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Further characterization of honey bees from the Iberian Peninsula by allozyme, morphometric and mtDNA haplotype analyses

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Summary

It has been proposed that the Iberian Peninsula served as an important refuge for honey bees during periods of European glaciation. The discovery of genetic markers originating from both African and European honey bee lineages in extant Iberian populations led to hypotheses that the region may be a zone of hybridization or reflect the action of human-assisted translocations. In the present study, we investigated allozyme, mitochondrial and morphological variation in honey bee populations sampled from southern France to northern Morocco. Morphological data supported the interpretation that the bees of Iberia, south and west of a line from Zaragoza to Barcelona, can be characterized as the subspecies *Apis mellifera iberica* (*A. m. iberiensis*). There appears to be a narrow demarcation between this subspecies and *A. m. mellifera,* rather than a gradual cline. The persistence of significant subpopulation differentiation indicates that migratory beekeeping has not strongly influenced the genetic characteristics of local populations. Allozyme data were consistent with previous studies showing north – south changes in allele frequencies, possibly related to subspecific origins or selection. Although mtDNA haplotypes of presumptive A lineage origin were found to be prevalent in Iberian honey bee populations, morphological analysis and published microsatellite evidence does not show a comparatively strong influence from the nearby African subpopulation (Morocco).

Keywords: honey bee, *Apis mellifera,* mtDNA, morphometry, allozyme, Iberian Penninsula

Introduction

In an endemic distribution that includes Europe, Western and Central Asia and Africa, *Apis mellifera* has been classified into 26 subspecies. Intraspecific taxonomy in the honey bee is based on morphometric characters and subspecies show strong correlation with distinct geographic areas (Ruttner, 1988). The subspecies can be further grouped into 4 main evolutionary "lineages" related to their phylogeographic distribution *(ibid).* The origin of a number of the subspecies of Europe has been associated with periods of geographic isolation during glaciation, with the Iberian, Ligurian and Greek peninsulas figuring prominently as refugia (Ruttner, 1988). The origins of African subspecies may be more likely related to adaptation to stable disparate ecological zones, with current distributions reflecting vegetation shifts through sidereal time (Potts and Behrensmeyer, 1992). It has been suggested that the term morphoclusters may be more appropriate to describe variation in honey bees from some regions of Africa (Radloff and Hepburn, 2000).

Although the basis of subspecific classification derives from the accumulation of genetic changes in isolated populations, hybridization can occur between subspecies of the same or even different lineages through secondary contact during interglacial periods (Badino et al., 1984; Comparini and Biasiolo, 1991; Meixner et al., 1993; Franck et al., 2000). For example, throughout much of their common border, the subspecies *A. mellifera mellifera* and *A. mellifera ligustica* are separated by the Alps. However, in an area of northwestern Italy where the mountains are less than 2000m in elevation, a hybrid zone has been described (Badino et al., 1983; Badino et al., 1984; Manino and Marletto, 1984). Similarly, in northeastern Italy, introgression of morphology and mitochondrial DNA markers has been reported between the Italian subspecies *A. m. ligustica* and neighboring *A. m. carnica* (Meixner et al., 1993).

Recently, Franck et al (2000) hypothesized that isolation of honey bee populations containing multiple mtDNA haplotypes into glacial refugia in southern Liguria best explained the distribution of mtDNA polymorphisms found in *A. m ligustica.*

In some cases, interactions between subspecies can be attributed to human activities. In Africa the subspecies *A. m. scutellata* and *A. m. monticola* are distinctly distributed in savanna and mountain areas, respectively. However, in certain mountain locales where the forests were devastated, genetic introgression of one subspecies into the other appears to have been accelerated or skewed (Meixner et al., 2000). The introduction of honey bee subspecies into different geographic areas by beekeepers also has produced subspecies admixtures in many parts of the world. One outstanding example is the past and present process of "Africanization" in the Americas, whereby African *A. m. scutellata* were introduced into Brazil in the 1950s and their descendents expanded a New World range to include most of south and central America and parts of north America (Rinderer et al., 1993a). Interest in the population genetic processes of this expansion and the extent to which genes from previously introduced European subspecies were incorporated has stimulated extensive research on behavioral, morphological and molecular genetic characteristics of the expanding population (Del Lama et al., 1988; Lobo et al., 1989; Smith et al., 1989; Hall and Muralidharan, 1989; Rinderer et al., 1991a; Sheppard et al., 1991a; Sheppard et al., 1991b; Quezada-Euan and Medina, 1998; Quezada-Euan 2000; Sheppard and Smith, 2000; Clarke et al. 2002; Pinto et al., 2005).

