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A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L.

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Abstract. The COI-COII intergenic region of *Apis mellifera* mitochondrial DNA contains an important length polymorphism based on a variable number of copies of a 192–196 bp sequence (Q) and the complete or partial deletion of 67 bp sequence (Po). This length variability has been combined with a restriction site polymorphism to produce a rapid and simple test for the characterization of mtDNA haplotypes. This test includes the amplification by the polymerase chain reaction of the COI-COII region followed by a *Dra*I restriction of the amplified fragment. In a survey of 302 colonies belonging to 12 subspecies, 21 different haplotypes have been found which have been unambiguously allocated to one of the 3 mtDNA lineages of the species. Although all colonies of lineage C exhibit the same pattern (C1), each one of lineages A and M presents up to 10 different haplotypes, opening the way to studies on the genetic structure and the evolution of a large fraction of the species. This test also differentiates southern Spanish and South African colonies, which can be of great interest for the Africanized bee problem.

Key words. Apis mellifera; honeybee; mitochondrial DNA; restriction fragment length polymorphism; *Dra*I; polymerase chain reaction; COI-COII intergenic region.

Mitochondrial DNA has proven to be a remarkable tool for studies on the differentiation and the evolution of honeybees[1–5]. These studies, based on restriction and sequence data, have shown that *Apis mellifera* evolved in three distinct branches which diverged about 1 Myr ago. The first branch (M) corresponds to the Western European subspecies *A. m. mellifera*. The second one (C), extending from the Middle East to Italy, encompasses the subspecies *meda*, *caucasica*, *cecropia*, *carnica* and *ligustica*. The last one (A) includes all African subspecies studied so far, except *lamarkii*[4], as well as honeybees from some Mediterranean islands such as Sicily (*sicula*). Secondary contacts between branches result in introgression areas where two categories of mtDNA haplotypes coexist. For instance, there is a progressive transition in Spain toward the north from branch A to branch M[6].

Surveys on large samples, which are necessary for various purposes (Africanization of American populations, studies of introgression areas between mitochondrial types) are not suited to whole mtDNA restriction fragment analyses and methods for rapid testing have been developed which are based on the polymerase chain reaction (PCR). Crozier et al.[7] proposed a test using the amplification of a fragment in the cytochrome b gene and its digestion by *Bgl*II. This test discriminates the African lineage (including American Africanized honeybees) from the other two. Hall and Smith[8] could distinguish 8 different haplotypes and assign them to one of the three branches by amplifying three mtDNA fragments and digesting each of them with a specific restriction enzyme. We propose an even more discriminatory test which requires the amplification and restriction by a single enzyme of only one PCR fragment.

This fragment contains the COI-COII intergenic region which has received special attention[2,9]. This region exhibits at least 7 length variants which can be explained by the combinations of 3 related sequences Po (67 bp), P (54 bp) and Q (192–196 bp): PoQ, PoQQ, PoQQQ, PQ, PQQ, PQQQ and Q. The three mtDNA lineages corresponding to the aforementioned three branches are characterized by Po (A), P (M) and neither Po nor P (C). The examination of the available COI-COII sequences led to the conclusion that *Dra*I, the recognition site of which is TTTAAA, should show a significant amount of polymorphism.

Material and methods

Samples. A total of 302 colonies were collected from a wide range of sites in the natural distribution of the species. The subspecies *Apis mellifera mellifera* was represented by 138 colonies from Scandinavia (41 colonies), England (18) and France (79), *A. m. iberica* by 75 colonies from Spain, *A. m. sicula* by 2 Sicilian colonies, *A. m. intermissa* by 2 Algerian and 4 Moroccan colonies, *A. m. scutellata* by 20 colonies from South

Africa and 2 colonies from Malawi, *A. m. monticola* by 11 colonies from Malawi, *A. m. adansonii* by 2 colonies from Congo, *A. m. caucasica* by 3 colonies from Caucasus, *A. m. ligustica* by 12 Italian colonies, *A. m. meda* by 2 Iranian colonies, *A. m. carnica* by 2 Austrian, 1 Hungarian and 1 Swiss colony and *A. m. anatoliaca* by 2 Turkish colonies. In addition, some colonies of unidentified subspecies were analysed: 9 from Mauritius, 7 from various Greek Islands, 1 from Crete, 2 from Cephalonia and 4 from the Canary Islands.

DNA extraction. After preservation in absolute ethanol, bees were rinsed for one hour at room temperature in a solution containing NaCl 128mM, $CaCl_2$ 1.5mM, KCl 5mM, pH 7.4 and vacuum dried overnight prior to DNA extraction. Total DNA was extracted from single bee thorax as described[10] and suspended in 1 ml of sterile water.

