

Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands

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Abstract

The genetic structure of *Apis mellifera* populations from the Canary Islands has been assessed by mitochondrial (restriction fragment length polymorphisms of the intergenic transfer RNA^{leu}-COII region) and nuclear (microsatellites) studies. These populations show a low level of genetic variation in terms of average number of alleles and degree of heterozygosity. Significant differences in the distribution of alleles were found in both data sets, confirming the genetic differentiation among some of the islands but not within them. Two mitochondrial haplotypes characteristic of the Canary Islands are found at high frequencies, although populations are introgressed by imported honeybees of eastern European C lineage. This introgression is rather high on Tenerife and El Hierro and low on Gran Canaria and La Gomera, whereas on La Palma it has not been recorded. The finding of microsatellite alleles characteristic of the eastern European lineage corroborates the genetic introgression. Phylogenetic analyses indicate that the Canarian honeybees are differentiated from other lineages and provide genetic evidence of their African origin.

Keywords: *Apis mellifera*, Canary Islands, genetic differentiation, microsatellite, mitochondrial DNA, population structure

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Introduction

The Canary Islands are located in the Atlantic Ocean 100 km from the coast of Africa. They have a volcanic origin and constitute a natural laboratory for studying the evolution and the speciation of numerous organisms. Canarian populations of many species have diverged into endemic races due to their special environmental conditions (review in Juan *et al.* 2000). Recent studies have focused not only on among-island but also on within-island variation of different genera (*Gallotia*, Thorpe *et al.* 1996; *Hegeter*, Juan *et al.* 1998; *Chalcides*, Brown & Pestano 1998) and have showed that differentiation has occurred within and among islands, thus giving rise to the present biodiversity.

Honeybees (Hymenoptera, Apidae) were naturally distributed in Africa, Europe and Western Asia before they were spread around the world by human beekeeping activities. The distribution area of each of the 24 morphologically characterized subspecies (Ruttner *et al.* 1978;

Ruttner 1988) allowed the grouping of these geographical races into three lineages: African subspecies (lineage A), west European subspecies (lineage M) and east European and north Mediterranean subspecies (lineage C). These morphological branches were lately confirmed as evolutionary lineages by molecular studies using mitochondrial DNA (mtDNA) (Smith 1991; Cornuet *et al.* 1991; Garnery *et al.* 1992), allozyme electrophoresis (Cornuet 1982; Badino *et al.* 1984; Smith & Glenn 1995) and microsatellite markers (Estoup *et al.* 1995a).

Recently, Franck *et al.* (2000) and Palmer *et al.* (2000), using mitochondrial and microsatellite variability, have given full support to the hypothesis proposed by Ruttner (1988) and Arias & Sheppard (1996) about the existence of a fourth evolutionary lineage (O), comprising *Apis mellifera* subspecies from the Middle East area.

Ruttner (1975), using morphologically discriminant tests, studied Canarian honeybee populations pointing out the close relationship between these honeybees and the continental populations from Southern Iberia. Previous analysis at the mitochondrial level of the Canarian honeybee populations (De la Rúa *et al.* 1998) has shown that these

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honeybees bear mitochondrial haplotypes of African origin, as do south Iberian and north Moroccan populations. However, the most common haplotypes in the island populations have not been found in the populations from Iberia and Africa, and therefore could be a mitochondrial marker for the Canarian honeybee populations.

The *DraI* test [restriction fragment length polymorphism (RFLP) of the intergenic transfer RNA (tRNA)^{leu}-COII] has become a widely used approach in the biogeography of the *A. mellifera* subspecies complex (Garner *et al.* 1993, 1995; 1998a; De la Rúa *et al.* 1998, 1999; 2000; Franck *et al.* 1998; 2000; Palmer *et al.* 2000). This polymerase chain reaction (PCR)-based technique distinguishes evolutionary lineages encompassing different subspecies depending on the sequence and length polymorphism of this intergenic region. In this way, African subspecies bear a P₀ sequence (62–69 bp), west European M subspecies carry a P sequence (52–56 bp) (Cornuet *et al.* 1991) and Canarian populations are characterized by a P₁ sequence (51 bp) (De la Rúa *et al.* 1998). These honeybee populations have also up to three copies of a Q sequence, whereas subspecies forming the east European and north Mediterranean C lineage show only one Q sequence and no P sequence.

Microsatellites are tandemly repeated sequences of 1–5 bp in length scattered throughout the nuclear genome of organisms (Estoup *et al.* 1993; Hughes & Queller 1993) and have proved to be highly efficient for differentiating populations and groups of populations and for detecting recent bottleneck events (Cornuet & Luikart 1996). These nuclear markers have been successfully used in studies at the population level of honeybees (Estoup *et al.* 1995a,b; Franck *et al.* 1998; Garner *et al.* 1998b) and bumblebees (Estoup *et al.* 1996; Widmer *et al.* 1998).

In this study, we attempt to characterize the genetic structure of the honeybee populations from the Canary Islands and their phylogenetic relationships using molecular approaches. These purposes are of particular interest due to the recent introduction of foreign honeybees that may change the gene pool of native Canarian honeybees adapted to the specific environmental conditions of these islands. The relationships between these islands and

continental populations of *A. mellifera* were investigated in order to test alternative hypotheses about the origin and evolution of the Canarian populations.

