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Stable genetic diversity despite parasite and pathogen spread in honey bee colonies

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Abstract In the last decades, the rapid spread of diseases, such as varroosis and nosemosis, associated with massive honey bee colonies mortality around the world has significantly decreased the number and size of honey bee populations and possibly their genetic diversity. Here, we compare the genetic diversity of Iberian honey bee colonies in two samplings performed in 2006 and 2010 in relation to the presence of the pathogenic agents *Nosema apis*, *Nosema ceranae*, and *Varroa destructor* in order to determine whether parasite and pathogen spread in honey bee colonies reflects changes in genetic diversity. We found that the genetic diversity remained similar, while the incidence of *N. ceranae* increased and the incidence of *N. apis* and *V. destructor* decreased slightly. These results indicate that the genetic diversity was not affected by the presence of these pathogenic agents in the analyzed period. However, the two groups of colonies with and without *Nosema/Varroa* detected showed significant genetic differentiation (G test). A detailed analysis of the allelic segregation of microsatellite loci in *Nosema/Varroa*-negative colonies and parasitized ones revealed two outlier loci related to genes involved in immune response.

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Introduction

The International Union for Conservation of Nature (IUCN) recognizes genetic diversity as one of the three forms of biodiversity worthy of conservation (McNeely et al. 1990). Indeed, the need to conserve genetic diversity within populations is based on two arguments: the importance of genetic variability for evolution to occur and the tight relationship between heterozygosity and population fitness (Reed and Frankham 2003). In the case of honey bees, within-colony genetic diversity has proven to reduce the negative impacts of pathogens and parasites (Crozier and Page 1985; Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001; Oldroyd and Fewell 2007). Meanwhile, the increased adaptive capacity associated with wider genetic diversity at the population level allows short-term environmental perturbations to be withstood, such as the emergence of new diseases, enabling bee populations to evolve and adapt to long-term environmental changes (Frankham et al. 2010).

Human management often brings profound changes in the genetic variation of species, and accordingly, the effect of domestication on the genetic diversity of honey bee populations has recently been addressed (Harpur et al. 2012; De la Rúa et al. 2013). In contrast to other livestock species, honey

bees are not fully domesticated (Oxley and Oldroyd 2010) given the lack of control that beekeepers have on the natural mating of honey bee queens with unselected drones during the breeding season. Nevertheless, honey bee populations have been severely affected by human activities, and in recent decades, there has been an overall reduction in the number of colonies from different regions (Biesmeijer et al. 2006; vanEngelsdorp and Meixner 2010), potentially affecting the diversity of honey bee populations. The importation of foreign queens in an attempt to replace lost colonies has become an important driver of these changes (Muñoz et al. 2013).

Different factors have been analyzed and discussed in the literature for their potential influence on the decline and eventual disappearance of honey bee colonies. The most significant of them is possibly the increased incidence of parasites and pathogens (vanEngelsdorp et al. 2009). Particularly, virulent combinations of different pathogenic agents, instead of a single pathogenic factor, are thought to be the most likely explanation for this decline (Chen and Evans 2007; Johnson et al. 2009). Pathogens like the *Varroa destructor* mite (Anderson and Trueman 2000) and its associated viruses, as well as the microsporidia of the genus *Nosema*, are currently considered to be the most harmful pathogens of honey bees (Higes et al. 2006; Cox-Foster et al. 2007; Johnson et al. 2009; Rosenkranz et al. 2010; de Miranda and Genersch 2010; de Miranda et al. 2010), while other agents that have been around since the 1950s (American foulbrood; chalkbrood) are reported much less often nowadays.

