



HAL
open science

Mitochondrial structure of Eastern honeybee populations from Syria, Lebanon and Iraq

Mohamed Alburaki, Sibyle Moulin, H el ene Legout, Ali Alburaki, Lionel Garnery

► **To cite this version:**

Mohamed Alburaki, Sibyle Moulin, H el ene Legout, Ali Alburaki, Lionel Garnery. Mitochondrial structure of Eastern honeybee populations from Syria, Lebanon and Iraq. *Apidologie*, 2011, 42 (5), pp.628-641. 10.1007/s13592-011-0062-4 . hal-01003598

HAL Id: hal-01003598

<https://hal.science/hal-01003598>

Submitted on 11 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin ee au d ep ot et  a la diffusion de documents scientifiques de niveau recherche, publi es ou non,  emanant des  tablissements d'enseignement et de recherche fran ais ou  trangers, des laboratoires publics ou priv es.

Mitochondrial structure of Eastern honeybee populations from Syria, Lebanon and Iraq

Mohamed ALBURAKI^{1,4}, Sibyle MOULIN¹, H el ene LEGOUT¹, Ali ALBURAKI²,
Lionel GARNERY^{1,3}

¹CNRS, Laboratoire Evolution, G enomes et Sp eci ation, UPR 9034, CNRS Avenue de la Terrasse, B atiment 13, Boite Postale 1, 91198 Gif-sur-Yvette, France

²Faculty of Agriculture, University of Damascus, P.O. Box 30621, Damascus, Syria

³Universit e de Versailles Saint-Quentin-en-Yvelines, 45 Avenue des Etats-Unis, 78000 Versailles, France

⁴National Commission for Biotechnology NCBT, Damascus, Syria

Received 8 November 2010 – Revised 21 January 2011 – Accepted 22 January 2011

Abstract – Honeybees (*Apis mellifera*) from Syria ($n=1,624$), Lebanon ($n=177$), and Iraq ($n=35$) were collected for genetic analysis. These samples were analyzed by the cleaved amplified polymorphisms technique using mitochondrial DNA (mtDNA) as a molecular marker in their COI–COII intergenic region (Cytochrome Oxidase I and II). Polymerase chain reaction amplicons (500–950 bp) were subjected to restriction analysis by *Dra*I enzyme. The restriction profiles were analyzed and compared with the existing mtDNA markers on polyacrylamide gels. Sixteen new haplotypes were identified, 14 of them on the African lineage A, one new haplotype on the North Mediterranean lineage C and one other on the West Mediterranean lineage M. DNA sequences of all these haplotypes were subsequently determined. We also identified five other haplotypes that had been described in a previous study. These five haplotypes were renamed and grouped more appropriately with the subgroup Z, rather than with the Oriental lineage O. The sequence structures of these novel haplotypes clearly display the specific characteristics of the African branch (A). However, they differ from the African haplotypes by a new restriction site; therefore, they have been moved into a new subgroup. We conclude that *A. mellifera syriaca* subspecies does not belong to the Oriental lineage O, but is situated geographically in an area of contact between the A and O lineages.

Apis mellifera syriaca / CAPS / haplotype / honeybee / intergenic region COI–COII / Cyt b / mtDNA

1. INTRODUCTION

Based on morphometrical approaches, the honeybee (*Apis mellifera* L.) has been subdivided into four different lineages: (1) the West Mediterranean lineage (M), which includes the Western European subspecies *A. mellifera mellifera*; (2) the North Mediterranean lineage (C); (3) the African lineage (A), which groups the

African honeybee subspecies; and (4) the Oriental lineage (O), which is distributed in the Middle East and represented mainly by Caucasian and Turkish honeybees *A. mellifera caucasica* and *A. mellifera anatoliaca*, respectively (Ruttner 1988). The Syrian honeybee subspecies (*A. mellifera syriaca*), present in the valleys east of the Mediterranean (Syria, Lebanon, and Jordan), was described by Bodenheimer (1941). It is the smallest of all Oriental and European races of *A. mellifera*; only African races, including *yemenitica*, are smaller (Ruttner 1988). The Syrian honeybee subspecies, and its relationship to the lineages

Corresponding author: M. Alburaki,

alburaki@legs.cnrs-gif.fr

Manuscript editor: David Tarpy

described above, is of interest for a number of reasons. First, it is evident from the distribution map of honeybee races of the Near East published by Ruttner (1988) that honeybees in parts of Syria, Jordan, and Iraq have not yet been studied. It is therefore unknown whether the Syrian bee, reported in Lebanon and the far west of Syria and Jordan, also occur east of these areas. Second, the fairly compact *A. mellifera syriaca* cluster is positioned peripheral to the oriental lineage and very close to the African races in all of the multivariate analyses presented by Ruttner (1988). Third, in the 3-D phenogram of *A. mellifera* that characterizes the four evolutionary lineages (Ruttner 1988), the Syrian honeybee clearly appears in the A branch next to *A. mellifera meda*. According to Ruttner's classification, the honeybee subspecies *A. mellifera anatoliaca*, which is naturally distributed over most of the Turkish territory, belongs to the O branch. This Turkish bee comes into contact with the *meda* bee in southeastern Turkey, but we do not know the exact limits of this contact with the Syrian honeybee in the north of Syria. Molecular genetic techniques, including analysis of mitochondrial DNA (mtDNA) and microsatellite markers, allow reliable classification of honeybee subspecies (Smith et al. 1991; Estoup et al. 1995). Mitochondrial DNA is widely used as a molecular marker to study genetic diversity among populations (Hall and Smith 1991; Franck et al. 2000; Abrahamovich et al. 2007; El-Niweiri and Moritz 2008; Özdil et al. 2009); it is more suitable than the morphometrical approach, which can be affected by environmental factors (Avisé et al. 1987). Hence, the first three lineages (A, C, and M) were later confirmed using molecular techniques that involved mtDNA (Garnery et al. 1991, 1995; Smith et al. 1991) and microsatellite markers (Estoup et al. 1993, 1995). Based upon 75 Lebanese honeybee samples and only a few Syrian samples, Franck et al. (2000) suggested that these populations could belong to the fourth lineage (O), as the mtDNA sequence

structures of these samples differed sufficiently from the three other described lineages (A, C, and M). On the other hand, Kandemir et al. (2006) expressed concern that designating this distinct mitochondrial lineage as "O" may cause confusion with the O morphological lineage described by Ruttner (1988). However, using a large number of single-nucleotide polymorphism markers, Whitfield et al. (2006) sorted the four honeybee evolutionary lineages and, based on nine Syrian honeybee samples, placed Syrian bees in the Oriental branch O along with the *anatoliaca*, *pomona*, and *caucasica* subspecies. Given these contradictory views, it appears that sampling honeybees from Syria could provide useful insight into the diversity of the O lineage of *A. mellifera*. The different opinions on Syrian honeybee classification that have arisen from previous studies might have been inevitable because subspecies located in geographical contact zones or in zones of secondary contact between lineages are not easy to identify. An in-depth analysis of Syrian honeybee populations was necessary for all of these reasons and also to allow us to delimit the contact zones between lineages and to understand the mitochondrial structure of honeybee populations in Syria. Based on an mtDNA marker, we analyzed a large number of populations from three different countries (Syria, Lebanon, and Iraq) and have generated a thorough description of Syrian honeybee genetic diversity and distribution.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