Materials and methods

Honeybee samples

Apis mellifera populations were sampled from five islands: Tenerife, Gran Canaria, La Gomera, La Palma and El Hierro. At present, Lanzarote and Fuerteventura do not have honeybee populations due to the climate conditions. Padilla-Alvarez *et al.* (1997) carried out morphometrical analyses of some of these samples, the haplotype composition of which was determined by De la Rúa *et al.* (1998). Details about the number of colonies and localities sampled per island are given in Fig. 1. From five to 20 workers per colony were kept in absolute ethanol at –4 °C until they were processed in the laboratory. Analyses of mitochondrial and microsatellite data were carried out including and excluding honeybees bearing the east European C1 haplotype, in order to have an insight into the effects of queen importation in population parameters (haplotype diversity, pairwise F_{ST} and phylogenetic relationships).

DNA extraction

Bees were rinsed for 1 h in rinse buffer (Garner *et al.* 1993). DNA was extracted from the worker thorax following standard organic methods (Sambrook *et al.* 1989) or the Chelex method (Walsh *et al.* 1991) with minor modifications.

PCR amplification

The mitochondrial analysis was carried out following Garner *et al.* (1993) with a PCR amplification of the intergenic tRNA^{leu}-COII region, followed by a restriction of the amplified product with the *DraI* enzyme. The resulting fragments were electrophoretically separated in 8% acrylamide gels that were stained with ethidium bromide and photographed under UV light.

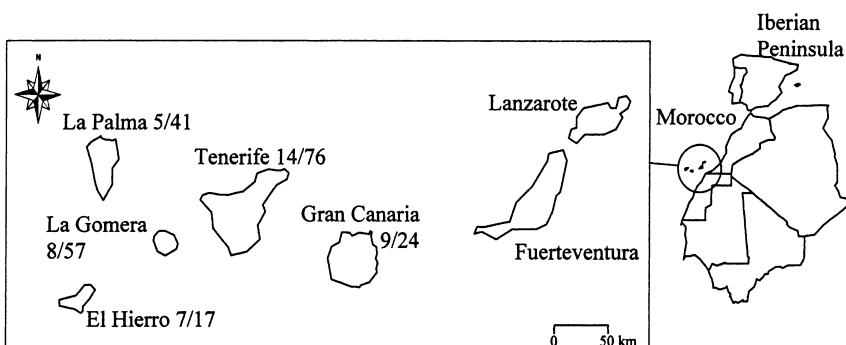


Fig. 1 Number of localities and colonies (values given as locality/colony) of *Apis mellifera* sampled on the Canary Islands for mitochondrial and microsatellite analyses.

Eight polymorphic microsatellite loci (Estoup *et al.* 1994; Estoup *et al.* 1995b; Franck *et al.* 1998) were scored: B124, A113, A7, A35, A24, A28, A88 and A8. The annealing temperature was changed according to the different conditions for the PCR reactions. Multiplex PCRs were conducted with two triplets and one pair of loci, i.e. with three or two pairs of primers (Simon 1998). PCR reactions were performed in 10 μ L volumes containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl₂, 200 μ M of each dNTP, 333 nM of each of the primers (one of each pair labelled at the 5'-end with one of the fluorescent dyes 6-FAM, HEX and TET, Perkin Elmer), 0.25 units of Golden *Taq* polymerase (Perkin Elmer) and 1 μ L DNA extract. Two μ L of the mixture was added to 10 μ L formamide containing 0.3 μ L of the TAMRA 500 standard (Perkin Elmer). The samples were run on a DNA sequencer (ABI 310, Perkin Elmer) with the POP4 polymer and a capillary (ϕ 50 μ m, 47 cm), 5-s injection and a total running time of 21 min.

Mitochondrial analysis

Unbiased estimates and standard deviations of gene diversity of mtDNA (Nei & Tajima 1981) and estimations of the population subdivision were documented using the ARLEQUIN software package (Schneider *et al.* 1997). When multiple comparisons were performed, *P* values were adjusted using the sequential Bonferroni procedure to control for Type I error (Rice 1989).

Microsatellite analysis

Microsatellite allele sizes were scored by comparison of the lengths of the PCR products with those of the standards added in each run. The POPGEN package was used for estimating population parameters, and unbiased estimates and standard deviations of gene diversity or expected heterozygosity were calculated following Nei (1973). The GENEPOP package (GENEPOP on the web <http://wbiomed.curtin.edu.au/genepop>; Raymond & Rousset 1995) was used for computing exact tests for Hardy-Weinberg equilibrium, genotypic linkage disequilibrium

and genetic structure (genotypic differentiation). The cyto-nuclear disequilibrium was analysed by performing a probability test (or Fischer exact test) for either alleles or genotypes at each locus-haplotype combination, using a Markov chain (Asmussen *et al.* 1987).

Variation of microsatellite within and between populations (F_{IS} and F_{ST}) was analysed with the FSTAT program (Goudet 1995). We set the significance value for multiple significance tests using the sequential Bonferroni procedure (Rice 1989).

