ORIGINAL RESEARCH ARTICLE

Effects of queen importation on the genetic diversity of Macaronesian island honey bee populations (*Apis mellifera* Linneaus 1758)

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Summary

Beekeeping practices such as the importation of non-native honey bee queens may interact with the conservation of honey bee biodiversity. Island honey bee populations are particularly appropriate to test the impact of the introduction of foreign subspecies into their genetic diversity and structure. Here we have used microsatellite markers to evaluate the temporal genetic variation over the last decade in Macaronesian honey bee populations, which have been exposed to different beekeeping strategies regarding queen importation as previously revealed by mitochondrial determination of the evolutionary lineage: a high level on Tenerife and São Miguel and absent or low on La Palma and Madeira. While genetic diversity remained low (from 0.402 to 0.483 in the previous survey and from 0.390 to 0.513 in the current survey), genetic introgression from foreign honey bees was detected on Tenerife and São Miguel based on Bayesian structure analysis. Nevertheless, the existence of endemic honey bee populations deserving conservation on the Macaronesian Islands can still be inferred from the aforementioned analyses.

Efecto de la importación de reinas en la diversidad genética de poblaciones de abejas insulares de la Macaronesia (*Apis mellifera* Linneaus 1758)

Resumen

Prácticas apícolas como la importación de abejas reinas no-nativas pueden interactuar con la conservación de la biodiversidad de abejas. Las poblaciones de abejas insulares son especialmente adecuadas para analizar el impacto de la introducción de subespecies foráneas en su diversidad y estructura genéticas. En este trabajo hemos utilizado como marcadores los microsatélites para evaluar la variación genética temporal en los últimos diez años en las poblaciones de abejas de la Macaronesia que implementan diferentes estrategias de gestión de la apicultura en relación con la importación de reinas: a un mayor nivel en el caso de Tenerife y San Miguel y ausente o reducida en La Palma y Madeira. Si bien la diversidad genética se ha mantenido baja, (de 0,402 a 0,483 en el muestreo antiguo y de 0,390 a 0,513 en el muestreo actual) se han detectado señales de introgresión genética de abejas foráneas en las poblaciones de Tenerife y San Miguel a partir de los resultados del análisis bayesiano de la estructura poblacional. No obstante, todavía se puede inferir de los análisis mencionados la existencia de poblaciones de abejas endémicas en las islas macaronésicas adecuadas para ser conservadas.

Introduction

The Atlantic archipelagos of volcanic origin located next to Europe and North Africa (Azores, Madeira and Savage Islands (Portugal), Canary Islands (Spain) and Cape Verde) are collectively known as the Macaronesia. The biodiversity of this region consists of a blend of taxa usually found in the North Atlantic region, the Mediterranean area and Africa. Despite representing only 0.2% of the territory of the EU, the Macaronesian region harbours no less than 19% of habitat types and 28% of plant species (Annex II of the Habitats Directive, European Union, 2010). As a result of isolation and adaptation to particular environmental island conditions, many organisms have given rise to locally adapted populations, such is the case of the honey bee, *Apis mellifera* Linneaus 1758. Indeed, honey bee populations locally adapted to endemic Macaronesian flora and particular weather and orography conditions are characterized by particular mitochondrial haplotypes (A11, A14, A15, and A16) belonging to the African evolutionary sub-lineage with Atlantic distribution (De la Rúa *et al.*, 1998, 2001, 2006).