PCR conditions and DraI restriction. The mtDNA fragment including the COI-COII intergenic region was amplified by PCR, using primers E2 and H2 (ref. 2). The reaction was performed in 25 µl containing 25 pmoles of each primer, 25 nmoles of each dNTP, 0.6 unit of Promega *Taq* polymerase, 1.5mM $MgCl_2$ and 0.5 µl of DNA extract. Reaction tubes were submitted to 30 cycles (1 min at 92 °C, 45 s at 48 °C, 2 min at 62 °C) in a Techne PHC2 thermocycler. A first aliquot of 10 µl was electrophoresed through a 1% agarose gel in order to determine the size category of the fragment. Ten µl of sterile water containing 4 units of restriction enzyme *Dra*I were added to the remaining 15 µl and this mix was incubated at 37 °C for 4 h. Restricted DNA fragments were seperated on 5% and 10% acrylamide gels and stained with ethidium bromide.

Results and discussion

As expected from the sequences, this test revealed an important amount of variability: in addition to 4 distinguishable lengths of non-restricted amplified DNA (fig. 1), 18 different *Dra*I restriction patterns were observed. A total of 21 different haplotypes were present in the 302 sampled colonies. All patterns have been unambiguously attributed to an mtDNA lineage. Consequently, they have been named with the letter relative to their lineage.

The *Dra*I restriction exhibit from 2 to 5 bands (figs 2 and 3) with sizes ranging from 12 to 880 bp, some of which correspond to 2 or 3 restriction fragments of the same size. Restriction maps (fig. 4) were developed from the following data: length of the PCR product, length of *Dra*I restriction fragments, and the positions of actual and potential *Dra*I sites in available sequences[9,11]. The variability is generated by 8 restriction sites and by the size variation which results from two sources; number of sequences Q and optional sequence P or Po.

An intriguing result is the homogeneity of mtDNA lineage C in which only one pattern (C1) has been found, whereas the other two lineages include 8 (M) and 9 (A) different patterns. Two explanations are proposed. The first one is that this lineage is underrepresented in the whole sample (only about 1/10 of sampled colonies) although it is present in 5 different subspecies. The second explanation, due to the short size and absence of length variability in the COI-COII region, is that the mtDNA of lineage C has a reduced potential for pattern variability. However, ignoring length variability and sites 3 and 4 (located in Po or P) which cannot be found in lineage C, there are still 4 or 6 site combinations found in lineages M and A as opposed to a single one in lineage C. Therefore, these two explanations may not be sufficient to explain the relative homogeneity of this lineage with our test.

The table shows the distribution of all patterns in the 302 colonies. The presence of pattern C1 in a few

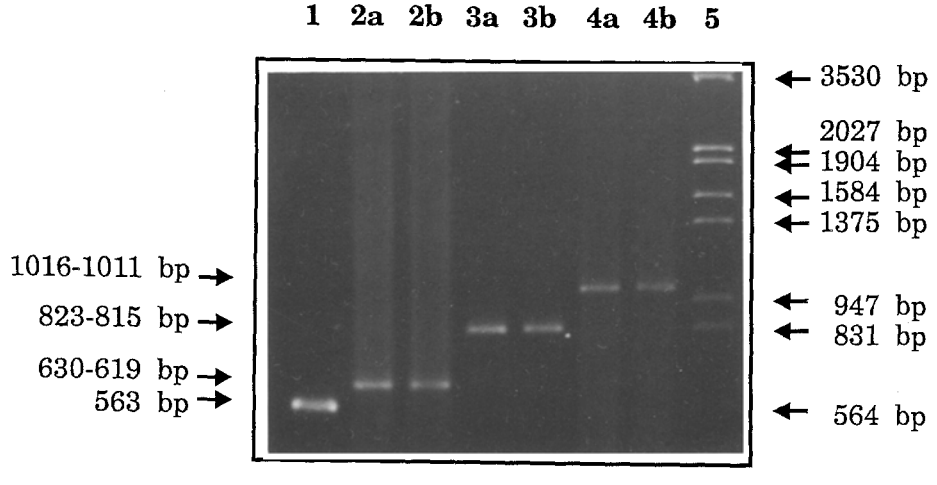

Figure 1. PCR amplification of the COI-COII mtDNA region provides four length categories of fragments corresponding respectively to the following combinations (1) Q, (2a) PQ, (2b) PoQ, (3a) PQQ, (3b) PoQQ, (4a) PQQQ and (4b) PoQQQ. Lane (5) is $\lambda/(HindIII + EcoRI)$ as a DNA molecular weight marker.