In Europe, another area of particular interest regarding secondary contact among honey bee subspecies is the Iberian peninsula. The subspecies endemic to this portion of south-western Europe, *A. m. iberica* (or *A. m. iberiensis* Engel 1999) has been shown to have a morphology somewhat intermediate between the subspecies *A. m. mellifera* that occurs in France and *A. m. intermissa,* that occurs in Morocco (Ruttner et al., 1978; Ruttner, 1988; Cornuet and Fresnaye, 1989; Hepburn and Radloff, 1996). Allozyme data (MDH-1) also have been reported to be consistent with a south-north cline. The allele Mdh^{100} appears to be fixed or nearly fixed in *A. m. intermissa* (Cornuet, 1982) and shows a high frequency in southern Spanish honey bee populations. The frequency of Mdh^{100} decreases in northern Spain, where allele Mdh^{80} predominates (Cornuet, 1982; Smith and Glenn, 1995). Mdh^{80} occurs in high frequency in samples of A. m. mellifera throughout France and northwestern Europe (Sheppard and Berlocher, 1984; Cornuet et al., 1986).

Mitochondrial genetic markers originating from both African (A) and western European (M) honey bee lineages can be found in Iberia. Initial studies revealed two different haplotypes, one predominant in the south (*A. m. intermissa*-like) and the other in the north (*A. m. mellifera*-like) (Smith et al., 1991; Garnery et al., 1995). Such results reinforced the hypothesis that Spain constituted a hybrid zone of secondary contact between *A. m. intermissa* and *A. m. mellifera* (Cornuet and Garnery, 1991; Smith et al., 1991; Smith and Glenn, 1995). However, recent authors have suggested that the introgression originated through human importation of African lineage honey bees into Iberia, based on fine scale analysis of mtDNA haplotypes (Garnery et al., 1998) and assessment of microsatellite variation (Franck et al., 1998). However, De la Rua and colleagues (2002, 2005) reported an absence of cyto-nuclear disequilibrium in honey bees from eastern Spain and postulated that *A. m. iberiensis* may derive from a complex of events, including multiple hybridizations, selection and human manipulation.

In the present study, we investigated allozyme, mtDNA and morphological variation in honey bee populations sampled from southern France to northern Morocco. The results are discussed in view of current hypotheses concerning the Iberian Peninsula as a glacial refuge and secondary contact between subspecies lineages.

Materials and Methods

Sample Collection

Adult workers from a total of 606 colonies were sampled from apiaries in Portugal, Spain, southern France and northern Morocco. Only colonies reported by beekeepers as being of local origin and from apiaries uninvolved in migratory or requeening operations were sampled. Sample sizes and collecting locations are described in Table 1. The honey bees were frozen in liquid nitrogen and later stored at -80°C until analysis.

Morphometric Analysis

The standard character set of 23 characters used in USDA-ID procedures (Rinderer et al., 1993b) was measured for 10 worker bees of each of the 606 colonies. Bees preserved in 95% ethanol were dissected and the right forewing, right hindwing, right metathoracic basitarsus, tibia and femur, and fourth abdominal sternite were mounted on glass slides (Rinderer et al., 1991b) and measured using computer assisted techniques (Daly et al., 1982; Rinderer et al., 1993b).

Protein Electrophoresis

We analyzed one adult worker honey bee from each colony sampled using methods described previously (Sheppard and Berlocher, 1984; 1985). The samples were screened for the following 4 enzymatic systems known to be polymorphic in European honey bees: *malate dehydrogenase* (MDH-1), *malic enzyme* (ME), *phosphoglucomutase* (PGM-1) and *hexokinase* (HK). Electrophoresis was performed in 11% horizontal starch gels using 2 different buffers. MDH-1 and ME were scored in continuous Tris-citrate, pH 8.6 and PGM and HK were scored in Tris-EDTA-maleate, pH 7.4. Standard histochemical stains were used to visualize the enzyme activity (Harris and Hopkinson, 1976).

mtDNA Analysis

Total nucleic acids were extracted from the thorax of 1 or 2 workers using techniques described elsewhere (Sheppard and McPheron, 1991). The extracted DNA was digested with *Eco*R I restriction enzyme following manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). The fragments were separated in 1.0% TAE agarose gel, stained with ethidium bromide, visualized under UV light and photographed. The fragments were transferred to nitrocellulose membranes using Southern blot technique (Sambrook et al., 1989). Cesium chloride gradient centrifugation was used to isolate pure mtDNA from a pooled sample of honey bee thorax and head (~ 2g). Pure mtDNA was labeled with ^{32}P, using nick translation method and hybridized (~ 16h) to the fragments on the filters. The filters were washed twice in 2X SSC, 0.1% SDS at room temperature, dried and exposed to X-ray film.

