmentation of tergite 4
13	Longitudinal diameter of tergite 3	*14*	Longitudinal diameter of tergite 4
15	Longitudinal diameter of sternite 3	*16*	Wax mirror, longitudinal
17	Wax mirror transversal	*19*	Sternite 6, longitudinal
20	Sternite 6, transversal	*21*	Forewing length
22	Forewing width	*27*	Cubital vein a
28	Cubital vein b	*6+7+8*	Length of hind leg

et al. (1978). Body parts were mounted on slides and were then scanned using a high-resolution scanner (600 ppi) connected to a desktop computer system supported with image tool software (Image tool® 3.0). The classical morphological traits used in this analysis included honey bee size and cuticular pigmentations. In total, 24 morphometric characteristics that were previously reported as highly discriminatory (Ruttner, 1988) were measured (table 1). Colony sample means were calculated for each characteristic of each bee sample. Reference bee data on the corresponding characteristics for seven other subspecies, namely: *Apis mellifera carnica* Pollmann, *Apis mellifera ligustica* Spinola, *Apis mellifera meda* Skorikov, *Apis mellifera syriaca* Skorikov, *Apis mellifera lamarkii* Cockerell, *Apis mellifera jemenitica* Ruttner and *Apis mellifera litorea* Smith, were obtained from the Oberursel Bee Research Institute (Frankfurt, Germany) and were included in the data set (N = 86). Subsequently, discriminate analysis using Wilk's lambda was used to verify reallocation probabilities and cluster distances. Analysis was performed using PASW 118 (2009).

Genetic study

The same samples used in the morphometric analysis were also used in the genetic characterization ($N = 179$). Samples were preserved in absolute ethanol and stored at −20 °C until DNA extraction. Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The COI-COII intergenic region of the *mt*DNA was then amplified using gene-specific primers (E2) 5'-GGCAGAATAAGTGCATTGGGC-3' and (H2) 5'-CAATATCATTGATGACCTTA-3' (Cornuet *et al.*, 1991; Garnery *et al.*, 1992) and a GeneAmp 9700 thermocycler (Applied Biosystems), as described by Garnery *et al.* (1992). Polymerase chain reaction (PCR) products were then sequenced in both directions using an automated 96 capillary ABI 3730XI DNA genetic analyzer (Applied Biosystem). Sequences were manually checked and assembled using Geneious version 5.5.2 software (Drummond *et al.*, 2011). Sequences were then exposed to two procedures. First, sequences were aligned using CLUSTAL*W* software (Thompson *et al.*, 1994), BLASTed and compared with other sequences available on the GenBank database (NCBI). Second, *In silico Dra*I restriction analysis was applied to the sequences using Geneious software version 5.5.2 (Drummond *et al.*, 2011). The resulting fragments were used to score different haplotypes according to Garnery *et al.* (1998). Similarities were calculated by the simple matching method, and a phylogenic tree was constructed using the Maximum Parsimony method (MP) (Chouhan and Pardasani, 2008). Parsimony analyses were un-weighted and used branch and bound searches. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 53% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 2 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 56 nucleotide sequences. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The various new sequences obtained in this study were deposited into GenBank.

Results

Morphometric study

Discriminant analysis of the reference groups confirmed their reallocation to their original subspecies. However, when the groupings were cross-validated, one set of measurements of the reference Yemeni honey bees, *A. m. jemenitica*, was allocated to *A. m. litorea* reference group. One hundred fifty samples (79%) of the Saudi honey bees analyzed in this study were grouped with the reference Yemeni honey bee cluster, *A. m. jemenitica*. The rest of the samples (n = 40, 21%) were clustered with the reference honey bee, *A. m. litorea*.

The analysis indicated that most Saudi honey bee samples are very similar to the Yemeni honey bee, *A. m. jemenitica*, but some are more similar to the other honey bee, *A. m. litorea*. Other Saudi honey bee samples clustered very close to the Egyptian honey bee reference group, *A. m. lamarckii* (figure 2).

