

The Atlantic side of the Iberian Peninsula: a hot-spot of novel African honey bee maternal diversity

Maria Alice PINTO¹, Irene MUÑOZ², Julio CHÁVEZ-GALARZA¹, Pilar De la RÚA²

¹Mountain Research Centre (CIMO), Polytechnic Institute of Bragança, Campus de Sta. Apolónia, Apartado 1172, 5301-855 Bragança, Portugal

²Área de Biología Animal, Dpto. de Zoología y Antropología Física, Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain

Received 10 November 2011 – Revised 14 April 2012 – Accepted 2 May 2012

Abstract – The Iberian honey bee has been one of the most surveyed subspecies for genetic diversity. Yet, previous studies have missed an important component of Iberian honey bee variation harbored by populations inhabiting the Atlantic side of the Iberian Peninsula. Herein, we provide a fuller picture of the Iberian honey bee maternal diversity by revealing 16 novel haplotypes detected in honey bees from Portugal. Of the 16 haplotypes, all of African ancestry, 15 belong to the Atlantic sub-lineage A_{III} while only one fits the most common sub-lineage A_I. This level of new variation is remarkable as it represents a 59% increase in the wide-range African lineage and a 188% in sub-lineage A_{III}. Our findings further highlight the complexity of the Iberian honey bee diversity patterns and reinforce the importance of this southernmost European territory as a reservoir of *Apis mellifera* genetic diversity, a resource increasingly important in a rapidly changing and demanding world.

Iberian honey bee / genetic diversity / mtDNA / *DraI* test / Portugal

1. INTRODUCTION

Understanding patterns and underlying processes of diversity of the western honey bee (*Apis mellifera* L.) has been a major goal of numerous genetic studies, which in a population declining scenario is becoming increasingly important because it can provide a stronger scientific basis for management and conservation decisions. Among the 29 recognized honey bee subspecies (Engel 1999; Sheppard and Meixner 2003), the

Iberian honey bee has been one of the most intensively surveyed for diversity patterns. Indeed, studies of Iberian honey bees have been performed since the 1970s (Ruttner et al. 1978) using morphology (Cornuet and Fresnaye 1989; Arias et al. 2006; Miguel et al. 2010), allozymes (Smith and Glenn 1995; Arias et al. 2006), mitochondrial DNA (Smith et al. 1991; Garnery et al. 1995, 1998a; Franck et al. 1998; De la Rúa et al. 2001, 2004, 2005; Arias et al. 2006; Miguel et al. 2007; Cánovas et al. 2008), and microsatellites (Franck et al. 1998; Garnery et al. 1998b; De la Rúa et al. 2002, 2003; Miguel et al. 2007; Cánovas et al. 2011). Differential and complex patterns of diversity have emerged from these surveys, which have yet to be fully resolved.

The maternally inherited mitochondrial DNA (mtDNA) has been the marker of choice for

Electronic supplementary material The online version of this article (doi:10.1007/s13592-012-0141-1) contains supplementary material, which is available to authorized users.

Corresponding author: M.A. Pinto,
apinto@ipb.pt
Manuscript editor: Marina Meixner

assessing Iberian honey bee variation, particularly the PCR–RFLP of the intergenic tRNA^{leu}-cox2 region, also known as the *Dra*I test (Garnery et al. 1993). Over 2,500 colonies, mostly sampled in the eastern half of the Iberian Peninsula, have been screened with the *Dra*I test (Garnery et al. 1993, 1995, 1998a; Miguel et al. 2007; Cánovas et al. 2008). The data generated by this massive sampling confirmed early findings of co-existence of African (A) and western European (M) lineages, forming a south–north cline (Smith et al. 1991), and revealed unparalleled levels of haplotype diversity and complexity (Franck et al. 1998; Cánovas et al. 2008). Accordingly, it has been suggested that, as for other animal and plant species, the Iberian Peninsula served as a glacial refuge (Hewitt 1999, 2001; Arias et al. 2006; Gómez and Lunt 2007), and as a place of secondary contact between European and African evolutionary lineages (Smith et al. 1991; Garnery et al. 1995; Cánovas et al. 2008), and therefore it has been a stage for historical processes possibly involving recurring phenomena of local adaptation, contraction, fragmentation, expansion, and admixture.