Honeybee workers ($N=1,836$) were collected from managed colonies in 2007 and 2008. Samples were collected from 12 Syrian regions, four Lebanese regions, and one Iraqi region. One worker was collected from each colony and 1,634 colonies were sampled in Syria, 167 colonies were sampled in Lebanon, and 35 colonies were sampled in Iraq. The

location of each of these sampling regions, as well as the number of samples taken in each region, is shown in Figure 1.

Each worker bee was individually preserved in absolute ethanol, and wings were removed and preserved in ethanol for further morphometrical analysis. DNA was extracted from the thorax using the Chelex method (Walsh et al. 1991). Briefly, the thoraxes were ground and added to 0.1-M Tris-HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid, 100 mM NaCl, 0.1% sodium dodecyl sulfate, 50 mM dithiothreitol and 0.25 mg/mL proteinase K. The extractions were placed at 50°C for 1 h followed by 30 min at 95°C, to eliminate the enzyme. DNA extractions were centrifuged for 15 min at 12,000 rpm and were preserved at -20°C.

2.2. PCR amplification and gel electrophoresis

Standard methods for polymerase chain reaction (PCR) amplification in honeybees were used. PCR amplification of the COI-COII intergenic region of the mtDNA was performed using PCR primers E2: 5'-GGCAGAATAAGTGCATTG-3' and H2: 5'-CAATATCATTGATGACC-3' (Garnery et al. 1993). PCR was carried out in a total volume of 25 µL, containing

2.5 µL of Taq 5× buffer (Promega), 1.5 mM MgCl₂, 25 pmol of primers E2 and H2 (Garnery et al. 1992), 25 nmol of each dNTP, 0.6 unit of Promega *Taq* polymerase, and 1.0 µL of DNA extract. Each PCR reaction was subjected to an initial denaturation cycle at 92°C for 3 min, followed by 30 cyclic reactions. Each of these cycles consisted of three steps: (1) denaturation of the DNA for 30s at 92°C, (2) hybridization of the primers to the template DNA for 1 min and 30s at 47°C, and (3) elongation of the primer template hybrid for 2 min at 63°C. Finally, the mixture was kept at 63°C for 10 min and PCR products were stored at -20°C. PCR results were resolved using 1.4% agarose gel electrophoresis with the molecular marker MIII. PCR products were photographed under UV light after immersion in ethidium bromide. This migration according to the molecular weight of the molecular marker MIII (Roche Diagnostics GmbH) enabled us to distinguish the organization of the intergenic region COI-COII.

2.3. CAPS technique

Evolutionary lineage and the haplotypic polymorphisms were characterized using the cleaved amplified polymorphisms technique. Briefly, PCR

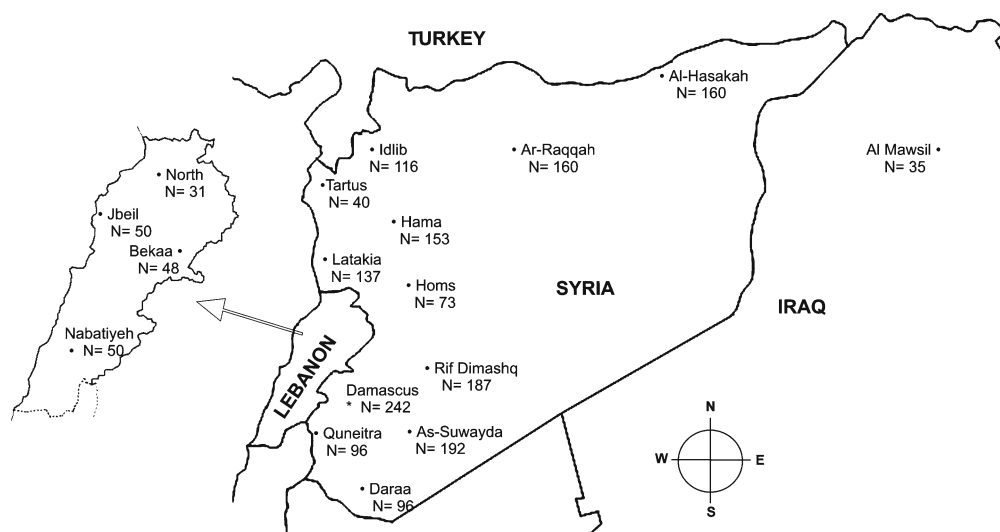


Figure 1. Locations of the sampled regions in Syria, Lebanon, and Iraq. They comprise 12 populations from Syria, one from Iraq, and four from Lebanon. *N* is the number of honeybees sampled in each population.

amplicons were cleaved by the restriction enzyme *DraI* and were incubated 48 h at 37°C. The restricted DNA was loaded on a 7.5% polyacrylamide gel and run at 150 V for 3 h. The differentiation in the various sizes of the DNA fragments allows the identification of three mitochondrial lineages and also reveals intralinear polymorphisms (Garnery et al. 1993; Moritz et al. 1994; Sheppard et al. 1999). Unbiased estimates of haplotype diversity (Nei and Tajima 1981) and the corrected haplotype diversity (Garnery et al. 1998) were calculated based on the total number of haplotypes in each population. Estimated values of introgression for each population were also calculated. The correlation coefficient of the north–south gradient for the A and C lineages was calculated after omitting the imported haplotypes in each of the populations.