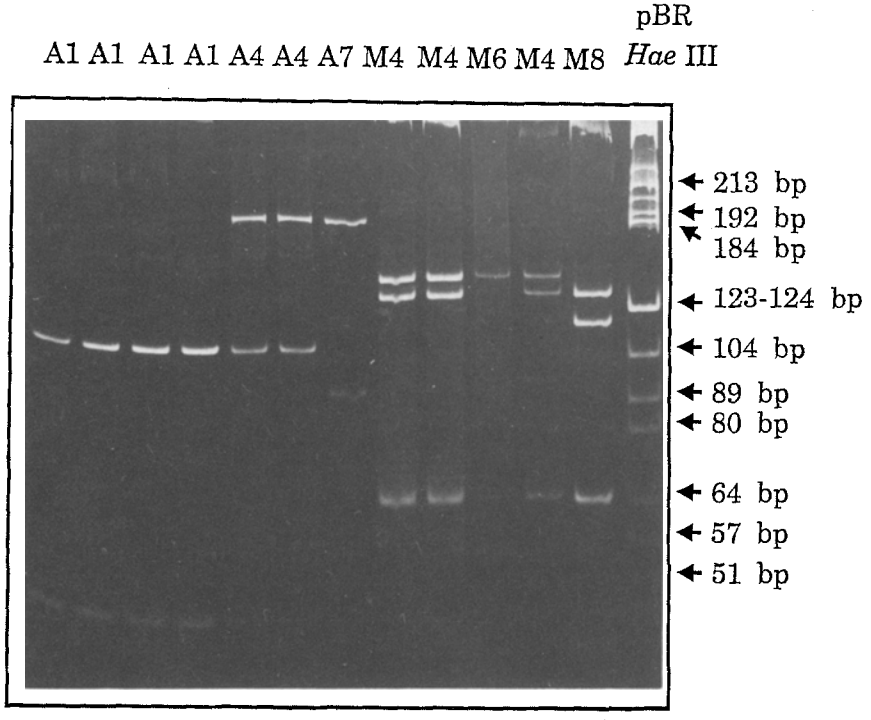

Figure 2. *Dra* I restriction patterns of the COI-COII mtDNA region of various honey bee samples in 10% polyacrylamide gel. Only fragments under 200 bp are resolved and shown in this picture. Larger fragments were analyzed on a 5% polyacrylamide gel.

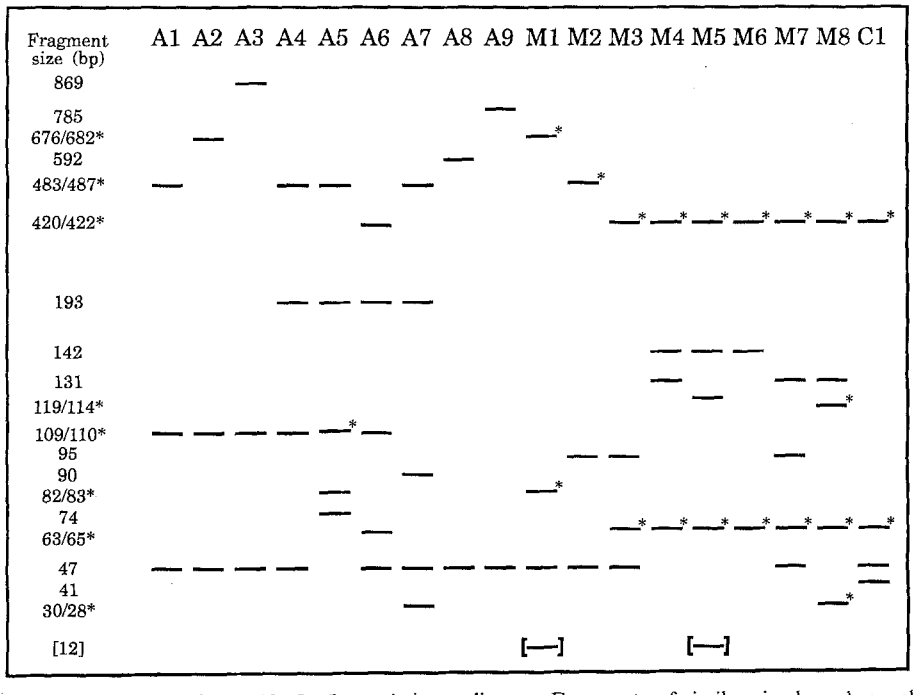

Figure 3. Schematic representation of the 18 *Dra*I restriction patterns of the amplified fragment in the honey bee mtDNA COI-COII intergenic region. Patterns A1 to A9 correspond to the African mtDNA lineage, patterns M1 to M8 to the Western European lineage and pattern C1 to the northern Mediterranean lineage. Fragments of similar size have been placed on the same line and noted with/without an asterisk. The 12 bp fragments are enclosed in brackets because they were inferred from sequences but not observed.

