J. Zool. Syst. Evol. Research 40 (2002) 164–168
© 2002 Blackwell Verlag, Berlin
ISSN 0947–5745

Received on 7 June 2001

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Microsatellite analysis of non-migratory colonies of *Apis mellifera iberica* from south-eastern Spain

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Abstract

Forty-five unmanaged honeybee colonies from the south-east of the Iberian Peninsula (*Apis mellifera iberica*) were selected for analysing their genetic structure using eight microsatellite loci. These colonies were not subjected to management for queen replacement, rearing or migratory movements and previous studies showed that they had mitochondrial DNA (mtDNA) of African origin. Six of the microsatellite loci show intermediate levels of polymorphism with a total number of alleles detected per locus ranging from 4 to 10. Microsatellite data relate these Iberian populations to the African *A. m. intermissa*, although the presence of some alleles and the observed heterozygosity are characteristic of the European *A. m. mellifera*, thus corroborating the postulated hybrid origin of *A. m. iberica*. The results suggest that no recent introgression from Africa has happened and that the populations of *A. m. iberica* are differentiated in many demes.

Key words: *Apis mellifera iberica* – microsatellites – genetic variability – biogeography – south east Spain

Introduction

The honeybees from the Iberian Peninsula are currently classified as the subspecies *Apis mellifera iberica* Goetze 1964, on the basis of morphological, ethological and geographical characters (Ruttner 1973). Ruttner (1988) proposed that the subspecies of West Europe (*A. m. mellifera* and *A. m. iberica*) make up a distinct evolutionary branch – the M branch – all the African subspecies belong to the branch A, and the subspecies distributed in the northern and eastern Mediterranean are included in the evolutionary branch C. Biometric studies have been carried out in different honeybee populations across the Iberian Peninsula and different morphological groups were found regarding the geographical variability (Izquierdo et al. 1985; Santiago et al. 1986; Cornuet and Fresnaye 1989; Orantes-Bermejo and García-Fernández 1995).

Smith et al. (1991) underlined the hybrid status of *A. m. iberica* populations between the European *A. m. mellifera* and the African *A. m. intermissa* by analysing mitochondrial DNA (mtDNA) variability. They concluded that mtDNA from western European M and African A lineages coexisted in the Iberian Peninsula. Garnery et al. (1995) characterized the mtDNA of Iberian honeybee colonies using a test based on the analysis of the intergenic COI–COII region (Garnery et al. 1993). They found a decreasing gradient of African haplotypes from south (86.4%) to north (0%). Franck et al. (1998) corroborated these findings in samples from French and Iberian populations and also found that haplotype diversity in *A. m. iberica* is markedly higher than in geographically related subspecies. They postulated that the introduction of foreign queens from Africa and the subsequent positive selection for African haplotypes might have increased mitochondrial variability. Based on the sequence of the ND2 and the $tRNA^{ile}$ genes, Arias and Sheppard (1996) hypothesized an African origin of the southern Iberian populations and postulated that the present situation is the result of a secondary contact between *mellifera* and *intermissa*-derived populations.

Smith and Glenn (1995) analysed the allozyme polymorphism of *A. m. iberica* populations and interpreted the results as congruent with the hypothesis of a hybrid zone between honeybees from western European and African lineages. The morphometric and pheromonal analyses of Hepburn and Radloff (1996) performed on *A. mellifera* colonies along a transect from the Sahara to the Pyrenees, were also consistent with such interpretation. Recently, Franck et al. (1998) have studied the distribution of eight microsatellite loci along a transect of honeybee populations from France to Morocco. Their results showed that there is no gradual modification or frequency cline of alleles across the Iberian populations. The three studied Spanish populations (located in the Basque Country, Castilla and Andalucia) were similar to the French populations and did not show introgression of African alleles. These authors concluded that the Iberian Peninsula does not seem to be an intergradation zone between European and African subspecies.