2.4. Sequence of mtDNA haplotypes

To interpret and confirm the observed polymorphism using the results of PCR restriction fragment polymorphisms obtained in the COI–COII intergenic region, at least one sample from each newly obtained restriction pattern was sequenced. This step allowed an exact estimate of the size of *DraI* restriction fragments by eliminating probable mismatch artifacts between the observed restriction fragment pattern and the haplotype reference. It also allowed us to detect small insertions or deletions and substitutions. Sequencing was performed by standard PCR using E2, H2 primers, followed by PCR sequencing with Big Dye Mix and both E2 and H2 primers. Analysis was done using a four-capillary ABI genetic analyser 3130. Restriction maps and other alignments were performed using BioEDIT and SEcentral software. Sequences for new haplotypes were submitted to GenBank. In addition to the COI–COII haplotypes sequences, the Cytochrome *b* (Cyt *b*) gene of two haplotypes of the C lineage and six other Z haplotypes belonging to the A lineage have been sequenced. Based on these sequences and other reference sequences from GenBank (Pinto et al. 2003), a neighbor-joining tree has been performed on nucleotide distances computed by Kimura's (1980) formula through PAUP* program, version Beta 4.0 (Swofford 2002).

3. RESULTS

3.1. Structure of the COI–COII intergenic region

For all studied samples, the migration profiles of the amplified fragments obtained on agarose 1.4% gel exhibit four different types of fragments. These four types of fragments correspond to the structures: Q, P/PoQ, P/PoQQ, and P/PoQQQ (Figure 2).

Five different COI–COII intergenic region lengths were observed within the Syrian honeybee populations and the vast majority were the PoQ length (524 Q, 895 PoQ, 16 PQQ, 178 PoQQ, and 25 PoQQQ). Four different lengths were observed for the Lebanese populations, most of which were also PoQ (20 Q, 112 PoQ, 31 PoQQ, and 4 PoQQQ), while Q length was observed for all Iraqi bee samples (35 Q), Table I.

3.2. Novel haplotypes identified

DraI restriction analysis performed on all samples revealed 27 different haplotypes. Among these haplotypes, 22 belonged to the African lineage A, four to the North Mediterranean lineage C, and another one (M63) to the West Mediterranean lineage M. A detailed description of the 17 studied populations is shown in Table I.

Of the 22 haplotypes belonging to the A lineage, 14 haplotypes are new and have not been described in previous studies. Five other haplotypes detected in our samples have been previously described but were classified as belonging to the Oriental lineage, O (Franck et al. 2001). The three remaining haplotypes (A1, A2, and A3) are classical mitotypes of the African lineage A. The North Mediterranean lineage C was found in almost all of the populations. Among the classical haplotypes of the C branch (C1, C2, and C3) found in our samples, one new restriction haplotype pattern belonging to this branch was found in the Hama honeybee population, and was named C8. The numbering of new haplotypes found in our

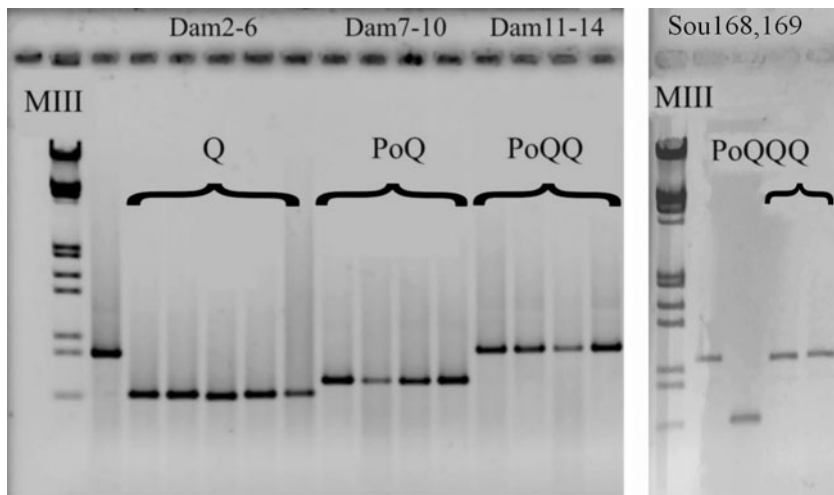


Figure 2. Structural organization of the COI–COII intergenic region of mtDNA migrated on 1.4% agarose gel. *MIII* is a molecular-size marker, *Q* is the specific sequence of the C lineage, while *PoQ*, *PoQQ*, and *PoQQQ* correspond to the African lineage A.

study was based on the number of the last haplotype found in each evolutionary lineage, according to the established rules for defining a new haplotype in the COI–COII marker (Garnery et al. 1992, 1993). The percentage presence of each evolutionary lineage found in each population is presented in Figure 3. We found that C lineage frequencies ranged from 100% of honeybees belonging to this branch in Al-Mawsil (Iraq) to only 6% in both the Daraa (Syria) and Nabatiyeh (Lebanon) populations (Figure 3). On the other hand, the African lineage A was the most observed lineage in all samples, ranging from 94% presence in both the Daraa and Nabatiyeh populations down to 9% in the Al-Hassaka population. The A lineage was not found at all in the Al-Mawsil population. The calculated correlation coefficient of the north–south gradient for the A and C lineages was highly significant ($R=0.778$, $P<0.001$, Figure 7). The M mtDNA lineage was only recorded in low amounts in two Syrian populations: Damascus and Rif Damashq (1% and 7%, respectively; Figure 3). This was a single, restricted, and previously unknown haplotype pattern, which we named M63 (Table I). Table II presents haplotype diversity for each population, reflect-

ing the level of population diversity. The lowest diversity was observed in Al-Mawsil (Iraq, 0.057) and the highest value in Rif Dimashq (Syria, 0.878). However, the presence of imported or non-local haplotypes in each population can bias diversity values. Therefore, corrected haplotype diversity values were calculated by removing the five known imported haplotypes (C1, C3, A2, A3, and M63) from the calculation (Table II). Surprisingly, corrected haplotype diversity was very low in some populations.

3.3. COI–COII mtDNA sequencing

At least one sample of each new haplotype belonging to the African lineage A was sequenced for the intergenic region COI–COII. The C8 and M63 haplotypes belonging to the C and M branches, respectively, were also sequenced. The sequences are available in NCBI GenBank under the following access numbers: M63, HM236202; C8, HM236203; Z1, HM236204; Z1', HM236205; Z2, HM236206; Z3, HM236207; Z4, HM236208; Z7, HM236209; Z8, HM236210; Z9, HM236211; Z12, HM236212; Z13, HM236213; Z15, HM236214; Z16,

Table 1. Number and distribution of COI-COII/*DraI* haplotypes found in Syrian, Lebanese, and Iraqi honeybee populations.

