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Human-mediated introgression and *Varroa destructor* shaped the genetic structure of honey bee populations in the Azores

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The evolutionary trajectory of island populations can be rapidly altered by human-mediated migration, a process further exacerbated when immigrants introduce invasive parasites, creating new selective pressures. Using customised SNP panels constructed with genome-wide diagnostic loci, we describe the genetic changes in honey bee populations inhabiting the Azores archipelago. As part of a breeding initiative in the 1980s, these populations were recurrently exposed to beekeeper-mediated gene flow from a highly divergent commercial line (C lineage) until the arrival of the *Varroa* mite to the Azores in 2000, which prompted a honey bee importation ban. Admixture analysis revealed a spatially heterogeneous introgression landscape in the Azores. Four of the five mite-free islands (Santa Maria, São Miguel, Terceira, and São Jorge) presented negligible levels of C-lineage introgression (mean Q-value: 0.004–0.091) despite repeated C-lineage importations in the past. In contrast, the three mite-infested islands (Pico, Faial, and Flores) presented high levels of introgression (mean Q-value: 0.156–0.261). The mite-free island of Graciosa harboured the most admixed population (mean Q-value: 0.392), which is consistent with efforts to eradicate the historical population and replace it with C-lineage honey bees during the implementation of the breeding program. Bayesian inference modelling indicated that the presence of a C-lineage maternal origin and *Varroa* were associated with increased introgression proportions (100% posterior probability), increasing the mean Q-value by 0.049 and 0.118, respectively. Our findings indicate that these anthropogenic processes altered the historically introduced gene pool and provide a foundation for developing effective conservation strategies to protect honey bees in the Azores.

Keywords *Apis mellifera*, *Varroa destructor*, Introgression, Conservation, Azores

Owing to their small size, spatial isolation, and diverse range of habitats, islands are excellent natural laboratories for studying evolutionary processes. When individuals from a mainland population migrate to islands, they can interbreed with local populations if reproductive isolation has not yet developed, and this hybridisation can profoundly shape population diversity[1]. If migration (gene flow) occurs repeatedly, it can lead to introgressive hybridisation, whereby the genomes of island individuals can be partially or completely replaced[2]. If gene flow is bidirectional, a swarm of variants, combining the mitotypes of the residents with the nuclear alleles of the migrants and vice versa, can be produced[2]. With time, migration can fundamentally alter the genetic makeup and evolutionary trajectory of island populations[1,3]. This change can occur more rapidly when migrants are introduced and favoured by humans and are accompanied by parasites and/or pathogens that are novel to the receiving population[4,5]. This is the case of the highly polytypic honey bee, *Apis mellifera* L., which humans have transported, along with its major enemy, the ectoparasitic mite *Varroa destructor*, across large geographical scales since historical times[6]. These movements have impacted the genetic integrity[7–10] and the health of many insular and mainland honey bee populations and subspecies[11–16].

The endemic range of *A. mellifera* spans the African, European, and western parts of the Asian continent, as well as several islands in the Mediterranean Sea and the Indian Ocean. Within this broad geographic and environmentally diverse area, honey bees differentiated into 31 subspecies[17–20], mostly belonging to four evolutionary lineages: M (western and northern Europe and north-western China), C (central and southeastern

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Europe), A (Africa), and O (the Middle East and western Asia) sensu Ruttner[18]. However, over the past four centuries, humans have introduced honey bees to all continents (except Antarctica), as well as numerous oceanic islands and this extraordinary range expansion has utilised a sliver of *A. mellifera* subspecific diversity[21,22]. Later, this narrow subspecific gene pool was expanded by honey bee breeders to include other subspecies of the four lineages, although the O-lineage *A. m. caucasia* and especially the C-lineage *A. m. ligustica* and *A. m. carnica* were preferred and intensively bred by beekeepers because of their perceived milder temper and superior honey yield[18].

