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Biodiversity of *Apis mellifera* populations from Tenerife (Canary Islands) and hybridisation with East European races

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Abstract. The biodiversity of honeybee (*Apis mellifera*) populations from Tenerife (Canary Islands, Spain) has been assessed by restriction analysis of a mitochondrial non-coding intergenic region. Seventy-nine colonies were analysed from thirteen apiaries in six populations that have been kept from recent queen introduction. The length and restriction pattern of the PCR amplified products of the intergenic region identified four mitochondrial haplotypes. One of these haplotypes shows the same restriction pattern and composition of the intergenic region carried by honeybees belonging to the African lineage. Two haplotypes are characterised by a particular intergenic region found with high frequency in the Canarian populations. The haplotype representative of the East European honeybee lineage shows a frequency of 35%, thus indicating introduction of queen honeybees. The finding of this haplotype in Canarian honeybees suggests that hybridisation between the endemic *Apis mellifera* populations and imported bees is occurring in Tenerife.

Key words: biodiversity, Canary Islands, conservation, honeybee, introgression, mtDNA

Introduction

The Canary Islands are well known by their rich endemic biota (e.g. Kunkel 1976). Studies about the diversity of Canarian endemisms have recently increased at both morphological and molecular levels (review in Juan et al. 2000).

The honeybee *Apis mellifera* is represented in the Canaries by populations that, according to beekeepers, correspond to a local form with a predominant black coloration. De la Rúa et al. (1998) have shown on the basis of the mitochondrial DNA variation that there is one set of populations which is probably native to the islands. In the same study a surprisingly high level of gene introgression from imported colonies of East European subspecies was detected within local populations. Beekeeping is very active in the island of Tenerife, which harbours 45% of all Canarian hives (Cañas 1998). Traditional beekeeping has recently changed to modern techniques including seasonal movements on the island and generalised queen importation, mostly of European honeybee races such as *A. m. ligustica* and *A. m. carnica*. The risk of gene introgression and of substitution of these island populations is high,

as evidenced by the complete replacement of local populations in Germany by imported *A. m. carnica* (Kauhausen-Keller and Keller 1994) and by the occurrence of this subspecies in French colonies located at the German border (Garnery et al. 1998).

The assessment of mitochondrial introgression has been carried out using a molecular test based on the mitochondrial sequence variability (Garnery et al. 1993). This test has been widely used for studies on the biogeography of the subspecies and races of *A. mellifera* (e.g. Garnery et al. 1993, 1995, 1998; Moritz et al. 1994; De la Rúa et al. 1998, 1999; Franck et al. 1998; Palmer et al. 2000), as well as for other *Apis* species (Smith and Hagen 1997; De la Rúa et al. 2000). This test distinguishes different haplotypes grouped into mitochondrial lineages, in accordance with previous morphological analyses (Ruttner et al. 1978) and the geographical distribution of the subspecies of *A. mellifera* (Smith 1991a,b; Garnery et al. 1992). The West European lineage (M) includes the subspecies *A. m. mellifera*; the East European lineage (C) comprehends *A. m. carnica* and *A. m. ligustica* among others, and a third lineage of African bees (A) is made up of honeybee subspecies from Africa. These authors underlined the hybrid status of *A. m. iberica* populations between the European *A. m. mellifera* and the African *A. m. intermissa* and concluded that the western European and the African lineages came into contact throughout the Iberian Peninsula. Each lineage has a characteristic non-coding intergenic region between the leucine tRNA gene and the cytochrome oxidase II gene (COII), which varies in length and sequence. The presence or absence of a 'P' element (67 or 52 bp), and of tandem repeats (from 1 to 3) of a 'Q' element with about 200 bp, cause length variation. Sequence variation is due to base substitutions, insertions and deletions. The native populations of Canarian honeybees (mainly bearing the haplotypes named A14 and A15) correspond to a particular subset of the African (A) lineage, since they have a peculiar P_1 region and two (A14) or three (A15) Q regions (De la Rúa et al. 1998).

The aim of this study was to analyse in detail the level of introgression of imported honeybees on the genetic (mitochondrial) pool of native honeybees from Tenerife, and to evaluate the danger that this process represents for the preservation of the diversity of such populations.

Materials and methods

Collection of honeybee samples

Samples of honeybee populations were collected from different localities throughout the island of Tenerife (Figure 1). Apiaries with known recent introductions of Italian honeybees were discarded, in order to focus the analysis on 'spontaneous' (not directed or man-favoured) introgression of foreign mtDNA haplotypes into local Canarian populations.

