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Short Communication

Hinf-I digestion of *cytochrome oxidase I* region is not a diagnostic test for *A. m. lamarckii*

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Abstract

Restriction fragment length polymorphism of whole mitochondrial DNA or PCR amplified mtDNA regions are known to be useful in discriminating among honey bee lineages and also some individual subspecies. In this study, PCR-amplified fragments of *cytochrome oxidase I* (*CO-I*) and *cytochrome B* (*Cyt B*) of honey bees sampled from different countries (Cyprus, Turkey, Ethiopia, Syria and Egypt) were digested with *Hinf I* and *Bgl II* restriction enzymes, respectively. Eastern Europe and Mediterranean honey bee subspecies were separated by the *Cyt B/Bgl II* analysis, although *Hinf I* digestion of the *CO-I* region yielded much finer resolution within different honey bee lineages. Here we report that *CO-I*/*Hinf-I* is a discriminative test for the mitochondrial "O" lineage, rather than a diagnostic site for *A. m. lamarckii*.

Key words: mtDNA, *cytochrome oxidase I*, *Apis mellifera lamarckii*, O lineage.

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Based on multivariate statistical analysis of morphometric characteristics, 26 honey bee subspecies have been recognized, divided into four evolutionary lineages (Ruttner, 1988; 1992; Sheppard *et al.*, 1997; Sheppard and Meixner, 2003). For the most part, these evolutionary groupings have been supported by mtDNA and microsatellite analysis (Cornuet and Garnery, 1991; Garnery *et al.*, 1992; Estoup *et al.*, 1995). Most individual subspecies cannot be distinguished based on current mtDNA or nuclear DNA protocols. However, PCR amplification of the *COI-COII* intergenic region and digestion with *Dra-I* restriction enzyme has proven useful to differentiate among some honey bee subspecies. There are other diagnostic sites useful to differentiate evolutionary lineages, group of subspecies and a few individual subspecies (Sheppard *et al.*, 1994; Palmer *et al.*, 2000; Sheppard and Smith, 2000; Pinto *et al.*, 2003). One of these subspecies, *A. m. lamarckii*, has been reported to be distinguishable by *CO-I* region amplification and *Hinf-I* digestion (Nielsen *et al.*, 2000). *A. m. lamarckii* was one of several African subspecies introduced into the United States prior to the arrival of Africanized honey bees (derived from *A. m. scutellata*). Here we investigated whether the phylogeographic distribution of this restriction fragment pattern is limited to *A. m. lamarckii* (Egypt) or whether it can be found in other subspecies including *A. m. syriaca* (Syria and Turkey), *A. m. meda* (Syria and Iran), *A. m. cypria* (Cyprus) and *A. m. yemenitica* (Ethiopia and Yemen).

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Adult honey bee samples were collected from several countries, including Turkey (Kandemir *et al.*, 2006), Northern Cyprus, Syria, Iran, Egypt and Ethiopia. Honey bees were stored either in 80% ethanol or frozen until laboratory analysis. Total nucleic acids were extracted following the methods of Sheppard and McPheron (1991) and Doyle and Doyle (1987). An approximately 1000 bp region of the *cytochrome oxidase I* (*COI*) gene and 800 bp region of the *cytochrome B* gene were amplified using primer pairs and PCR conditions previously reported (Sheppard *et al.*, 1994; Nielsen *et al.*, 2000; Crozier *et al.*, 1991). After PCR, the amplified fragments for *COI* and *CytB* were digested with *Hinf-I* and *Bgl II* restriction enzymes, respectively, according to manufacturer's recommendations. Amplified PCR products were separated on a 1.5% agarose gel (Bio-Rad) and restriction enzyme digested fragments were separated on a 2.5% mixed agarose gel consisting of 1% agarose (Bio-Rad) and 1.5% Nu-Sieve agarose (Nu-Sieve). Gels were stained with ethidium bromide, destained with distilled water and photographed under UV light.

With the exception of some samples from Ethiopia, all samples analyzed by *CytB* amplification and *Bgl II* di-

Table 1 - Restriction analysis of PCR amplified *CO I* and *Cyt B* regions of mtDNA of honey bee samples collected from different countries.

COI/HinfI *Cut/Uncut* ±	*CytB/BglII* *Cut/Uncut* ±	Syria (N = 35) *A. m. syriaca, A. m. meda*	Turkey(N = 334) *A. m. syriaca, A. m. meda, A. m. anatoliaca*	Cyprus (N = 101) *A. m. cypria*	Iran (N = 174) *A. m. meda*	Ethiopia (N = 109) *A. m. yemenitica, A. m. scutellata, A. m. monticola*	Egypt (N = 62) *A. m. lamarckii*
+	+	28	6	2	0	8	62
-	+	7	328	99	174	83	0
-	-	0	0	0	0	18	0

gestion (*Cyt B/Bgl II*) exhibited a two-banded mitochondrial restriction fragment pattern (mitotype). This mitotype is generally considered to exclude *A. m. scutellata* as the possible subspecies of origin. However, when the *cytochrome oxidase I* (*CO I*) region of the same samples was digested with *Hinf I* (*CO I*/*Hinf I*), further resolution of the middle eastern and east African honey bee population was achieved.