In contrast with the populations inhabiting the eastern side of the Iberian Peninsula, Portuguese honey bees have been largely under-sampled. Yet, the few samples collected in Portugal (Garnery et al. 1998a; Arias et al. 2006; Miguel et al. 2007) suggest that the Atlantic side of Iberia may harbor an important component of the Iberian honey bee maternal diversity. Therefore, a fuller understanding of diversity patterns of the Iberian honey bee requires further surveys of Ibero-Atlantic populations. As part of an ongoing genetic study of the honey bees occupying the Portuguese territory, we have detected 16 novel haplotypes with the *Dra*I test. This finding is non-trivial as this small corner of southern Europe is contributing with 59% of new variation to the wide-range African lineage. Herein, the novel haplotypes are fully described by the RFLP approach and by sequence data. Our findings

suggest that the Atlantic side of the Iberian Peninsula harbors important genetic resources, especially in face of the escalating threats to the honey bee diversity.

2. MATERIALS AND METHODS

2.1. Samples and RFLP analysis

As part of an ongoing study of the Portuguese honey bee populations, over 950 stationary colonies were sampled, between 2008 and 2010, covering every district of continental Portugal and the archipelagos of Azores and Madeira (sample sizes and locations are provided in Online resource 1). Honey bee workers were collected from the inner part of the hives, placed in absolute ethanol, and then stored at -20°C until molecular analysis. The maternal ancestry of the 950 workers, each representing a single colony and a single apiary, was assessed using the *Dra*I test (Garnery et al. 1993), which consists on PCR amplification of the tRNA^{leu}-cox2 intergenic region, using the primers E2 and H2 (see PCR details in Garnery et al. 1993), followed by digestion with the restriction enzyme *Dra*I (see digestion and gel electrophoresis details in Cánovas et al. 2008). Of the 950 individuals scored using the complete set of restriction maps and restriction fragment sizes reported to date (De la Rúa et al. 1998, 2005; Franck et al. 2001; Collet et al. 2006), 43 exhibited a total of 16 unreported PCR–RFLP patterns. Location of the 43 colonies, from which the individuals were sampled, is shown in Figure 1. In this study, the 16 novel PCR–RFLP patterns were fully characterized by sequencing.

2.2. Sequencing and sequence analysis

The tRNA^{leu}-cox2 intergenic region analyzed herein contains a non-coding sequence which size depends on the forms of the P element and the number of repeats of the Q element. In the African lineage, the P element can display two different forms: P₀ (sub-lineage A_I and A_{II}) and P₁ (sub-lineage A_{III}). The P₀ differs from P₁ by a 15-bp deletion. The Q element can be repeated in tandem one to four times. A further distinction between sub-

(Zymo Research®) and sent to Secugen S. L. (Madrid, Spain) or Macrogen (Seoul, Korea) for direct sequencing in both directions with primers E2 and H2. The sequences were checked for base calling using SeqMan® version 7.0.0 and then deposited in GenBank (<http://www.ncbi.nlm.nih.gov>).

The 23 sequences, plus the published sequences of haplotypes A29a (FJ890930.1; Szalanski and Magnus 2010) and A30 (EF033654.1; Collet et al. 2006), belonging to sub-lineage A_{III}, were aligned using MEGA version 5.03 (Tamura et al. 2011). Proximity among the haplotypes was established using the median-joining network algorithm (Bandelt et al. 1999) as implemented in the program Network version 4.6.0.0 (Fluxus Engineering, Clare, UK; <http://www.fluxus-engineering.com>), with epsilon set to zero and downweighting the four most variable sites (weight=0 for the most variable site; weight=5 for the remaining three most variable sites). Indels (e.g., P₁ element, third and fourth Q element, 15-deletion of A40') were considered as a single mutational step, being therefore coded as a 1-bp gap. Variable characters within the first (Q1), second (Q2), and third Q (Q3) elements were included in the analysis.

3. RESULTS

The *Dra*I test performed on colonies surveyed across Portugal (continent and archipelagos of Azores and Madeira) identified 16 novel PCR-RFLP patterns (haplotypes) carried by 43 colonies (Figure 1). The restriction maps and length of restriction fragments (Figure 2) suggest that they all fit within the African evolutionary lineage (A). Following the nomenclature established earlier (Garnery et al. 1998a) and recently reviewed for lineage M (Rortais et al. 2011), the 16 haplotypes were numbered sequentially from A31 to A46 (accession numbers JQ746684–JQ746701). Haplotypes showing the same RFLP pattern but bearing three or four Q elements were further distinguished by addition of the symbols ' and ", respectively, after the haplotype number (Garnery et al. 1998a; Rortais et al. 2011).

Figure 2 shows that the 16 patterns are dramatically distinct from those previously

reported for the African lineage. The number of restriction fragments varied between three (A31, A41', A45'') and five (A32, A35, A37, A40'), with most haplotypes exhibiting a four-fragment pattern (A33, A34, A36, A38', A39', A42', A43', A44', A46'). The shortest band (28 bp) was displayed by four haplotypes (A35, A36, A37, A39') whereas the longest (1,064 bp) was unique to A45''.