Swedish, English and French colonies is interpreted as the evidence of queen importations (especially *ligustica* queens) which occurred in these countries. Data concerning lineage A show an interesting geographical structure for this lineage which did not appear in previous studies[2,4]. There is a clear distinction between colonies of the Mediterranean, including southern Spain/Mediterranean islands (Greek Islands and Sicily),

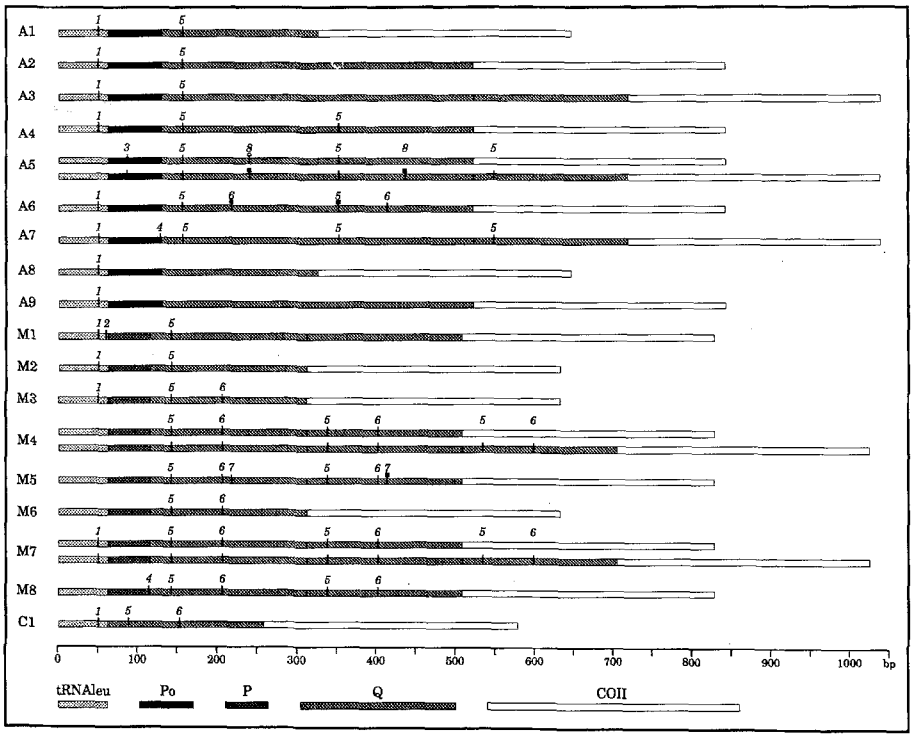

Figure 4. Restriction maps corresponding to the 18 *Dra*I restriction patterns of figure 1. Restriction sites are numbered from 1 to 9. In some cases (A5, M4, M7), intergenic regions containing 2 or 3 Qs result in the same restriction pattern. In the haplotype A5/QQ found in two *monticola* colonies from Malawi, heteroplasmy at site 5 (noted with an open circle) has to be admitted to explain the *Dra*I pattern. Restriction sites noted with a black square (A5/QQQ and A6/QQ) correspond to alternate solutions. In patterns M5, site 8 of the second Q may or may not be present and therefore has been noted by a black square.

and southern Africa. Pattern A2 is the most common in the first group but absent in the second one whereas the opposite situation is found for pattern A4. The low number of colonies sampled in northern or central Africa prevents any clear conclusion concerning these areas. No such geographical structure for lineage M is revealed by our test. However, some haplotypes are restricted to only one area, such as M5 in Sweden or M8 in Spain.

Spanish populations result from a secondary hybridization between populations of lineages M and A[6]. With whole mtDNA restriction analyses, only 5 different haplotypes (2 M and 3 A types) were discriminated[2], whereas the present study reveals 11 different haplotypes (6 M and 5 A types). This makes our test particularly suited to examine this hybrid zone.

Previous studies based on morphometry[12] or allozymes[13] indicated the existence of phylogenetic links between *A. m. sicula* (Sicily) and *A. m. intermissa* (Algeria). Both subspecies belong to the mtDNA lineage A, but our results suggest that Sicilian colonies are more related to southern Spanish than to Algerian populations. Colonies from Greek Islands also share the southern Spanish/Sicilian A2/QQ haplotype. So, applied to a larger set of data, this test should provide some new insight into the colonization of Mediterranean Islands by honeybees. This study could be extended to other islands such as the Canary Islands where some populations might have originated from southern Spain.