Transhumance is a widespread practice among beekeepers in southern Spain because of the dry summer conditions; beekeepers move their colonies northward from May to September. This study is focused on non-migratory colonies in apiary sites that have been kept from introductions of foreign queens, so that it may well reflect the ancestral genetic characteristics of honeybees in south-east Iberia. These samples have been previously submitted to the *Dra*I mitochondrial test, showing a rather homogeneous high frequency (91.11%) of the haplotype A2 of African origin (De la Rúa et al. 1999) in contrast with the high level of variation described in other Iberian populations (Garnery et al. 1995; De la Rúa et al. 1998, 2001; Franck et al. 1998). In this study we analyse the microsatellite distribution in these colonies in order to characterize their genetic structure and the contribution of geographically close populations to their gene pool.

Materials and methods

Material

Forty-five colonies from eight apiaries located in south-eastern Spain were studied. These apiaries belong to beekeepers that maintain their colonies without performing queen replacement or migratory movements. The number and distribution of colonies per apiary are

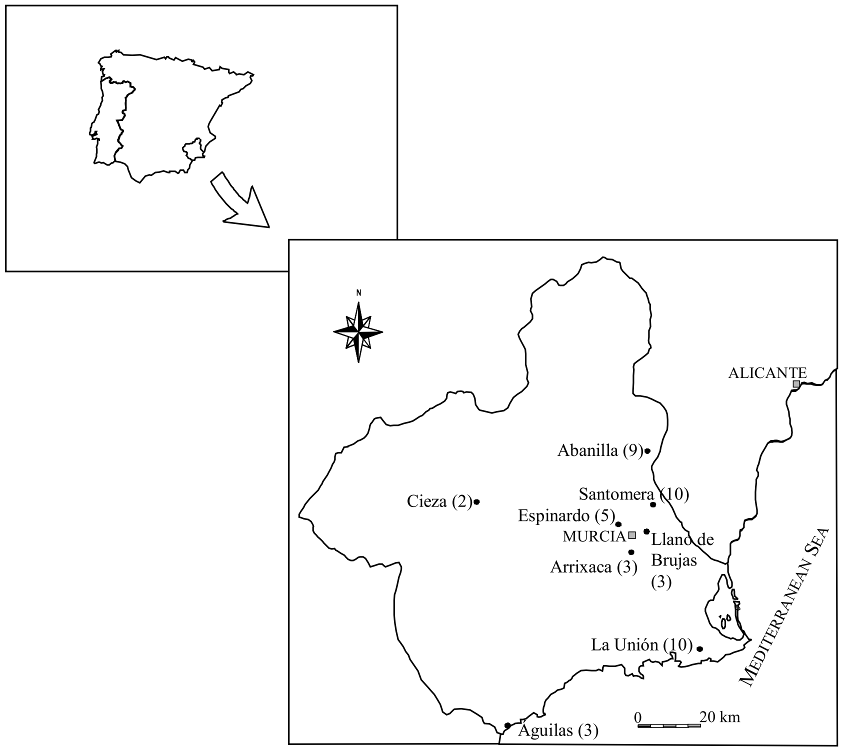

Fig. 1. Geographical location of sampled apiaries of *Apis mellifera iberica* from Murcia. The number of sampled colonies in each apiary is indicated in brackets

shown in Fig. 1. Honeybee samples were preserved at −20°C in pure ethanol.

Microsatellite amplification

One worker bee per colony ($n = 45$ colonies) was analysed. DNA isolation was performed from the thorax of each individual following standard organic methods (Sambrook et al. 1989), after 1 h in rinse buffer (Garnery et al. 1995). Polymerase chain reactions (PCR) were carried out in a Perkin-Elmer Cetus 9600 thermocycler with 0.2 μM fluorescence labelled primers (Perkin-Elmer, Foster City, CA, USA). Eight microsatellite loci were scored, B124, A113, A35, A7, A24, A28, A88 (Estoup et al. 1994, 1995), and A8 (Franck et al. 1998). Multiplex PCRs were run for combinations B124–A28, A113–A7–A35 and A24–A88–A8, following Simon (1998) at the appropriate annealing temperature for each loci combination. The reactions were run on a ABI PRISM 310 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The size of some alleles at loci A88, A28, A7 and A8 was determined by sequencing directly the PCR products using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq DNA Polymerase, in an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Statistical analyses

The results have been analysed using GENEPOP package (version 1.2; Raymond and Rousset 1995a). Hardy–Weinberg test was performed by the Markov chain method for every locus in each locality (Guo and Thompson 1992). The different apiaries have been tested for population differentiation with the Fisher exact test (Raymond and Rousset 1995b). The cyto-nuclear disequilibrium has been analysed with the same test (Asmussen et al. 1987). The proportion of heterozygous and the gene diversity (Nei 1973) were obtained with the program POPGEN.