Location of the 43 colonies, and corresponding distribution of the 16 haplotypes depicted in Figure 1, shows that haplotype A42' was the most widespread and common (16 colonies) followed by A40' (four colonies). Haplotypes A39', A43', and A45'' were carried by three colonies whereas A33, A34, and A36 by two colonies. The remaining eight haplotypes were singletons (A31, A32, A35, A37, A38', A41', A44', and A46'). While most haplotypes were detected in the center of continental Portugal, A34 and A40' were private to the islands of San Miguel (Azores) and Madeira, respectively. A42' was the only haplotype detected in both mainland and island (Madeira) populations.

Sequence data (Figure 3) confirmed the novelty of the 16 haplotypes of African ancestry and allowed identification of two additional variants of haplotypes A42' and A43', which were distinguished by a lowercase letter (A42'a, A43'a), as suggested by Rortais et al. (2011). Among the 16 haplotypes, 15 contained the P₁ element whereas only one (A46') exhibited the P₀ element. The length of the sequenced region ranged from 803 bp to 1,204 bp, depending on the number of Q elements and presence of indels. Fifteen haplotypes contained either two (A31, A32, A33, A34, A35, A36, A37) or three Q elements (A38', A39', A40', A41', A42', A43', A44', A46'). Only one haplotype (A45'') displayed a sequence with four Q elements (Figure 2). As typical for this intergenic region, numerous indels of variable sizes were detected within the P and Q elements (Figure 3). In addition to the 15-bp deletion characteristic of the P₁ element (marked as "dl" in Figures 2 and 3), large indels were displayed by A31, A34, A40', and A41'. The largest fragment (33 bp, marked as "c" in Figure 3) was inserted in the

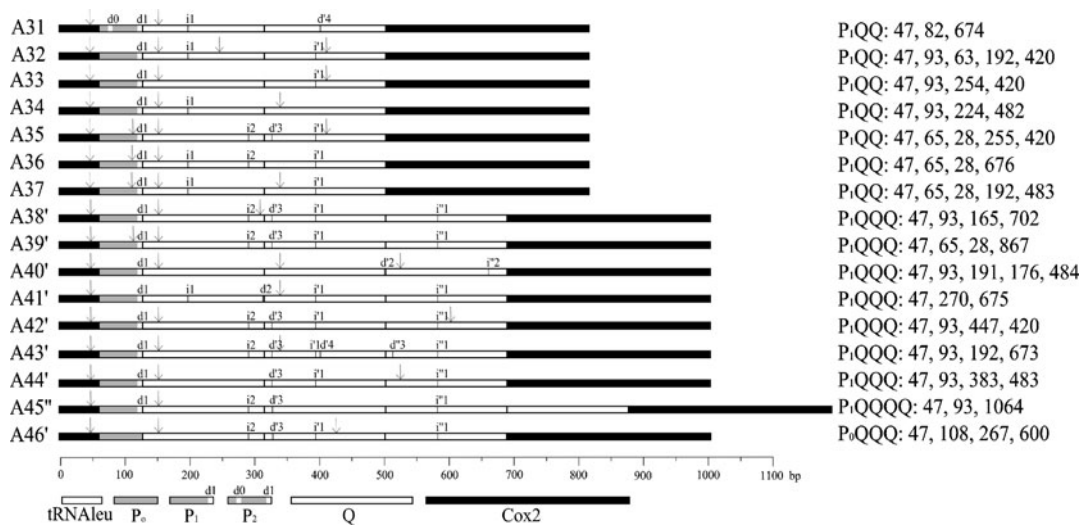


Figure 2. Restriction maps (*left*) and length of restriction fragments (*right*), deduced from *DraI* restriction patterns and sequences of the tRNA^{Leu}-cox2 intergenic region, of the 16 novel haplotypes found in Portugal. The *DraI* recognition site is denoted with an *arrow*. Deletions and insertions are numbered preceded by the letters *d* and *i*, respectively. Deletion marked as *d1* is specific of the P₁ element which defines sub-lineage A_{III}, as in Franck et al. (2001).