Data Analysis

The honey bee samples were divided in 18 subpopulations roughly corresponding to the geographical proximity of collecting sites for analytical purposes (Table 1). Discriminant analysis was applied to the mean colony vectors for each colony using a pooled variance-covariance matrix. Three a *priori* groups (Morocco, Portugal and France) were used to classify all remaining Iberian colonies. Discriminant analysis was applied to the colony centroids for each colony at these locations to derive classification criteria. The criteria allowed classification of each Iberian colony into one of these established groups. Mahalanobis distance (D^2) was calculated between all 18 population centroids using a pooled variance-covariance matrix and these were compared to their corresponding *F* values. Average Mahalanobis distance (D) was calculated between each of the three poles (France, Morocco, Portugal) and all locations on the continent (average distances between Morocco and all locations, average distances between France and the remaining locations on the continent or average distance between Portugal and the remaining locations on the continent).

Allozyme data analysis and genetic distance measures were performed with BIOSYS-1 (Swofford and Selander, 1981). Heterogeneity among populations for allozyme and mtDNA data were examined using chi-square test and the correlation between mtDNA haplotypes and alleles was analyzed by SAS.

Results

Morphometric analysis

Descriptive statistics of morphometric characteristics for 133 colonies representing 4 reference population groups (France, Morocco, southern Spain and Portugal) are shown in Table 2. Mehalanobis distances from a common analysis comparing the size of average multivariate distances between and among various populations are presented in Table 3. Pairwise squared Mehalanobis distances between groups from the discriminant analysis of 18 subpopulations are presented in Table 4. A discriminant analysis classification of colonies from the subpopulations is shown in Figure 1.

Protein electrophoresis

The 4 different allozyme systems were assayed in 585 samples. The loci MDH-1 and PGM were polymorphic in most of the subpopulations, whereas HK and ME were monomorphic. The allozyme frequencies and their distributions in the 18 subpopulations are shown in Table 5. Chi-square heterogeneity tests showed that the overall sampled population was not homogeneous. Group comparisons showed that there was no significant difference among the subpopulations from south and central Spain (SW1, SW2, SS1, SS2, ES1, CWS, WSP, SES) and Portugal (PO1, PO2) based on analysis of combined allozyme loci

Table 1. Locations of the samples (subpopulations) used in this study, their respective names and the number of colonies used in allozyme and mtDNA analyses.

Sample names	Collecting locations	Number of colonies analyzed	
		ALLOZYME	mtDNA
PO1	Malveria – Portugal	32	32
PO2	Evora, Serpa and Moura – Portugal	45	45
SW1	Lora Del Rio – Spain	32	32
SW2	Pilas – Spain	46	46
SS1	Bosque, Ubrique, Los Barrios and.Mijas – Spain	63	64
SS2	Lecrin – Spain	26	26
MRC	Asilah and Had El Ghardih – Morocco	13	12
SES	Sorbas and Vera – Spain	17	17
ES1	Orba – Spain	46	47
ES2	Serra – Spain	34	34
NE1	Pobla de Montornes and Breda – Spain	38	38
NE2	Torroella de Montgri – Spain	50	53
NE3	Garriguella – Spain	45	53
FRC	Ille-sur-Tete – France	21	21
NSP	Coll de Nargo – Spain	4	5
CNS	Calatayud – Spain 28	28	31
CWS	Avila – Spain	27	30
WSP	Aliseda – Spain	18	20
TOTAL		585	606

Table 2. Descriptive statistics (mean ± STD) of 23 morphometric characteristics (described in Rinderer *et al.*, 1989) of 10 worker bees each from (*n*) colonies of four populations of honey bees used as reference populations in Figure 1. Lengths are mm and angles are degrees. Population values for each morphological characteristic within a row followed by unlike letters (a,b,c) are significantly different ($P = 0.05$) as judged by a post-ANOVA Duncan's Multiple Range Test.

