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Note: Cytonuclear patterns of a honey bee population from the Azores show a stable population at the nuclear but not at the mitochondrial DNA level

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ABSTRACT

The Azores archipelago has been the stage for multiple introductions of *Apis mellifera* from varying origins, which have led to widespread admixture and the existence of phenotypically and genotypically heterogeneous populations. This is evident on the São Miguel Island, where the historically introduced black phenotype of *A. m. iberiensis* (lineage M) co-exists with the contemporaneously introduced yellow phenotype of C-lineage ancestry. Interestingly, the cytonuclear markers used herein revealed that C-lineage ancestry is residual at the nuclear level for both the black ($5.82 \pm 1.66\%$) and yellow ($5.91 \pm 1.85\%$) phenotypes, although this is more pronounced at the mitochondrial level (27.27% for black and 14.74% for yellow). While the C-lineage contribution has remained stable at the nuclear level for over 20 years, there has been a recent decrease in the proportion of C-derived mitotypes.

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Humans have long moved the honey bee (*Apis mellifera*) across large geographical scales, expanding its range, which was originally confined to Europe (home to the M-western European, C-eastern European, and A-African lineages, *sensu* Ruttner, 1988), Africa, and the Middle East, to a global distribution. At the same time, the multiple migration events have led to widespread admixture and introgressive hybridisation between previously allopatric subspecies and lineages, with possible consequences for local adaptation due to the breakdown of gene complexes fine-tuned by natural selection (De la Rúa et al., 2009, 2013). While several subspecies have been moved around over the past 150 years, the Italian yellow honey bee *A. m. ligustica* and the Carniolan honey bee *A. m. carnica* (both belonging to the C-lineage, *sensu* Ruttner, 1988) have been preferentially introduced by beekeepers worldwide (De la Rúa et al., 2009; Jensen et al., 2005; Pinto et al., 2014). As a result of this process, the genetic integrity of many subspecies and historical populations has been threatened primarily by C-derived introgression at both mitochondrial and nuclear levels due to gene flow mediated by both queens and drones (De la Rúa et al., 2009; Jensen et al., 2005; Pinto et al., 2014).

In the sixteenth century, Spanish and Portuguese settlers extended the range of *A. m. iberiensis* (M-lineage, *sensu* Ruttner, 1988) from Iberia to the Canary Islands, Madeira, and the Azores. While the signature of this historical human-mediated migration is still evident in the extant populations (Ferreira et al., 2020; Henriques et al., 2024; Miguel et al., 2015; Muñoz et al., 2013), several islands of these archipelagos harbour populations with varying levels of C-lineage introgression (De La Rúa et al., 1998, 2001, 2006; Ferreira et al., 2020; Miguel et al., 2015). This is the case in the Azores, where distinct patterns of introgression are observed across different islands. Santa Maria, São Miguel, and São Jorge are home to populations exhibiting high levels of *A. m. iberiensis* genetic integrity with a residual background of nuclear C-lineage ancestry (Ferreira et al., 2020; Henriques et al., 2024). In contrast, populations in Pico, Faial, and Graciosa exhibit relatively strong introgression signatures, particularly at the mitochondrial level, with 89%, 75%, and 62% of the colonies carrying C-lineage mitotypes, respectively (Ferreira et al., 2020; Henriques et al., 2024). The presence of C-derived genes in the Azores populations is explained by beekeeper-mediated importations and a breeding program in the 1980s, where *A. m. ligustica* and *A. m. carnica* queens were reared on

Santa Maria and Graciosa and crossed on Pico for later distribution of the hybrids to the beekeepers of the other islands (Ferreira et al., 2020; Lh  r  t  , 1987).