The biodiversity of the Macaronesian honey bee populations has been threatened by beekeeping practices such as the introduction of commercial honey bee subspecies that may lead to introgressive hybridization and also to the spread of parasites and pathogens (De la Rúa *et al.*, 2009; Muñoz *et al.*, 2014). Such introduction events have been inferred through concurrent analyses of mitochondrial DNA (De la Rúa *et al.*, 1998, 2002) and microsatellite markers (De la Rúa *et al.*, 2001, 2006). Furthermore, recent temporal analyses revealed differential patterns of change in mitochondrial diversity: whereas an increase of the introduction rate was observed on the Canary Islands such as El Hierro and La Gomera, diversity has remained stable in the other Macaronesian islands (Muñoz *et al.*, 2013). Detected foreign C1, C2 and M7 European haplotypes characterize Italian (*A. m. ligustica*), Balkan (like *A. m. carnica*) and western European (northern *A. m. iberiensis* and *A. m. mellifera*) honey bee populations respectively (Franck *et al.*, 2000; Muñoz *et al.*, 2009).

The subspecies *A. m. ligustica* and *A. m. carnica* are globally imported by beekeepers because of their presumed docility and productivity (Moritz *et al.*, 2005). Although it is known that introduction of foreign queens is a serious threat to the genetic integrity of local honey bee subspecies, populations and ecotypes (De la Rúa *et al.*, 2009; Mutinelli, 2011), there is only one conservation programme in Macaronesia for selecting and preserving the local black honey bee on La Palma (Canary Islands), which was initiated in 2001. Since then, the introduction of foreign subspecies has been prohibited and a natural mating area for local queens was established at the northeast section of the island. While mitochondrial diversity increased significantly in this area over an eight year period, such temporal pattern was not observed at the nuclear level, providing full support for the conservation measures adopted (Muñoz and De la Rúa, 2012).

Previous analysis of mitochondrial variation has shown that Macaronesian honey bee populations have been exposed to different strategies of beekeeping management: whereas on La Palma and Madeira queen importation has been be absent or low, on Tenerife and São Miguel, around 50% of the colonies carried foreign haplotypes (Muñoz *et al.*, 2013). Here we have quantified the level of introgression of non-native subspecies into local honey bees, and tested the impact of such introduction in the genetic diversity and population structure of those Macaronesian populations by analysing 12 microsatellite *loci*.

Material and methods

Population sampling

A subset (448 colonies) of a Macaronesian sampling (Fig. 1) performed during 1998-2010 (Muñoz *et al.*, 2013) has been included in this analysis in order to compare islands with known importation of honey bee queens (Tenerife and São Miguel) with an island with an established conservation programme (La Palma) and another where introduction of honey bee queens of Eastern European origin is virtually unknown (Madeira). Worker bees were collected from the inner frames and kept in absolute ethanol at -20ºC until laboratory processing. A reference data set with a total of 40 individuals of lineage C ancestry (N = 20 *A. m. ligustica* and N = 20 *A. m. carnica*) was also included to perform the introgression analyses.
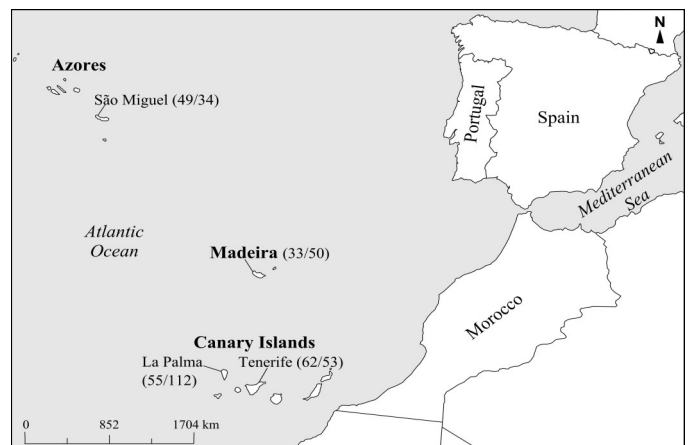

Fig. 1. Sampling locations for Macaronesian island honey bee populations. The number of analyzed colonies in each island for each survey (previous / current) is indicated in parentheses.