Q1 element of haplotype A34. This 33-bp insertion produced the longest Q element (227 bp) ever reported. The 15-bp deletion motif of A40' (marked as "l" in Figure 3) and A41' (marked as "h" in Figure 3) was a perfect match of *d1*, the only difference being the position. Indeed, while *d1* was located at the 3' end of the P element, deletions of A41' and A40' were at the 3' end of Q1 and Q2, respectively. In addition to the variation originated from large indels, there were 10 short indels (1–2 bp) and 17 single-base substitutions, of which nine resulted in a gain/loss of the *DraI* recognition site. The most striking mutation, displayed by seven haplotypes, is the "AG" inserted at the 3' end of Q1 element (marked as "f" in Figure 3). Interestingly, except for the Q3 of haplotype A41', no other Q element carried this mutation.

A median-joining network (Figure 4) based on 36 variable sites (18 coded indels and 18 substitutions) illustrates the relationships among the novel and previously described haplotypes of sub-lineages A_I (A3) and A_{III} (A14, A16, A29a, A30). Two distinct clusters, separated by the number of Q elements, are

represented in the network. The more poorly resolved right-hand side cluster connects the haplotypes with two Q elements. One unresolved connection and four hypothetical haplotypes (unsampled or extinct) link this group to A35, the closest haplotype of the left-hand side cluster. Haplotypes A36 and A37 are the closest in the group with only one mutational step (a transition in A36 that led to loss of *DraI* restriction site) separating them. The haplotype pairs A30/A37 and A32/A33 are separated by one hypothetical haplotype and two mutational steps (one is a transversion that led to a gain/loss of *DraI* restriction site). The previously described A14 and A34, which are derived from the same hypothetical haplotype, are the most distant in the group. A minimum of eight mutations, including the duplication of the Q element, and two hypothetical nodes separate the closest haplotypes of both clusters (A35 and A43'a).

The better resolved left-hand side cluster connects the haplotypes with three and four Q elements. The 14 haplotypes are separated from each other by one to eight mutational steps and three hypothetical nodes. The previously de-

	76	77	78	79	80	81	82	83	84	85	86	102	111	112	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	158	197	198	199	200	201	
	a																	1	2	3	dl	4											c			
A3	A	T	A	A	A	A	T	A	A	A	A	T	T	A	A	T	T	T	A	A	T	T	A	T	T	A	A	A	A	A	-	-	-	-	-	-
A14	A	T	A	A	A	A	T	A	A	A	A	C	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A16	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A29a	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A30	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A31	-	-	-	-	-	-	-	-	-	-	-	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A32	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A33	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A34	A	T	A	A	A	A	T	A	A	A	A	C	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A35	A	T	A	A	A	A	T	A	A	A	A	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A36	A	T	A	A	A	A	T	A	A	A	A	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A37	A	T	A	A	A	A	T	A	A	A	A	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A38'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A39'	A	T	A	A	A	A	T	A	A	A	A	T	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A40'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A41	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A42'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A42'a	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A43'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A43'a	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A44'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A45''	A	T	A	A	A	A	T	A	A	A	A	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A46'	A	T	A	A	A	A	T	A	A	A	A	T	T	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	A	-	-	-	-	-	-

	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	252	275	303	304	326	327	
																													d	5	6	e	7	f		
A3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A29a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A34	T	T	T	T	A	T	A	T	T	T	A	A	T	A	A	A	A	T	A	A	A	T	A	A	T	T	A	A	-	-	-	-	-	-	-	-
A35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A38'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A39'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A40'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A42'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A42'a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A43'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A43'a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A44'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A45''	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A46'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3. Variable sites of tRNA^{leu}-cox2 intergenic region of the 16 novel haplotypes, the two additional sequence variants (A42'a and A43'a), and the previously reported African haplotypes (A3, A14, A16, A29a, A30). Sequences of A29a (FJ890930.1) and A30 (EF033654.1) were obtained from GenBank. Sequences of A3, A14, and A16 were obtained from individuals collected in Portugal. The numbers on the top line represent the nucleotide position within the tRNA^{leu}-cox2 sequenced region. Position 1 marks the beginning of the tRNA^{leu} gene, corresponding to position 3370 of the honey bee mitochondrial genome (Crozier and Crozier 1993). Substitution sites are numbered from 1 to 17. Indels (marked with a dash) are denoted by letters from a to q. The 15-bp deletion characteristic of the P1 element is marked as dl (position 115). Variable sites between positions 158–358, 365–550, and 551–714 are within Q1, Q2, and Q3 elements, respectively. Position 745 identifies the beginning of Q4. Full sequences are available in GenBank under accession numbers JQ746684–JQ746701.

