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Evidence for sub-populations of *Apis mellifera jemenitica* colonies along the Red Sea coast of Saudi Arabia

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Abstract

Characterization of *Apis mellifera jemenitica* Ruttner populations along the Red Sea Coast of Saudi Arabia revealed two distinct sub populations. Principal component analysis of 28 morphometric characters revealed that wing venation characters (factor one) accounted for >91% of the variation among our samples and reference subspecies, and for 98% of the variation between both Saudi sub-populations. Separation of the study samples into two clusters based on colony means of wing venation was highly significant. Principal coordinate analysis using forewing landmarks confirmed the allocation of the samples into two clusters. Coordinates associated with factor one accounted for >99% of the variance between both clusters. Results indicated significant correlation between morphometric and geometric approaches in allocating samples into the same cluster ($R = 0.474$; $P < 0.0001$). Analysis of *mt*DNA tRNAleu-COII showed high similarity among our sequences and reference sequences of the sub-lineage Z (previously O lineage) and of the lineage C (*Apis mellifera anatoliaca* Maa and of the M lineage *Apis mellifera mellifera* L.). Results revealed 24 variable haplotypes, 22 of which were previously unreported. Our study shows that *A. m. jemenitica* samples of non-migratory honey bee colonies along the Saudi Arabia Red Sea coast represent two sub-populations and phylogenetically clustered with subspecies from two different lineages.

Key words: *A. m. jemenitica*, Saudi Arabia, lineage, geometry, *mt*DNA.

Introduction

Apis mellifera jemenitica Ruttner is naturally distributed in Asia and Africa, where it has the widest natural distribution range among *Apis mellifera* L. subspecies (Ruttner, 1988). Within Asia it represents the endemic honey bee of the Arabian Peninsula. It is characterized by distinctive traits (Ruttner, 1988), which result in high adaptation to extreme temperatures (Ruttner, 1988; Alattal and Alghamdi, 2015). In Saudi Arabia it is assumed that *A. m. jemenitica* has been used in apiculture as early as 2000 BC (Crane, 1983; Alqarni *et al*., 2011), and spread along the western Red Sea coast around the Sarawat Mountains Range, from the Jordanian borders in the north to the Republic of Yemen in the south (Ruttner, 1988) with more than 1,550 km long and from 10-140 km wide. The northern half of this range, known as Sarat al-Hejaz, rises approximately to 2,100 m a.s.l. in some portions, while the middle and southern portions (Sarat 'Asir and Sarat al-Yemen, respectively) can reach altitudes over 3,600 m (Scoville, 1995). Most Saudi Arabian beekeeping occurs around these mountain ranges. Yet, official records have shown that over the last 50 years huge importations of exotic honey bee subspecies have been increasingly taking place. More than two million bee packages of foreign subspecies were introduced in 2019 (mostly from Egypt, according to MoWEA, 2019). These imports strongly impact the conservation status of the native subspecies. Based on geographical distribution and body traits, *A. mellifera* populations have been classified into different subspecies which are grouped into four main evolutionary lineages i.e., Africa (A), Western Europe (M), South-Eastern Europe (C) and Middle East (O) (Ruttner, 1988). However, morphological characterization is impacted by adaptation to local environment and hybridization with imported non-native subspecies (Alqarni *et al*., 2013), which may lead to improper identifications. Nowadays, molecular methods such as analyses of mitochondrial DNA have been widely used to support identification of honey bee subspecies and classification based on the different lineages (Garnery *et al*., 1992). Previous studies based on morphometric characterization (Alghamdi *et al*., 2012), and mitochondrial DNA analyses (Alattal *et al*., 2014; Alghamdi and Alattal, 2020) including migratory and non-migratory colonies revealed that the honey bee of Saudi Arabia, *A. m. jemenitica*, belongs exclusively to A lineage (Z sub-lineage, previously O lineage), and is separated into 3 distinctive clusters, with a change of body size and colour from far North near the Syrian honey bee distribution range to the far South near Yemen. However, this variation in size and colour among clusters may be associated with hybridization with imported subspecies. Recently, a comprehensive phylogenetic analysis of *A. m. jemenitica*, based on complete mitochondrial DNA sequences (n = 14) revealed three sub-populations within Saudi Arabia, with very close evolutionary relationship with the Syrian honey bee, *Apis mellifera syriaca* Skorikov, the Egyptian honey bee *Apis mellifera lamarckii* Cockerell, and other east African subspecies such as *Apis mellifera capensis* Eschscholtz and *Apis mellifera scutellata* Lepeletier (Alghamdi and Alattal, 2020). These subpopulations vary, indeed, in several behavioural traits such as productivity, calmness and swarming tendency. In this study we used body morphometry, wing landmarks, as well as *mt*DNA tRNAleu-COII analyses from 133 non-migratory endemic honey bee colonies distributed along the Red Sea coast to explore variation among subpopulations and further confirm their lineages.