There is accumulating evidence that genetic variation can influence host susceptibility to pathogens (Reed and Frankham 2003; Spielman et al. 2004; Whitehorn et al. 2011). However, we still have a limited and insufficient understanding of the interaction of these pathogenic organisms with their host, *Apis mellifera* (Martinson et al. 2011). In a recent study (Jara et al. 2012), the distribution of the *V. destructor* mite was not significantly related to that of the two evolutionary lineages making up the Iberian honey bee population, *Apis mellifera iberiensis* (M, Western European lineage at the North-West, and A, African lineage at the South-East; Cánovas et al. 2008). This data coincided with that obtained in the original host species of this mite, *Apis cerana* (Rueppell et al. 2011). However, a significantly higher incidence of the microsporidian *Nosema apis* was detected in northern Iberian honey bee populations belonging to the M lineage, a result that changed due to the rapid expansion of *Nosema ceranae* that now affects more than 50 % of Iberian colonies (Jara et al. 2012; Martín-Hernández et al. 2012; Botías et al. 2012).

The wide dispersion of *N. ceranae*, both in Spain and worldwide (Fries 2010; Higes et al. 2010a, b; Muñoz et al. 2014), is indicative of the successful colonization of this new pathogen and the limited adaptation of its new host, *A. mellifera*. A potential effect of this rapid expansion of

N. ceranae is a reduction in genetic diversity, since invasive species may affect certain genotypes more than others, and the high risk of collapse in infected colonies (including asymptomatic colonies during the incubation period: Martín-Hernández et al. 2007; Higes et al. 2008). To assess the relationship between the spread of three major pathogens (*N. ceranae*, *N. apis*, and *V. destructor*), and the genetic diversity in Iberian honey bee colonies (*A. mellifera iberiensis*), we used microsatellite markers to analyze the genetic diversity of *A. m. iberiensis* in two different samplings obtained in 2006 and 2010. The null hypothesis tested was that changes in the frequency of pathogens and parasites are unrelated to the changes in genetic diversity of honey bee colonies. In addition, because transmissible diseases or pests are an important and universal selective evolutionary force (Seal 1991), we assessed whether any of the microsatellite alleles analyzed was associated to genes possibly involved in the adaptive response of honey bees to these harmful agents.

Materials and methods

Sampling

The samplings of this study were designed to be representative of Spanish honey bees at a country scale including samples from the peninsula and from islands such as Majorca and Tenerife. We analyzed adult worker honey bees from 228 colonies sampled in 41 Spanish provinces, corresponding to two surveys carried out in spring 2006 (113 colonies) and 2010 (115 colonies: Fig. 1). Each sample contained about 150 adult inner worker honey bees from each colony. An etiological analysis was performed for some of the major pathogenic agents affecting honey bees, and subsequently, one adult worker honey bee from each colony was placed directly into 100 % ethanol and stored at −20 °C for later DNA analysis (Department of Zoology and Anthropology, Faculty of Veterinary Medicine, University of Murcia). The evolutionary lineage of each colony was determined previously by analyzing the mitochondrial DNA variation (Jara et al. 2012).

DNA extraction and microsatellite analysis

The two left legs were removed from one worker honey bee per colony, and the total DNA was extracted in a 5 % Chelex solution according to a modified version of the protocol published by Walsh et al. (1991). The final solution of DNA (2 μl) was then used for PCR amplification, performing two multiplex PCR reactions in order to study a total of 12 microsatellite loci in the genotype of the worker honey bees (Chahbar et al. 2013; Evans et al. 2013). PCR products were visualized by capillary electrophoresis (ABI-3730, Applied Biosystems) and sized with an internal standard (Servei Central de Suport