The presence of C-derived genes in the populations of S  o Miguel is also evident, particularly at the mitochondrial DNA (mtDNA) level. However, a temporal survey has shown varying levels of C-lineage introgression in both mitochondrial and nuclear compartments (de la De La R  a et al., 2006; Mu  oz et al., 2013, 2014). The gene pool of the population of S  o Miguel was sampled in 2001 and 2010 using mtDNA and microsatellites (de la De La R  a et al., 2006; Mu  oz et al., 2013, 2014) and in 2014 using single nucleotide polymorphisms (SNPs) (Henriques et al., 2024). In 2001, the population exhibited a 35% prevalence of C-lineage mitotypes and 2% C-lineage introgression in the nuclear compartment (Mu  oz et al., 2013, 2014). In 2010, the proportion of C-lineage mitotypes was 38%, accompanied by a 1.7% C-lineage nuclear introgression (Mu  oz et al., 2013, 2014). In 2014, the proportion of C-lineage mitotypes reduced to 27%, and the C-lineage nuclear introgression inferred from SNPs was 3.4% (Ferreira et al., 2020; Henriques et al., 2024).

Abdominal pigmentation is a conspicuous trait in honey bees, often interpreted by beekeepers as an indicator of subspecies purity and used to select colonies for breeding. However, previous work showed that this trait is not a reliable predictor of genetic purity in *A. m. mellifera* and *A. m. iberiensis* (Henriques, 2020). In the Azores, particularly in S  o Miguel, there is an ongoing dispute between the beekeepers who favour the yellow phenotype and believe that they breed a descendant of *A. m. ligustica* and the beekeepers who prefer the black phenotype characteristic of the historically introduced M-lineage *A. m. iberiensis* (sensu Ruttner, 1988).

To address this dispute, in 2023, an unprecedented sampling was undertaken across S  o Miguel (one colony per apiary from 192 apiaries; Figure 1). The genetic background of its population was studied using the tRNA^{Leu}-cox2 mitochondrial region (Garnery et al., 1993) and a customised SNP assay. SNP genotyping was performed on pools of 15 workers, representing the most common phenotype within each colony: entirely black metasoma (BLK; N=95 colonies, Supplementary Table S1) or yellow