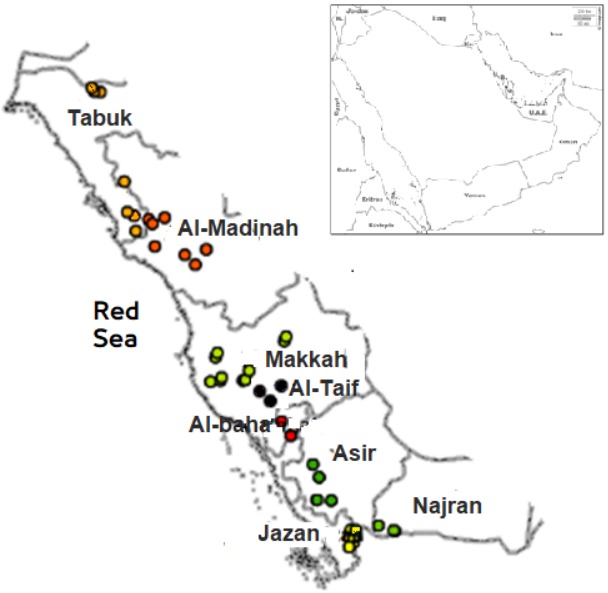

Figure 1. Location of sampled apiaries along the Red Sea coast of Saudi Arabia; Tabuk (n = 18): 28.387028 36.855194; Al-Madinah (n = 17): 25.411561 37.520384; Makkah (n = 20): 21.911416 39.753706; Al-Taif (n = 14): 21.472722 40.551694; Al-Baha (n = 15): 19.852472 41.585611; Asir (n = 19): 18.256722 42.229028; Jazan (n = 16): 17.517472 43.075000; Najran (n = 14): 17.625917 43.754611.

Materials and methods

One hundred thirty-three honey bee samples were collected from non-migratory native honey bee colonies distributed along the Red Sea coast of Saudi Arabia (figure 1). Each sample consisted of 15 workers. Workers were immersed directly in hot water for several second to ensure proboscis elongation and were then preserved in 70% ethanol. Ten workers from each sample were dissected according to Ruttner *et al*. (1988). Body parts were mounted on slides, and scanned using a high resolution scanner (Image resolution = 600 dpi) connected to a desk-top computer system supported with an image tool software (Image tool® 3.0). Body characters associated with body size and pigmentations (table 1), wing angles (Meixner *et al*., 2013), and 20 wing landmarks (Kandemir *et al*., 2011) were also measured using MorphoJ (v1.07a) and were used separately in the analysis part (Goetze, 1964; Ruttner, 1988; Kandemir *et al*., 2011). Colony means were then calculated for each character. Afterwards, reference data representing the measurements of the corresponding characters for four other *A. mellifera* subspecies from six countries (Syria, Egypt, Sudan, Uganda, Somalia, Italy) obtained from the Oberursel Bee Research Institute (Frankfurt, Germany) were included in the data set (table 2). Subsequently, discriminant analysis using Wilk's lambda was used to verify reallocation probabilities and cluster distances. Analysis was performed using Past 4.03 (Hammer *et al*., 2001). To explore variation within honey bee population along the Red Sea Coast of Saudi Arabia, principal component analysis of the 28 morphometric characters using colony means was performed. For *mt*DNA analyses, DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Cat No./ID: 69506) and then sequenced using BGISEQ system. Pair-end short reads (150 bp) DNA libraries were sequenced by BGI (http://www.bgi.com, Hong Kong, China) and were subjected to three-step filtration, started with adaptor trimming. Any reads with adaptor mapping rate higher than 50% was removed. Then, low quality reads with more than 50% of low quality bases (Q20 < 50%) were removed. Finally, contiguous reads

Table 1. List of morphometric characters used in this analysis and their numbers as given by Ruttner (1988).