Country/ Population	Evolutionary lineage, COI-COII structure organization, mtDNA haplotype																			M lineage								
	African Lineage A																			North Mediterranean Lineage C or Oriental lineage O								
PoQQ	PoQQ	PoQ																	Q			PQQ						
Z1' /O1''	Z2' /O1'	Z6'	Z8	Z11	Z12	Z13	Z4 /O3	Z5	Z7 /O1	Z9	Z1	Z3	Z15	Z16	Z17	Z19	Z21	A1	A2	A3	C8	C2	C1	C3	M63	Total		
Syria																												
Damascus	2	18	-	-	-	-	30	-	17	-	12	43	-	57	1	-	-	-	7	1	-	-	32	1	-	3	224	
Rif Dimashq	-	20	-	-	-	-	3	1	32	-	27	36	-	16	-	4	1	19	-	-	-	-	12	3	-	13	187	
As-Suwayda	12	59	-	25	-	-	-	2	12	12	5	27	-	-	-	-	-	-	-	-	-	25	13	-	-	192		
Hama	-	-	-	-	-	-	-	2	-	-	-	4	-	26	-	-	-	-	-	-	2	95	24	-	-	153		
Daraa	1	15	-	-	-	-	23	-	4	-	4	19	-	24	-	-	-	-	-	-	-	6	-	-	-	96		
Al-Hasakah	-	1	-	-	-	-	-	2	3	1	3	-	2	-	-	3	-	-	-	-	-	145	-	-	-	160		
Homs	-	7	-	-	-	-	-	5	2	-	12	-	25	-	-	-	-	-	-	-	-	20	1	1	-	73		
Idlib	-	-	-	-	-	-	3	14	1	-	-	7	-	17	-	-	-	-	-	-	-	72	2	-	-	116		
Quneitra	-	14	-	-	-	-	-	-	1	20	37	2	12	-	-	-	-	-	-	-	-	10	-	-	-	96		
Latakia	6	2	-	-	-	-	5	2	47	-	1	43	-	8	-	-	-	-	-	-	-	17	3	3	-	137		
Ar-Raqqa	-	16	-	-	-	-	1	18	-	-	90	-	7	-	1	-	-	-	-	-	-	20	-	7	-	160		
Tartus	-	1	-	-	-	-	1	-	12	-	-	5	-	11	-	-	-	-	-	-	-	6	3	1	-	40		
Lebanon																												
Nabatiye	2	11	1	-	-	-	2	-	6	-	18	4	-	-	-	-	-	-	-	-	-	-	3	-	-	48		
North	-	-	-	-	-	-	-	-	-	-	3	7	-	4	-	-	-	-	-	-	-	3	4	-	-	21		
Jbeil	1	4	-	-	1	5	-	-	-	-	18	8	-	8	-	-	-	-	-	-	-	-	5	-	-	50		
Bekaa	-	1	-	5	1	-	6	-	4	-	6	16	-	4	-	-	-	-	-	-	-	3	2	-	-	48		
Iraq																												
Al-Mawsil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	34	1	-	-	35		
Total	4	21	169	1	30	2	5	2	72	46	138	15	115	361	2	221	1	5	4	19	7	1	2	500	65	12	16	1,836

The evolutionary lineages and the structural organization of each haplotype are shown in the first three lines. Haplotype nominations are indicated in bold, old haplotype nominations are mentioned under the new haplotype names preceded by a slash. The last line shows the total number of each haplotype found in all populations, while the last column represents the total number of all haplotypes found in each population

(-) This haplotype was not found in the population

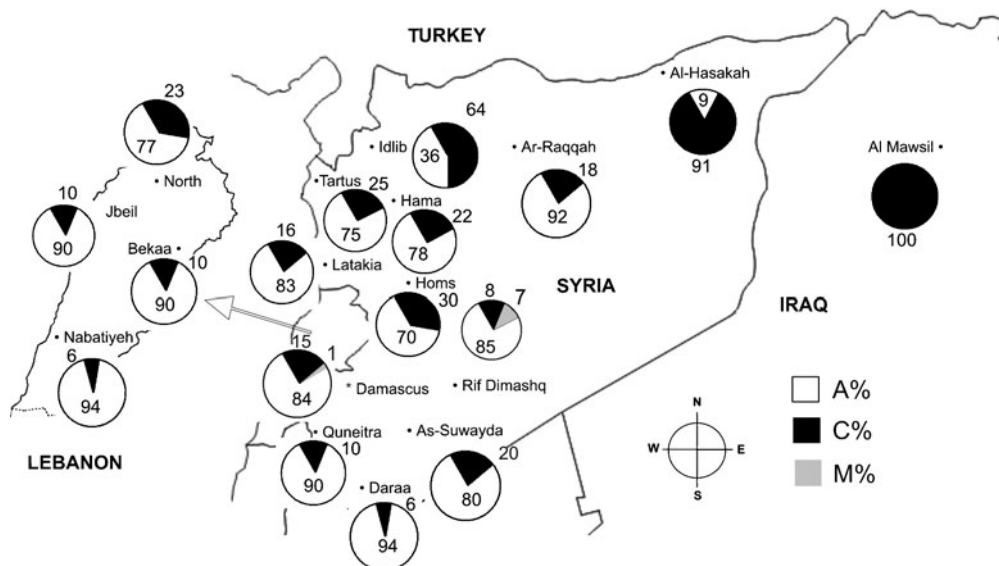


Figure 3. Pie charts of the percentage of the three mtDNA lineages *A*, *C*, and *M* in the different Syrian, Lebanese, and Iraqi populations.

HM236215; Z17, HM236216; Z19, HM236217; Z21, HM236218; Z6', HM236219.

Restriction maps for each new haplotype are shown in Figure 4. Based on the sequence results, the length of the COI–COII region for each haplotype found in our study (Q, PoQ, PQQ, PoQQ, and PoQQQ) have been determined, along with the restricted *DraI* sites. All haplotypes belonging to the African lineage A contain the fragment Po in their sequence. This is not the case for the M63 haplotype of the West Mediterranean lineage M, in which Po is replaced by a P fragment (Figure 4). The sequence of the unique new haplotype found in the North Mediterranean branch (C8) is presented in this paper as an example (Figure 5). The new restriction pattern of the C8 haplotype is due to a deletion of 10 nucleotides in the Q sequence. Sequencing of this haplotype showed a substitution in the Q fragment at the 203 bp position (Figure 5).