Relationships among honeybee samples from Murcia and those studied by Franck et al. (1998) were analysed by the neighbour-joining method (NJ; Saitou and Nei 1987) and the chord distance of Cavalli-Sforza and Edwards (1967), using the procedures of the PHYLIP version 3.5 package (Felsenstein 1993). Bootstrap values were obtained after 2000 iterations of the data set (Hedges 1992). The loci that were studied only in Murcia (A35) or in the other populations but not in Murcia (A43) were not included in the analysis.

Results

Genetic diversity

The number of alleles per locus varied between 10 (loci A113 and A35) and 4 (loci A8 and A28) (Table 1). Mean expected and observed heterozygosity for all apiaries and loci ranged from 0.108 to 0.816 and from 0.067 to 0.867, respectively. The observed heterozygosity was lower than expected under Hardy–Weinberg equilibrium for every locus except for A35 and A28.

The Hardy–Weinberg test performed for every locus or apiary shows no significant departure from the equilibrium

Table 1. Genetic characteristics of eight microsatellite loci variation of *Apis mellifera iberica* from Murcia

Locus	Sample size	Total no. alleles	H_o	H_e
B124	41	9	0.781	0.809
A113	40	10	0.525	0.704
A7	39	7	0.333	0.437
A35	45	10	0.867	0.816
A28	45	4	0.111	0.108
A24	45	7	0.333	0.388
A88	45	5	0.067	0.129
A8	44	4	0.067	0.192
Over all loci	42.95 ± 2.5	7.0 ± 2.5	0.386 ± 0.314	0.448 ± 0.297

(0.06 < p < 0.99 and 0.07 < p < 1, respectively), giving an overall probability of 0.69 over all loci and apiaries. The unbiased estimate of the Fisher exact test for population differentiation was tested for every analysed locus considering all the apiaries as a single population. It only gave a significant result (p = 0.008) when the locus A113 was tested. Therefore we consider all the sampled apiaries as a single honeybee population.

The exact test for genotypic disequilibrium gave no significant results (p > 0.33) in any of the 28 loci combinations, when the disequilibrium was tested for the whole population.

Characteristic alleles

Some alleles of the loci A24 (Fig. 2a), A28 and A88 were previously observed only in populations belonging to the African lineage (*A. m. major, A. m. intermissa* and *A. m. sahariensis* from Morocco; Franck et al. 1998). These alleles are 100, 102 and 104 at the locus A24, 131 and 140 at the locus A28, and 144 at the locus A88. On the other hand, the alleles 232, 234 at the locus A113 (Fig. 2b) were previously detected only in west European populations (*A. m. mellifera* from France; Franck et al. 1998). Finally, some alleles are reported for the first time, particularly at the loci A88 and A8, when our data are compared with those of Franck et al. (1998) and Garnery et al. (1998). These alleles, and perhaps others at the loci A113, A28 and A7 with an unusual high frequency, may have a diagnostic value for the population of Murcia.

The results of the Fischer exact test for disequilibrium between nuclear (microsatellites) and cytoplasmic genotypes (mitochondrial haplotypes, data from De la Rúa et al. 1999) were not significant, neither for haplotypes-microsatellite alleles (p = 0.87) nor haplotypes-microsatellite genotypes (p = 0.78) associations.