Microsatellite genotyping

DNA was extracted from a pair of legs of a single worker per colony following the Chelex® extraction protocol described by Walsh *et al.*, (1991). Individuals were genotyped at 12 microsatellite *loci* in two different multiplex PCRs (see Evans *et al.*, 2013 for details). Multiplex 1 included A7, A113, Ap43, Ap55 and B124 whereas multiplex 2 included A8, A79, A88, Ac11, Ap224, Ap249 and Ap274; Estoup *et al.*, 1995; Garnery *et al.*, 1998; Solignac *et al.*, 2003). PCR reactions were

performed in a thermocycler PTC 100 (MJ Research) in a total volume of 10 µl with 1X reaction buffer, 1.2 mM MgCl$_2$, 0.3 mM of each dNTP, 0.4 µM of each primer, 1.5 U Biotools® DNA polymerase (BIOTOOLS B&M Labs, S.A.; Madrid, Spain) and 2 µl of extracted DNA. Annealing temperature was set at 54°C (multiplex 1) and at 55°C (multiplex 2). Capillary electrophoresis was performed on an ABI® 3730 DNA analyser (APPLIED BIOSYSTEMS; Foster City, California, USA) at the University of Valencia (Servei Central de Suport a la Investigació Experimental; Valencia, Spain). Alleles were scored using GeneMapper® v3.7 (APPLIED BIOSYSTEMS).

Population genetic analysis

Each sampled island per survey was treated as a single population. Number of alleles (nA), number of effective alleles (eA), observed (Ho) and expected (He) heterozygosity, and inbreeding coefficient ($F$is) per population were calculated with GENALEX v 6.41 (Peakall and Smouse, 2006). Allelic richness (Ar) and private allelic richness (pAr) were estimated using rarefaction (standardized to 12 genes per population) in HP-RARE (Kalinowski, 2005). Significance of population parameters differences was tested with Student's t-test.

Deviations from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) and genic and genotypic differentiation were tested in GENEPOP v.4.0 (Rousset, 2008) using Markov chain approximation to exact tests. Population pairwise genetic differentiation (F_{ST}) and the analysis of molecular variance (AMOVA) were performed to evaluate spatial and temporal genetic differences between Macaronesian honey bee populations with ARLEQUIN v3.1 (Excoffier et $al.$, 2005) using 10,000 permutations.

The software STRUCTURE v2.3.3 (Pritchard et $al.$, 2000) was used to infer the origin of honey bee queen importations into local island honey bee populations and the admixture proportions (q). We evaluated K = 1 to 10 using default parameter settings to assign individuals to populations (correlated allele frequencies and admixture model were

assumed) with 100,000 burn-in steps and 1,000,000 MCMC (Markov Chain Monte Carlo algorithm) iterations for five simulations per K. The most probable number of clusters was estimated using the ΔK method described by Evanno et $al.$ (2005).

Results

Genetic diversity

All microsatellite $loci$ in the honey bee populations from the Macaronesian islands were found to be polymorphic with a total number of 143 alleles, ranging from 5 (Ap249 and Ap274) to 23 (Ap43) alleles per $locus$ (Table 1). After rarefaction, the mean allelic and private allelic richness showed the lowest values in the population from La Palma in both surveys and the highest in the population from São Miguel in 2010. The average genetic diversity, measured as expected heterozygosity (He), ranged from 0.390 ± 0.293 (La Palma-2006) to 0.513 ± 0.307 (São Miguel-2010). A decrease in allelic richness and genetic diversity (Table 1) was observed in the Canarian populations (La Palma and Tenerife), while an increase was detected in the populations of Madeira and São Miguel, although this variation was non-significant in any case (Student's t-test, $P > 0.05$).

All populations studied showed significant deviations from Hardy-Weinberg equilibrium ($P < 0.001$) probably due to a significant deficit of heterozygotes as inferred by the F_{IS} values ($P < 0.001$). When testing for linkage disequilibrium (LD) for all pairs of $loci$, 39 pairwise combinations were found to be significant out of 465 possible comparisons (although non-significant after Bonferroni correction). Twenty one pairs of $loci$ with LD were found on Tenerife (increasing from 8 in 1998 to 13 in 2008) and 11 on São Miguel (also increasing from 5 in 2001 to 6 in 2010) whereas La Palma and Madeira showed 3 (2 in 1998 and 1 in 2006) and 4 (3 in 2001 and 1 in 2010) pairs of loci with LD, respectively.