3.4. Z haplotype subgroup

The result of neighbor-joining tree analysis is shown in (Figure 6). The topology of this tree discriminates clearly a subgroup of haplotype Z,

since all Z haplotype samples formed an independent subgroup Z of the African lineage A.

4. DISCUSSION

Numerous morphometrical studies have attempted to classify the *A. mellifera* subspecies. These studies have mainly led to the identification of four different lineages A, M, C, and O (Ruttner et al. 1978; Ruttner 1988). In one of these studies (Ruttner 1988), *A. mellifera syriaca* consistently clustered with the African bees, regardless of the samples used (24 races of *A. mellifera* vs. exclusively Eastern Mediterranean bees) or the method used to analyze the data (principal component analysis vs. phenogram). An important point is that in his studies, Ruttner first described and grouped many subspecies by geographical locations; using this method the Syrian honeybee is grouped as an Oriental honeybee subspecies. But, after statistical analyses, some of these subspecies clustered in different groups from those described geographically; the Syrian honeybee clustered with the African group. This has created some confusion between

Table II. Number of samples, haplotype diversity and corrected haplotype diversity for each Syrian, Lebanese, and Iraqi honeybee population.

Country/ population	Number of sample	Haplotype diversity	Corrected haplotype diversity	Excluded haplotype	Num. of excluded haplotype	Estimated introgression %
Syria						
Damascus	224	0.847	0.834	C1, A2, A3, M63	1, 7, 1, 3	5.35
Rif Dimashq	187	0.878	0.875	C1, M63	3, 13	8.55
As-Suwayda	192	0.839	0.820	C1	13	6.77
Hama	153	0.564	0.400	C1	24	15.68
Daraa	96	0.818	0.818	–	–	0
Al- Hasakah	160	0.178	0.178	–	–	0
Homs	73	0.776	0.763	C1	1	1.36
Idlib	116	0.579	0.564	C1	2	1.72
Quneitra	96	0.768	0.768	–	–	0
Latakia	137	0.766	0.745	C1, C3	3, 3	4.37
Ar-Raqqah	160	0.645	0.614	C3	1	0.62
Tartus	40	0.809	0.768	C1, C3	3, 1	10
Lebanon						
Nabatiye	48	0.793	0.767	C1	3	6.25
North	21	0.814	0.757	C1	4	19.04
Jbeil	50	0.808	0.773	C1	5	10
Bekaa	48	0.844	0.831	C1	2	4.16
Iraq						
Al Mawsil	35	0.057	0.00	C1	1	2.8

Non-local haplotypes (excluded haplotypes) found in each population are cited as well as their numbers and the estimated introgression in each population

(–) Means not found

the subspecies names based on geographical location and the Oriental morphometrical lineage O that was also described by Ruttner (this confusion is discussed in Kandemir et al. (2006)).

Adding to the confusion, the COI–COII test is not able to discriminate the Oriental lineage O from the C lineage. Therefore, the increase of C lineage in the north and northeast of Syria could be related to an increase in the undetectable O lineage haplotypes originally located in Turkey and Caucasus.

Our data show that the African lineage A is present in all Syrian and Lebanese honeybee

populations; these samples universally contain the Po sequence within their mtDNA COI–COII sequence (Cornuet et al. 1991; Gamery et al. 1993). This specific fragment, which characterizes the A lineage, is not present in the honeybees of Turkey, Caucasus, or Iran (Palmer et al. 2000; Gruev et al. 2005) where the O morphometrical lineage is expected to be (Ruttner 1988). Moreover, the region inhabited by Syrian bees is not part of the geographical area of the morphometrical O lineage described by Ruttner (1988). However, based on samples collected from Lebanon and Syria, Franck et al. (2000) designated new restriction fragment patterns to a putative