Phylogenetic analysis

Results obtained in this work were combined with those from other locations in Iberia (Andalucia, Castilla, Portugal) and Morocco (South-west Rif, Northern Rif, Southern Morocco), to establish phylogenetic relationships among populations with different origins (number of analysed individuals ranges from 42 to 58; data from Franck *et al.* 1998). We used the maximum likelihood approach performed on the frequency of haplotypes present in each population, and neighbour-joining (Saitou & Nei 1987) and the chord distance of Cavalli-Sforza & Edwards (1967) based on the microsatellite variation. Bootstrap values (in percentage) were obtained after 2000 iterations of the data set in every case (Hedges 1992). Programs included in the package PHYLIP (version 3.5c, Felsenstein 1993) were used for these purposes.

Results

Mitochondrial data

Six different mitochondrial haplotypes were observed in the 215 investigated colonies, all of them already described (Garnery *et al.* 1993, 1995; De la Rúa *et al.* 1998) (Table 1). Haplotypes A15, A14 and A11 have a similar composition, bearing the P₁ sequence and three (A15), two (A14) or one (A11) Q copies. The African haplotypes A9 and A1 are characterized by the presence of the P₀ sequence and two or one Q copy, respectively. The European C1 haplotype has only one Q sequence.

Table 1 Mitochondrial DNA variability in the honeybee populations from the Canary Islands, showing the intergenic region composition (type of P and number of Q sequences) and frequency of each haplotype. The unbiased estimates of haplotype diversity (*D*) and sampling variance are shown for the overall samples and also excluding those samples with C1 haplotype. (*N* is the sample size)

Population	<i>N</i>	A15 P ₁ QQQ	A14 P ₁ QQ	A11 P ₁ Q	A9 P ₀ QQ	A1 P ₀ Q	C1 Q	<i>D</i>	<i>D</i> _{without C1}
Gran Canaria	24	0.42	0.04	0.13	0.08	0.25	0.08	0.371 ± 0.219	0.357 ± 0.213
Tenerife	76	0.37	0.12			0.16	0.35	0.355 ± 0.206	0.278 ± 0.169
La Gomera	57	0.68	0.18			0.12	0.02	0.208 ± 0.134	0.195 ± 0.127
La Palma	41	0.27	0.02			0.71		0.278 ± 0.169	0.278 ± 0.169
El Hierro	17	0.82					0.18	0.206 ± 0.139	0.000 ± 0.000

	Gran Canaria	Tenerife	La Gomera	La Palma	El Hierro
Gran Canaria		0.015*	0.076*	0.166	0.281
Tenerife	0.048*		0.009	0.227	0.201
La Gomera	0.079*	0.139		0.364	0.120*
La Palma	0.167	0.256	0.355		0.603
El Hierro	0.149	0.154	0.058*	0.502	

Values computed excluding the C1 haplotypes are shown above the diagonal, values obtained from the overall haplotypes are shown below the diagonal. * $P > 0.005$.

The haplotype A15 was observed in every island, whereas A14 was not detected in El Hierro, and A11 was only present in Gran Canaria. The haplotype A9 was detected exclusively in samples from Gran Canaria (8%); the haplotype A1 was found in every island except El Hierro and was the most frequent haplotype in La Palma (71%).

The haplotype C1 was detected on every island except on La Palma. Samples bearing this haplotype are probably the result of many recent introductions of queens from the C lineage (mainly from Italy, E. González and F. González, personal communication). The proportion of introduced haplotypes was higher on Tenerife (35%) and El Hierro (18%) than on Gran Canaria (8%) and La Gomera (2%).

No haplotypes of the west European M lineage were found in the overall Canarian sample.

The unbiased estimates of haplotype diversity and the sampling variance for the populations studied have been calculated for each island, either including or excluding the bees with the east European haplotype. The diversity values (Table 1) only show significant changes in Tenerife and El Hierro. In both cases, the values are similar to those found in Crete and Sicily (Garnery *et al.* 1993; Franck *et al.* 2000) and lower than those reported for Iberian and Moroccan colonies (Franck *et al.* 1998). A gradient related to island size and distance to the African mainland can be observed in the genetic diversity values, as the small western islands (La Palma, La Gomera and El Hierro) are less variable than Gran Canaria and Tenerife, although this difference was not statistically significant.

We found significant genetic differentiation using population pairwise F_{ST} significance tests based on haplotype frequencies. From all possible pairwise comparisons three population pairs (Gran Canaria–Tenerife, Gran Canaria–La Gomera and El Hierro–La Gomera) were not significantly different after Bonferroni correction (Table 2). The same results were obtained after excluding the C1 haplotype from the analysis. The F_{ST} values as an estimate of genetic distance were high between La Palma–El Hierro (0.502) and La Palma–La Gomera (0.355) and low between Gran Canaria–Tenerife (0.048) and Gran Canaria–La Gomera (0.079).

The maximum likelihood tree (Fig. 2a) shows three groups of populations: Canarian, Moroccan and Iberian.