Human-mediated migration has also occurred within the honey bee's endemic range since at least the tenth–early ninth century BCE, when beekeepers of the Jordan Valley introduced the Turkish subspecies *A. m. anatoliaca* into the *A. m. syriaca* realm[23]. In recent decades, facilitated by globalisation and the rise of commercial beekeeping, human-mediated migration, primarily involving C-lineage genes, has reached an unprecedented geographical scale[8]. This has led to extensive introgressive hybridisation and even the replacement of many populations and subspecies, especially in Europe[10,24,25]. Of particular concern are *A. m. mellifera* and the subspecies endemic to Mediterranean islands[26–29]. Genetic surveys of *A. m. mellifera* have shown high levels of introgression and even replacement by C-lineage genes in large tracts of its range in northern and western Europe[10,24,25]. Similarly, genetic surveys of *A. m. siciliana* and *A. m. ruttneri* revealed extensive C-lineage introgression across most of their range in Sicily and Malta, respectively, whereas *A. m. adami* is believed to be extinct in Crete[26,29,30]. *A. m. cypria* is seemingly the best preserved of the four Mediterranean subspecies, with only a population in north-western Cyprus carrying alleles typical of mainland *A. m. anatoliaca*[27,28].

Other well-documented insular targets of C-lineage introgression are the historical populations inhabiting the Azores, Madeira, and Canary Islands—three of the four archipelagos that make up the Macaronesia biogeographic region located in the North Atlantic Ocean. These populations have been extensively surveyed using, individually or concurrently, morphology, mitochondrial DNA (mtDNA), and microsatellite variations[31–41]. However, most of these surveys commonly use the popular tRNA^{leu}-cox2 (leucine transfer RNA-cytochrome oxidase subunit 2) intergenic region as a marker of mtDNA variation. This region is ideal for tracing maternal gene flow because of its high degree of polymorphism, which allows for the identification of hundreds of mitotypes and discrimination among A, M, and C lineages as well as African sub-lineages (A$_I$, A$_{II}$, A$_{III}$, and Z)[42–44]. Multiple tRNA^{leu}-cox2 surveys revealed that mitotypes of sub-lineage A$_I$, A$_{II}$, and A$_{III}$ ancestries were spread across Macaronesia, with A$_{III}$ being the most abundant in the Canary Islands, Madeira, and Azores but not in Cape Verde, where mitotypes were mostly of A$_I$ ancestry, as in the closest African mainland populations[34,35,37–41]. Remarkably, the north-western corner of the Iberian Peninsula is a hot spot of sub-lineage A$_{III}$ maternal diversity[45–47], supporting the hypothesis that honey bees were originally introduced in Macaronesia by Spanish and Portuguese settlers approximately 400 years ago[35,39,40], as opposed to natural expansion from mainland Africa[37]. It was also the tRNA^{leu}-cox2 marker that revealed for the first time the presence of the C-lineage in the populations of the Canary Islands and the Azores, whereas those of Madeira and Cape Verde remained free of foreign mitotypes[34,35,37,38,40,41]. While these tRNA^{leu}-cox2 surveys have been enlightening, a complete view of the impact of C-derived migration on the genetic makeup of Macaronesian populations requires an assessment of nuclear variation. This has been done with microsatellites for a subset of islands and revealed signals of C-lineage introgression in the Tenerife and São Miguel populations but not in those of La Palma and Madeira, which is consistent with mitochondrial patterns[33,36,37].

In the endless quest for the "perfect" honey bee, humans have not only altered the gene pools of the Macaronesian population and many other populations and subspecies through C-lineage gene flow[10,21,24–26,31,33,35,37] but also facilitated the spread of *V. destructor* across large geographical scales[6]. Since the host shift from *Apis cerana* to *Apis mellifera* in the mid-twentieth century, *V. destructor* has invaded all continents inhabited by honey bees in less than 60 years[6,48]. Only a few geographically isolated territories in the world remain naïve to the mite, with most Azores islands among these rare exceptions, as recently recognised by the European Commission[49]. *Varroa destructor* invaded three of the nine Azorean islands following the illegal importation of C-lineage queens to Pico and Flores in the early 2000s and a natural expansion from Pico to Faial in 2008[40]. The combination of mite-free and mite-infested islands with geographical isolation, along with a relatively well-documented history of human-mediated migration, creates a unique natural laboratory for understanding how human activities can alter the evolutionary trajectory of a population. A compelling example is provided by a temporal tRNA^{leu}-cox2 survey in the Azores, which revealed dramatic maternal turnover on Faial, shifting from predominantly A-lineage to predominantly C-lineage in less than 6 years after mite invasion, whereas the other islands remained stable[40].