Samples were collected from 13 different apiaries in six populations. We have followed the definition of population given by Garnery et al. (1998), who considered a

FIGURES

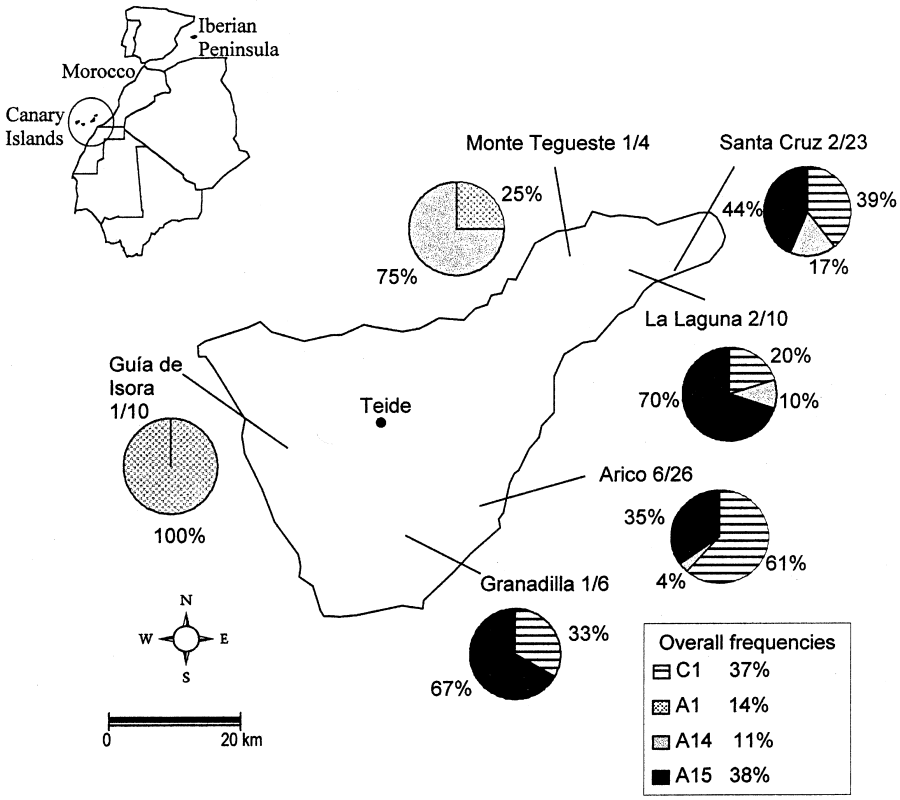

Figure 1. Geographical localisation of the island of Tenerife and mtDNA haplotype distribution and relative frequencies (in %) for each sampled population from Tenerife. The number of apiaries and colonies (values before and after the dash, respectively) of *Apis mellifera* sampled on Tenerife is also indicated.

honeybee population as 'a set of colonies whose fertile members (queens and drones) mate in the same drone congregation area(s)'. A total amount of 79 colonies were sampled. Eleven colonies sampled in 1994 and 1997 have been previously analysed (De la Rúa et al. 1998). Sixty-eight new colonies were sampled in 1998. The hives were opened and the bees were trapped from the inner frames to prevent the collection of drifting bees. Bees were immediately killed by immersion in absolute ethanol and kept at −20 °C until they were processed in the laboratory.

DNA isolation and PCR amplification

Total DNA was extracted from thoraces after rinsing the bees during 1 h in rinse buffer following Garnery et al. (1993). DNA isolation was performed following standard

organic methods (Sambrock et al. 1989) or the Chelex method (Walsh et al. 1991) with slight modifications.

PCR amplification was performed following Garnery et al. (1992) with the primers E2 (5′-GGCAGAATAAGTGCATT-G3′) located at the 5′ end of the gene $tRNA^{Leu}$, and H2 (5′-CAATATCATTGATGACC-3′) located close to the 5′ end of the COII gene (positions 3363 for the E2 primer and 3937 for the H2 following Crozier and Crozier 1993).

The amplified resulting products were visualised after electrophoretic separation on 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

Restriction analysis

Ten μl aliquots of the resulting PCR products were digested following manufacturer instructions, with 5 units of the enzyme *Dra* I purchased either from Pharmacia Biotech or Gibco BRL. The restriction reactions were kept at 37 °C during 4–12 h and the resulting fragments were visualised in 8% acrylamide gels stained with ethidium bromide. Photographs under UV light were taken for documentation.

Statistical analysis

The Fischer exact test has been performed to test for differences in the distribution of haplotypes and evolutionary lineages among populations. The AMOVA test for population structure was calculated using the ARLEQUIN program (Schneider et al. 1997).