Table 2 Pairwise F_{ST} values computed from haplotype frequencies between pairs of populations of *Apis mellifera* from the Canary Islands

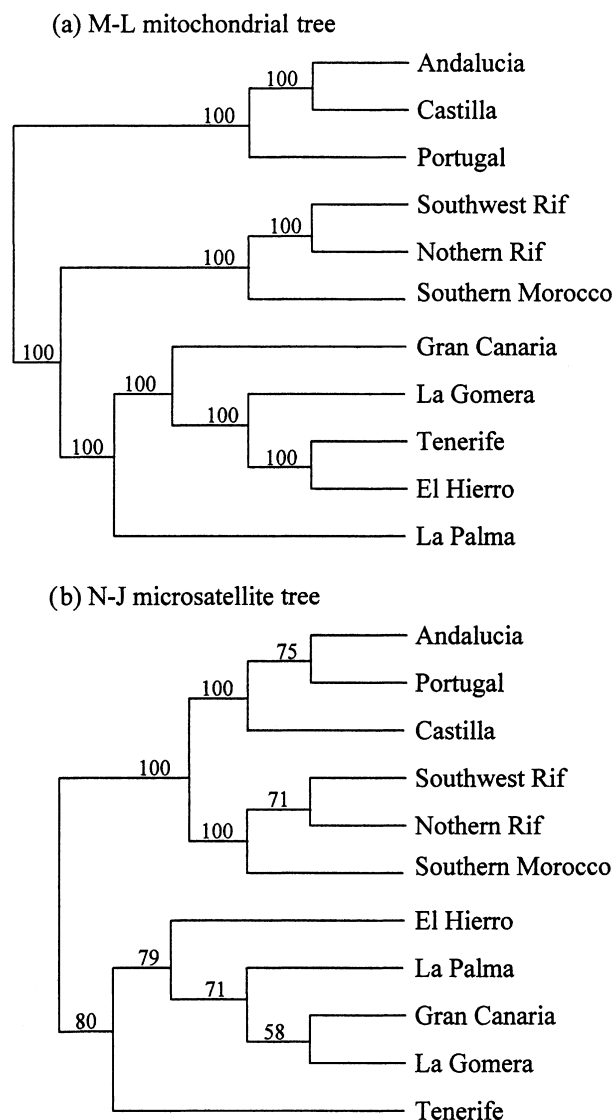


Fig. 2 Phylogenetic (unrooted trees) analyses of the honeybee populations from the Canary Islands (data from the present paper), and Iberia and Africa (data from Franck *et al.* 1998) based on the mitochondrial (a) and microsatellite (b) variation, and inferred from the maximum-likelihood and neighbour-joining methods, respectively. Bootstrap values are noted as percentages and were computed over 2000 replications of the data sets.

Locus/alleles	Gran Canaria	El Hierro	La Gomera	La Palma	Tenerife
B124	(17)	(13)	(46)	(38)	(62)
Alleles	7	5	11	8	10
H_O	0.882	0.308	0.696	0.737	0.790
H_E	0.809	0.491	0.784	0.636	0.750
A113	(18)	(16)	(50)	(40)	(71)
Alleles	6	4	5	5	9
H_O	0.722	0.750	0.800	0.850	0.704
H_E	0.620	0.662	0.733	0.707	0.616
A7	(21)	(16)	(56)	(40)	(72)
Alleles	4	2	6	5	7
H_O	0.571	0.062	0.571	0.425	0.431
H_E	0.489	0.060	0.625	0.421	0.367
A35	(21)	(16)	(55)	(40)	(67)
Alleles	7	6	10	6	11
H_O	0.524	0.500	0.800	0.850	0.627
H_E	0.698	0.578	0.748	0.727	0.697
A28	(23)	(17)	(56)	(41)	(75)
alleles	2	2	2	1	3
H_O	0.043	0.176	0.107	0.000	0.093
H_E	0.042	0.161	0.101	0.000	0.089
A24	(23)	(17)	(53)	(39)	(74)
Alleles	3	5	6	3	4
H_O	0.348	0.294	0.302	0.051	0.108
H_E	0.355	0.358	0.441	0.099	0.555
A88	(24)	(17)	(54)	(39)	(72)
Alleles	3	4	4	3	9
H_O	0.000	0.235	0.093	0.077	0.181
H_E	0.156	0.216	0.089	0.075	0.474
A8	(15)	(12)	(30)	(30)	(70)
Alleles	4	2	3	4	6
H_O	0.267	0.167	0.067	0.467	0.371
H_E	0.340	0.226	0.238	0.507	0.361

Table 3 Number of alleles detected, observed and expected heterozygosities (H_O and H_E) by microsatellite locus in *Apis mellifera* populations from the Canary Islands

The sample size in each location is indicated in brackets. Detailed data for each locality are available upon request.

The Canarian honeybee populations branch in a way that suggests a closer relationship to north African populations, despite the fact that this is an unrooted tree. The same topology was obtained analysing only the frequencies of African and Canarian haplotypes in each population.

Microsatellite data

The eight loci scored display different numbers of alleles in the sampled populations (Table 3), ranging from 11 (loci B124 and A35) to one (locus A28). The island of Tenerife exhibited a higher level of polymorphism, with an average of 7.2 alleles per locus, whereas in El Hierro this average is 3.9 alleles (Table 4). The expected heterozygosity per locus as a measure of gene diversity ranges from 0.489 ± 0.215 (Tenerife) to 0.344 ± 0.237 (El Hierro). The average observed

heterozygosity ranges from 0.432 ± 0.359 (La Palma) to 0.312 ± 0.219 (El Hierro).

The pattern of locus-specific variability was different for each locus but similar among the populations of the Canary Islands. Loci A28, A7, A24, A88 and A8 were less polymorphic (with some alleles near to fixation) than B124, A113 and A35. Some alleles at loci A113, A88 and A8, considered as diagnostic of east European bee races by Garnery *et al.* (1998b), have been detected on Tenerife and El Hierro, where the proportion of introgressed apiaries is higher.