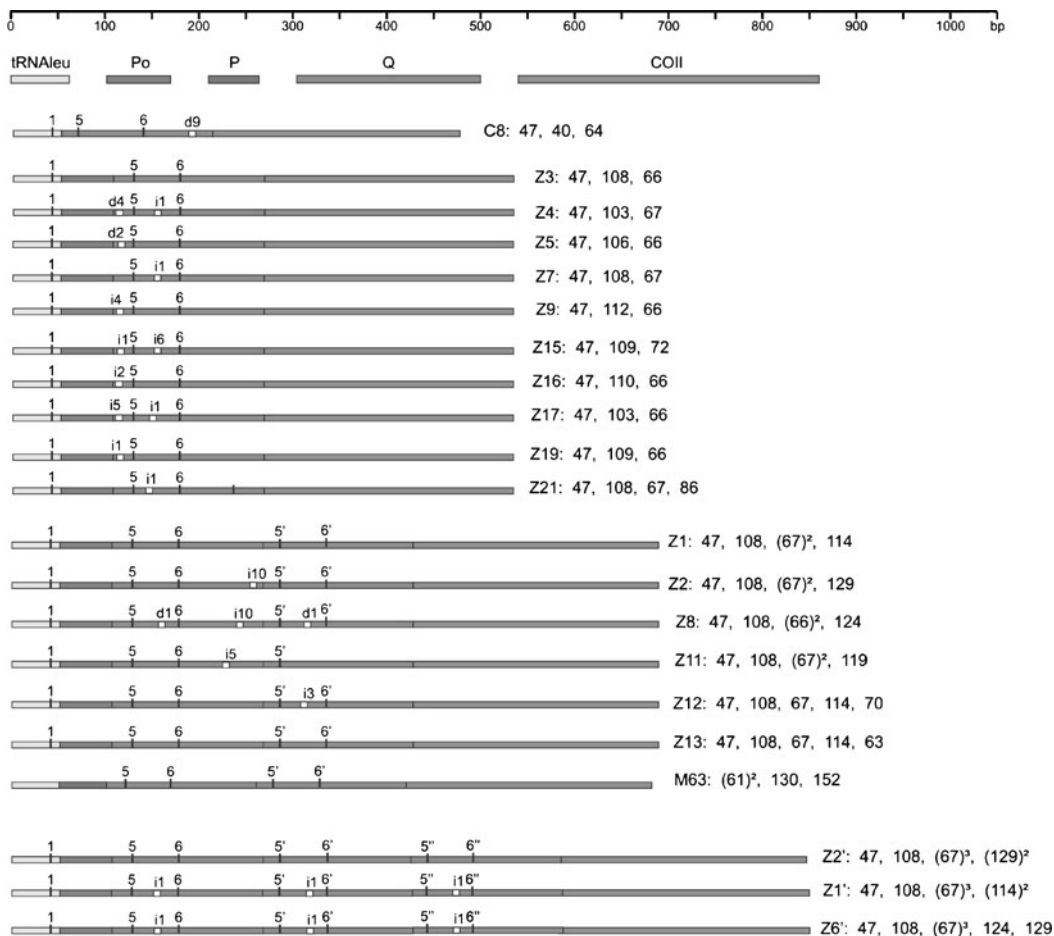


Figure 4. Restriction map corresponding to 21 *DraI* restricted mtDNA haplotypes containing the *Q* intergenic COI–COII region length for C8, PQQ for M63 and PoQ, PoQQ, and PoQQQ for the rest. Length of restriction fragments in base pairs are shown with haplotype names on the right. Restriction sites are indicated by vertical line, *d* and *i* indicate the number of deletions and insertions in the sequences, respectively.

mitochondrial lineage O. This interpretation is no longer valid. We conclude that Franck et al.’s designation of an O lineage creates confusion with the O morphometrical lineage described by Ruttner (1988) and should be avoided.

Our mtDNA results show that the Syrian honeybee has the African structural organization in its COI–COII intergenic region. We consider the populations of *A. mellifera syriaca*, which exhibit these new distinct fragment patterns on the mtDNA in our results, as a subgroup (named Z) of the African lineage A. Therefore,

the few Syrian haplotypes previously described as O digitals by Franck et al. (2001) have been renamed as a Z subgroup of the A lineage (Table I). Z haplotypes are sufficiently differentiated to be considered an independent subgroup; they belong to the A branch according to the molecular classification of the three evolutionary lineages A, C, and M (presence of the fragment Po) but their sequences differ sufficiently from traditional A haplotypes (Franck et al. 2000). They all contain a new restriction site in position 6 of their COI–COII mtDNA

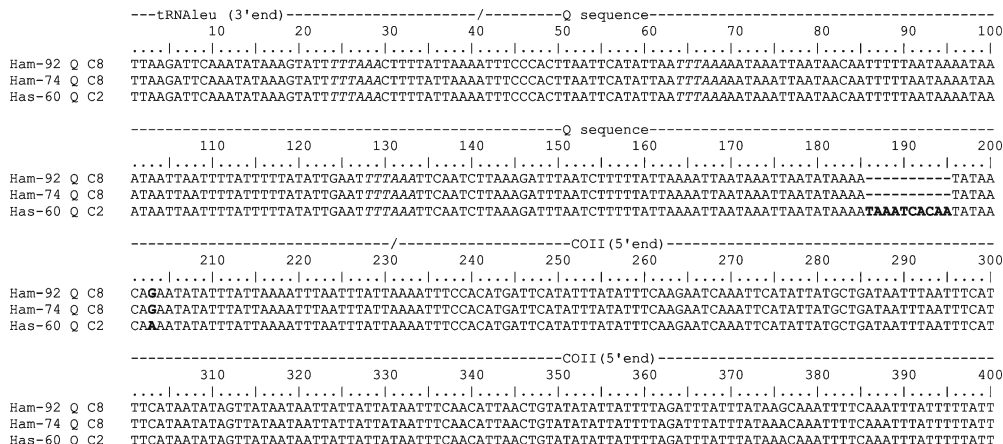


Figure 5. tRNA^{Leu}, Q and COII sequences of the C8 haplotype (Ham-92, Ham-74) and of the C2 haplotype (Has-60). *Dra*I restriction sites are shown in *italics* while the insertion, the deletion and the substitution are shown in *bold*.

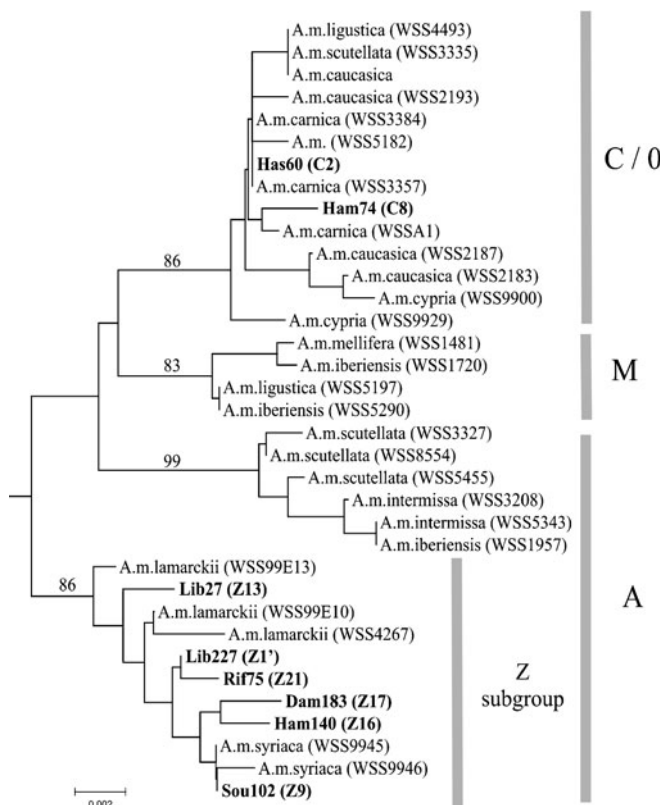


Figure 6. A neighbor-joining tree based on *Cyt b* sequences performed on nucleotide distances computed by Kimura's (1980) formula through PAUP* program, version Beta 4.0 (Swofford 2002). *Apis cerana* has been taken as an outgroup, bootstrap values are indicated for the main nodes. C, O, M, and A refer to North Mediterranean, Oriental, West Mediterranean, and African lineages, respectively. Z subgroup is a haplotype subgroup of the African lineage A. Samples in *bold* are our samples from Syria and Lebanon, other data are from GenBank (Pinto et al. 2003).