Morphologica characteristic (P of F)	**Population**			
	Portugal (32)	Southern Spain(64)	Morocco (15)	France (22)
Fore wing length (0.0001)	9.22 ± 0.10a	9.22 ± 0.10a	9.09 ± 0.56b	9.28 ± 0.09a
Fore wing width (0.003)	3.12 ± 0.05a	3.09 ± 0.04b	3.11 ± 0.06ab	3.13 ± 0.05a
Hind wing length (0.038)	4.39 ± 0.06a	4.37 ± 0.06ab	4.34 ± 0.09b	4.39 ± 0.06a
Hind wing width (0.0001)	1.80 ± 0.04a	1.76 ± 0.03b	1.76 ± 0.04b	1.81 ± 0.04a
Hamuli number (0.0081)	22.34 ± 0.80a	22.00 ± 0.81a	21.42 ± 1.06b	21.94 ± 0.89a
Angle 29 (0.4755)	31.64 ± 1.37	31.36 ± 1.05	31.61 ± 1.14	31.74 ± 1.15
Angle 30 (0.0038)	107.55 ± 2.68a	106.63 ± 3.48ab	104.65 ± 2.12c	104.89± 3.34bc
Angle 31(0.001)	104.20 ± 1.95a	103.93 ± 2.01a	101.89 ± 1.41b	103.72 ± 1.67a
Angle 32 (0.0001)	18.44 ± 0.69c	18.93 ± 0.72b	19.77 ± 0.68a	18.76± 0.68cb
Angle 33 (0.0001)	94.74 ± 1.81c	96.80 ± 1.85b	98.91 ± 1.17a	98.21 ± 1.86a
Angle 34 (0.0001)	46.70 ± 1.31b	46.74 ± 1.61b	50.49 ± 0.78a	47.09 ± 1.61b
Angle 35 (0.0001)	20.66 ± 0.95b	19.94 ± 0.90c	24.30 ± 1.11a	20.51 ± 0.99b
Angle 36 (0.0001)	61.13 ± 1.87a	59.65 ± 1.74b	57.81 ± 1.27c	57.52 ± 1.80c
Cubital vein A (0.0001)	0.27 ± 0.02b	0.28 ± 0.01a	0.22 ± 0.02c	0.28 ± 0.02a
Cubital vein B (0.0001)	0.48 ± 0.02c	0.48 ± 0.03c	0.55 ± 0.02a	0.50 ± 0.02b
Tibial length (0.0001)	3.33 ± 0.05a	3.34 ± 0.45a	3.20 ± 0.07b	3.32 ± 0.05a
Femur length (0.0001)	2.72 ± 0.04a	2.71 ± 0.04a	2.62 ± 0.06c	2.67 ± 0.04b
Trochanter length (0.0001)	2.15 ± 0.04a	2.14 ± 0.04a	2.04 ± 0.06c	2.09 ± 0.04b
Trochanter width (0.0001)	1.12 ± 0.02b	1.13 ± 0.02b	1.08 ± 0.02c	1.15 ± 0.02a
Sternal length (0.0001)	2.88 ± 0.04a	2.83 ± 0.04b	2.70 ± 0.07c	2.84 ± 0.03b
Wax mirror length (0.0001)	1.43 ± 0.03a	1.39 ± 0.04b	1.29 ± 0.04c	1.42 ± 0.03a
Wax mirror width A (0.0001)	2.47 ± 0.03a	2.44 ± 0.05b	2.32 ± 0.06c	2.47 ± 0.04a
Wax mirror width B (0.0001)	0.25 ± 0.03b	0.22 ± 0.03c	0.28 ± 0.04a	0.23 ± 0.03c

($P = 0.187$). Thus, these samples can be considered part of one homogenous population. However when this group was compared with samples from the north (NE1, NE2, NE3, NSP and FRC) or from Morocco (MRC) the tests showed significant heterogeneity ($P = 0.0002$ and $P = 0.0004$, respectively). Interestingly, two subpopulations (ES2 and CNS) were significantly different from both the southern and northern Iberian subpopulations ($P = 0.005$ and $P = 0.0002$, respectively), consistent with their location in a transitional zone.

The Mdh^{100} allele occurred in high frequency in the Morocco subpopulation, decreasing northward across Spain. Conversely the frequency of Mdh^{80} increased from south to north, reaching its maximum in the subpopulations of northern Spain and southern France (Fig. 2). Two alleles, Mdh^{65} and Mdh^{87}, were found in low frequency in some subpopulations (Table 5).

Unlike MDH, PGM did not show a pronounced south-north variation. Subpopulations from the extreme northern and southern locations, MRC, NE1, NE2, NE3, NSP and FRC, were fixed or almost fixed for the common allele (Pgm^{100}), whereas the subpopulations of south and central Spain were more heterogenous (Fig. 3). For example, in south and central Spain, the slow allele Pgm^{77} occurred commonly and some rarer alleles (Pgm^{90} and Pgm^{114}) were also detected (Table 5).

mtDNA haplotypes

Within the 606 samples analyzed, three different *Eco*R I haplotypes were found and their frequencies are presented in Table 5. The pattern designated here as P (Portuguese) was previously described from Africanized honey bees in Argentina ("new African" pattern, Sheppard et al., 1992) and is equivalent to the A16 haplotype resolved by *Dra*-I analysis of the mitochondrial intergenic spacer (Garnery et al., 1998, unpublished data). The two other haplotypes, A (African) and E (European), are typically characteristic of African and west European (M lineage) honey bees, respectively (Smith et al., 1991). The 18 subpopulations analyzed were not homogenous overall (heterogeneity test, $P < 0.05$). The distribution of the P

haplotype was mainly restricted to Portugal, although it also occurred in samples collected in western Spain. The African pattern predominated in south and central Spain and the European haplotype was widely distributed among the northeastern Spain and southern France subpopulations (Fig. 4). When the subpopulations from Portugal (PO1, PO2), south Spain (SW1, SW2, SS1, SS2, SES, ES1) and center Spain (CWS, WSP) were grouped together and compared with a group of samples from north Spain (NE1, NE2, NE3, NSP, CNS, ES2) and south France (FRC), the chi-square test was significant (P <0.05). A similar result was obtained when the two Portugal subpopulations were compared with those from southern Spain. Based on the distribution of mtDNA haplotypes, we could divide the 606 samples into 3 distinct groups: southern Spain/Morocco, northern Spain/France and Portugal.