	328	329	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	365	387	406	429	435	448	457	471	536	537	538	539	540	541	542	543	544
	g		h		8																i	9	10	j	k	11	12	13	l					
A3	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	C	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A14	-	-	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	A	T	-	T	T	C	C	A	T	T	T	A	A	T	T	T
A16	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	-	T	T	C	C	A	T	T	T	A	A	T	T	T
A29a	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A30	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A31	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	G	T	T	-	T	C	C	A	T	T	T	A	A	T	T	T
A32	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	G	T	T	T	A	C	C	A	T	T	T	A	A	T	T	T
A33	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	G	T	T	T	A	C	C	A	T	T	T	A	A	T	T	T
A34	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	A	T	-	T	T	C	C	A	T	T	T	A	A	T	T	T
A35	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	A	C	C	A	T	T	T	A	A	T	T	T
A36	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A37	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	A	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A38'	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A39'	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A40'	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	C	A	T	-	T	T	C	C	-	-	-	-	-	-	-	-	-
A41	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	A	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A42'	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	T	A	T	T	T	A	A	T	T	T
A42'a	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A43'	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	A	T	T	-	T	C	C	A	T	T	T	A	A	T	T	T
A43'a	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	A	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A44'	-	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	T	T	T	C	C	A	T	T	T	A	A	T	T	T
A45''	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	T	-	T	T	C	C	A	T	T	T	A	A	T	T	T
A46'	G	A	A	T	T	T	A	A	T	T	T	A	T	T	A	A	A	-	G	C	T	T	T	T	C	A	T	T	T	A	A	T	T	T

	545	546	547	548	549	550	551	557	579	593	621	640	709	713	714	745
	m n 14 15 o 16 17 p q															
A3	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A14	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A16	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A29a	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A30	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A31	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A32	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A33	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A34	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A35	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A36	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A37	A	T	T	A	A	A	-	-	-	-	-	-	-	-	-	-
A38'	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A39'	A	T	T	A	A	A	A	C	G	T	T	T	A	-	-	-
A40'	-	-	-	-	-	-	A	C	A	C	-	T	A	A	G	-
A41	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A42'	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A42'a	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-
A43'	A	T	T	A	A	A	A	-	G	C	T	T	A	-	-	-
A43'a	A	T	T	A	A	A	A	-	G	C	T	T	G	-	-	-
A44'	A	T	T	A	A	A	A	C	A	C	T	T	A	-	-	-
A45''	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	A
A46'	A	T	T	A	A	A	A	C	G	C	T	T	A	-	-	-

Figure 3. (continued).

scribed A29a is central to this group, showing considerably more connections (eight) than any other haplotype in the network. This central haplotype is connected to the closest ones by a single mutation (1-bp deletion in Q2 for A16, and a substitution in Q3 for A42'a) and to the most distant (A41') by eight mutational steps

and one hypothetical node. The previously described A3 haplotype of sub-lineage A_I (as defined by Franck et al. 2001) and the novel A46' are together in a branch that is separated from A29a by three and four mutations, respectively. These two sub-lineage A_I haplotypes differ from each other by one transition

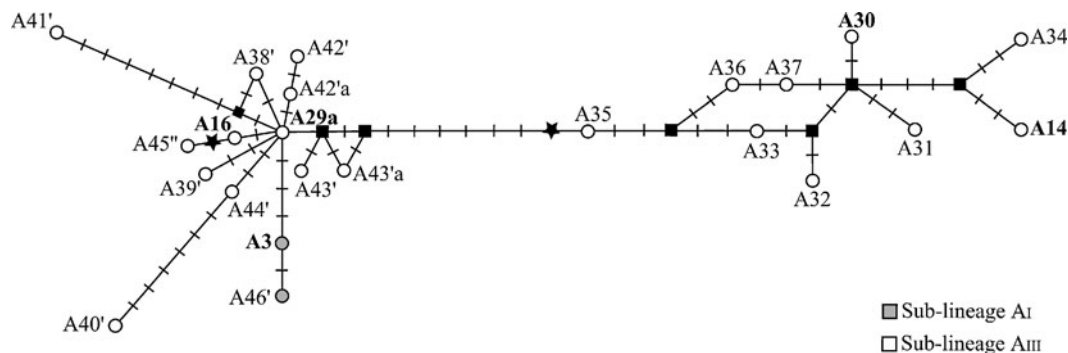


Figure 4. Median-joining network of the novel and previously described African haplotypes (marked in *bold*) that were identified from the tRNA^{leu}-cox2 region. Haplotypes are denoted as *circles*. Hypothetical (unsampled or extinct) haplotypes are symbolized as *filled squares*. The *cross lines* along branches represent mutational steps between nodes. The duplication of the Q element is represented by a *star*.

mutation (C/T), which accounted for an additional *Dra*I recognition site in Q2 of haplotype A46' (substitution 12 in Figure 3).