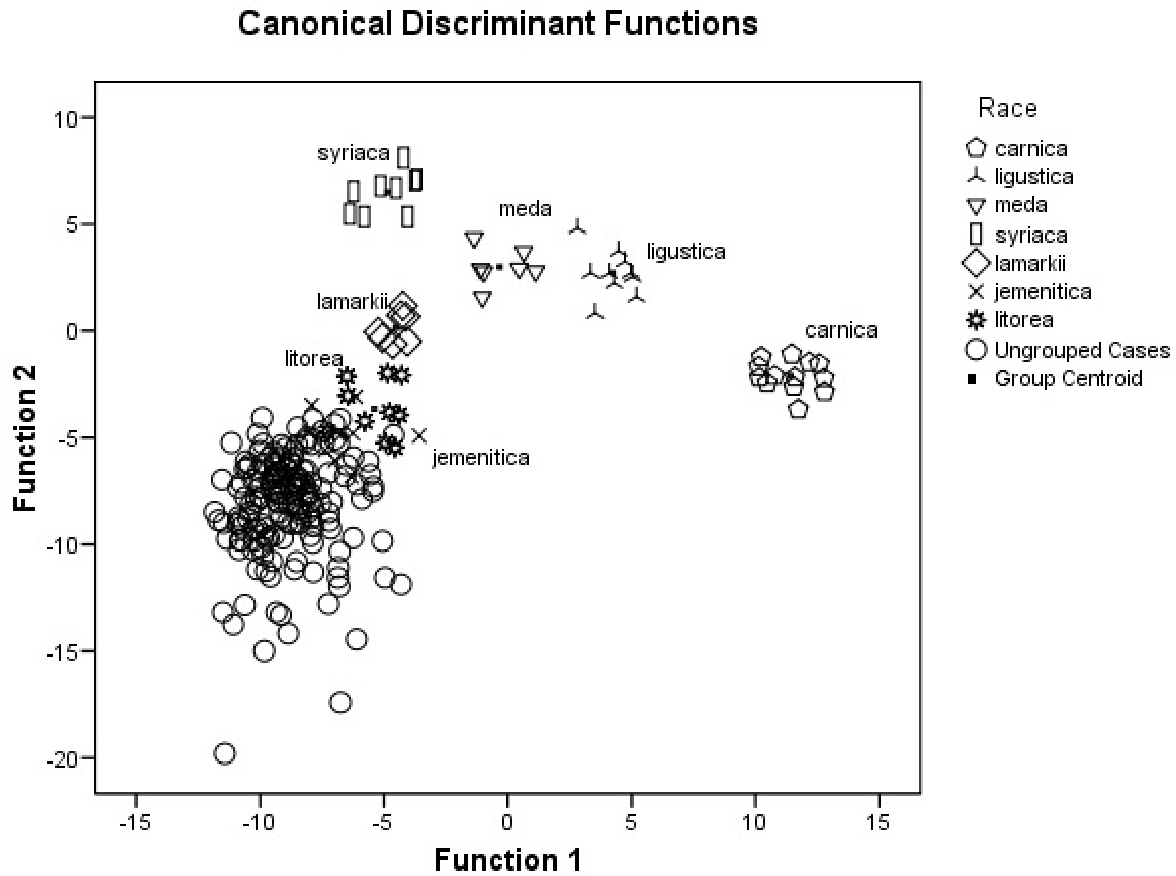

Figure 2. Discriminant analysis of the Saudi honey bees based on seven reference subspecies.

Genetic study

Based on the presence and absence of *P* and *Q* sequences, the COI–COII intergenic region revealed three different amplicon sizes (612 bp *PoQ*, 812 bp *PoQQ* and 1000 bp *PoQQQ*) (figure 3). Most of the samples (75%) contained the PoQ sequences. The PoQQ sequences were found in Asir, Albahah and Altaif and represented 23% of the samples. Only a few samples from Albaha had the PoQQQ sequences, representing 2% of the entire population (table 2). *In silico Dra*I restriction analysis revealed seven different haplotypes (figure 2); six of them belonged to the O lineage, and one belonged to the A lineage. Three of these haplotypes were novel and

Table 2. Distribution of Saudi honey bee haplotypes based on the number of repeats of the Q element.