Fig. 1 Map showing the location of the *Apis mellifera iberiensis* colonies sampled in 2006 and 2010 (from Jara et al. 2012)
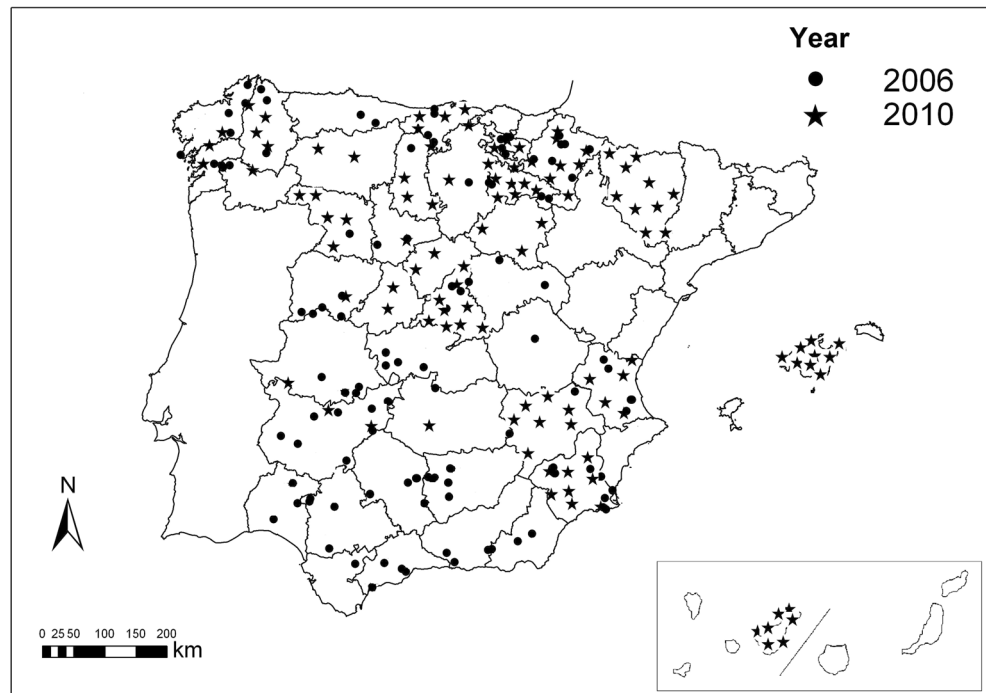
a la Investigació Experimental, University of Valencia, Spain). The individual alleles were subsequently scored using GeneMapper v3.7 software (Applied Biosystems).

Detection of pathogenic agents

All the samples came from colonies treated against *Varroa* (mandatory in the Spanish beekeeping regulation); therefore, the finding of *Varroa*-positive samples indicates that the treatment was not successful or incorrectly applied (>50 % of the treatments are applied improperly in Spain: see Garrido Bailón 2012). Conversely, *Varroa*-negative colonies indicate either the result of a recent treatment (reducing infestation to levels below the threshold of detection) or the development of natural tolerance to *Varroa* as that reported by other authors (Rinderer et al. 2001; Büchler et al. 2002, 2008; Le Conte et al. 2007; Seeley 2007; Rosenkranz et al. 2010; Locke et al. 2012).

Nosema spp. and *V. destructor* presence had already been analyzed in these colonies (Jara et al. 2012). Briefly, about 150 inner worker honey bees from each colony were pooled and analyzed to detect the presence of *Nosema* spp. and *V. destructor* according to OIE recommendations (2008). To assay *Nosema* species, DNA extraction and PCR reactions were performed as described previously, using an internal PCR control to determine the reliability of the analysis (Martín-Hernández et al. 2007; Botías et al. 2011). Therefore, *Nosema* spp.- and *V. destructor*-negative colonies

are defined here as colonies in which none of these parasites were detected in a sample of 150 bees.

Genetic and statistical analysis

Population genetic parameters were calculated for the samples in the two samplings (2006 and 2010) using GenAlex (Peakall and Smouse 2006). Given the notable genetic homogeneity found in the microsatellite analyses of Iberian honey bees across the Peninsula (Cánovas et al. 2011), we considered that all samples collected in the same year make up a single population. Genetic diversity was evaluated by calculating allele frequencies, comparing the observed (H_o) and expected (H_e) heterozygosity values. Population genetic differentiation was tested using Genepop software (Raymond and Rousset 1995; Rousset 2008), and the relationship between genetic diversity in the population and the prevalence of the pathogenic elements (*V. destructor* and *Nosema* spp.) was analyzed with the Pearson's chi-squared and Fisher exact tests, as implemented in SPSS 19 for Windows.