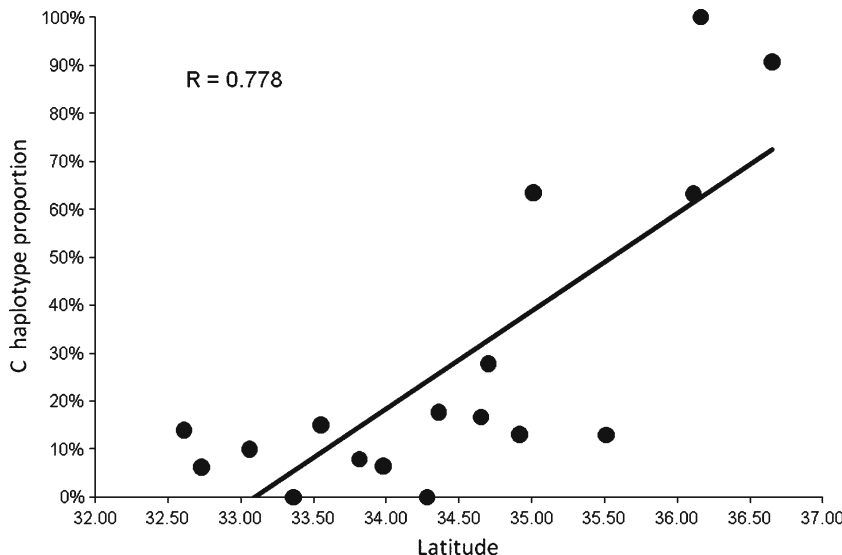


Figure 7. Representation of the north–south gradient of the A and C lineages found in the studied populations. The proportion of C haplotypes in the population is plotted as a function of the latitude of the locality (in degrees above the equator). The correlation coefficient R is highly significant ($P < 0.001$).

sequence, which gives a new African pattern on polyacrylamide gels. Moreover, the neighbor-joining tree performed based on six Cyt *b* sequences of Z haplotypes grouped them in an independent subgroup (Figure 6).

The five haplotypes found and previously classified by Franck et al. (2001) on *A. mellifera syriaca* are O1, O1' (duplication of Q sequence), O1'' (two duplications of Q sequence), O2 and O3. Based on the evidence generated by our study, and particularly our finding that the Syrian honeybee does not belong to the O morphometrical lineage, we have reclassified these haplotypes as follows (Table 1 and Figure 3):

- O1 (PoQ) with restriction *Dra*I fragments (47, 108, 67) corresponds to Z7;
- O1' (PoQQ; 47, 108, (67)², 114) corresponds to Z2;
- O1'' (PoQQQ; 47, 108, (67)³, (129)²) corresponds to Z2';
- O2 (PoQQ; 47, 108, (67)², 114) corresponds to Z1;
- O3 is the haplotype Z4 (PoQ; 47, 103, 67).

The A lineage is more common in south-west populations (94% for the Daraa region in Syria and 94% in the Nabatiyeh region in Lebanon) than in the north and northeast where the C lineage is dominant (91% for the Al-Hasakah region). Our analysis indicated a significant correlation between the percentage of Z haplotypes (African lineage) in the population and the latitude of the sampling locality. In contrast, for the northeast of Syria, going from Tartus to Idlib and Al-Hassaka, the C lineage is clearly dominant; Al-Mawsil in Iraq is 100% C lineage (Figure 4). The C2 haplotype was observed at a very high frequency (34/35 samples) in the Iraqi honeybee population from Al-Mawsil.

The C2 haplotype is present at a high frequency in the Al-Hasakah population. This haplotype is found in Turkish populations of *A. mellifera anatoliaca* in the north as well as in the populations of *A. mellifera meda* present in northeastern Iran (Palmer et al. 2000; Gruev et al. 2005); this haplotype probably spread from northeastern Iran into the North Syrian populations. On the other

hand, the C2 haplotype is also present in southern Syrian and Lebanese populations far away from the Turkish border. The C8 haplotype found in the Hama honeybee population differs from the C2 haplotype by a deletion of 10 nucleotides (185–195 bp) in its Q sequence (Figure 5).

Haplotype diversities were estimated for each population and were corrected by eliminating all nonlocal haplotypes present in each of the populations from the calculation (Table II). The lowest haplotype diversity (0.057) and corrected haplotype diversity (0.00) are found in the Al Mawsil population in Iraq (Table II). The highest haplotype diversities are found in Rif Damascus and Damascus, in Syria (Table II), where the most important beekeeping activity is found. The fairly stable corrected haplotype diversities in our study indicate very weak queen importation in those two regions; this same conclusion can be made for the Nabatiyeh and Bekaa populations. In contrast, the Hama population presents the most significant divergence between haplotype and corrected haplotype diversity (0.564 and 0.400, respectively); this explains why this population also has the highest level of introgression observed in our study (15.68%; Table II).

The results of this study have revealed evidence of introgression of commercially imported honeybees. The M63 haplotype, which corresponds to the European honeybee (*A. mellifera mellifera*), has surprisingly been found in two Syrian populations (Damascus and Rif Dimashq, Table II). It is not always easy to detect queen importation; but in this case, the phenomenon was easy to detect because the subspecies belongs to a separate evolutionary lineage (M lineage). Two haplotypes (A2 and A3) from the AII group (Franck et al. 2001) have been found in the Damascus population at a low level relative to the number of samples coming from this region. These two haplotypes are not naturally distributed in this area and are more commonly observed in Spain and

Sicily (Franck et al. 2001). These haplotypes were, therefore, imported to the Damascus population (Table II). Imported Buckfast honeybees (Garnery, personal communication), or at least *A. mellifera carnica* (C3 haplotype), were detected at a low level in Latakia, Ar-Raqqah, and Tartus populations. Imported Italian, C1 haplotype honeybees corresponding to *A. mellifera ligustica* were detected in almost all populations (Table II).

The mtDNA results of the Syrian and Lebanese populations clearly show that *A. mellifera syriaca* is situated in a geographical contact area between the A and O lineages. The number of C2 haplotypes increases from south to north and are expressed as a proportional increase of the C lineage (this is because it is not possible to discriminate between the C and O lineages using the mtDNA marker). The north–south gradient of A and C lineages, presented in Figure 7, underscores the importance of the sampling location; the variation in sampling locations in previous studies might help explain the contradictory views of Syrian honeybee classification. We found no evidence of a conservation threat to the local Syrian honeybee due to massive importation of foreign honeybee subspecies; in fact, we found no evidence of any importation in some populations (Daraa, Quneitra, and Al-Hasakah; Table II). These areas may be good candidate regions for establishing genetic conservatories for the local honeybee (*A. mellifera syriaca*) that can help preserve both inter- and intra-population mitochondrial diversity of this honeybee subspecies in each area of the Syrian territory.