Discussion

Morphological analyses support the conclusion that the honey bees of Morocco are very different from the honey bees of south-western Europe. Only four of the colonies of Iberia showed a morphological similarity to Moroccan honey bees (Fig. 1). The Moroccan honey bees were morphologically distinct for 16 of the 23 morphological variables listed in Table 2. Moreover, the average Mehalanobis distance from "Morocco to all other sites" was more than twice the distance among all Iberian sites (Table 3). Mehalanobis distances are directly comparable if they come from the same analysis and comparative Mahalanobis distance differences reflect the magnitudes of comparative morphological differences. Hence, the honey bees of Morocco

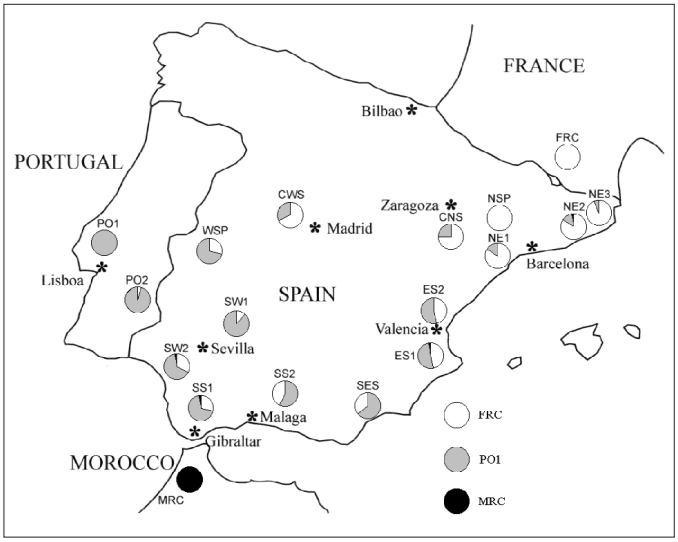

Fig. 1. Discriminant analysis classification of colonies in 15 populations in Iberia according to the greatest similarity to one of three reference populations. The three reference populations are the honey bee samples from Morocco (MRC), Portugal (PO1), and France (FRC).

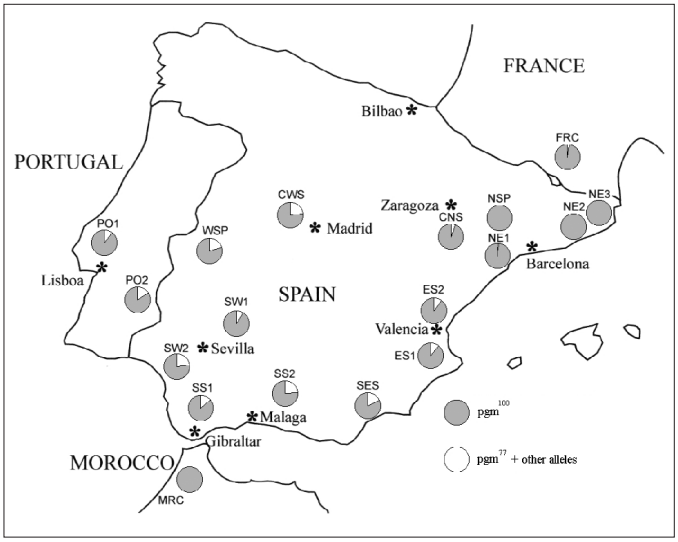

Fig. 3. Geographic distribution of the Pgm allele frequencies. Locations are named as in Table 1.

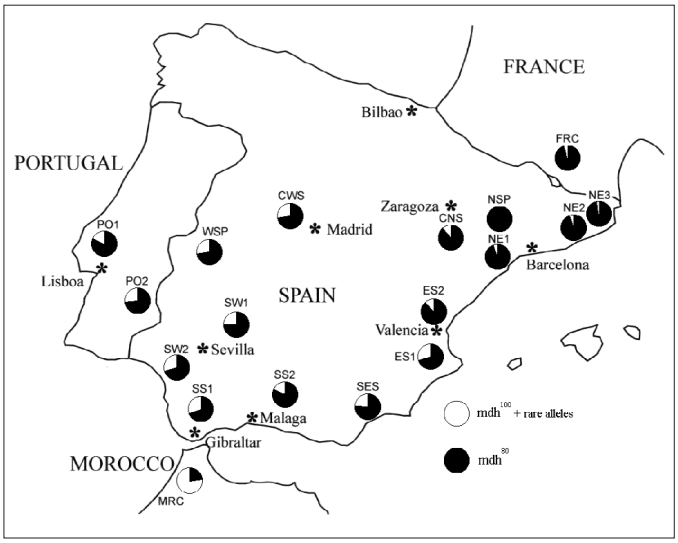

Fig. 2. Geographic distribution of the Mdh – 1 allele frequencies. Locations are named as in Table 1.