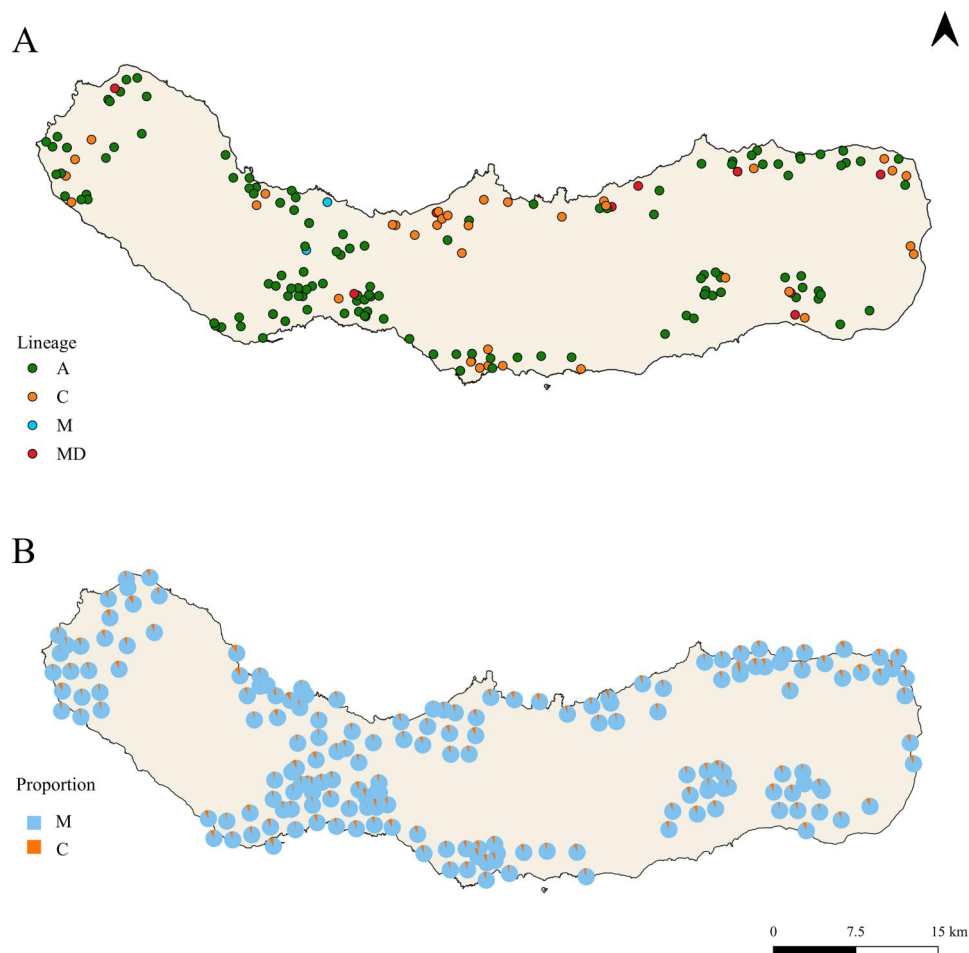


Figure 1. Geographical distribution and genetic characterization of 192 colonies sampled on S  o Miguel (Azores, Portugal) in 2023. (A) The mitochondrial lineage (A-African, C-Central, and Southeastern Europe, M-Western and Northeastern Europe, and Northwestern China) of each colony was classified using the tRNA^{Leu}-cox2 mitochondrial region. The missing data (MD) is represented by red dots. (B) Proportion of nuclear C- and M-alleles for each colony.

bands on the abdominal tergites (B2T; $N = 97$ colonies, [Supplementary Table S1](#)), as in Henriques (2020). DNA was extracted from the pools as described in [Supplementary Information 1](#). The samples were genotyped for 130 diagnostic (M/C-lineage) SNPs distributed across the 16 chromosomes ([Supplementary Figure S1A](#)) in the Illumina MiSeq platform, and the libraries were constructed using the NEBNext Direct Genotyping Solution (New England BioLabs® Inc.). The pools were demultiplexed with Illumina Bcl2fastq2 (v2.20), and the data were processed as described in [Supplementary Information 2](#). The $tRNA^{leu}\text{-cox2}$ region was PCR-amplified with the primers E2 and H2 using the conditions of Garnery et al. (1993). PCR products were Sanger-sequenced by Stabvida (Portugal), and the sequences were aligned with MEGA 11 (Tamura et al., 2021). The sequences of nine samples failed quality standards and were discarded from the mtDNA analysis. To verify if there is a relationship between nuclear C-allele frequency, phenotype, and mitotype, a Bayesian GLMM (General Linear Mixed Models) framework using a Normal distribution was employed (see [Supplementary Information 3](#)).

The average number of reads per sample and locus was 45.29 ([Supplementary Table S2](#)). A total of 41 loci were removed because they contained ≤ 15 reads in $\geq 25\%$ of the samples ([Supplementary Table S2](#)). From the initial 130 SNPs, 58 were excluded from the analysis, and the proportion of the C-alleles was calculated in each pool using the remaining 72 SNPs ([Supplementary Figure S1B](#)). One sample was not analysed because it showed ≤ 15 reads in 70.3% of the loci ([Supplementary Tables S1 and S2](#)).