Detecting loci under selection

To investigate whether selection affected any of the loci analyzed, we used the Lositan software (Antao et al. 2008). Adaptive differentiation has traditionally been identified from the differences in allele frequencies among different populations, summarized by an estimate of the F_{ST} (Beaumont 2005). Low-frequency outliers relative to an appropriate

neutral population genetic model indicate loci subject to balancing selection, whereas high-frequency outliers suggest adaptive (directional) selection. Lositan is based on a method that describes the expected distribution of allele frequencies (F_{ST}) vs H_e under an island model of migration with neutral markers (Wright 1931). This distribution is used to identify outlier loci that have excessively high or low F_{ST} scores compared to neutral expectations. Such outlier loci are therefore candidates to be subject to selection.

To minimize the bias on the estimation of the mean neutral F_{ST}, Lositan was run using all loci through 50,000 simulations. After the first run, those loci that appeared outside the desired confidence intervals (95 % CIs) were removed and the mean neutral F_{ST} was computed again. In this way, a more accurate estimate of the neutral F_{ST} value was obtained using only neutral loci. This refined estimate was used for a final set of 50,000 simulations over all loci, and the estimated selection status of each of them was reported.

The outlier loci were submitted to a sequence similarity search using Blast (www.ncbi.nlm.nih.gov) and BeeBase (http://hymenopteragenome.org/beebase/), and likewise, they were screened for annotation of genes using the Map Viewer tool (NCBI; Amel 4.5). As the functional annotation of the honey bee genome is incomplete, putative Gene Ontology classifications were ascribed to genes based on homology to *Drosophila melanogaster* FlyBase (www.flybase.org), complemented by NCBI annotation.

Results

Of the 12 microsatellite loci analyzed, one (AC011) was removed from the analysis due to the poor efficiency of amplification and posterior fragment detection. For the 11 remaining microsatellite loci, we found a total of 122 different alleles within the 228 samples studied (Table 1). All the loci were polymorphic in both the 2006 and 2010 samplings, and the allele frequencies for all of them are listed in Supplementary material, including the observed (H_o) and expected (H_e) heterozygosity values.

There was allelic loss at the Ap43 and Ap55 loci in 2010 compared to 2006, and whereas five loci exhibited an increase (A8, Ap274, A79, A88, and Ap249), the remaining four maintained the same number of alleles in both samplings (A113, A7, B124, and Ap224). Of the 87 alleles identified in 2006, only 63 were found in samples collected in 2010, a loss of 24 alleles all of which had a low frequency (below 0.015). Moreover, of the 98 alleles recorded in 2010, 35 corresponded to alleles not detected in 2006, and again, these were alleles with a low frequency (between 0.005 and 0.018).

Highly significant genetic differentiation was found between 2006 and 2010, as evident with the G test implemented

Table 1 Microsatellite descriptive statistics for 2006 and 2010 samples of *Apis mellifera iberiensis*

Locus	Na		H_o		H_e	
	2006	2010	2006	2010	2006	2010
A113	13	13	0.604	0.588	0.592	0.619
A7	7	7	0.459	0.330	0.478	0.416
Ap43	16	14	0.735	0.489	0.719	0.669
Ap55	12	11	0.673	0.689	0.744	0.755
B124	11	11	0.815	0.806	0.834	0.821
A79	9	11	0.146	0.194	0.294	0.301
A8	3	7	0.062	0.172	0.145	0.278
A88	3	7	0.081	0.400	0.132	0.363
Ap224	6	6	0.545	0.578	0.614	0.631
Ap249	2	5	0.033	0.058	0.464	0.122
Ap274	5	6	0.042	0.051	0.081	0.108
All[a]	7.9	8.9	0.381	0.396	0.463	0.462

Columns indicate the total number of alleles observed (Na), observed heterozygosity (H_o), and expected heterozygosity (H_e) estimates per locus

[a] Average values across loci

in the Genepop software ($p < 0.001$). However, genetic diversity levels (H_e) were similar in both surveys (H_e in 2006= 0.463 ± 0.080 and 0.462 ± 0.075 in 2010). These values are within the expected range of genetic diversity for *A. m. iberiensis* (Cánovas et al. 2011).