Character	No.
Length of proboscis	*4*
Length of femur	*5*
Length of tibia	*6*
Length of metatarsus	*7*
Width of metatarsus	*8*
LW_MTAR	*7:8*
Length of hind leg	*5+6+7*
Tergite 3 longitudinal	*9*
Tergite 4 longitudinal	*10*
Forewing length	*17*
Forewing width	*18*
LWFW	*17:18*
Cubital 1	*19*
Cubital 2	*20*
Pigmentation of tergite 2	*32*
Pigmentation of tergite 3	*33*
Pigmentation of tergite 4	*34*
Wing angle (a4)	21
Wing angle (b4)	22
Wing angle (d7)	23
Index of slenderness	*19:20*
Wing angle (e9)	24
Wing angle (g18)	25
Wing angle (j10)	26
Wing angle (j16)	27
Wing angle (k19)	28
Wing angle (l13)	29
Wing angle (n23)	30
Wing angle (o26)	31
Wing landmarks (20)	see figure 3

Table 2. Name, number (N) and origin of reference honey bee subspecies included in the morphometric analysis of honey bee along the Red Sea Coast of Saudi Arabia, data were obtained from the Oberursel Bee Research Institute, Frankfurt, Germany.

Subspeices	(N)	Bees/ colonies	Country
A. m. jemenitica (Africa)	50	20	Sudan, Tschad, Somalia, Uganda
A. m. syriaca	12	20	Syria
A. m. lamarckii	28	20	Egypt
A. m. ligustica	50	20	Italy

with more than 2% N bases were removed. Filtration was performed using Flexbar v3.4.0 (Roehr *et al*., 2017). Clean *mt*DNA reads were then mapped and annotated in Geneious Prime 2020.1.2 (http://www.geneious.com) using the mitogenome of *A. m. jemenitica* (GenBank: MN714161), derived from a sample collected from Yemen in 1988 as reference (Boardman *et al*., 2020). Sequences of COI and the tRNAleu-COII for each sample were then extracted in FASTA format and were aligned with other reference sequences (n = 19) of other *A. mellifera* subspecies using BioEdit v7.2.5 (Hall, 1999). The phylogenetic tree was constructed and tested over1000 bootstrap replicates (Felsenstein, 1985), evolutionary distances as the number of base substitutions per site were calculated in MEGA7 (Kumar *et al*., 2016). To explore new haplotypes, variable sequences were additionally analysed with Basic Local Alignment Search Tool (BLAST) search program (National Center for Biotechnology Information site - NCBI), and compared with other sequences available in GenBank, then previously unreported haplotypes were uploaded into NCBI.

Results

Discriminant analysis of our samples and the reference groups confirmed the affiliation of the latter to their original subspecies (n = 140). However, in cross-validating grouping one set of measurements of our samples (<0.01) was allocated with the *Apis mellifera ligustica* Spinola reference group and three sets of measurements of the reference samples (2.0%) were not coherently allocated with original classification. Principal component analysis of the 28 characters (associated with size, pigmentation and wing venation) using sample means revealed that the first four factors with eigenvalues >1 accounted for >99% of the variation among the different groups. Factor one and factor two which are associated with size and wing venation (angles) accounted for approximately 91% of the variation among subspecies and for 65% of the variation within populations. Although the analysis indicated some proximity of our samples to reference samples from neighbouring regions. Our samples were distinctly separates from African *A. m. jemenitica* reference data-sets (n = 50) (figure 2). Table 3 shows squared Mahalabonis distances among different data sets. Results revealed that factors associated with wing venation accounted for 98% of the variation, while a slight variation was associated with pigmentation of tergite 2 and other characters associated with size. The K-means clustering procedure showed that the number of groups which gave highest mean F values for the considered characters was two. Variation based on means for both clusters (table 4) was significantly different ($P \leq 0.0001$,