Here, we build upon previous knowledge of mtDNA variation to provide a more comprehensive view of the impact of both contemporary human-mediated gene flow and mite invasion on the genetic structure of honey bee populations originally introduced to the Azores by Portuguese settlers[40]. To achieve this goal, we used genome-wide, highly informative single-nucleotide polymorphism[50] panels to interrogate the previously mitotyped 474 honey bee samples collected from across the Azores. These panels were developed in-house from whole genomes to accurately estimate C-lineage introgression into *A. m. iberiensis*, the subspecies historically introduced to the Azores[40]. We combined the mtDNA dataset with the newly developed SNPs and addressed the following questions: (1) Do SNPs detect signals of introgression in the Azores populations? (2) If so, which islands are more threatened by introgression? (3) Are there still islands free of foreign alleles? (4) Are the introgression signatures inferred from SNPs paralleled by mtDNA? (5) Is the maternal turnover detected on Faial mirrored by nuclear DNA? (6) Is there any relationship between introgression patterns and mite invasion? By answering these questions, we aim to provide an unprecedented view of mitonuclear diversity patterns in Macaronesian honey bees. This will reveal the extent to which contemporary introductions of commercial lines and *V. destructor* have altered the historically introduced gene pool and serve as a foundation for future conservation programs.

Results

Quality control of SNP genotyping

Among the 79 highly informative SNPs[50] used to genotype the 474 workers, six did not pass the established threshold for missing data (> 30%) and were removed from the analysis (Table S1; Additional file 1). After this quality control step, the percentage of missing data varied between 0% and 16.4%, with 93.2% of the workers exhibiting less than 5% missing data (Table S1; Additional file 1). Among these, 73 SNPs were successfully genotyped in more than 90% of the samples, highlighting the high success rate achieved by these assays. The observed heterozygosity ranged from 0 for the SNP located on LG1 at position 8,125,946, where all individuals carried the A allele (the same as the reference *A. m. iberiensis*), to 0.27 for the SNP located on LG2 at position 9,154,694. The average heterozygosity was 0.18 (Table S1; Additional file 1).

Introgression and differentiation

Most individuals (313, 66.0%) exhibited varying proportions of C-lineage introgression ($0.051 \leq$ Q-value ≤ 0.702; Tables 1 and S2; Additional file 1), and their distribution was heterogeneous across the Azores (Fig. 1 and Table 1). Santa Maria and São Miguel harboured the lowest percentage of introgressed individuals: 0% and 25%, respectively. The mean Q-value for Santa Maria was 0.004 ± 0.007 ($\pm$ SD), whereas for São Miguel, it was 0.034 ± 0.023 (Table 1). In contrast, most individuals ($\geq 66\%$; Table 1) from the other islands were introgressed. The most extreme case was observed for Graciosa and Pico, where all individuals showed signs of introgression, with Q-values ranging from 0.131 to 0.547 (Table 1). Faial also harboured a highly admixed population, with 98% introgressed individuals and the highest Q-value (0.702). However, the highest mean Q-value was observed for Graciosa (0.392 ± 0.073), whereas Pico and Faial had similar mean Q-values (0.261 ± 0.078 and 0.216 ± 0.148, respectively).

The pairwise F_{ST} values revealed that all island populations exhibited closer genetic affinity to the *A. m. iberiensis* reference population ($0.035 \leq F_{ST} \leq 0.476$; Fig. 2) than to the C-lineage reference population ($0.569 \leq F_{ST} \leq 0.961$). São Miguel had the population with the highest genetic affinity for *A. m. iberiensis* ($F_{ST} = 0.035$). On the other side of the spectrum is Graciosa, with the population most closely related to the C-lineage reference ($F_{ST} = 0.569$). Within the archipelago, Santa Maria harboured the most differentiated population, reaching an F_{ST} value of 0.533 in the pairwise comparison with Graciosa. In contrast, Pico and Faial were home to the most closely related populations, with an F_{ST} value of 0.016 (Fig. 2).