Out of 334 colonies from Turkey, 6 from the very southern end (within the geographic range of *A. m. syriaca*) showed the typical "*A. m. lamarckii*" *CO I/Hinf I* mitotype (Kandemir *et al.*, 2006). Honey bee samples from northern Cyprus and Iran, *A. m. cypria* and *A. m. meda*, respectively, exhibited European mitotypes for both *Cyt B*/*Bgl II* and *CO I*/*Hinf I* digestions, except two of the *A. m. cypria* colonies which had the *A. m. lamarckii CO I/Hinf I* mitotype. All samples from Egypt (*A. m. lamarckii*) showed the *A.m. lamarckii CO I/Hinf I* mitotype. Out of 35 samples from Syria (*A. m. syriaca*), 28 of them had the *A. m. lamarckii CO I/Hinf I* mitotype. Out of 109 Ethiopian samples, eight had *A. m. lamarckii* mitotypes (Table 1).

The analysis of the *COI-COII* intergenic region of honey bees from Syria and Turkey showed similar mtDNA restriction fragment patterns as has been found in *A. m. lamarckii*. Palmer *et al.* (2000) mentioned the presence of a fourth mtDNA lineage, but did not discuss the similarity to *A. m. lamarckii*. Similar findings were reported by Franck *et al.* (2000) based on *COI-COII* analysis of Lebanese honey bees. The results of their analysis of the *COI-COII* intergenic region indicated that honey bees from Lebanon (*A. m. syriaca*) had the same *Dra-I* restriction digestion pattern found in *A. m. lamarckii*. Due to the distinction of this pattern from African (A) restriction fragment patterns (other than those found in Egypt), they designated these new restriction fragment patterns as belonging to a mitochondrial lineage O.

Our results show that the distribution of the *CO I/Hinf I* mitotype "typical" of *A. m. lamarckii* is not restricted to Egypt, but is dispersed along the Nile river south toward Ethiopia and to the east to Yemen on the Arabian Peninsula. To the north, it extends to Turkey where the mitotype was found in the Hatay province that borders to Syria. Quite likely, this mitotype occurs in Israel, Palestine and Jordan as well. Although morphological data supports the placement of *A. m. lamarckii* within the African lineage, accumulated molecular data appears to contradict this grouping and suggests placement of *A. m. lamarckii* within a distinctive lineage (Arias and Sheppard, 1996; Franck *et al.*, 2000). The use of O as the designation for this distinctive mitochondrial lineage (Franck *et al.*, 2000) may present confusion with the O morphological lineage described by Ruttner (1988). The distinctive morphological lineages C and O cannot be resolved easily using mitochondrial analysis and, thus, are both grouped within the C mtDNA lineage. Nonetheless, the "*A. m. lamarckii*" restriction fragment pattern (*CO I/Hinf I*) or "O" mitochondrial lineage (Franck *et al.*, 2000) appears to be a widespread genetic variant. Further study of the distribution of this variant among the subspecies of the A and C morphological lineages will be important to fully understand the phylogeography of *Apis mellifera*.

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References

Arias MC and Sheppard WS (1996) Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. Mol Phylogenet Evol 5:557-566.

Cornuet JM and Garnery L (1991) Mitochondrial DNA variability in honeybees and its phylogenetic implications. Apidologie 22:627-642.

Crozier YC, Koulianos S and Crozier RH (1991) An improved test for africanized honeybee mitochondrial DNA. Experientia 47:968-969.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11-15.

Estoup A, Garnery L, Solignac M and Cornuet JM (1995) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: Hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140:679-695.

Franck P, Garnery L, Solignac M and Cornuet JM (2000) Molecular confirmation of a fourth lineage in honeybees from the Near East. Apidologie 31:167-180.

Garnery L, Cornuet JM and Solignac M (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. Mol Ecol 1:145-154.

Kandemir I, Kence M, Sheppard WS and Kence A (2006) Mitochondrial DNA variation in honey bee (*Apis mellifera* L.) populations from Turkey. J Apicult Res 45:33-38.

Nielsen DI, Ebert PR, Page Jr RE, Hunt GJ and Novoa-Guzman E (2000) Improved polymerase chain reaction-based mitochondrial genotype assay for the identification of the africanized honey bee (Hymenoptera, Apidae). Ann Entomol Soc Am 93:1-6.

Palmer MR, Smith DR and Kaftanoglu O (2000) Turkish honeybees: Genetic variation and evidence for a fourth lineage of *Apis mellifera* mtDNA. J Hered 91:42-46.

Pinto MA, Johnston JS, Rubink WL, Coulson RN, Patton JC and Sheppard WS (2003) Identification of Africanized honey bee (Hymenoptera, Apidae) mitochondrial DNA: Validation of a rapid PCR-based assay. Ann Entomol Soc Am 96:679-684.

Ruttner F (1988) Biogeography and Taxonomy of Honeybees. Springer-Verlag, Berlin, 284 pp.

Ruttner F (1992) Naturgeschichte der Honigbienen. Ehrenwirth, Munich, 357 pp.

Sheppard WS and McPheron BA (1991) Ribosomal RNA diversity in *Apis*. In: Smith DR (ed) Diversity in the Genus *Apis*. Westview Press, Boulder, pp 89-102.

Sheppard WS and Meixner MD (2003) *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. Apidologie 34:367-375.

Sheppard WS and Smith DR (2000) Identification of African-derived bees in the Americas: A survey of methods. Ann Entomol Soc Am 93:159-176.

Sheppard WS, Arias MC and Shimanuki H (1994) Determination of mitochondrial DNA haplotypes from sting remnants of the honey bee *Apis mellifera* (Hymenoptera, Apidae). Bull Entomol Res 84:551-554.

Sheppard WS, Arias MC, Grech A and Meixner MD (1997) *Apis mellifera ruttneri*, a new honey bee subspecies from Malta. Apidologie 28:287-293.

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