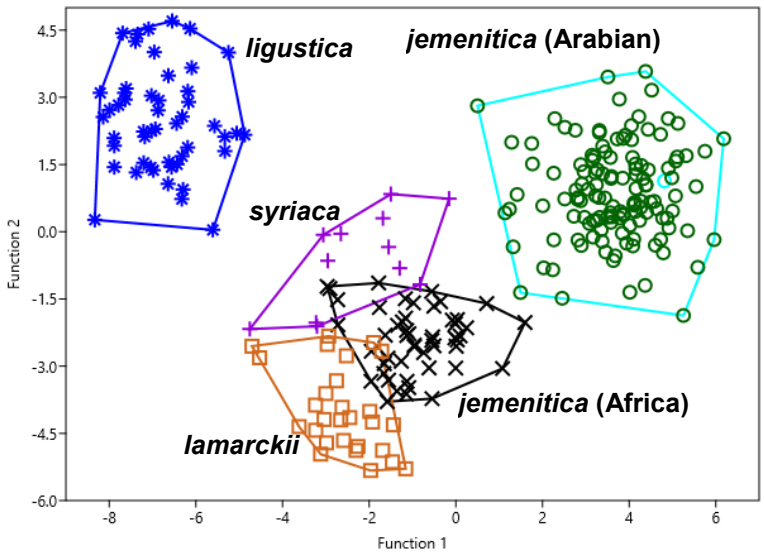

Figure 2. Discriminant analysis of morphological data. Samples include the measurements of the Arabian honey bee samples and the reference data of four other honey bee sub-species obtained from Oberursel Bee Research Institute, Frankfurt, Germany. Reference subspecies are the Italian honey bee (*A. m. ligustica*); the Syrian honey bee (*A. m. syriaca*); the Yemeni honey bee (*A. m. jemenitica*) and the Egyptian honey bee (*A. m. lamarckii*).

Table 3. Squared Mahalabonis distances among Saudi Arabian *A. m. jemenitica* samples and the other four reference honey bee subspecies based on morphometric data analysis of 28 characters.

Subspeices group	*A. m. jemenitica* (Africa)	*A. m. syriaca*	*A. m. lamarckii*	*A. m. ligustica*
Our samples	29.1	36.7	47.0	91.4
A. m. jemenitica (Africa)	-	30.9	30.3	52.4
A. m. syriaca		-	43.1	49.7
A. m. lamarckii			-	67.1

Table 4. Colony means and medians of significant characters used to separate the two clusters of the native *A. m. jemenitica* population from the Red Sea Cost of Saudi Arabia providing Kruskal-Wallis P value for that character.

Character	Cluster -1		Cluster -2		Kruskal - Wallis P
	Mean ± SE	Median	Mean ± SE	Median	
CUB1	0.48 ± 0.006	0.48	0.51 ± 0.004	0.51	0.0012
T3	8.77 ± 0.36	8.82	8.70 ± 0.03	8.78	0.05
A4	33.66 ± 0.25	33.52	34.92 ± 0.14	34.81	<0.0001
B4	101.62 ± 0.29	101.00	95.39 ± 0.27	95.27	<0.0001
T4	5.07 ± 0.06	5.08	4.96 ± 0.03	5.00	0.01
D7	101.64 ± 0.31	101.83	100.17 ± 0.24	100.28	0.00012
E9	18.91 ± 0.10	18.85	18.38 ± 0.86	18.44	0.00012
J10	54.51 ± 0.32	54.19	55.69 ± 0.25	55.58	0.004
Slenderness	2.09 ± 0.03	2.08	2.21 ± 0.03	2.22	0.005
LWFW	33.62 ± 0.07	33.53	33.57 ± 0.1	33.56	0.03
FEM	2.52 ± 0.023	2.52	2.51 ± 0.02	2.53	0.021
Prob	5.42 ± 0.009	5.40	5.39 ± 0.06	5.41	0.05