4. DISCUSSION

This study further highlights the Atlantic side of the Iberian Peninsula as an important repository of Iberian honey bee maternal diversity. Analysis of Portuguese honey bee populations with the *Dra*I test revealed 16 novel haplotypes of African ancestry, which were confirmed by sequence data of the tRNA^{leu}-cox2 intergenic region. These haplotypes join the 27 African haplotypes previously described (De la Rúa et al. 1998, 2005; Franck et al. 2001; Collet et al. 2006) of which 17 have been found in the Iberian Peninsula (Garnery et al. 1998a; De la Rúa et al. 2004, 2005; Cánovas et al. 2008), representing an increase of 59% and 94%, respectively. Most haplotypes (15) contained the P₁ element, typical of sub-lineage A_{III}, whereas only one was assigned to sub-lineage A_I, as defined by Franck et al. (2001). The 15 haplotypes were added to the eight previously reported for sub-lineage A_{III} (De la Rúa et al. 1998; Franck et al. 2001; Collet et al. 2006), representing an increase of 188%.

The Iberian honey bee has been one of the most intensively surveyed subspecies in its natural range (Garnery et al. 1995, 1998a; Franck et al.

1998; Arias et al. 2006; Miguel et al. 2007; Cánovas et al. 2008). Therefore, detection of such a remarkable number of novel haplotypes, mostly of sub-lineage A_{III} ancestry, was unexpected and suggests that prior studies have missed an important diversity component held by the western populations of Iberia. This study not only adds to the complexity of the Iberian honey bee diversity patterns (Franck et al. 1998; Cánovas et al. 2008) but also reinforces the Atlantic distribution proposed for sub-lineage A_{III} (De la Rúa et al. 1998, 2006; Franck et al. 2001), as most colonies were detected in the north of continental Portugal (Figure 1), which exhibits a more Atlantic climate contrasting with the more Mediterranean southern Portugal. Our findings further support an ancient natural colonization of the Iberian Peninsula by African swarms (De la Rúa et al. 2002, 2004; Cánovas et al. 2008) as the hypothesis of historical human-mediated multiple introductions (Franck et al. 1998; Garnery et al. 1998a) is untenable with such complex levels of diversity.

Among the 16 novel haplotypes, A42 is probably the oldest because of its higher frequency and wider geographical distribution (sole haplotype shared between mainland and island colonies). Alternatively, it could have been disseminated by human-assisted colony transportation, as occurred with A29. While the history of introductions of A29 (Collet et al.

2006; Prada et al. 2009) and its variant A29a (Szalanski and Magnus 2010) is unknown, we postulate that these haplotypes descend from colonies of Portuguese origin. The PCR–RFLP patterns of A29 (47/93/866) and A29a (47/93/867) are virtually indistinguishable from those of A16 (47/93/866). Additionally, sequence data shows that they are closely related (Figure 4 and sequences on GenBank), suggesting that A29 (Collet et al. 2006) and 29a (Szalanski and Magnus 2010) are merely sequence variants of A16. The PCR–RFLP haplotype A16 has only been reported in the Portuguese territory, including mainland (Garnery et al. 1998a; Miguel et al. 2007) and islands (De la Rúa et al. 2006). Therefore, it is possible that haplotypes A29 and A29a descend from Portuguese colonies introduced by settlers in South America in historical times and later expanded to North America (Szalanski and Magnus 2010) by Africanized honey bees. Alternatively, there were multiple independent, historical or recent, introductions in North and South America of putatively Portuguese-derived colonies.

Similarities in primary and secondary structures between the Q element and the 3' end of COI gene (5' end of Q), tRNA^{Leu} gene (middle part of Q), and the P sequence (3' end of Q) led Cornuet et al. (1991) to propose an origin of the Q element by tandem replication. The first and the second Q of haplotypes A41' and A40', respectively, bear a 3' end similar to P₁ whereas those of the other Q's are similar to P₀. Interestingly, the 3' end of Q1 of some lineage M haplotypes (M34-HQ337456.1; M43Q-HQ260365.1) and Z haplotypes (Z1-HM236204.1; Z12-HM236212.1; Z13-HM236213.1; Z1Q-HM236205.1) also bear a deletion motif similar to the P₁ element. While this deletion has probably multiple independent origins, this finding deserves further investigation as it may shed some light in the evolution of this complex region.