Table 1. Population genetic parameters for island honey bee populations from the Macaronesian region per survey. Number of sampled colonies (N) per island and year and the average over $loci$ (mean ± SD) of number of alleles (nA), number of effective alleles (eA), allelic richness (Ar), private allelic richness (pAr), observed and expected heterozygosity (Ho and He), inbreeding coefficient ($F$is), proportion of inferred admixture (q) calculated in STRUCTURE considering four clusters (K = 4) between the honey bee populations from Macaronesian islands and eastern European (A. m. ligustica and A. m. carnica) honey bee populations.

Island	Year	N	nA	eA	Ar	pAr	Ho	He	F_{IS}	q
La Palma	1998	55	3.667 ± 1.923	2.024 ± 0.843	2.563 ± 1.122	0.004 ± 0.010	0.360 ± 0.290	0.402 ± 0.281	0.199 ± 0.284	0.004
	2006	112	4.000 ± 2.216	1.999 ± 0.839	2.492 ± 1.177	0.006 ± 0.016	0.318 ± 0.248	0.390 ± 0.293	0.123 ± 0.294	0.005
Tenerife	1998	62	6.083 ± 2.314	2.328 ± 1.155	3.136 ± 1.167	0.154 ± 0.192	0.381 ± 0.274	0.471 ± 0.245	0.200 ± 0.247	0.017
	2008	53	5.833 ± 2.290	2.179 ± 1.238	2.973 ± 1.271	0.033 ± 0.064	0.369 ± 0.266	0.420 ± 0.257	0.120 ± 0.260	0.025
Madeira	2001	33	4.917 ± 2.778	2.680 ± 1.800	3.217 ± 1.667	0.047 ± 0.090	0.388 ± 0.306	0.467 ± 0.302	0.238 ± 0.308	0.005
	2010	50	6.333 ± 2.995	2.747 ± 1.865	3.360 ± 1.666	0.123 ± 0.248	0.443 ± 0.315	0.481 ± 0.305	0.102 ± 0.308	0.005
São Miguel	2001	49	6.333 ± 2.674	2.596 ± 1.354	3.301 ± 1.388	0.092 ± 0.195	0.437 ± 0.288	0.483 ± 0.285	0.089 ± 0.288	0.020
	2010	34	6.500 ± 3.177	2.908 ± 1.572	3.582 ± 1.636	0.211 ± 0.246	0.434 ± 0.321	0.513 ± 0.307	0.149 ± 0.312	0.017

Table 2. Differentiation between islands and surveys estimated by pairwise F_{ST} values based on microsatellite *loci*. All population pairwise F_{ST} values across all *loci* were highly significant ($P < 0.001$, Fisher's test), except the values marked as ns (non-significant). LP98, La Palma 1998; LP06, La Palma 2006; TF98, Tenerife 1998; TF08, Tenerife 2008; MD01, Madeira 2001; MD10, Madeira 2010; SM01, São Miguel 2001; SM10, São Miguel 2010.

	LP98	LP06	TF98	TF08	MD01	MD10	SM01
LP06	0.006^{ns}	-					
TF98	0.100	0.079	-				
TF08	0.132	0.090	0.025	-			
MD01	0.110	0.089	0.037	0.039	-		
MD10	0.081	0.076	0.044	0.078	0.010^{ns}	-	
SM01	0.167	0.155	0.090	0.080	0.030	0.075	-
SM10	0.112	0.127	0.085	0.141	0.056	0.035	0.062

Population differentiation

Temporal genetic variation analyses were performed by comparing the data between the two surveys on each island. Significant genic and genotypic differentiation were detected on every island, except on La Palma (Fisher's test, $P > 0.05$). Pairwise F_{ST} values (Table 2) showed a significant differentiation in most population comparisons, except between the surveys conducted on La Palma and Madeira. The AMOVA showed that most variation was due to individuals (90.89%). A lower fraction of the variation was attributed to islands (6.99%) and temporal differences within the Macaronesian region (2.12%).