Kruskal-Wallis test). Discriminant analysis of sample colony means using Wilks Lambda test confirmed their affiliation of the samples to their original clusters (A = 0.26; F = 8.28, P < 0.0001). However, in cross-validating grouping four sets of measurements (~3%) were assigned incorrectly (2 data sets from Jazan and 2 from Najran). Figure 3 shows factor scores of the colony means for factor one and two, labelled according to their K-means clustering and sample allocations. In principal component analysis of the 20 forewing landmarks, coordinates associated with factor one accounted for >99% of the variance between both clusters. Geometric analysis of forewing landmarks confirmed the allocation of the samples into two clusters using K-means clustering procedure with highly significant correlation between morphometric and geometric approaches in separating samples into the same clusters (R = 0.474; P < 0.0001). Table 5 shows colony means and medians of characters with significant variation between both clusters. Figure 4 shows factor scores of colonies means presented according to their K-mean clustering affiliation with a slight degree of overlapping at margins. Discriminant analysis using Wilks' Lambda test demonstrated that differences between both clusters are highly significant (A = 0.22, F = 8.28, P < 0.0001). In cluster one 98.5% of the colonies were reclassified correctly, in cluster two 97.2% of the colonies were reclassified correctly. Mahalanobis distance between both clusters was 11.7. Geographical discrimination indicated that 84% of the samples from Al-Madinah and Al-Taif (northern part) are members of cluster two and 82% of the samples from Asir and Jazan (southern part) are located in cluster one.

Sequences alignment showed high similarity among our sequences and reference sequences of the sub lineage Z (previously O lineage) and of the lineage C (*Apis mellifera anatoliaca* Maa) and of the lineage M (*A. m. mellifera*), and revealed 24 variable sequences, 22 of them were previously unreported. New haplotypes were uploaded into NCBI (supplemental material table S1). Sampled sequences could be divided into three groups, sequences with few numbers of nucleotide substitution (haplotypes 1-4 and 19), sequences with additional insertions of nucleotides (haplotypes 6-18), and the last group that comprises two haplotypes (haplotype 20-21) with a high rate of substitution. Sequences of the second group are restricted to northern regions, while those of group three are restricted to the far southern part (table 6). The evolutionary distances were computed using Maximum Likelihood method, and showed that group one and two are very close with evolutionary distance P = 0.001, while group three is not close to the other two groups with a distance of P = 0.036 and 0.035 for group one and two respectively. This may indicate that group three is not part of the Z sub-lineage. Figure 5 shows the evolutionary history among our sequences and reference samples.

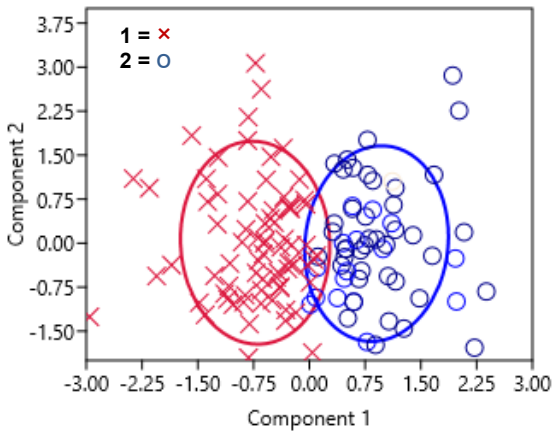

Figure 3. Principal factor analysis scores plotted using K-means clusters with 75% confidence ellipses. Sample affiliation revealed that 69 of the samples from the southern region (Najran, Jazan, Makkah and Al-Baha) were members of cluster one, while 59% of the samples from the northern areas (Al-Madinah and Tabuk) grouped in cluster two.

Table 5. Forewing landmarks coordinates (Mean ± SE; Median) that contributed significantly to discriminate native *Apis mellifera* population of Saudi Arabia into two clusters.