We first tested if the honeybees on each island could be considered as one single population. Therefore, an exact test for population differentiation (Fischer's method) was performed among the different localities within islands to evaluate if the allelic composition was independent of the

Population	<i>N</i>	<i>n</i>	<i>H_O</i>	<i>H_E</i>
Gran Canaria	20.01 ± 3.24	4.5 ± 1.9	0.419 ± 0.313	0.439 ± 0.266
Tenerife	70.25 ± 4.19	7.2 ± 2.9	0.413 ± 0.274	0.489 ± 0.215
La Gomera	49.15 ± 8.75	5.9 ± 3.2	0.429 ± 0.323	0.469 ± 0.294
La Palma	38.22 ± 3.51	4.4 ± 2.1	0.432 ± 0.359	0.396 ± 0.299
El Hierro	15.39 ± 1.93	3.9 ± 1.4	0.312 ± 0.219	0.344 ± 0.214

Values are the average and the standard deviation in each population (*N* is the mean sample size, *n* is the mean observed number of alleles per locus, *H_O* and *H_E* are the observed and the expected heterozygosity).

	Gran Canaria	Tenerife	La Gomera	La Palma	El Hierro	<i>F_{IS}</i>
Gran Canaria		0.073	0.033*	0.056*	0.076	0.071
Tenerife	0.049		0.107	0.113	0.097	0.129
La Gomera	0.034*	0.087		0.052	0.127	0.097
La Palma	0.057*	0.082	0.052		0.106	-0.076
El Hierro	0.074	0.081	0.135	0.121		0.162

Values below the diagonal with the C samples and above without them. Multilocus *F_{IS}* values for the complete sample set from each island are given to the right. **P* > 0.005.

locality assignment on each island. Only on Tenerife was the hypothesis of lack of intrapopulation differentiation not corroborated (*P* < 0.0005).

Four of the 40 island–locus combinations showed significant departures from Hardy–Weinberg equilibrium, but only two of them remained significant after applying the Bonferroni correction to the significance level due to the multiple comparisons (Tenerife locus A24 *P* = 0.000001 and locus A88 *P* = 0.000039). When the 14 localities from Tenerife were tested individually, only one of them (San Andrés de Tigueste) showed a significant departure from Hardy–Weinberg equilibrium (*P* = 0).

Exact tests for genotypic disequilibrium for each pair of loci in each island population resulted in eight significant values out of 129 comparisons, six of them were found in Tenerife and two in La Gomera.

From the overall allele–haplotype combinations at the eight loci, significant cyto–nuclear disequilibrium was only found in four: Gran Canaria at the locus A28 (*P* = 0.04), La Palma at the locus A113 (*P* = 0.02) and Tenerife at the loci A24 (*P* = 0.004) and A88 (*P* = 0). From the 293 genotype–haplotype combinations, we found significant disequilibrium in the same islands and loci as before and also in Tenerife at the locus A28 (*P* = 0.009).

Pairwise *F_{ST}* multilocus values performed as in the mitochondrial analysis, are shown in Table 5. Values below the diagonal were obtained considering those samples bearing the C1 haplotype and those above the diagonal were obtained excluding them. From all possible pairwise comparisons the higher genetic distances were observed between El Hierro–La Gomera and El Hierro–La Palma

(0.135 and 0.121, respectively). The *F_{ST}* estimates were lower between Gran Canaria and the other four islands, ranging from 0.034 (La Gomera) to 0.074 (El Hierro). Multiple comparisons after the Bonferroni correction showed significant population subdivision for every pair of islands except for Gran Canaria–La Gomera and Gran Canaria–La Palma. *F_{IS}* estimates gave positive values for every island except La Palma, thus indicating an excess of heterozygotes on this island, whereas on Tenerife and El Hierro the high values may suggest a deficit of heterozygotes. As in the mitochondrial analysis, the pairwise comparison showed the same significant results whether or not the samples with the C1 haplotype were considered.

The observed pattern in the neighbour-joining (NJ) tree from the microsatellite data (Fig. 2b) showed, as in the population mtDNA phylogeny, that honeybee populations clustered in three groups depending on their origin: Iberia, Africa or the Canaries. Moroccan and Iberian populations clustered together whereas the Canarian populations made up a separate branch. Within the Canarian group, Tenerife and El Hierro branched separately at the base while in the maximum-likelihood (ML) mtDNA tree they clustered together.

Discussion

Genetic variability within islands

Canarian honeybee populations are characterized by a low variability in terms of number of alleles per microsatellite locus and heterozygosity. These values are similar within

Table 4 Multilocus microsatellite variability in the honeybee populations from the Canaries

Table 5 Pairwise *F_{ST}* values between pairs of Canarian populations of *Apis mellifera* calculated from microsatellite variation.

the islands and coincide with the results observed in the endemic Canarian bumblebee *Bombus canariensis* (Estoup *et al.* 1996; Widmer *et al.* 1998) and in honeybee populations from Crete and Sicily (Garnery *et al.* 1993; Franck *et al.* 2000). Island populations usually have lower levels of genetic variation than equivalent continental populations (Frankham 1997), which is observed in this study when these first data on Canarian honeybees are compared with those from Iberia and Morocco (Franck *et al.* 1998; Garnery *et al.* 1998b). The overall heterozygosity deficiency could be due to a strong deficit at some loci (A24, A88 and A8) that themselves vary among the islands. These results do not seem to be due to null alleles because the expected same deficiency at a given locus was not observed in all the islands. Inbreeding and pooling of populations (Wahlund's effect) are more plausible explanations, particularly in Tenerife and El Hierro where the finding of two mitochondrial lineages points towards a subdivision of populations.