Admixture patterns

Bayesian admixture analyses performed with STRUCTURE using the local Macaronesian and reference populations are shown in Fig. 2. The most probable number of clusters was two after Evanno *et al.* (2005) correction ($K = 2$, mean LnP(D) = −11984.98, mean Var[LnP(D)] = 216.26, Fig. 2A). Honey bees were probabilistically assigned to Macaronesian cluster (cluster-1) with an average proportion of membership of 0.990. Cluster-2, which corresponded to the reference populations of C lineage ancestry (*A. m. carnica* and *A. m. ligustica*), showed a mean proportion of membership of 0.990.

STRUCTURE analysis for $K = 4$ (Fig. 2B) showed differentiation among all Macaronesian islands, except between Madeira and São Miguel, which were grouped in one cluster. Cluster-1 grouped honey bees from La Palma (79% and 61% of individuals from 1998 and 2006, respectively). Cluster-2 included mainly honey bees from Tenerife (67% and 80% of individuals from 1998 and 2008, respectively) but also some individuals from La Palma (18% and 36% of individuals from 1998 and 2006, respectively). Honey bees from Madeira (68% and 73% of individuals from 2001 and 2010, respectively) and São Miguel (83% and 81% of individuals from 2001 and 2010, respectively) belonged to the cluster-3.

Admixture was observed in these clusters (Fig. 2B), especially in the more recently surveyed populations of Tenerife and La Palma. A residual level of nuclear introgression (q) from the C-lineage populations was detected (Table 1 and Fig. 2A), which ranged from 0.004 on La Palma in 1998 to 0.025 on Tenerife in 2008.

When comparing the degree of nuclear C-admixture (Fig. 3) calculated in this study with the frequency of Eastern European haplotypes detected by Muñoz *et al.*, (2013) for the same samples, significant differences were detected on the islands with known introduction of foreign honey bee queens (t-test, $t = 17.7611$, $P = 0.0004$) while islands with no signs of foreign honey bee queens showed no significant differences (t-test, $t = 1.0835$, $P = 0.3579$).
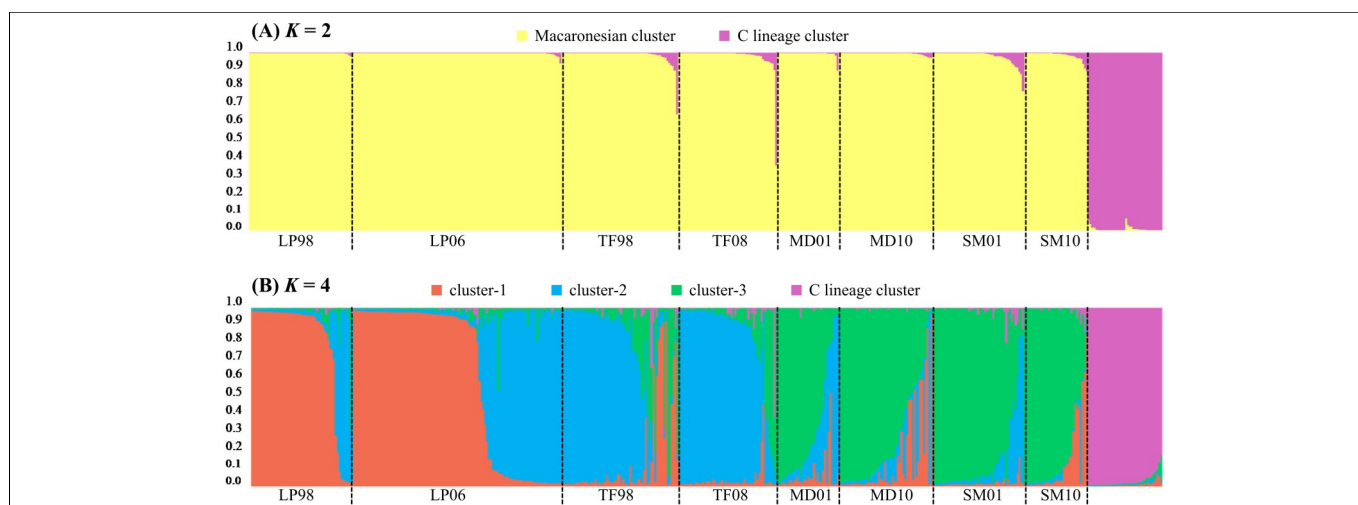