Forewing landmark	Axes	Cluster -1		Cluster -2		Kruskal - Wallis P
		Mean ± SE	Median	Mean ± SE	Median	
3	Y(+)	0.01936 ± 0.0002	0.0191	0.01815 ± 0.0002	0.0180	0.00002
4	X(-)	0.1608 ± 0.0003	0.1609	0.1594 ± 0.0002	0.1592	0.00006
4	Y(+)	0.0713 ± 0.0002	0.0714	0.0703 ± 0.0002	0.0702	0.00009
5	X(-)	0.1054 ± 0.0004	0.1055	0.1028 ± 0.0003	0.1029	0.0000007
7	Y(+)	0.0544 ± 0.0004	0.0543	0.0573 ± 0.0003	0.0573	0.0000001
8	X(+)	0.2047 ± 0.0004	0.2047	0.2093 ± 0.0004	0.2089	1×10^{-12}
10	X(+)	0.2456 ± 0.0003	0.2457	0.2448 ± 0.0003	0.2448	0.03
10	Y(-)	0.0602 ± 0.0002	0.0603	0.0591 ± 0.0002	0.0594	0.0002
11	X(+)	0.1833 ± 0.0003	0.1834	0.1843 ± 0.0003	0.1826	0.03
12	X(+)	0.0795 ± 0.0004	0.0792	0.0808 ± 0.0003	0.0809	0.01
13	X(+)	0.0184 ± 0.0003	0.0185	0.0195 ± 0.0001	0.0762	0.002
14	X(-)	0.0446 ± 0.0003	0.0446	0.0423 ± 0.0003	0.0426	0.0000003
14	Y(+)	0.0470 ± 0.0002	0.0467	0.0460 ± 0.0002	0.0459	0.0009
16	X(-)	0.0677 ± 0.0002	0.0675	0.0686 ± 0.0002	0.0687	0.002
17	X(-)	0.0776 ± 0.0002	0.0776	0.0791 ± 0.0002	0.0016	0.000002
18	X(-)	0.1059 ± 0.0002	0.1059	0.1089 ± 0.0002	0.1089	1×10^{-13}
19	X(-)	0.0832 ± 0.0003	0.0832	0.0879 ± 0.0004	0.0877	1×10^{-14}
20	X(+)	0.1599 ± 0.0003	0.1604	0.1570 ± 0.0003	0.1571	5×10^{-9}

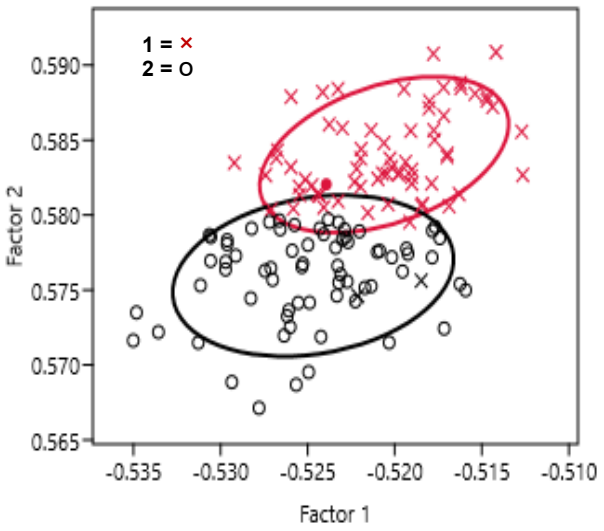

Figure 4. Principal factor analysis scores of colonies forewing landmarks coordinates plotted using K-means clusters with 80% confidence ellipses. Most samples from the northern regions (Al-Madinah - Al-Taif) group in cluster two and most samples from the southern part (Asir - Jazan) group in cluster one.

Discussion and conclusion

Using classical morphometry, forewing landmarks and *mt*DNA tRNAleu-COII analyses of the same samples was very useful to characterize the honey bee population in the study area and to explore its relationship with other subspecies. Both morphometric and geometric approaches were successful in grouping the samples into two clusters with highly significant correlation in allocating the samples into the same cluster, with cluster one dominating the southern regions near Yemen and cluster two with lighter pigmentations and larger size dominates the northern regions which are closer to natural distribution range of *A. m. syriaca*. Sequence analysis of the *mt*DNA tRNAleu-COII of 133 colonies sampled along the Red Sea Cost of Saudi Arabia revealed 22 novel haplotypes, 91% of them (20 haplotypes) assigned to the African Z sub-lineage and clustered phylogenetically with the *A. m. syriaca* and *A.m. lamarckii* reference sequences. Out of the 20 haplotypes, the ones which geographically resemble the northern region of the Red Sea Coast (Al-Madinah and Tabuk) are almost restricted by morphometry and geometry to cluster two, while the others are dominant in cluster one.