C-lineage allele frequency ranged from 1.61% to 10.42%, with a mean (\pm SD) of $5.87 \pm 1.75\%$, indicating a predominance of *A. m. iberiensis*-derived nuclear alleles in the population of São Miguel ([Supplementary Table S1](#)). The pools prepared with workers displaying the black (BLK) phenotype showed a mean proportion of $5.82 \pm 1.66\%$ for C-derived nuclear alleles, similar to the pools prepared with workers displaying the yellow (B2T) phenotype, which had a mean proportion of $5.91 \pm 1.84\%$ ([Figure 2](#)). Comparable C-derived proportions in the São Miguel population were reported by Muñoz et al. (2014) using 12 microsatellites in samples collected in 2001 (2.0%) and 2010 (1.7%), and by Henriques et al. (2024) using diagnostic SNPs in samples collected in 2014 (3.4%). Moreover, the Bayesian inference modeling showed no differences or relationship between the C-derived allele frequencies at the nuclear compartment and phenotypes ([Supplementary Table S3](#)). In contrast, a significant relationship between the mitotype and the proportion of nuclear C-lineage alleles was detected by the model. However, the

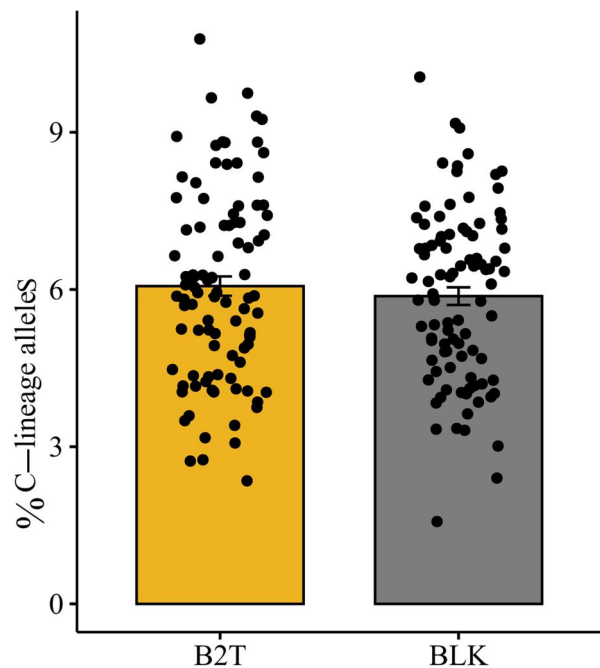


Figure 2. Bar charts showing the average proportion (%) of C-alleles in the colonies sampled in 2023. The black dots represent the proportion (%) of C-alleles for each sampled individual. The samples were classified as BLK (metasoma entirely black) or B2T (presence of yellow bands in abdominal tergites).

increase in nuclear C-lineage proportion in individuals carrying the C-lineage mitotype was only 0.43% ([Supplementary Table S3](#)).

The frequency of C-derived mitotypes was 20.8% ([Supplementary Table S1](#)), over threefold higher than the mean proportion of C-derived SNP alleles (5.87%). However, this frequency was unexpectedly lower than the 35% observed in 2001, 38% in 2010, and 27% in 2014 (Ferreira et al., 2020; Muñoz et al., 2013, 2014), suggesting that C-derived mitotypes have been lost by genetic drift and/or selection.

We showed that the population of São Miguel still exhibits a high proportion of *A. m. iberiensis*-derived ancestry. Over time, the proportion of C-derived alleles introduced in the 1980s has declined for the mitochondrial compartment while it has remained stable for the nuclear compartment (Ferreira et al., 2020; Henriques et al., 2024; Muñoz et al., 2013, 2014). Of note is the similar C-lineage nuclear proportions inferred from a single individual (Henriques et al., 2024) or a pool (this study). This suggests that in a closed and stable population, a single individual is sufficient to reliably estimate the introgression proportions of the colony, regardless of abdominal pigmentation phenotype. The conspicuous difference between yellow and black bees was not reflected by the SNPs, which showed similar C-derived proportions for both phenotypes. This finding confirms that colour alone is not a reliable indicator of subspecies purity in the São Miguel population. Therefore, the ongoing dispute between beekeepers favouring

yellow or black bees as a basis for colony selection is overstated.

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Disclosure statement

The authors declare no conflicts of interest.

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Data available statement

All data used in this work is available in the [supplementary materials](#).

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