Changes in the beekeeping management on the Canary Islands due to importation of queens from other regions, and the known movements of colonies within each island have probably influenced the patterns of genetic variation. This is shown by the increase in the estimates of genetic diversity observed in those populations that include east European colonies.

Severe bottleneck events may have happened in the three smallest islands. In La Gomera 86% of the samples carries haplotypes A14 and A15, in El Hierro every sample except those bearing the east European haplotype shows the haplotype A15, and in La Palma 71% of them shows the haplotype A1. The possibility of this population event is reinforced by the finding of a generalized deficiency of genetic diversity in microsatellite data (Tables 3 and 4). In the island of La Palma, this bottleneck might have happened during the 1950s due to a severe infestation of *Acarapis woodi*.

Population differentiation

The results of the genetic differentiation tests corroborated by the *F*-statistic values, showed similar patterns either with mitochondrial or microsatellite data, and indicate that each island population has undergone its own degree of genetic differentiation. Thus all the colonies situated on one island can be considered as a single population except for Tenerife.

The genetic distance observed between Gran Canaria and La Gomera at both mitochondrial and microsatellite levels indicates a remarkable degree of gene flow between them. Gran Canaria has probably played a major role in the honeybee distribution in the Canary Islands, as suggested by the low genetic distance values found. This hypothesis is in agreement with a directional east-west colonization

pattern of the islands from the mainland, postulated for other organisms (review in Juan *et al.* 2000).

The F_{ST} values indicate that honeybee differentiation between islands is lower than in other animal groups. In particular, speciation or marked differentiation has been reported for the Coleopteran genera *Pimelia* (Juan *et al.* 1995) and *Hegeter* (Juan *et al.* 1998), the lizard genus *Gallotia* (Thorpe *et al.* 1994) and the skink genus *Chalcides* (Brown & Pestano 1998). This lower differentiation in honeybees is probably due to several factors, including different migration abilities as noticed for the bumblebee *Bombus canariensis* (Widmer *et al.* 1998) and human activities that may homogenize the gene pool, thus reducing intra- and perhaps inter-island variability.

Genetic introgression

The genetic introgression of foreign queens has first been estimated from the mtDNA diversity. The maternal inheritance of this molecule (Meusel & Moritz 1990), combined with the haploid system and its high variability in some regions, make mitochondrial analysis a suitable tool for characterizing colonies within populations and consequently detecting foreign queen introduction. The frequency of the east European C1 haplotype in the Canarian honeybee populations may be used for estimating the levels of queen importation and genetic introgression due to beekeeping activities and/or natural processes.

Except on La Palma, the C1 haplotype appeared in every island, and especially on Tenerife and El Hierro where frequent importations have taken place recently (F. González, personal communication). The finding of this haplotype (characteristic of yellowish east European honeybees) in morphologically black Canarian honeybees indicates that hybridization and mixing of populations is occurring within the Canarian populations. The impact of the introgression of imported honeybees on the genetic pool of native honeybees from Tenerife has been analysed in detail (De la Rúa *et al.* 2001) and needs further analyses (morphometry, allozymes, etc.) in order to establish the genetic basis for protecting the local ecotypes.

On the other hand, the mitochondrial diversity found in Gran Canaria is probably due to introductions from other sources, i.e. from the Iberian mainland or the near coast of Southern Morocco. Although Gran Canaria is located about 200 km away from the African coast, natural colonization events via the north trade winds should be considered for explaining this mitochondrial diversity, with the closer islands—Fuerteventura and Lanzarote—acting as stepping stones in this process. Nowadays, in these two islands there are no honeybee populations due to the climate conditions, but in a geologically recent past they had a more humid climate and even a laurisilva forest (Machado 1976), which probably harboured environmental conditions suitable for honeybees.

This high level of east European mtDNA coincides with the presence of diagnostic microsatellite alleles (as defined by Garnery *et al.* 1998b) on Tenerife and El Hierro. In Tenerife, significant departures from the Hardy–Weinberg and genotypic linkage equilibria at some microsatellite loci have been detected. These findings are characteristic of populations that have undergone migratory movements and/or gene flow from other races or populations. The effect of these honeybee introductions is also reflected in the significant cyto-nuclear disequilibrium estimates, which indicate a recent introgression of European honeybees in Canarian populations.

According to Garnery *et al.* (1998b), when importations are reiterated every year, mtDNA is more quickly introgressed than nuclear genes. This distinctness of the marker distribution is related to the social system of *Apis mellifera* in which the individual and colony reproduction levels affect differentially both types of genomes (Franck *et al.* 1998). This difference in the rate of introgression might cause the differences in the tree topologies (Fig. 2), and is especially noticeable on the islands of Tenerife and El Hierro. Both islands clustered together in the mitochondrial ML tree, while in the NJ microsatellite tree they swapped their positions, being more distantly related to each other.