All samples from Al-Madinah and most samples from Tabuk (northern regions) were collected from historical colonies spread in caves along the Sarat Alhejaz mountain chain and are being maintained by the same family and continued for generation spanning several hundreds of years, which may partly explain the discrepancy of their *mt*DNA tRNAleu-COII sequences compared to other samples. The other 2 haplotypes (haplotype 20 and 21) clustered closer to *A. m. anatoliaca* and other *A. m. syriaca* reference sequences from southern Turkey, which are members of Z sub-lineage. Both haplotypes were assigned morphometrically to *A. m. jemenitica* samples and clustered geometrically in cluster two. Alghamdi and Alattal (2020) based on analysis of complete mitochondrial PCGs of *A. m. jemenitica* for the same samples reported a third group that was apparently not a member of Z sub-lineage. This may indicate that both analyses based on complete mitogenomes and *mt*DNA tRNAleu-COII using large sample size were highly related except for two samples from (haplotypes 20 and 21). We assessed that our samples correspond to two distinct clusters reporting for the first time two haplotypes (20 and 21)

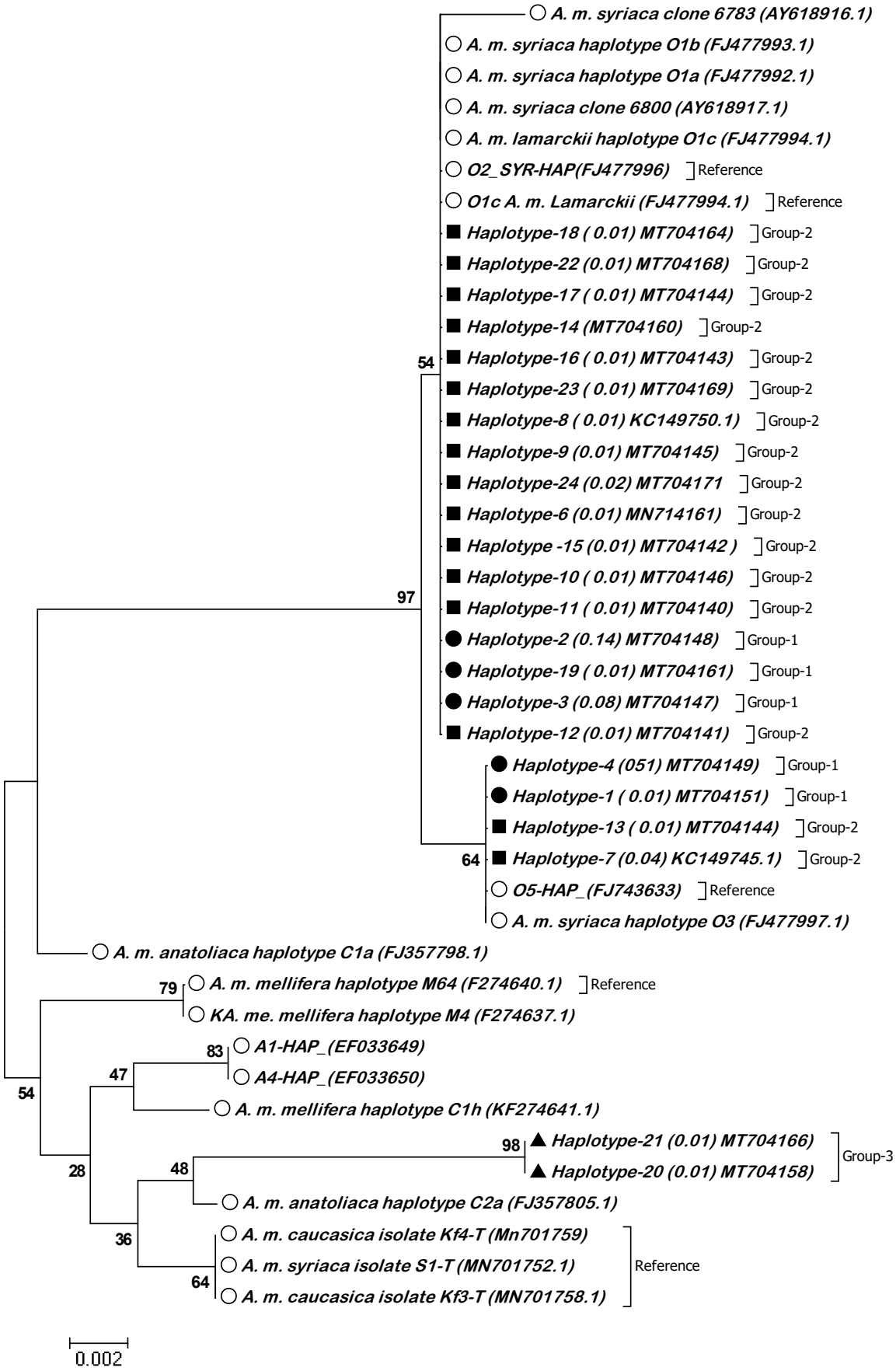

Figure 5. Minimum evolution phylogenetic tree for COI-COII intergenic region sequences of new haplotypes of our samples and 19 sequences of other subspecies representing other honey bee lineages. The evolutionary distances were computed using maximum composite. Evolutionary analyses were conducted in MEGA7.

Table 6. New haplotypes (based on mtDNA analyses) distribution in different regions along the Red Sea Coast of Saudi Arabia and their membership (%) into either of both morphometric and geometric clusters.

Haplotype	Accession No.	Freq. (%)	Region one								Wing geometry class (%)		Morphometry class (%)	
			Makkah	Al-Madinah	Al-Taif	Jazan	Najran	Tabuk	Al-Baha	Asir				
			No. (%)								I	II	I	II
1	MT704151	0.01	-	-	-	-	-	-	-	1	100	-	100	-
2	MT704148	0.15	3	-	9	1	1	1	1	4	77	33	82	18
3	MT704147	0.08	3	-	1	2	-	1	-	4	60	40	60	40