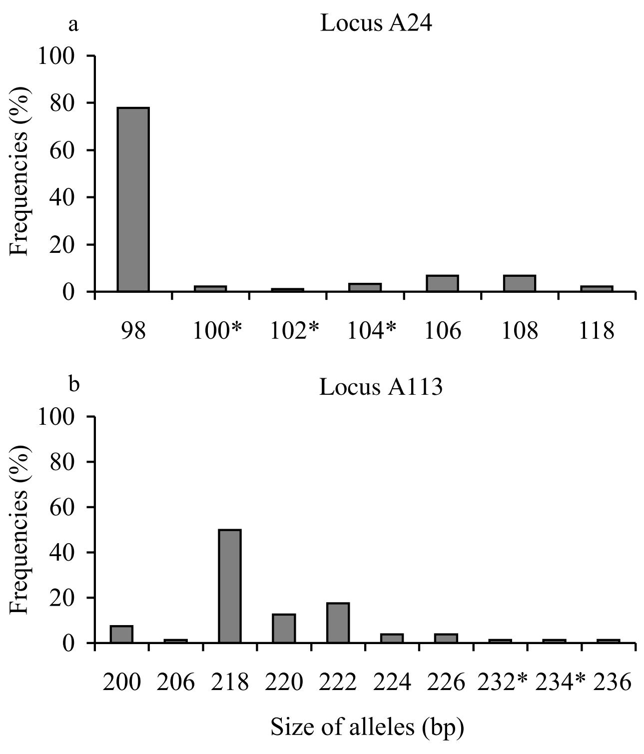

Fig. 2. Allele frequency distribution at the loci A24 (a) and A113 (b) for *Apis mellifera iberica* from Murcia. Asterisks indicate characteristic alleles of either the A (locus A24) or the M lineage (locus A113)

Relationships between populations

The results from Murcia were compared with those from other regions making up a latitudinal transect from northern France to Guinea (Franck et al. 1998) and including other Spanish populations (Basque Country, Castilla and Andalucia). The resulting NJ tree (Fig. 3) was based on the allele frequency of seven common loci for both data sets. Murcia is the Spanish population most closely related to the clade made up by the four African populations. However and since the tree is unrooted, the possibility exits that Murcia could be included within the European clade.

Discussion

Genotypic frequencies in the colonies from south-eastern Spain show no departure from the Hardy–Weinberg equilibrium, suggesting that no local differentiation is occurring among them. Exact test for population differentiation indicates that all the studied apiaries from Murcia behaves as a single population. This eases the comparison of the honeybee population of Murcia with another *iberica*, *intermissa* and *mellifera* populations based on microsatellite data.

The genetic variation in terms of heterozigosity values and number of microsatellite alleles found in the population of *A. m. iberica* from south-eastern Spain, is similar to that found in populations from western Europe. Estoup et al. (1995) and Franck et al. (1998) hypothesized a Pleistocene bottleneck for explaining the low variability of these populations in comparison with the African ones, a hypothesis that agrees with the previously reported mitochondrial and the present microsatellite analyses for the south-eastern Iberian population.

The population from Murcia shows characteristic alleles of both African *A. m. intermissa* and French *A. m. mellifera* populations, that explains its intermediate position in the cladogram of Fig. 3. These findings agree with the postulated hybrid nature of the Iberian honeybee populations (Smith et al. 1991; Smith and Glenn 1995; Arias and Sheppard 1996; Hepburn and Radloff 1996).

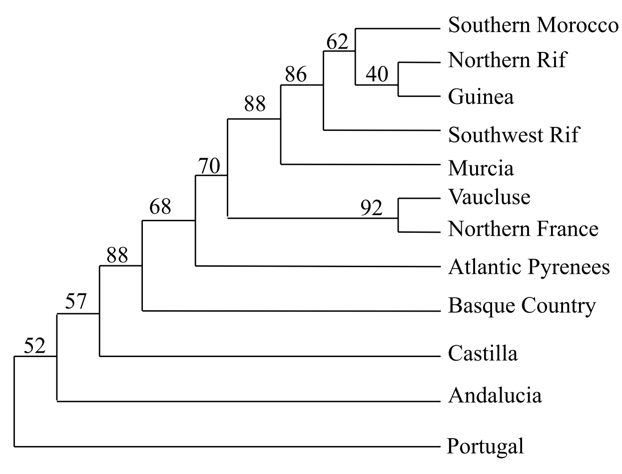

Fig. 3. Microsatellite analysis: neighbour-joining unrooted tree based on the Cavalli-Sforza and Edward's chord distance. Bootstrap values were computed from 2000 replications