Comparative analysis of SNPs and mtDNA

Among the 313 introgressed individuals, 132 (42%) were of C-lineage mitochondrial ancestry. This proportion increased up to 79.7% (47 of 59) for the individuals with a Q-value > 0.30 and was only 16% (25 of 161) for the purebred (Q-value < 0.05) individuals (Table S2; Additional file 1). The analysis by island revealed a moderate correlation ($r = 0.51$; Spearman's rank correlation test), albeit not significant (p value $= 0.158$), between the proportion of introgressed individuals at the nuclear and mitochondrial levels (Table 1). For São Miguel and Santa Maria, the differences in the introgression proportions between the two compartments were minimal: 0% versus 2% and 25% versus 27%, respectively. In contrast, on Terceira and São Jorge, there were no colonies of C-lineage maternal ancestry, yet 86% and 81% of the individuals showed signals of introgression, albeit with Q values ≤ 0.181 (Table 1). Santa Maria was unique in exclusively hosting pure individuals ($0.000 \leq$ Q value ≤ 0.031), with only one (2%) carrying a C-lineage mitotype.

Bayesian inference modelling revealed that introgression is affected by both *V. destructor* and the mitotype. Compared with individuals with other mitotypes, those with C-lineage mitochondrial ancestry have a mean increase in the Q-value of 0.049 ± 0.013 (100% probability of increase). For islands with *V. destructor*, the mean increase in the Q-values was 0.118 ± 0.014, and the Bayesian probability of increase was 100% (Table 2).

Island	N	Mean ± SD Q-value	Median Q-value	Minimum Q-value	Maximum Q-value	% of Introgressed individuals (ncDNA)	% of C-lineage individuals (mtDNA)
Santa Maria	57	0.004 ± 0.007	0.000	0.000	0.031	0	2
São Miguel	99	0.034 ± 0.023	0.036	0.000	0.111	25	27
Terceira	78	0.091 ± 0.038	0.097	0.000	0.181	86	0
Graciosa	21	0.392 ± 0.073	0.397	0.131	0.493	100	62
São Jorge	37	0.088 ± 0.040	0.086	0.000	0.157	81	0
Pico	75	0.261 ± 0.078	0.254	0.137	0.547	100	89
Faial	60	0.216 ± 0.148	0.177	0.045	0.702	98	75
Flores	47	0.156 ± 0.101	0.183	0.000	0.344	77	9
Total	474	0.034 ± 0.023	0.036	0.000	0.111	25	27