Results

Diversity of mitochondrial DNA

PCR amplification of the $tRNA^{Leu}$-COII intergenic region yielded products with four different sizes. These PCR-fragments correspond to different combinations of the sequences P_0, P_1 and Q. After the *Dra* I restriction of the amplified fragments, four different haplotypes were determined; in this case each PCR product with a different size corresponds to one distinct haplotype (Figure 2). The composition was determined by comparison with the restriction pattern revealed by samples that were previously sequenced (De la Rúa et al. 1998).

Haplotypes A15 and A14, show the sequence P_1 and three or two copies of the Q sequence respectively. The sequence P_1 shows a deletion of 17 bp in the 3′ end of the P_0 sequence (De la Rúa et al. 1998). The haplotype A1 has the sequence P_0 and one Q sequence; these colonies belong to the African lineage of honeybee races. None

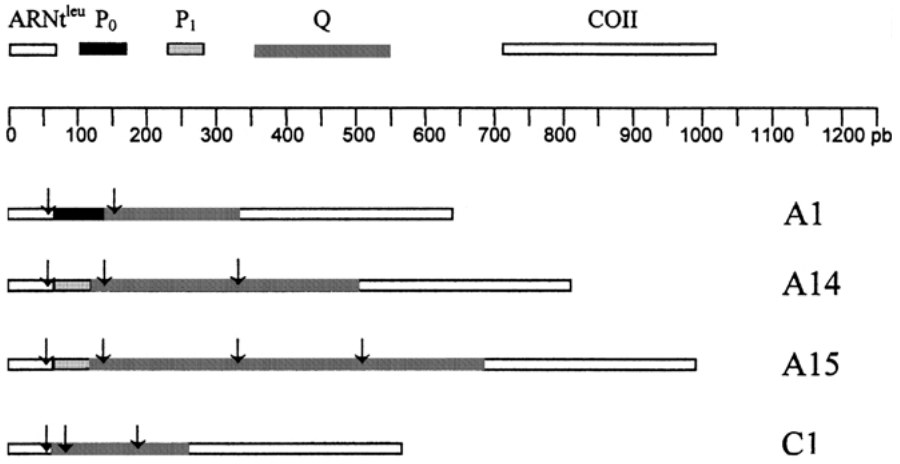

Figure 2. Restriction maps of the haplotypes found on Tenerife (following Garnery et al. 1993). Arrows indicate restriction sites.

of these P sequences appears in the haplotype C1, which shows only one Q sequence in its intergenic region. Bees bearing this haplotype belong to the East European evolutionary branch that includes the subspecies *A. m. ligustica* and *carnica.*

Distribution and frequencies of haplotypes

The distribution and the relative frequencies of these four haplotypes in the sampled populations are given in Figure 1. Haplotypes A15 and A14 have a high frequency making a total of 38% and 11% respectively of the sampled colonies. Haplotype A1 has been detected in two populations: Monte Tegueste and Guía de Isora where the ten sampled colonies show the A1 haplotype. The C1 haplotype has been found in four populations and shows an overall frequency of 37%, ranging from 61 to 20%. The C1 haplotype was present in 4 of the 13 sampled apiaries, including the one maintained as a reservoir of the Canarian bee by the local beekeepers in Arico. In other five apiaries, C1 was detected together with the Canarian haplotypes (A15 and A14). None of the studied populations show the simultaneous presence of the four haplotypes.

Population structure

A comparative analysis of the foreign haplotype introgression among and within populations has been done using the Fischer exact test. We have compared the distribution of the haplotypes and mitochondrial lineages among the apiaries and populations. The results were significant ($P = 0$) either for haplotypes or mitochondrial lineages, indicating differentiation among the populations.

North to the Teide peak, the frequency of A1 and A14 is higher than in the south, whereas the haplotypes C1 and A15 are more frequent in the south (Figure 1). In order to check whether the frequency and distribution of haplotypes vary significantly in relation to the geographical distribution of the colonies, an AMOVA test was performed. Two groups were detected: Monte Tegueste and Guía de Isora (north of Mount Teide) and Santa Cruz, La Laguna, Arico and Granadilla (south of Mount Teide). Results are shown in Table 1. Fifty-two percent of the total variance is due to the differences among the apiaries within populations, whereas 36% of the variance depends on the differences between the two groups, thus confirming the influence of the geographical localisation in the haplotype distribution. Only 10% of the total variance is due to the differences among the populations forming the two groups, which suggests a certain level of homogeneity within these groups.