Fig. 2. Results of probabilistic assignment to the genetic clusters inferred by the Bayesian analysis implemented in STRUCTURE with $K = 2$ (A) using Evanno *et al.,* correction (2005) and $K = 4$ (B). Each individual is represented by a vertical line fragmented into two (A) or four (B) sections that are relative to their membership proportion in the genetic clusters. LP98, La Palma 1998; LP06, La Palma 2006; TF98, Tenerife 1998; TF08, Tenerife 2008; MD01, Madeira 2001; MD10, Madeira 2010; SM01, São Miguel 2001; SM10, São Miguel 2010.
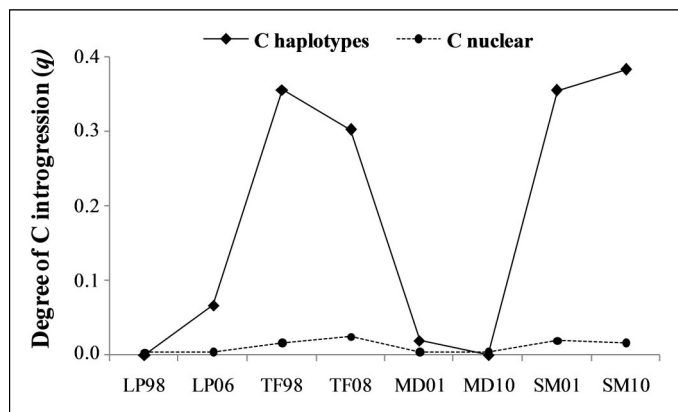
Fig. 3. Temporal pattern of introgression of Eastern European genes into the Macaronesian populations as given by the frequency of C haplotypes (Muñoz *et al.,* 2013) and nuclear admixture (12 microsatellite *loci*) estimated with STRUCTURE. LP98, La Palma 1998; LP06, La Palma 2006; TF98, Tenerife 1998; TF08, Tenerife 2008; MD01, Madeira 2001; MD10, Madeira 2010; SM01, São Miguel 2001; SM10, São Miguel 2010.

Discussion

Monitoring genetic variation of populations is an important step towards conservation of their diversity, and this is especially important in honey bees in the light of recent declines (Potts *et al.*, 2010). Earlier microsatellite studies found that the genetic diversity of Macaronesian honey bees is characterized by low values, as reflected by the number of alleles (nA) and heterozigosity (He) values (De la Rúa *et al.*, 2001, 2006). Despite significant temporal mitochondrial changes detected in the Macaronesian honey bee populations (Muñoz *et al.*, 2013), our present study suggests that nuclear genetic diversity has not varied significantly and has remained relatively low. The low level of genetic diversity observed in this study is consistent with the results reported for other honey bee island populations such as Crete, Sicily and the Balearic Islands (Garnery *et al.*, 1993; Franck *et al.*, 2000; De la Rúa *et al.*, 2003, respectively). It is known, not only for insects but also for mammals, that island populations have lower level of genetic diversity than their continental counterparts (Frankham, 2008) mainly due to genetic drift and differential selection inherent to the colonization process.