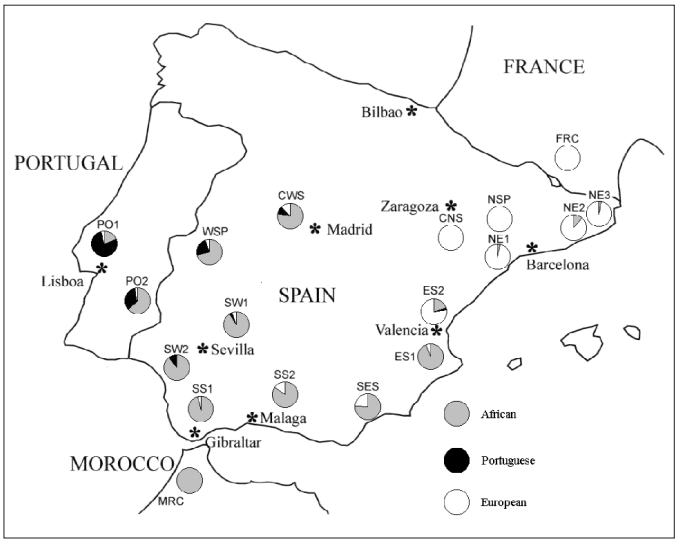

Fig. 4. Geographic distribution of mtDNA haplotype frequencies. Locations are named as in Table 1.

Table 3. Mehalanobis distances from a common analysis comparing the size of average multivariate distances between and among various populations; *n* = number of comparisons that were used to produce the Mehalanobis value or average value.

Comparison	**Mehalanobis Distance (D)**	
	Value or Average (*n*)	Range
Among all sites	3.96 (153)	1.80 – 9.13
Among all Iberian sites	3.40 (136)	1.80 – 5.48
Morocco to all other sites	8.38 (17)	7.69 – 9.13
Portugal to all other Iberian sites	4.42 (16)	2.82 – 4.91
France to all other Iberian sites	4.11 (16)	1.79 – 5.24
France to Portugal	3.50 (1)	–
France to Southern Spain	4.22 (1)	–
France to Morocco	8.26 (1)	–
Portugal to Southern Spain	3.50 (1)	–
Portugal to Morocco	8.12 (1)	–
Southern Spain to Morocco	7.70 (1)	–
Each Iberian site to the nearest site	2.49 (16)	1.76 – 3.99
Each Iberian site to the next nearest site	2.89 (17)	2.04 – 3.98

were more than twice as morphologically different as the average difference among Iberian sites, supporting the interpretation that they are members of the subspecies *A. mellifera intermissa.*

Among the bees of south-western Europe, almost every collection site proved to be associated with Mehalanobis distances which differed significantly from all other sampled European populations (Table 4). The exceptions to this were the populations NE1, NE2, NE3, and FRC, all of which had a similarity with NSP, and to lesser degrees with each other. This supports the interpretation that the honey bees in the north-eastern areas of our survey were (or were genetically influenced by) *A. m. mellifera.* These results were consistent with a suggested gradient in morphological variation between Iberian and French honey bees, but at variance with the extension of the morphological gradient southward to include the honey bees of Morocco (Cornuet and Fresnaye, 1989). The remaining subpopulations were homogeneous in the sense that they did not differ along direction or altitude, were usually identifiable according to their collection location, and when confused with other groups mostly were confused with nearest neighbours ("nearest neighbour vs other" discriminant analysis, not shown). Overall, the morphometric observations support the interpretation that the bees of Iberia south and west of a line from Zaragoza to Barcelona are best characterized as *A. m. iberiensis.* This subspecies is characterized by having distinct local subpopulations (Table 4). The finding of significant subpopulation differentiation is supported also by previous morphological studies of honey bees from southern (Orantes-Bermejo and Garcia-Fernandez, 1995) and northern Spain (Izquierdo et al., 1985). Evidently, the migratory beekeeping habits of modern Iberian beekeepers have not strongly influenced the genetic characteristics of the local populations that are not moved or subjected to intensive requeening procedures.