The relationships of Murcia with other Iberian populations are somewhat uncertain because the relationships suggested in Fig. 3 do not show geographic congruence. Although mtDNA and allozyme data are consistent with a north–south gradient (Garnery et al. 1995; Smith and Glenn 1995), other studies based on microsatellite markers do not show a cline distribution but a reticulate and complex pattern (Franck et al. 1998). In fact, data on morphological characters (Izquierdo et al. 1985; Santiago et al. 1986; Orantes-Bermejo and García-Fernández 1995), and microsatellites (Franck et al. 1998), suggest that the populations of *A. m. iberica* are differentiated in many demes, being in any case, the southern Iberian populations more related to the African races than to the M lineage.

Franck et al. (1998) postulated that populations of *A. m. iberica* have been introgressed by African populations due to beekeeping in historical times. However, the expected cyto-nuclear disequilibrium has not been found in the population of Murcia. The same authors postulated a positive selection (direct or indirect) within Iberian populations for explaining the prevalence of African mitochondrial haplotypes and the dilution of neutral loci such as microsatellites. However, this dilution is not observed in the population from Murcia.

Therefore we postulate that (1) populations of *A. m. iberica* have a hybrid origin, and (2) these populations have undergone a fragmented evolutionary process because of selection, population bottleneck, and perhaps, further introgression events. To these natural factors it must be added the influence of beekeeping techniques, especially trashumance during the last 20 years. All these factors result in a complex scenario for the honeybee evolution in the Iberian Peninsula.

Further comparison of the data here reported from the non-migratory colonies of Murcia to those from neighbouring moving colonies, will allow to determine the extent of gene flow between them. This will provide the rationale for establishing breeding and conservation policies based on local populations.

Acknowledgements

Thanks to A. Franco and U. E. Simon for their valuable comments that clearly improved this work and to M. Meixner for her help with the graphics programs. We are grateful to the beekeepers that contributed with samples (A. Franco, R. Rosa, A. Ramirez, F. Fernández, A. Calvo, M. Morante and J. Soler). We also thank two anonymous referees for their valuable comments to the manuscript. This work was partly supported by Fundación Séneca (project 00712/CV/99) and by EVK-2000-00628 (Fifth European Framework), and the project RZ00-013 (INIA, MCYT, Spain).

Zusammenfassung

Mikrosatelliten-Analyse an standortfesten Apis mellifera iberica Kolonien aus dem Südosten Spaniens

Fünfundvierzig Bienenvölker (*Apis mellifera iberica*) auf acht extensiv bewirtschafteten Bienenständen im Südosten der iberischen Halbinsel wurden mit acht DNA- Mikrosatelliten-Loci genotypisch untersucht. Königinnenzucht, Völkerwanderungen oder andere züchterische Maßnahmen waren nicht bekannt und die Völker waren zuvor über mitochondriale DNA Marker als typisch (d.h. afrikanischen Ursprungs) klassifiziert worden. Acht der Mikrosatelliten-Loci zeigten allelische Polymorphismen zwischen vier und zehn Allelen pro Locus. Der Heterozygotiegrad und die Anzahl der beobachteten Allele war ähnlich wie in anderen europäischen Populationen der Honigbiene aber niedriger als in afrikanischen Populationen. Die gefundenden Allele zeigten sowohl Ähnlichkeit zu *Apis mellifera intermissa* aus Nordafrika sowie zu *Apis mellifera mellifera* aus Westeuropa. Dies bestätigt die Hybridstellung zwischen den beiden benachbarten Rassen. Es konnten keine Hinweise auf rezente Introgressionen aus Afrika oder anderen Bereichen Europas gefunden werden. Wir schließen hieraus, dass keine der zuvor gemachten Hypothesen zum Ursprung von *A. m. iberica* die komplexen evolutiven Prozesse der Honigbienen auf der iberischen Halbinsel hinreichend erklären.

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