Of the 228 colonies randomly sampled in 2006 and 2010, at least one of the pathogenic agents analyzed was detected in almost 80 % of them (*V. destructor*, *N. apis*, and *N. ceranae*), although they were less prevalent in 2006 (74 %) than in 2010 (83 %). About 31 % of the colonies analyzed in 2006 were parasitized by *V. destructor*, which decreased to 19 % in 2010 (Table 2), while *Nosema* microsporidia were more prevalent than the *V. destructor* mite in 2006 (64 %) and 2010 (72 %). In 2006, *N. ceranae* was detected in 50 % of the colonies while *N. apis* was less frequent (13 %). However, by 2010, the presence of *N. ceranae* reached to 59 %, whereas in only three of the colonies sampled in 2010, *N. apis* was detected alone (3 %). Indeed, while *N. apis* and *N. ceranae* were detected together in only 1 % of the colonies in 2006, this had risen to 10 % in 2010 (Table 2).

Since the genetic diversity of the *A. m. iberiensis* population was similar in 2006 and 2010, no temporal correlation could be established between this parameter and the prevalence of pathogens and parasites in the Iberian honey bee. Nevertheless, we analyzed the allelic patterns in the colonies parasitized by *Varroa* or *Nosema* separately vs *Nosema/Varroa*-negative colonies (i.e., without any of these pathogenic agents). Greater genetic diversity was found in these *Nosema/Varroa*-negative colonies ($H_e = 0.480 \pm 0.075$) than

Table 2 Presence of *Nosema* spp. and *Varroa destructor* by sampling year in *Apis mellifera iberiensis* colonies

	% Positive colonies (number of positive colonies)	
	2006 (*N*=113)	2010 (*N*=115)
N. ceranae	50 % (56)	59 % (68)
N. apis	13 % (15)	3 % (3)
Co-infection by *N. apis*+*N. ceranae*	1 % (1)	10 % (12)
Total prevalence of *Nosema* spp.	64 % (72)	72 % (83)
Prevalence of *Varroa* mite	31 % (35)	19 %[a] (21)
Presence of at least one of the analyzed agents	74 % (84)	83 % (95)

[a] *Varroa* presence was not analyzed in six of the 115 colonies from the sampling in 2010. Furthermore, the percentage is calculated in this case based on 109 colonies analyzed

in the parasitized colonies (H_e=0.464±0.075), and although these differences did not appear to be statistically significant (Student t=−0.149, p=0.441), the G test revealed highly significant genetic differentiation between these two groups of colonies (p<0.001).

To further analyze these genetic differences, we studied the allele frequency patterns of each locus in colonies sampled in 2006 vs colonies sampled in 2010. Coalescent simulations performed with Lositan software detected an outlier locus (Ap249) as a candidate of being subjected to positive selection (Table 3). The comparison between *Nosema*/ *Varroa*-negative colonies vs colonies parasitized by *Varroa* revealed another outlier locus, B124, as a candidate for positive directional selection (Table 4). No outliers were detected when comparing *Nosema*/*Varroa*-negative colonies vs positive colonies to *Nosema* spp., irrespective of the sampling year. Screening for annotated genes (Map Viewer tool NCBI; Amel 4.5) showed that the Ap249 locus is located on chromosome LG2 of *A. mellifera* and that it is linked to the protein-coding gene *Aos1* related to protein degradation,

whereas B124 locus is located on chromosome LG13 and it is associated with the *dpr7* gene that encodes immunoglobulin proteins.