Evolutionary relationships to continental honeybee populations

Both mitochondrial and microsatellite data sets indicate that the Canarian populations are differentiated from continental populations. Honeybees from the Canaries show a closer relationship to Moroccan than to Iberian populations, although Ruttner (1975) and Padilla-Alvarez *et al.* (1997) concluded that Canarian and Iberian populations are related on morphological grounds. The affinity between Canarian and Moroccan organisms on a molecular basis has also been reported for *Drosophila suboscuro* (Afonso *et al.* 1990) and *Bombus canariensis* (Widmer *et al.* 1998). It might be suggested that Canary Island honeybees were originally derived through early founder events from a stock having an African origin (much like Malta and Sicily), with many of the haplotypes now being found in common between the islands and continental locales. Novel variants (A15 and A14) may have arisen *in situ* through time but the other African or Iberian alleles and haplotypes that are found in the Canary Islands could have an equally ancient presence. The subsequent genetic differentiation of Canarian honeybee populations, probably as a consequence of their isolation from the continent, is corroborated by the existence of the haplotypes A14 and A15. These haplotypes have a characteristic composition of the tRNA^{leu}-COII intergenic region only found in Canarian honeybees (De la Rúa *et al.* 1998). Therefore, we might

consider the Canarian honeybees as a well-defined subset of the African evolutionary lineage of *Apis mellifera*.

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References

- Afonso JM, Volz A, Hernández M *et al.* (1990) Mitochondrial DNA variation and genetic structure in old-world populations of *Drosophila suboscuro*. *Molecular Biology and Evolution*, **7**, 123–142.
- Arias MC, Sheppard WS (1996) Molecular phylogenetics of honeybee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. *Molecular Phylogeny and Evolution*, **5**, 557–566.
- Asmussen MA, Arnold J, Avise JC (1987) Definition and properties of disequilibrium statistics for associations between nuclear and cytoplasmic genotypes. *Genetics*, **115**, 755–768.
- Badino A, Celebrano G, Manino A (1984) Population genetics of Italian honeybee *Apis mellifera ligustica* Spin. & its relationships with neighbouring subspecies. *Bulletin of the Museum of Science and Nature of Torino*, **2**, 571–584.
- Brown RP, Pestano J (1998) Phylogeography of skinks (*Chalcides*) in the Canary Islands inferred from mitochondrial DNA sequences. *Molecular Ecology*, **7**, 1183–1191.
- Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis models and estimation procedures. *Evolution*, **3**, 550–557.
- Cornuet J-M (1982) The MDH polymorphism in some West Mediterranean honeybee populations. In: *Proceedings of the IX Congress IUSSI* (eds Breed MD, Michener CD, Evans HE), pp. 415–416. Westview Press, Boulder, CO.
- Cornuet J-M, Garnery L, Solignac M (1991) Putative function of the intergenic region between COI and COII of *Apis mellifera* L. mitochondrial DNA. *Genetics*, **128**, 393–403.
- Cornuet J-M, Luikart G (1996) Description and power analysis of two tests for inferring recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.
- De la Rúa P, Galián J, Serrano J (1998) Mitochondrial variability of honeybees populations from the Canary Islands. *Molecular Ecology*, **7**, 1543–1547.
- De la Rúa P, Galián J, Serrano J (1999) Variabilidad mitocondrial en poblaciones de abejas de la miel del Sureste Peninsular. *Investigación Agraria Producción Y Sanidad Animal*, **14**, 24–30.
- De la Rúa P, Serrano J, Galián J (2001) Biodiversity of *Apis mellifera* populations from Tenerife (Canary Islands) and hybridisation with East European races. *Biodiversity and Conservation*, in press.
- De la Rúa P, Simon UE, Tilde A, Mortiz RFA, Fuchs S (2000) MtDNA variation in *Apis cerana* populations from the Philippines. *Heredity*, **84**, 124–130.