4	MT704149	0.51	13	-	2	10	11	10	14	8	63	37	69	31
5	MT704156	0.01	-	-	-	1	-	-	-	-	-	100	-	100
6	MN714161	0.02	-	-	-	-	-	-	-	2	100	-	100	
7	KC149745.1	0.02	-	3	-	-	-	-	-	-	100	-	100	-
8	KC149750.1	0.02	-	2	-	-	-	-	-	-	50	50	-	100
9	MT704145	0.02	-	2	-	-	-	-	-	-		100		100
10	MT704146	0.01	-	1	-	-	-	-	-	-	100		100	
11	MT704140	0.01	-	1	-	-	-	-	-	-		100		100
12	MT704141	0.02	-	2	-	-	-	-	-	-	50	50		100
13	MT704162	0.01	-	1	-	-	-	-	-	-		100		100
14	MT704160	0.01	-	1	-	-	-	-	-	-	100		100	
15	MT704142	0.02	-	2	-	-	-	-	-	-		100		100
16	MT704143	0.01	-	1	-	-	-	-	-	-		100		100
17	MT704144	0.01	-	1	-	-	-	-	-	-		100		100
18	MT704164	0.01	1	-	-	-	-	-	-	-		100		100
19	MT704161	0.02	-	-	2	-	-	-	-	-		100		100
20	MT704158	0.02	-	-	-	2	-	-	-	-		100		100
21	MT704166	0.02	-	-	-	-	2	-	-	-	50	50		100
22	MT704168	0.02	-	-	-	-	-	2	-	-		100		100
23	MT704169	0.01	-	-	-	-	-	1	-	-	100		100	
24	MT704171	0.02	-	-	-	-	-	3	-	-	66	33	66	33

exhibiting features of both lineages A (Sub-lineage Z) and C. Future comparison of complete *A. m. jemenitica* mitogenomes of our samples with a suitable number of mitogenomes from same reference honey bee subspecies such as *A. m. syriaca* or *A. m. anatoliaca* and of the Socotra honey bee (Alattal *et al*., 2019) may enrich our knowledge about the phylogenetic relationships among these honey bee subspecies. We can conclude that Arabian *A. m. jemenitica* honey bee is clearly distinct from same subspecies populations of African origin as well as from the Syrian honey bee *A. m. syriaca*, and the Egyptian honey bee reference samples *A. m. lamarckii* with considerable evolutionary distance among the four subspecies. The name *A. m. jemenitica* was introduced in 1976 by Ruttner to describe a honey bee subspecies that is naturally distributed within several localities in Africa and Asia. Results of this study provide evidence that the honey bee population that exist along the Red Sea Coast of Saudi Arabia represent a distinctive population and might propose a new honey bee subspecies.

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