Analysis of temporal variation of genetic diversity may be a first step in detecting honey bee populations at risk, since a reduction on its levels is of concern, as it may act as a driver of colony decline (vanEngelsdorp and Meixner, 2010). This temporal survey suggests that the genetic diversity has not significantly changed on the Macaronesian populations during the last decade. We previously reported that genetic diversity (He) has not changed significantly over the last decade on Tenerife (Muñoz *et al.*, 2012) and on a mating area on La Palma (Muñoz and De la Rúa, 2012), but this more in-depth analysis (with additional microsatellite *loci*, colonies and populations) largely confirms those observations supporting the conclusions of De la Rúa *et al.* (2013) that admixture in managed populations does not necessarily lead to an increase in genetic diversity.

Genic and genotypic differentiation and *F*-statistics indicated the existence of spatial structure in the Macaronesian region. Therefore, colonies of each island may be considered as a different population. Temporal analysis of the genetic structure has revealed a small but significant variation in populations from islands with known introduction of foreign honey bee queens (Tenerife and São Miguel) while no temporal change was detected in populations from islands where beekeeping is practiced mainly with local ecotypes (Madeira) or implementing a conservation programme (La Palma). These patterns may be a consequence of different management practices performed by the beekeepers from each island, including the different origin and amount of imported honey bee queens (Muñoz *et al.*, 2013).

Despite the population differentiation, the existence of gene flow within honey bee populations from Macaronesia has been shown, especially between Tenerife and La Palma populations, probably due to the geographic proximity and also to the movement of colonies between the islands by beekeepers managing apiaries in both islands. Another source of admixture is the introduction of honey bees usually of eastern European ancestry, including *A. m. carnica* and *A. m. ligustica*. Native Macaronesian honey bees belong to the African mitochondrial evolutionary lineage A, more specifically to the Atlantic sub-lineage A_{III} (De la Rúa *et al.*, 1998, 2001, 2006). Detection of foreign haplotypes (C1, C2 and M7; Muñoz *et al.*, 2013) can provide an estimation of the level of mitochondrial introgression. Such introductions also have an impact on population structure at the nuclear level, as revealed by Hardy-Weinberg and linkage disequilibria and deficit of heterozygotes observed on Macaronesian populations.

A significant difference in the degree of C introgression was detected on Tenerife and São Miguel. These honey bee populations displayed a strong asymmetrical introgression of C-derived genes, which could be explained by introduction of eastern European queens. Asymmetrical introgression was also observed in Africanized honey bee populations (Kraus *et al.*, 2007). A possible explanation for these differences might be a partial reproductive isolation between native honey bees and introduced C subspecies, since it appears that reproduction occurs preferably with native drones better adapted to local environmental conditions. Partial and asymmetric reproductive isolation has also been observed between *A. m. carnica* and *A. m. mellifera* subspecies (Oleksa *et al.*, 2013).

Although there is a large human influence in the Macaronesian region, as suggested by the results of the introgression of European subspecies, there is still a continuing predominance of native haplotypes belonging to the sub-lineage A_{III} and a low level of nuclear admixture with introduced subspecies. Such introgressive hybridization may be further reduced through conservation policies aimed at protecting local ecotypes. The control of introgression by removing foreign queens and monitoring genetic variation by molecular analysis may be suitable measures to reduce and prevent the effects of introgression in the Macaronesian honey bee populations in order to conserve native ecotypes. These are essential reservoirs of local adaptations, and their

extinction thus implies the loss of a valuable combination of traits shaped by natural selection over extended periods of time (De la Rúa *et al.*, 2009). While this strategy requires effort from beekeepers it will generate a reliable situation between economic development of bee-keeping and conservation of honey bee biodiversity. Local honey bee genetic patrimony needs to be protected against the introduction of foreign subspecies, since its loss reduces the capacity to maintain and to improve farming productivity, diminishing the aptitude to face novel environmental or epidemiological conditions in the future.

Acknowledgments

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