This study further expands on the complexity of the Iberian honey bee patterns and reinforces the importance of this southernmost European territory as a reservoir of *A. mellifera* genetic diversity. In response to a rapidly changing world (e.g., new pests and parasites, land use

change), which has had severe consequences in apiculture, there is a growing alert for protecting honey bee genetic resources across its natural range (Jensen et al. 2005; De la Rúa et al. 2009; Dietemann et al. 2009; Haddad et al. 2009) and an increasing number of conservation programs, specially to protect *A. m. mellifera* (reviewed by De la Rúa et al. 2009). Preservation of honey bee genetic variation is a pre-requisite for long-term adaptive change and avoidance of fitness decline, through inbreeding depression, and thereby a guarantee of a sustainable apiculture. The Iberian Peninsula has been a stage for evolutionary events that have shaped the evolutionary history of western European honey bee lineage. Therefore, this territory certainly deserves special attention in both small- and large-scale conservation programs.

ACKNOWLEDGMENTS

We are indebted to numerous technicians of the *Divisões de Intervenção Veterinária (Direcção Geral de Veterinária)* and of the Beekeeping Associations, who kindly provided the honey bee samples. Financial support was provided by *Fundação para a Ciência e Tecnologia* and *COMPETE/QREN/EU* (project PTDC/BIA-BEC/099640/2008), Programa Apícola Nacional (Medida 6A) to M. A. Pinto, and by *Fundación Séneca* (project 11961/PI/09) to P. De la Rúa. We also thank the two anonymous reviewers for their valuable comments.

La façade atlantique de la péninsule ibérique : un « point chaud » pour une nouvelle diversité maternelle de type africain chez l'abeille

Abeille ibérique / diversité génétique / ADN mitochondrial / test *DraI* / Portugal

Die atlantische Seite der iberischen Halbinsel: ein Hotspot für bisher unbekannte Afrika-typische maternale Diversität bei Honigbienen

Iberische Honigbiene / genetische Diversität / mtDNA / *DraI*-Test / Portugal

REFERENCES

- Arias, M.C., Rinderer, T.E., Sheppard, W.S. (2006) Further characterization of honey bees from the Iberian Peninsula by allozyme, morphometric and mtDNA haplotype analyses. *J. Apic. Res.* **45**, 188–196
- Bandelt, H.J., Forster, P., Rohl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol.* **16**, 37–48
- Cánovas, F., De la Rúa, P., Serrano, J., Galián, J. (2008) Geographical patterns of mitochondrial DNA variation in *Apis mellifera iberiensis* (Hymenoptera: Apidae). *J. Zool. Syst. Evol. Res.* **46**, 24–30
- Cánovas, F., De la Rúa, P., Serrano, J., Galián, J. (2011) Microsatellite variability reveals beekeeping influences on Iberian honeybee populations. *Apidologie*. **42**, 235–251
- Collet, T., Ferreira, K.M., Arias, M.C., Soares, A.E.E., Del Lama, M.A. (2006) Genetic structure of Africanized honeybee populations (*Apis mellifera* L.) from Brazil and Uruguay viewed through mitochondrial DNA COI–COII patterns. *Heredity*. **97**, 329–335
- Cornuet, J.M., Fresnaye, J. (1989) Biometrical study of honey bee populations from Spain and Portugal. *Apidologie*. **20**, 93–101
- 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
- Crozier, R.H., Crozier, Y.C. (1993) The mitochondrial genome of the honey bee *Apis mellifera*: complete sequence and genome organization. *Genetics*. **133**, 97–117
- De la Rúa, P., Serrano, J., Galián, J. (1998) Mitochondrial DNA variability in the Canary islands honeybees (*Apis mellifera* L.). *Mol. Ecol.* **7**, 1543–1547
- De la Rúa, P., Galián, J., Serrano, J., Moritz, R.F.A. (2001) Molecular characterization and population structure of the honeybees from the Balearic islands (Spain). *Apidologie*. **32**, 417–427
- De la Rúa, P., Galián, J., Serrano, J., Moritz, R.F.A. (2002) Microsatellite analysis of non-migratory colonies of *Apis mellifera iberica* from south-eastern Spain. *J. Zool. Syst. Evol. Res.* **40**, 164–168
- De la Rúa, P., Galián, J., Serrano, J., Moritz, R.F.A. (2003) Genetic structure of Balearic honeybee populations based on microsatellite polymorphism. *Genet. Sel. Evol.* **35**, 339–350
- De la Rúa, P., Jiménez, Y., Galián, J., Serrano, J. (2004) Evaluation of the biodiversity of honey bee (*Apis mellifera*) populations from eastern Spain. *J. Apic. Res.* **43**, 162–166
- De la Rúa, P., Hernandez-Garcia, R., Jiménez, Y., Galián, J., Serrano, J. (2005) Biodiversity of *Apis mellifera iberica* (Hymenoptera: Apidae) from northeastern Spain assessed by mitochondrial analysis. *Insect Syst. Evol.* **36**, 21–28
- De la Rúa, P., Galián, J., Pedersen, B.V., Serrano, J. (2006) Molecular characterization and population structure of *Apis mellifera* from Madeira and the Azores. *Apidologie*. **37**, 699–708
- De la Rúa, P., Jaffé, R., Dall'Olio, R., Muñoz, I., Serrano, J. (2009) Biodiversity, conservation and current threats to European honeybees. *Apidologie*. **40**, 263–284
- Dietemann, V., Pirk, C.W.W., Crewe, R. (2009) Is there a need for conservation of honeybees in Africa?. *Apidologie*. **40**, 285–295
- Engel, M.S. (1999) The taxonomy of recent and fossil honey bees (Hymenoptera: Apidae; Apis). *J. Hym. Res.* **8**, 165–196
- 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., Loiseau, A., Oldroyd, B.P., Hepburn, H.R., Solignac, M., Cornuet, J.M. (2001) Genetic diversity of the honey bee in Africa: microsatellite and mitochondrial data. *Heredity*. **86**, 420–430
- 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, E.H., Oldroyd, B.P., Cornuet, J.M. (1995) Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Mol. Ecol.* **4**, 465–471
- Garnery, L., Franck, P., Baudry, E., Vautrin, D., Cornuet, J.M., Solignac, M. (1998a) Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *A. m. iberica*). I. Mitochondrial DNA. *Genet. Sel. Evol.* **30**, S31–S47
- Garnery, L., Franck, P., Baudry, E., Vautrin, D., Cornuet, J.M., Solignac, M. (1998b) Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *A. m. iberica*). II. Microsatellite loci. *Genet. Sel. Evol.* **30**, S49–S74
- Gómez, A., Lunt, D.H. (2007). Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. Springer, Dordrecht
- Haddad, N., Meixner, M.D., Fuchs, S., Migdadi, H., Garnery, L., Sheppard, W.S. (2009) Mitochondrial DNA support for genetic reserves of *Apis mellifera syriaca* in Jordan. *J. Apic. Res.* **48**, 19–22
- Hewitt, G.M. (1999) Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* **68**, 87–112
- Hewitt, G.M. (2001) Speciation, hybrid zones and phylogeography—or seeing genes in space and time. *Mol. Ecol.* **10**, 537–549