- 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 Research*, **21**, 1427–1431.
- Estoup A, Solignac M, Cornuet J-M (1994) Precise assessment of the number of patriline and of genetic relatedness in honeybee colonies. *Proceedings of the Royal Society of London B*, **258**, 1–7.
- Estoup A, Garnery M, Solignac M, Cornuet J-M (1995a) Microsatellite variation in honeybee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics*, **140**, 679–695.
- Estoup A, TAILLEZ C, Cornuet J-M, Solignac M (1995b) Size homoplasy and mutational processes of interrupted microsatellites in Apidae species, *Apis mellifera* and *Bombus terrestris*. *Molecular Biology and Evolution*, **12**, 1074–1084.
- Estoup A, Solignac M, Cornuet J-M, Goudet J, Scholl A (1996) Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molecular Ecology*, **5**, 19–31.
- Felsenstein J (1993) PHYLIP, phylogeny inference package, Version 3.5. University of Washington, Seattle, WA.
- Franck P, Garnery L, Solignac M, Cornuet J-M (1998) The origin of West European subspecies of honeybees (*Apis mellifera*) new insights from microsatellite and mitochondrial data. *Evolution*, **52**, 1119–1134.
- 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.
- Frankham R (1997) Do island populations have less genetic variation than mainland populations? *Heredity*, **78**, 311–327.
- Garnery L, Cornuet J-M, Solignac M (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Molecular Ecology*, **1**, 145–154.
- 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, Mosshine EH, Oldroyd BP, Cornuet J-M (1995) Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Molecular Ecology*, **4**, 465–471.
- Garnery L, Franck P, Baudry E *et al.* (1998a) Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *A. m. iberica*). I. Mitochondrial DNA. *Genetics Selection Evolution*, **30**, 31–47.
- Garnery L, Franck P, Baudry E *et al.* (1998b) Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *A. m. iberica*). II. Microsatellites. *Genetics Selection Evolution*, **30**, 49–74.
- Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-Statistics. *Journal of Heredity*, **86**, 485–486.
- Hedges SB (1992) The number of replications needed for accurate estimation of the bootstrap P value in phylogenetic studies. *Molecular Biology and Evolution*, **9**, 366–369.
- Hughes CR, Queller DC (1993) Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. *Molecular Ecology*, **2**, 131–137.
- Juan C, Oromí P, Hewitt GM (1995) Mitochondrial DNA phylogeny and sequential colonization of Canary Islands by darkling beetles of the genus *Pimelia* (Tenebrionidae). *Proceedings of the Royal Society of London B*, **261**, 173–180.
- Juan C, Ibrahim KM, Oromí P, Hewitt GM (1998) The phylogeography of the darkling beetle, *Hegeter politus*, in the eastern Canary Islands. *Proceedings of the Royal Society of London B*, **265**, 135–140.
- Juan C, Emerson BC, Oromí P, Hewitt GM (2000) Colonization and diversification: towards a phylogeography synthesis for the Canary Islands. *Trends in Ecology and Evolution*, **3**, 104–109.
- Machado A (1976) Introduction to a faunal study of the Canary Islands laurisilva, with special reference to the ground-beetles (Coleoptera, Caraboidea). In: *Biogeography and Ecology in the Canary Islands* (ed. Kunkel G), pp. 347–411. Dr W. Junk b.v. Publishers, The Hague.
- Meusel M, Moritz RFA (1990) Transfer of paternal mitochondrial DNA in fertilization of honeybee (*Apis mellifera* L.) eggs. In: *Social Insects and the Environment* (eds Veeresh GK, Malik B, Viraktamanth CA), pp. 135. Oxford IBH Publishing Co. PVT. LTD, New Delhi.
- Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science of the USA*, **70**, 3321–3323.
- Nei M, Tajima F (1981) DNA polymorphisms detectable by restriction endonucleases. *Genetics*, **97**, 583–590.
- Padilla-Alvarez F, Puerta-Puerta F, Flores-Serrano J-M, Bustos-Ruiz M, Hernández-Fernández R (1997) Biometrical study of bees on the island of La Palma. (I. Proboscis, hindleg, cubital index A/B, tergites and sternites 3 and 4). *Archivos de Zootecnia*, **46**, 21–30.
- Palmer MR, Smith DR, Kaftanoglu O (2000) Turkish honeybees: genetic variation and evidence for a fourth lineage of *Apis mellifera* mtDNA. *Journal of Heredity*, **91**, 42–46.
- Raymond M, Rousset F (1995) GENEPOP (vers 1.2) population genetics software for exact test and ecumenism. *Journal of Heredity*, **86**, 248–250.
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223–225.
- Ruttner F (1975) African races of honeybees. In: *Proceedings of the XXV International Beekeeping Congress* (Apimondia Publ. House), pp. 325–344. Bucharest, Romania.
- Ruttner F (1988) *Biogeography and Taxonomy of Honeybees*. Springer Verlag, Berlin.
- Ruttner F, Tassencourt L, Louveaux J (1978) Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L. *Apidologie*, **9**, 363–381.
- Saitou N, Nei M (1987) The neighbour joining method a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.
- Sambrook J, Fritsch E, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Schneider S, Kueffer JM, Roessler D, Excofier L (1997) ARLEQUIN: a software for population genetic data analysis, Version 1.1. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.
- Simon UE (1998) *Regulation of reproductive dominance hierarchies in A. m. capensis workers*. PhD Thesis, University of Halle-Wittenberg.
- Smith DR (1991) Mitochondrial DNA and honeybee biogeography. In: *Diversity in the Genus Apis* (ed. Smith DR), pp. 131–176. Westview, Boulder, CO.
- Smith DR, Glenn TC (1995) Allozyme polymorphism in Spanish honeybees (*Apis mellifera iberica*). *Journal of Heredity*, **86**, 12–16.
- Thorpe RS, McGregor DP, Cumming AM, Jordan WC (1994) DNA Evolution and colonization sequence of island lizards in relation to geological history mtDNA RFLP, Cytochrome B, Cytochrome Oxidase, 12S rRNA and nuclear RAPD analysis. *Evolution*, **48**, 230–240.
- Thorpe R, Black H, Malhotra A (1996) Matrix correspondence tests on the DNA phylogeny of the Tenerife Lacertid elucidate both

- historical causes and morphological adaptation. *Systematics and Biology*, **45**, 335–343.
- Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**, 506–512.
- Widmer A, Schmid-Hempel P, Estoup A, Scholl A (1998) Population genetic structure and colonization history of *Bombus terrestris* s.l. (Hymenoptera Apidae) from the Canary Islands and Madeira. *Heredity*, **81**, 563–572.

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