Discussion

This study indicates that the genetic diversity level among honey bee colonies in Spain has remained stable over the last decade, despite the colony losses recorded in this and other European countries (Higes et al. 2010a, b; vanEngelsdorp and Meixner 2010). Therefore, the initial hypothesis that the increased incidence of parasites (*V. destructor*) and pathogens (*N. apis* and *N. ceranae*) in recent years was associated with a decrease in genetic diversity of the Iberian honey bee population was not corroborated here. This result does not rule out the possibility of such effects at a local or intra-colony scale, an issue that deserves more specific research. However, the genetic diversity levels at the peninsular scale (H_e) remained similar in both years surveyed, 2006 (H_e=0.463±0.080) and

Table 3 Results of the outlier analysis of *Apis mellifera iberiensis* populations when comparing 2006 and 2010 samples

Locus	H_e	F_{ST}	P (SimulF_{ST}<sample F_{ST})
A113	0.606836	−0.003254	0.33287
A7	0.450309	0.002717	0.475918
Ap43	0.703883	0.008342	0.464157
Ap55	0.789031	0.045271	0.824397
B124	0.829635	−0.003618	0.142272
A79	0.29875	−0.002601	0.450995
A8	0.216294	0.017493	0.593753
A88	0.267323	0.069342	0.869986
Ap224	0.623494	−0.00542	0.30051
Ap249	0.39438	0.250268	0.999406
Ap274	0.094999	−0.001697	0.386087

Locus Ap249 shows a high probability of being subjected to selective pressures

Table 4 Results of the outlier analysis when comparing *Apis mellifera iberiensis* populations (2006 plus 2010 samples) negative for *Varroa*/ *Nosema* infestation vs colonies parasitized by *V. destructor*

Locus	H_e	F_{ST}	P (SimulF_{ST}<sample F_{ST})
A113	0.62406	−0.000357	0.613779
A7	0.478716	2.8e-05	0.69447
Ap43	0.681419	−0.007087	0.436818
Ap55	0.791319	0.001775	0.562544
B124	0.866319	0.047409	0.985782
A79	0.401316	0.021712	0.798066
A8	0.336097	0.029368	0.85626
A88	0.201974	−0.008837	0.470685
Ap224	0.648352	−0.00876	0.437697
Ap249	0.357919	0.025789	0.829389
Ap274	0.077703	−0.000715	0.595662

Locus B124 shows a high probability of being subjected to selective pressures

2010 (H_e=0.462±0.075). In fact, the genetic diversity recorded in this study is concordant with that obtained previously in the Iberian Peninsula studying microsatellite loci (Cánovas et al. 2011: H_e 0.401 to 0.518; Miguel et al. 2007: H_e 0.442 to 0.516). As discussed by these authors, honey bee populations in Spain might be considered to make up a single large population for certain purposes, given the large population interchange and gene flow derived from the extensive beekeeping mobility around the Peninsula (Cánovas et al. 2011; 2014). In fact, around 80 % of the estimated number of Spanish colonies move in a yearly cycle, which leads to a potential bi-directional genetic flow between migratory and resident colonies (Perrier et al. 2003; Hernández-García 2010), an effect that is enhanced by the lack of control that beekeepers have on natural honey bee mating (Baudry et al. 1998; Koeniger and Koeniger 2000; Hernández-García et al. 2009; Jaffé et al. 2009). Additional factors promoting the genetic homogeneity in the Iberian Peninsula are the capture of swarms and the purchase of colonies or mated queens, often originating from distant locations (Cánovas et al. 2008; Serrano et al. 2011). All these factors would account for the maintenance of genetic diversity in Iberian honey bee populations reported in this study, despite of the colony losses occurred in recent decades. However, there is an evident risk of disease spreading as a result of the intense colony movement between most regions in the Iberian Peninsula. In this sense, the increased prevalence of pathogens in Spain during the study period is not surprising, consistent with data found in other studies (Higes et al. 2010a, b; Botías et al. 2012; Martín-Hernández et al. 2012; Muñoz et al. 2014) and in other regions (Fries 2010; Traver and Fell 2011; Martin et al. 2013; Bekele et al. 2015). It is noteworthy that around 74 % (2006) or 86 % (2010) of the colonies presented at least one of the searched pathogenic agents (*V. destructor*, *N. apis*, and *N. ceranae*) showing *N. apis* a lower prevalence even though the samples were obtained in Spring, the peak season for *N. apis* infestation (Fries 2010). By contrast, *N. ceranae* was the pathogen that showed the greatest expansion with an increase in prevalence of 10 % in the 4 years of our study. This high prevalence of parasites and pathogens, and the fast expansion of *N. ceranae*, are of particular concern given the potential of *V. destructor* (Rosenkranz et al. 2010) and *N. ceranae* (Martín-Hernández et al. 2007; Higes et al. 2008) to produce colony collapse.