- Jensen, A.B., Palmer, K.A., Boomsma, J.J., Pedersen, B.V. (2005) Varying degrees of *Apis mellifera ligustica* introgression in protected populations of the black honeybee, *Apis mellifera mellifera*, in northwest Europe. *Mol. Ecol.* **14**, 93–106
- Miguel, I., Iriondo, M., Garnery, L., Sheppard, W.S., Estonba, A. (2007) Gene flow within the M evolutionary lineage of *Apis mellifera*: role of the Pyrenees, isolation by distance and post-glacial recolonization routes in the western Europe. *Apidologie*. **38**, 141–155
- Miguel, I., Baylac, M., Iriondo, M., Manzano, C., Garnery, L., Estonba, A. (2010) Both geometric morphometric and microsatellite data consistently support the differentiation of the *Apis mellifera* M evolutionary branch. *Apidologie*. **42**, 150–161
- Prada, Q.C.F., Duran, J.T., Salamanca, G.G., Del Lama, M.A. (2009) Population genetics of *Apis mellifera* L. (Hymenoptera: Apidae) from Columbia. *J. Apic. Res.* **48**, 3–10
- Rortais, A., Arnold, G., Alburaki, M., Legout, H., Garnery, L. (2011) Review of the DraI COI–COII test for the conservation of the black honeybee (*Apis mellifera mellifera*). *Conserv. Genet. Resour.* **3**, 383–391
- Ruttner, F., Tassencourt, L., Louveaux, J. (1978) Biometrical–statistical analysis of the geographic variability of *Apis mellifera* L. *Apidologie*. **9**, 363–381
- Sheppard, W.S., Meixner, M.D. (2003) *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. *Apidologie*. **34**, 367–375
- Smith, D.R., Glenn, T.C. (1995) Allozyme polymorphisms in spanish honeybees (*Apis mellifera iberica*). *J. Hered.* **86**, 12–16
- 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
- Szalanski, A.L., Magnus, R.M. (2010) Mitochondrial DNA characterization of Africanized honey bee (*Apis mellifera* L.) populations from the USA. *J. Apic. Res.* **49**, 177–185
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* doi:10.1093/molbev/msr121