Discussion

Since honeybee mitochondrial DNA appears to be exclusively maternally inherited (Meusel and Moritz 1990), the study of one worker per colony allows characterising the colony itself and the queen haplotype. Therefore we were able to evaluate the level of introgression of alien mitochondrial genes into the local populations.

The A1 haplotype has been detected in a low frequency in populations from South Spain (Garnery et al. 1995; De la Rúa et al. 1999), and in populations from North Africa (Morocco and Guinea) belonging to *A. m. intermissa* (Garnery et al. 1995). In fact this haplotype, which is rare in southern Spain, is the most frequent in southern Morocco, which is geographically close to the Canary Islands. This finding suggests a relationship between Canarian and African honeybee populations.

The most common and widespread mtDNA haplotypes (A15 and A14) in the island of Tenerife have the characteristic Canarian pattern. These haplotypes include the sequence P_1 in the intergenic region and can be distinguished from each other by the presence of one extra repetition of the Q unit (A15 with three and A14 with two Q sequences). Honeybees bearing these haplotypes could make up a particular subset of the African lineage, known to date only in the Canary Islands (De la Rúa et al.

Table 1. AMOVA design and results of the analysis of the population structure of *Apis mellifera* from Tenerife.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	12.221	0.444	36.77
Among populations within groups	4	8.997	0.129	10.67
Among apiaries within populations	73	46.351	0.635	52.56
Total	78	67.570	1.208	

1998). The P_1 sequence has also been observed in other haplotypes encountered in high proportion in Portugal (Franck et al. 1998) and in less extent in Spain (Garnery et al. 1995), but such haplotypes differ from the Canarian haplotypes by one or two restriction sites. These observations and the fact that the Canaries were a trading post on the way to South America during historic times, suggest alternative hypotheses about the origin of the Canarian honeybee populations: either they were derived from *A. m. iberica* populations introduced in the Canary Islands since the sixteenth century, or from African *A. m. intermissa* populations via the north trade winds, acting closer islands as stepping-stones in the colonisation process.

A fourth haplotype has the East European restriction site pattern and only one Q sequence. A number of different geographic populations and races of *A. mellifera* are characterised by such a composition of the intergenic region (Garnery et al. 1993), but with this test we cannot determine the geographical origin of the foreign population. *Apis mellifera carnica* from Germany and *A. m. ligustica* from Italy are included among them, the Italian yellow bee being the most widely imported (Bar-Cohen et al. 1978; Woodward 1993). The high frequency of this haplotype in Tenerife is surprising, as colonies with known introductions of foreign queens were not included in the analysis. This result can be explained by an active gene flow across the apiaries. The particular distribution of the C1 haplotype, more frequent in the south, may reflect the different intensity of beekeeping activities: amateur beekeepers with permanent local colonies in the north, and professional beekeepers who regularly import foreign queens and move their colonies through the island periodically, south of the Teide peak. Apart of this human-influenced factor it should be noted that Teide peak (3710 m) probably acts as an effective barrier to gene flow.

The protected apiary located in Arico (Taller Apícola) shows a marked introgression of East European mtDNA, although it was supposed to have only endemic honeybees. Thus in morphologically uniform black Canarian bee populations of Tenerife coexist Canarian, African or East European mitochondrial haplotypes. Since black Canarian bees of Tenerife show the East European mtDNA, it can be inferred that local drones have fertilised imported queens during successive generations. This causes dilution of nuclear genes responsible for the external morphology, but alien mitochondrial genes are kept and introgressed due to the maternal inheritance of this molecule.

Garnery et al. (1995) did not found European mtDNA in Morocco despite frequent introductions of queens from France. This difference with the situation found in the Canaries, can be explained because past introductions in Morocco might have been diluted over time, whereas in the Canaries the introgression is still occurring or it is very recent. On the other hand, East European haplotypes have also been detected in honeybee populations from France (Garnery et al. 1998) with different intensities. This fact has been interpreted as a result of present queen introductions of *A. m. carnica* and *A. m. ligustica* by the beekeepers, and of gene flow across hybrid zones between *A. m. mellifera* (lineage M) and eastern European populations (lineage C).

In this study we have detected a high level of mitochondrial introgression from East European honeybee races in the honeybee populations from the island of Tenerife. This finding is probably the result of random mating between imported queens and native drones. Thus, if importation of queens is maintained or increased on this island during the next years, the gene pool of native populations will be severely disrupted. This process could cause the extinction of ecotypes well adapted to the special environmental conditions of the Canaries. Further research must be done for evaluating this risk and establishing policies to preserve the native bees.

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