Location	N.	No. and % within population of individuals containing Q elements		
		PoQ	*PoQQ*	*PoQQQ*
Asir	31	26 (84)	5 (16)	-
Najran	13	13 (100)	-	-
Jazan	45	41 (91)	4 (9)	
Albahah	28	11 (39)	13 (47)	4 (14)
Altaif	52	30 (58)	22 (42)	-
Al-qaseem	4	4 (100)	-	-
Almadinah	6	6 (100)	-	-

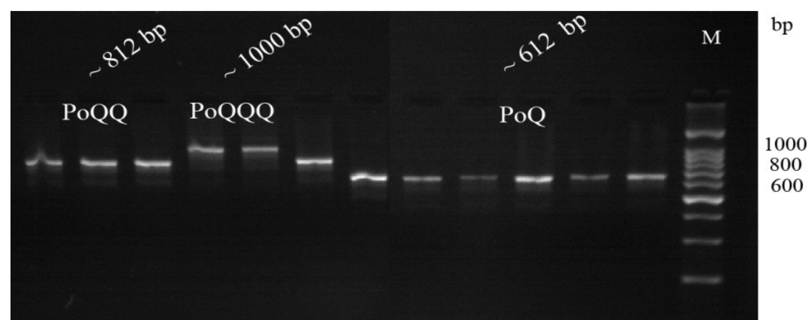

Figure 3. Structural organization of the COI–COII intergenic region of *mt*DNA migrated on 1.5% agarose gel. M is the molecular size marker; PoQ, PoQQ and PoQQQ sequences correspond to the O lineage.

Table 3. Haplotype based on fragment length of *mt*DNA COI-COII intergenic region.

*Dra*I Saudi haplotype	Location	Haplotype and percentage	Fragment length	N	Haplotype percentage (= N/the entire sample size)
Haplotype 1 (A1)	Najran Jazan Altaif Almadinah Asir Albaha Alqaseem	O1(Z7) (100%) O1(Z7) (87%) O1(Z7) (49%) O1(Z7) (50%) O1(Z7) (84%) O1(Z7) (39%) O1(Z7) (100%)	29,108,67,371 (PoQ)	122	67
Haplotype 2 (A2)	Jazan Altaif	O1d (4%) O1d (9%)	29,108,65,371 (PoQ)	6	3
Haplotype 3(A3)	Almadinah	New 1 (50%)	29,112,67,371 (PoQ)	3	2
Haplotype 4 (A4)	Asir Albaha Altaif	O1' (Z2) (16%) O1' (Z2) (42%) O1' (Z2) (21%)	30,108,67^2,129,375 (PoQQ)	29	16
Haplotype 5 (A5)	Altaif Albaha	New2 (21%) New2 (5%)	34,108,67,129,66,357(PoQQ)	15	8
Haplotype 6 (A6)	Jazan	New3 (9%)	34,108,66,129,67,357(PoQQ)	4	2
Haplotype 7 (A7)	Albaha	O1'' (Z2)' (14%)	12,108, 67^3, 129^2,357(PoQQQ)	4	2

Table 4. Number and distribution of Saudi honey bee haplotypes according to sequences of the COI-COII intergenic region.

Haplotype Accession No.	NCBI haplotype	Identical %	Najran	Asir	Jazan	Albaha	Altaif	Alqaseam	Almadinah	N.	Haplotype %
KC149745	O1	99.8	7	11	27	10	13	1	3	72	40.2
KC149747	O1a	99.8	2	12	8	3	10	3		38	21.2
KC149979	O1'	99.5	-	5	-	7	-	-	-	12	6.7
KC149746	O4b	99.1	3	3	3	1	-	-	-	10	5.6
KC149984	O1'	99.4	-	-	-	-	11	-	-	11	6.1
KC149989	O1'	99.3	-	-	-	-	7	-	-	7	3.9
KC149749	M4	97.5	-	-	-	-	4	-	-	4	2.2
KC149750	O1	99.3	-	-	-	-	-	-	3	3	1.7
KC149983	O1'	99.4	-	-	-	-	3	-	-	3	1.7
KC149985	O1'	99.3	-	-	4	-	-	-	-	4	2.2
KC176269	O1''	99.3	-	-	-	4	-	-	-	4	2.2
KC149748	A1	97.5	-	-	2	-	-	-	-	2	1.1
KC149987	O1'	99.4	-	-	-	-	2	-	-	2	1.1
Syrian(9)HM236209	O1	100	1	-	1	-	-	-	-	2	1.1
KC149980	O5a	99.5	-	-	-	1	-	-	-	1	0.6
KC149981	O1'	99.7	-	-	-	1		-	-	1	0.6
KC149982	O1'	99.6	-	-	-	-	1	-	-	1	0.6
KC149986	O1'	99.4	-	-	-	1		-	-	1	0.6
KC149988	O1'	99.2	-	-	-	-	1	-	-	1	0.6
Total			13	31	45	28	52	4	6	179	100