ACKNOWLEDGMENTS

We thank the Syrian beekeeper association and the Ministry of Agriculture in Syria for contributing and facilitating the sampling in most Syrian regions. We are grateful to Jean-Christophe Sandoz for reading this manuscript and making valuable suggestions.

Structure mitochondriale des populations de l'abeille 'syrienne' (Syrie, Liban et Iraq)

Apis mellifera syriaca / CAPS / haplotype / région intergénique COI–COII/cytochrome oxydase / ADN mitochondrial

Die Struktur von Populationen der Honigbiene in Syrien, dem Libanon und dem Irak anhand mitochondrialer DNA

Apis mellifera syriaca / CAPS / Haplotypen / Honigbiene / Zwischengenregion COI–COII / Cyt b / mtDNA

REFERENCES

- Abrahamovich, A.H., Atela, O., De La Rua, P., Galian, J. (2007) Assessment of the mitochondrial origin of honey bees from Argentina. *J. Apic. Res.* **46**, 191–194
- Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., Saunders, N. C. (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annu. Rev. Ecol. Syst.* **18**, 489–522
- Bodenheimer, F. (1941) Studies on the honeybee and beekeeping in Turkey. Merkez Ziraat Mücadela Enstitüsü, Ankara, Numune matbasi, İstanbul
- Cornuet, J.M., Garnery, L., Solignac, M. (1991) Putative origin and function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics* **128**, 393–403
- El-Niweiri, M.A.A., Moritz, R.F.A. (2008) Mitochondrial discrimination of honeybees (*Apis mellifera*) of Sudan. *Apidologie* **39**, 566–573
- Estoup, A., Solignac, M., Harry, M., Cornuet, J.M. (1993) Characterization of (GT)_n and (CT)_n microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Res.* **21**, 1427–1431
- Estoup, A., Garnery, L., Solignac, M., Cornuet, J.M. (1995) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics* **140**, 679–95
- Franck, P., Garnery, L., Solignac, M., Cornuet, J.M. (2000) Molecular confirmation of a fourth lineage in honeybees from the Near East. *Apidologie* **31**, 167–180
- Franck, P., Garnery, L., Loiseau, A., Oldroyd, B.P., Hepburn, H.R., Solignac, M., Cornuet, J.M. (2001) Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. *Heredity* **86**, 420–30
- Garnery, L., Vautrin, D., Cornuet, J.M., Solignac, M. (1991) Phylogenetic relationships in the genus *Apis* inferred from mitochondrial DNA sequence data. *Apidologie* **22**, 87–92
- Garnery, L., Cornuet, J.M., Solignac, M. (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Mol. Ecol.* **1**, 145–54
- Garnery, L., Solignac, M., Celebrano, G., Cornuet, J. M. (1993) A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. *Experientia* **49**, 1016–1021
- Garnery, L., Mossline, E.H., Oldroyd, B.P., Cornuet, J.M. (1995) Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Mol. Ecol.* **4**, 465–472
- Garnery, L., Franck, P., Baudry, E., Vautrin, D., Cornuet, J.M., Solignac, M. (1998) Genetic biodiversity of the West European honey bee (*Apis mellifera* and *A.m iberica*) I: mitochondrial DNA. *Evolution* **30**, 31–47
- Grucev E.B., Nikolova M., Donev A. (2005) Morphometric and mtDNA analysis in honeybee populations (*Apis mellifera* L.) of North and Northwest Iran, Proceedings of the Balkan scientific conference of biology in Plovdiv Bulgaria
- Hall, H.G., Smith, D.R. (1991) Distinguishing African and European honeybee matrines using amplified mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **88**, 4548–52
- Kandemir, I., Pinto, M.A., Meixner, M.D., Sheppard, W. S. (2006) *Hinf*-I digestion of cytochrome oxidase I region is not a diagnostic test for *A. m. lamarckii*. *Genet. Mol. Biol.* **29**, 747–749
- Kimura M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* **16**, 111–120.
- Moritz, R.F.A., Cornuet, J.M., Kryger, P., Garnery, L., Hepburn, H.R. (1994) Mitochondrial DNA variability in South African honeybees (*Apis mellifera* L.). *Apidologie* **25**, 169–178
- Nei, M., Tajima, F. (1981) DNA polymorphism detectable by restriction endonucleases. *Genetics* **97**, 145–63
- Özdil, F., Yıldiz, M., Ali, Hall, H., Glenn (2009) Molecular characterization of Turkish honey bee populations (*Apis mellifera*) inferred from mitochondrial DNA RFLP and sequence results. *Apidologie* **40**, 570–576
- Palmer, M.R., Smith, D.R., Kaftanoglu, O. (2000) Turkish honeybees: genetic variation and evidence for a fourth lineage of *Apis mellifera* mtDNA. *J. Hered.* **91**, 42–6
- Pinto, M.A., Johnston, J.S., Rubink, W.L., Coulson, R.N., Patton, J.C., Sheppard, W.S. (2003) Identification of Africanized honey bee (Hymenoptera: Apidae) mitochondrial DNA: validation of a rapid PCR-based assay. *Ann. Entomol. Soc. Amer.* **96**, 670–84
- Ruttner, F. (1988) Biogeography and taxonomy of honeybees. Springer, Berlin

- Ruttner, F., Tassencourt, L., Louveaux, J. (1978) Biometrical-statistical analysis of the geographic variability of *Apis mellifera*. *Apidologie* **9**, 363–381
- Sheppard, W.S., Rinderer, T.E., Garnery, L., Shimanuki, H. (1999) Analysis of Africanized honey bee mitochondrial DNA reveals further diversity of origin. *Genet. Mol. Biol.* **22**(n.1), 73–75
- Smith, D.R., Palopoli, M.F., Taylor, B.R., Garnery, L., Cornuet, J.M., Solignac, M., Brown, W.M. (1991) Geographical overlap of two mitochondrial genomes in Spanish honeybees (*Apis mellifera iberica*). *J. Hered.* **82**, 96–100
- Swofford, D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version Beta 4.0. Sinauer Associates. Sunderland, Massachusetts
- Walsh, P.S., Metzger, D.A., Higuchi, R. (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 506–13
- Whitfield, C.W., Behura, S.K., Berlocher, S.H., Clark, A. G., Johnston, J.S., Sheppard, W.S., Smith, D.R., Suarez, A.V., Weaver, D., Tsutsui, N.D. (2006) Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. *Science* **314**, 642–5