Compared to restriction analyses on the whole mtDNA molecule which discriminated 19 haplotypes among 68 colonies[2], the COI-COII/*Dra*I method presented here provides about the same amount of variability. The former methods are particularly suited to infer phylogenetic relationships, whereas the latter are not. As the major events of the evolutionary history of *Apis mellifera* has been established (see introduction), more specialized issues can be approached such as differentiation between particular subspecies or hybrid zones where the discrimination of haplotypes is more crucial than inferring phylogenetic links. These population studies require large surveys involving numerous samples, for which whole mtDNA studies are impractical. The present method, which combines simplicity, speed and resolution power, should allow new advances in the knowledge of the genetic structure of honeybee populations. For example, it could be useful in discriminating between Africanized and European matrilines in American Bees[14–16] since it distinguishes South African from southern Spanish mtDNA types[6].

Geographic distribution of mtDNA haplotypes. The haplotype combines the restriction pattern and the number of sequences Q deduced from the total length of the amplified fragment

Geographic origin	mtDNA haplotype A1 Q	A2 QQ	A3 QQQ	A4 QQ	A5 QQ	A5 QQQ	A6 QQ	A7 QQQ	A8 Q	A9 QQ	M1 QQ	M2 Q	M3 Q	M4 QQ	M4 QQQ	M5 QQ	M6 Q	M7 QQ	M7 QQQ	M8 QQ	C1 Q	Total
Sweden (Umea)														20	11	7					3	41
England (Alderney)														1	1			3	2			7
(Berkshire)														7	1			1	1		1	11
France (miscellaneous)														11	2			2			3	18
(Avignon)														25	2		2					29
(Vallouise: Alps)											1	1		29							1	32
Spain (northeast)														13	2			5		5		25
(northwest)															1					1		2
(central)		4							3					3	1			3	1	1		16
(south)	2	22	2	1									1	2				2				32
Greek Islands		7																				7
Sicily (Anka)		2																				2
Canary Islands		2						2														4
Algeria (Alger)				2																		2
Morocco (Rabat)	1								1	2												4
Congo (Brazzaville)	1			1																		2
Malawi	2			8	2	1																13
South Africa (Johannesburg)	1			18			1															20
Mauritius	8			1																		9
Italy (miscellaneous)																					12	12
Austria/Switzerland/Hungary																					4	4
Turkey (Ankara)																					2	2
Caucasus																					3	3
Iran (Astara)																					2	2
Cephalonia/Crete																					3	3
Total	15	37	2	31	2	1	1	2	4	2	1	1	1	111	21	7	2	16	4	7	34	302

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1 Cornuet, J. M., and Garnery, L., Apidologie *22* (1991) 627.
2 Garnery, L., Cornuet, J. M., and Solignac, M., Mol. Ecol. *1* (1992) 145.
3 Smith, D. R., and Brown, W. M., Experientia *44* (1988) 257.
4 Smith, D. R., in: Diversity in the genus *Apis*, p. 131. Ed. D. R. Smith. Westview Press, Boulder–San Francisco–Oxford 1991.
5 Smith, D. R., and Brown, W. M., Ann. ent. Soc Am. *83* (1990) 81.
6 Smith, D. R., Palopoli, M. F., Taylor, B. R., Garnery, L., Cornuet, J. M., Solignac, M., and Brown, W. M., J. Hered. *82* (1991) 96.
7 Crozier, Y. C., Koulianos, S., and Crozier, R. H., Experientia *47* (1991) 968.
8 Hall, H. G., and Smith, D. R., Proc. natl Acad. Sci. USA *88* (1991) 4548.
9 Cornuet, J. M., Garnery, L., and Solignac, M., Genetics *1128* (1991) 393.
10 Garnery, L., Vautrin, D., Cornuet, J. M., and Solignac, M., Apidologie *22* (1991) 87.
11 Crozier, R. H., Crozier, Y. C., and Mackinley, A. G., Mol. Biol. Evol. *6* (1989) 399.
12 Lebdigrissa, K., Msadda, K., Cornuet, J. M., and Fresnaye, J., Landbouwtijdschrift *44* (1991) 1231.
13 Cornuet, J. M., unpubl. data.
14 Taylor, O. R., Bee Wld *58* (1977) 19.
15 Smith, D. R., Taylor, O. R., and Brown, W. M., Nature *339* (1989) 213.
16 Hall, H. G., and Muralidharan, K., Nature *339* (1989) 211.