In the allozyme analysis, the patterns of allelic distribution for MDH and PGM were similar to the findings of Smith and Glenn (1995). The higher diversity of PGM alleles found in subpopulations from south and central Spain in this study, relative to those of northern Spain/France or Morocco supports the role of Iberia as a long term refugial area during glaciation (Ruttner, 1988). The relatively sharp demarcation in allele frequency shifts was consistent with the morphological evidence in delineating the transition zone between *A. m. mellifera* and *A. m. iberiensis.* While the MDH data were also consistent with the interpretation of a cline related to subspecific origin of alleles (Fig. 2), various allelic combinations of MDH are now known to present unequal thermostability profiles (Cornuet et al., 1995). Therefore, the role of selection rather than phylogeography in determining the geographic pattern of allele frequencies in Iberia cannot be discounted.

The distribution of mtDNA haplotypes found among the subpopulations sampled indicated that the bees of northeastern Spain were closely associated with the subpopulation from southern France (Fig. 4). Together with data from the morphometric and allozyme analyses, the mtDNA haplotype profiles support the concept of a narrow demarcation rather than gradual cline between *A. m. mellifera* and *A. m. iberiensis.* The results of fine-scale analyses of mtDNA haplotype variation, in Iberia and elsewhere, have been consistent with the interpretation that multiple introductions of various A lineage mtDNA haplotypes were made into Iberia. These may have been as a result of human activity (Garnery et al., 1998; Franck et al., 1998) or not (De la Rua et al., 2002, 2004). The discovery that some of the A mtDNA lineage haplotypes commonly found in Iberia were absent or rare in Morocco, yet common in other Mediterranean locales (Garnery et al., 1995), provides further support for the involvement of human transportation in relocating honey bees. Alternatively, the discovery of African-characteristic microsatellite alleles in Iberia (albeit in low frequency), together with a lack of cyto-nuclear disequilibrium was interpreted to support the importance of ancient introgression (De la Rua et al., 2002). The high frequency of the P haplotype we found in Portugal and its

Table 4. Pairwise squared Mehalanobis distances between groups from the discriminent analysis of 18 sub populations. Distances between groups are followed by probabilities that group centroids are different.

Name	PO1	PO2	SW1	SW2	SS1	SS2	SES	MRC	ES1	ES2	NE1	NSP	NE2	NE3	FRC	CNS	WSP	CWS
PO1	0 1.0	7.96 0.0001	13.53 0.0001	17.79 0.0001	12.28 0.0001	15.84 0.0001	12.98 0.0001	65.96 0.0001	10.51 0.0001	8.59 0.0001	16.65 0.0001	22.39 0.0001	24.09 0.0001	18.02 0.0001	22.17 0.0001	15.27 0.0001	15.92 0.0001	15.87 0.0001
PO2	–	0 1.0	5.21 0.000	6.85 0.000	12.06 0.000	14.40 0.000	10.91 0.000	80.22 0.000	10.01 0.000	6.43 0.000	17.49 0.000	20.82 0.000	27.66 0.000	21.54 0.000	23.55 0.000	11.15 0.000	7.02 0.000	9.42 0.0001
SW1	–	–	0 1.0	3.09 0.000 2	9.91 0.000	11.94 0.000	10.94 0.000	71.58 0.000	10.98 0.000	10.14 0.000	20.71 0.000	23.17 0.000	30.10 0.000	24.88 0.000	27.55 0.000	13.86 0.000	7.16 0.000	10.24 0.0001
SW2	–	–	–	0 1.0	8.83 0.000	10.77 0.000	10.01 0.000	75.05 0.000	11.65 0.000	11.14 0.000	19.31 0.000	19.98 0.000	28.08 0.000	22.18 0.000	24.19 0.000	11.74 0.000	6.22 0.000	9.76 0.0001
SS1	–	–	–	–	0 1.0	6.27 0.000	8.23 0.000	59.24 0.000	7.92 0.000	10.42 0.000	14.86 0.000	14.14 0.000	18.20 0.000	14.28 0.000	17.74 0.000	10.54 0.000	11.80 0.000	15.55 0.0001
SS2	–	–	–	–	–	0 1.0	6.47 0.000	68.43 0.000	4.17 0.000	8.00 0.000	10.56 0.000	9.61 0.000	13.82 0.000	11.59 0.000	13.26 0.000	9.03 0.000	12.60 0.000	11.79 0.0001
SES	–	–	–	–	–	–	0 1.0	73.51 0.000	4.83 0.000	6.33 0.000	8.95 0.000	13.40 0.000	15.32 0.000	10.15 0.000	11.43 0.000	9.77 0.000	11.04 0.000	12.85 0.0001
MRC	–	–	–	–	–	–	–	0 1.0	67.01 0.000	69.89 0.000	62.63 0.000	67.32 0.000	63.32 0.000	64.48 0.000	68.23 0.000	72.59 0.000	83.38 0.000	82.58 0.0001
ES1	–	–	–	–	–	–	–	–	0 1.0	3.28 0.000	5.99 0.000	9.51 0.000	10.65 0.000	8.08 0.000	9.64 0.000	6.86 0.000	10.43 0.000	11.62 0.0001
ES2	–	–	–	–	–	–	–	–	–	0 1.0	6.09 0.000	10.01 0.000	13.57 0.000	9.16 0.000	9.67 0.000	5.80 0.000	8.63 0.000	8.03 0.0001
NE1	–	–	–	–	–	–	–	–	–	–	0 1.0	4.43 0.127 2	4.25 0.000	4.42 0.000	4.51 0.000	5.77 0.000	16.18 0.000	12.69 0.0001
NSP	–	–	–	–	–	–	–	–	–	–	–	0 1.0	4.75 0.052 7	4.26 0.124 0	6.47 0.018 9	6.25 0.011 0	19.54 0.000	12.39 0.0001
NE2	–	–	–	–	–	–	–	–	–	–	–	–	0 1.0	4.68 0.000	4.38 0.000	10.93 0.000	28.10 0.000	20.98 0.0001
NE3	–	–	–	–	–	–	–	–	–	–	–	–	–	0 1.0	3.24 0.002 7	7.33 0.000	19.76 0.000	16.05 0.0001
FRC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0 1.0	9.29 0.000	20.95 0.000	17.58 0.0001
CNS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0 1.0	11.32 0.000	9.23 0.0001
WSP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0 1.0	7.93 0.0001