A relationship between honey bee genetic diversity and the prevalence of parasites and pathogens could not be established in this study due to the genetic stability observed. However, we did detect significant genetic differences in the surveys performed in 2006 and 2010 (G test, $p < 0.001$), as well as *Varroa*/*Nosema*-negative colonies and parasitized colonies (G test, $p < 0.001$), with tentatively higher H_e values in the *Varroa*/*Nosema*-negative colonies

compared to parasitized ones. This is consistent with studies showing that colonies of genetically diverse social insects have a selective advantage because of increased resistance to pests (Sherman et al. 1988; Liersch and Schmid-Hempel 1998; Tarpy 2003; vanBaalen and Beekman 2006; Mattila and Seeley 2007; Oldroyd and Fewell 2007). It is thought that different host genotypes have distinct susceptibilities to diverse parasitic strains; thus, parasitic infections are not likely to spread in genetically heterogeneous colonies as rapidly as in more homogeneous ones (Sherman et al. 1988; Schmid-Hempel 1998). Studies on different Hymenoptera species also support this hypothesis, including those on bumblebees like *Bombus terrestris* (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 2001) and *Bombus pascuorum* (Whitehorn et al. 2011) and on ants (Hughes and Boomsma 2004).

To further investigate the genetic differences between the groups of colonies, we studied whether any of the loci analyzed was subject to selective pressure. A comparison of allele frequencies between the samples collected in 2006 and 2010 showed that the Ap249 locus was subjected to positive selection during that period. Searches of the complete *A. mellifera* genome (Consortium HGS 2006) showed that this *locus* is located on the chromosome LG2 and that it is linked to the protein-coding gene *Aos1*. Experimental data in *D. melanogaster* indicate that *Aos1* (FlyBase: http://flybase. org/reports/FBgn0029512.html, 16/01/2015) is involved in the biological processes of protein sumoylation, a protein modification implicated in various cellular processes that include the stress response (Hay 2005; Paddibhatla et al. 2010), and the positive regulation of the NF-kappaB transcription factor, a modulator of the immune response to infection and of nervous system plasticity (Ghosh et al. 1998; Albensi and Mattson 2000; Li and Verma 2002; Meffert et al. 2003).

When comparing *Varroa*/*Nosema*-negative colonies vs colonies parasitized by *V. destructor*, we found a second outlier locus, B124. As noted before, *Varroa*-negative colonies might be false negatives because of a recent treatment, but also the result of the development of natural tolerance to the mite. It is interesting that the screening of B124 in the annotated genome of *A. mellifera* (Map Viewer tool, NCBI; Amel 4.5) suggested the linkage of this locus with the *dpr7* gene that encodes immunoglobulin proteins (FlyBase: http://flybase.org/reports/FBgn0053481.html, 16/01/2015).

In conclusion, in this study, we show that the genetic diversity in Spanish honey bee populations remained stable between 2006 and 2010, despite the increasing levels of pathogens and parasites. This result possibly reflects the multi-drone mating system of the queens and the high gene flow between colonies over most regions of the country. We identify two outlier loci related to genes that respond to stress and that could potentially reflect selective processes a hypothesis that should be assessed in more specific analyses.

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