have not been described in previous studies (Franck *et al.*, 2001; Alburaki *et al.*, 2011). One of these new haplotypes was found only in Almadinah (table 3).

Based on the sequence variations (SNP), 19 different haplotypes were distinguished. One haplotype was identical to a previously described haplotype (Acc. HM236209) from an eastern honey bee population (Syria, Lebanon and Iraq) (Alburaki *et al.*, 2011). The other 18 haplotypes were new and were added to GenBank (table 3). The number of SNPs per sample ranged from 1 to 20. The distribution of the different haplotypes between the sampling regions, based on the sequence variations (SNP), did not show a specific distribution pattern, and haplotypes overlapped between different sampling regions (table 4). However, the O lineage (O1) was the main haplotype and was

represented in all sampling locations. The phylogenic tree grouped members of the same linage together in the same branch. Most of the Saudi haplotypes clustered with other members of the O lineage. Two haplotypes (KC149748 and KC149748) were grouped very close to the C lineage. In reality, however, neither haplotype belongs to lineage C because both have complete *P* sequences. Figure 4 shows the phylogeny tree of many honey bee haplotypes, including different Saudi haplotypes.

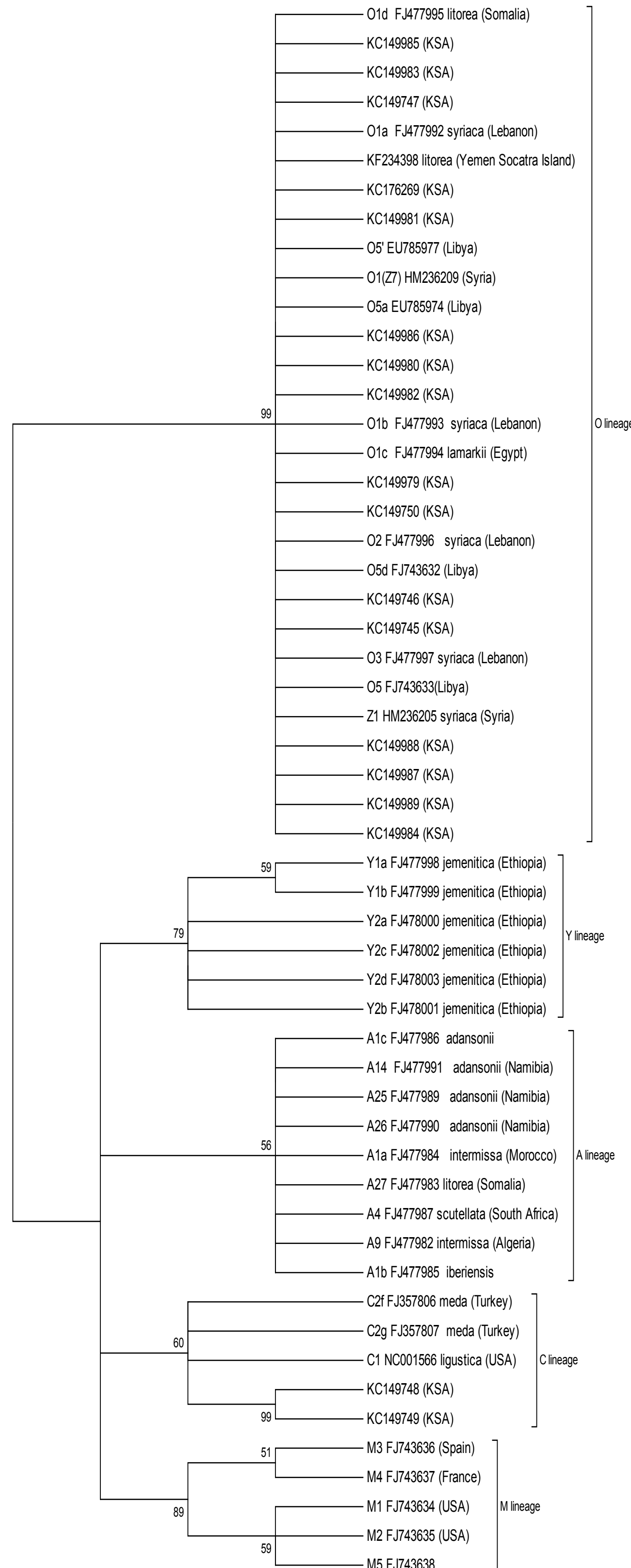

Figure 4. Maximum parsimony phylogenic tree among COI-COII intergentic region sequencing of samples collected from Saudi Arabia.

Discussion and conclusion

The morphometric analysis of 24 characteristics divided the Saudi honey bee into two subspecies. Most individuals clustered with the Yemeni honey bee, *A. m. jemenitica*; however, good portion clustered with the litorea honey bee reference samples, *A. m. litorea*. The reported distribution of *A. m. jemenitica* is extremely large, including Oman, Yemen, Saudi Arabia, Somalia, parts of Ethiopia, Sudan, Chad and Mali (AlGhamdi *et al*., 2013). The other subspecies, *A. m. litorea*, is distributed on the coastal region of East Africa, from Lamu, Mombasa, Tanga to Moçambique (Ruttner, 1988). Whether the Saudi honey bee represents a unique and well-separated cluster or a subspecies of either *A. m. litorea* or the *A. m. jemenitica* is an issue that can be resolved using the geometric approach of honey bee wing angles, as described by Ruttner (1988).