Table 5. Frequencies of isozyme alleles and mtDNA haplotypes in the 18 sub populations studied.

Samples	*Mdh* ALLELES				*Pgm* ALLELES			MTDNA HAPLOTYPES			
	100	*87*	*80*	*65*	*114*	*100*	*90*	*77*	A	P	E
PO1	0.199	0.000	0.78	0.02	0.00	0.91	0.03	0.06	0.19	0.78	0.03
PO2	0.27	0.00	0.73	0.00	0.01	0.84	0.02	0.12	0.62	0.36	0.02
SW1	0.25	0.00	0.75	0.00	0.00	0.92	0.00	0.08	0.91	0.03	0.06
SW2	0.30	0.00	0.70	0.00	0.00	0.77	0.01	0.22	0.90	0.10	0.00
SS1	0.30	0.00	0.70	0.00	0.00	0.86	0.03	0.11	0.95	0.00	0.05
SS2	0.17	0.00	0.81	0.02	0.00	0.75	0.02	0.23	0.85	0.00	0.15
SES	0.24	0.00	0.77	0.00	0.00	0.82	0.03	0.15	0.76	0.00	0.24
MRC	0.77	0.00	0.23	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00
ES1	0.30	0.00	0.70	0.00	0.02	0.89	0.00	0.09	0.94	0.00	0.06
ES2	0.12	0.00	0.88	0.00	0.00	0.91	0.04	0.04	0.21	0.03	0.76
NE1	0.03	0.00	0.97	0.00	0.00	0.99	0.01	0.00	0.03	0.00	0.97
NE2	0.00	0.02	0.98	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00
NE3	0.04	0.00	0.96	0.00	0.00	1.00	0.00	0.00	0.11	0.00	0.89
NE4	0.02	0.00	0.98	0.00	0.00	1.00	0.00	0.00	0.03	0.00	0.97
FRC	0.02	0.00	0.96	0.02	0.00	0.98	0.00	0.02	0.00	0.00	1.00
CNS	0.11	0.00	0.89	0.00	0.00	0.96	0.00	0.04	0.00	0.00	1.00
WSP	0.28	0.00	0.72	0.00	0.00	0.81	0.00	0.19	0.70	0.25	0.05
CWS	0.28	0.00	0.72	0.00	0.02	0.76	0.04	0.19	0.77	0.10	0.13

rapid frequency decline with distance (moving eastward into Spain) is consistent with either hypothesis above, in that the introduction of this haplotype into Iberia may have taken place through introduction into Portugal or the haplotype had a more ancient origin in Portugal *de novo*.

The Iberian peninsula is an area of particular interest to study the outcome of possible historic and prehistoric interactions between subspecies and lineages. Although mtDNA haplotypes of A lineage origin were found to be quite prevalent in Iberian honey bee populations, the morphological analysis in the present study did not show a comparably strong influence from the nearby African subpopulation (Morocco). This finding is consistent with nuclear marker (microsatellite) evidence presented by Franck et al. (1998) and supports an interpretation that the honey bees of Iberia (especially south of a line between Barcelona and Zaragoza) are a distinct subspecies. The frequency distribution of PGM alleles is also consistent with this conclusion. The apparent disparity between the results obtained with nuclear and mtDNA approaches may best be explained through the action of positive selection on African mtDNA haplotypes (Garnery et al., 1998; Franck et al., 1998).

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