The genetic data resulting from the amplification of *mt*DNA COI-COII intergenic region revealed three distinct amplicons, 612 bp, 812 bp and 1000 bp, which were consistent with a previous study performed on the Syrian honey bee, a member of the O lineage, in Jordan (Haddad *et al*., 2009). The mode by which the haplotypes with PoQ, PoQQ and PoQQQ sequences are distributed, suggests that different haplotypes coexist within the same geographical area. The same mode of distribution was reported for Iraqi and Syrian honey bee populations (Alburaki *et al.*, 2011).

Results also indicated that using the *Dra*I restriction enzyme is not sufficient to explore variation among samples. Thus, sequencing of this region contribute significantly to the exploration of further variation (Cornuet and Garnery, 1991; Muñoz *et al.*, 2009; Magnus *et al.*, 2011). Both the morphometric and genetic analyses provide evidence that the Saudi honey bee population from the Arabian Peninsula belongs to the O lineage. Additionally, these analyses confirm the previous hypothesis of Franck *et al.* (2000b) regarding the distribution ranges of the O lineage in the Middle East and African Horn. However, the presence of the O lineage in the hot and dry climate of Saudi Arabia suggests an adaptive evolution to such adverse conditions.

The situation in Saudi Arabia may be similar to that in Sudan, where most of the honey bees belong to the O lineage, but samples from dry savanna regions belong to the A lineage (El-Niweiri and Moritz, 2008). There are no genetic or comprehensive morphometric data on the land subspecies of Saudi Arabia. The high number of haplotypes resulting from this study supports the finding of Alburaki *et al.* (2011) on the high variability of this region. In contrast, the high genetic variability found within the Saudi honey bee population supports a previous hypothesis on the center of the O lineage (Franck *et al.*, 2000b). Nevertheless, the classification of the Saudi honey bee originating from this study could be used to group other bee populations in neighboring countries.

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