Table 1. Introgression statistics for the honey bee populations of the Azores. The sample size (N); the mean ± standard deviation (SD), median, minimum, and maximum levels of introgression (Q-value); the percentage of introgressed individuals using SNPs as nuclear markers (ncDNA); and the percentage of individuals carrying C-lineage mtDNA (retrieved from Ferreira et al., 2020) are shown for each island.
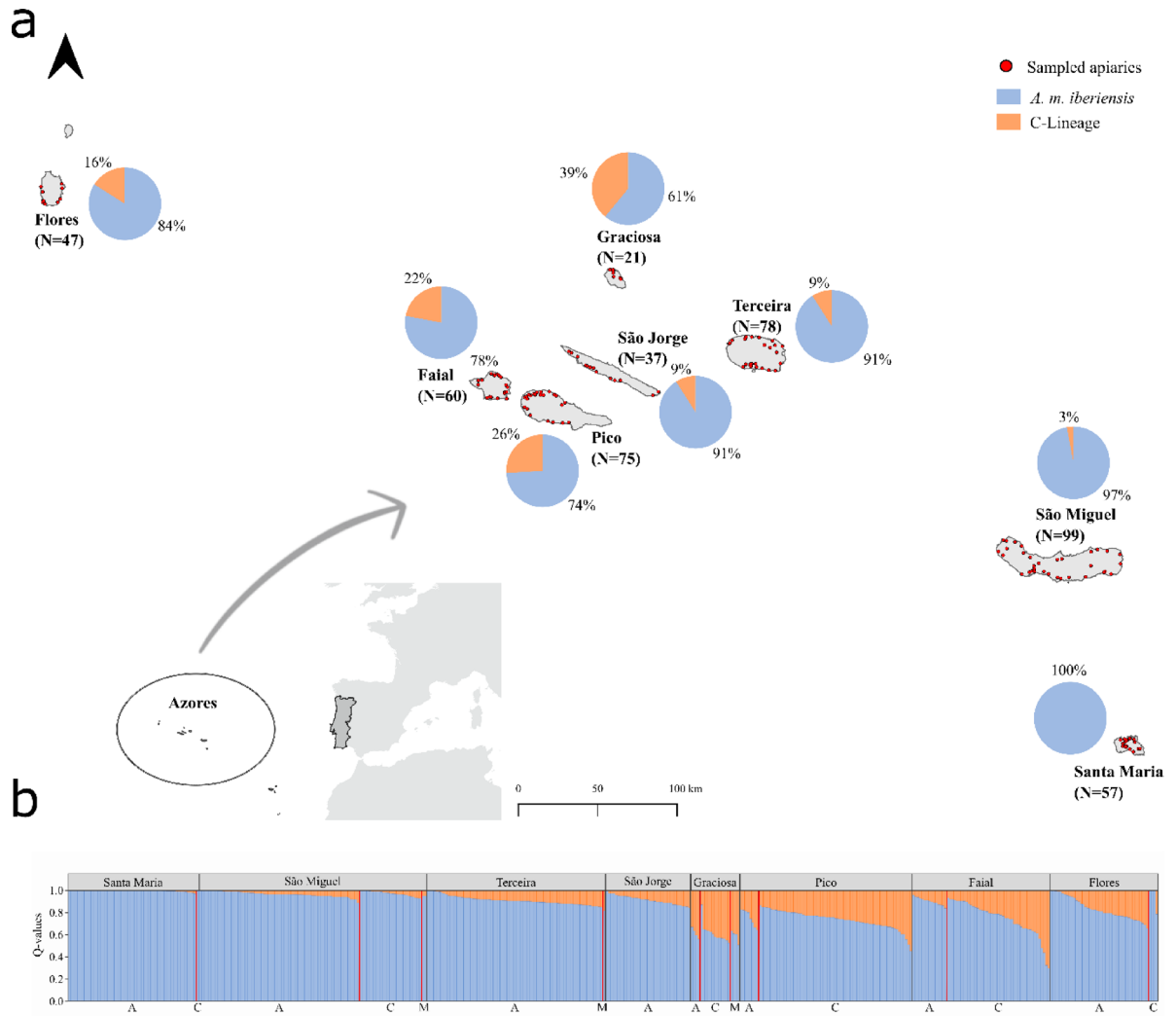
Fig. 1. Map of sampled Apiaries and ADMIXTURE Plot inferred from SNPs in the Azores. (**a**) Map displaying the location of the apiaries (red dots) sampled across the Azores. Each island includes sample sizes (in parentheses) and a pie chart illustrating the average membership proportions (Q-values) of *A. m. iberiensis* (blue) and the C-lineage (orange), which were calculated using customised highly informative SNP panels. (**b**) The ADMIXTURE plot illustrates the genome partitioning into two clusters (K = 2) for each of the 474 individuals sampled across the Azores. The C-lineage cluster is depicted in orange, whereas the *A. m. iberiensis* cluster is shown in blue. The vertical solid lines separate populations between islands, whereas the red lines separate groups of individuals by mitochondrial DNA lineage (A—African, C—Central and Southeastern European, and M—Western and Northeastern European) within each island.

Discussion

The C-lineage introgression patterns of honey bee populations in the Macaronesia biogeographic region have been extensively surveyed using the maternal marker tRNA[leu]-cox2 alone[34,35,41] or in combination with microsatellites[33,36–38] and/or morphometry[31,32,39,40]. Here, we used customised diagnostic SNP panels covering all 16 honey bee chromosomes[50] to genotype 474 individuals from all the bee-populated islands of the Azores. This effort allowed us to uncover the impact of contemporary C-lineage migration with unprecedented genomic and spatial resolution, representing a significant advancement over previous studies from the archipelagos of Madeira[37], the Canary[33,36,38], and Cape Verde[41], which relied on a few non-diagnostic microsatellite loci genotyped in a limited number of island populations. While microsatellites have been the nuclear marker of choice in numerous population genetics studies of honey bees[27,41,51–54], SNPs have demonstrated superior performance in inferring C-lineage introgression in M-lineage honey bees[55,56]. Therefore, our study also provides the most accurate estimates of C-lineage admixture proportions in island populations to date[26,30,33,36,57].

Our data revealed that individuals with C-lineage maternal ancestry tended to be slightly more admixed than those with M- or A-lineage maternal ancestry. However, there were also many instances of mitonuclear incongruence: 16% of individuals exhibiting pure *A. m. iberiensis*-like nuclear DNA (ncDNA) carried C-lineage mtDNA, whereas 58% of admixed individuals had A- or M-lineage mitotypes, which are typically found in the native range of *A. m. iberiensis*[45–47]. These findings demonstrate that relying solely on mtDNA can provide an
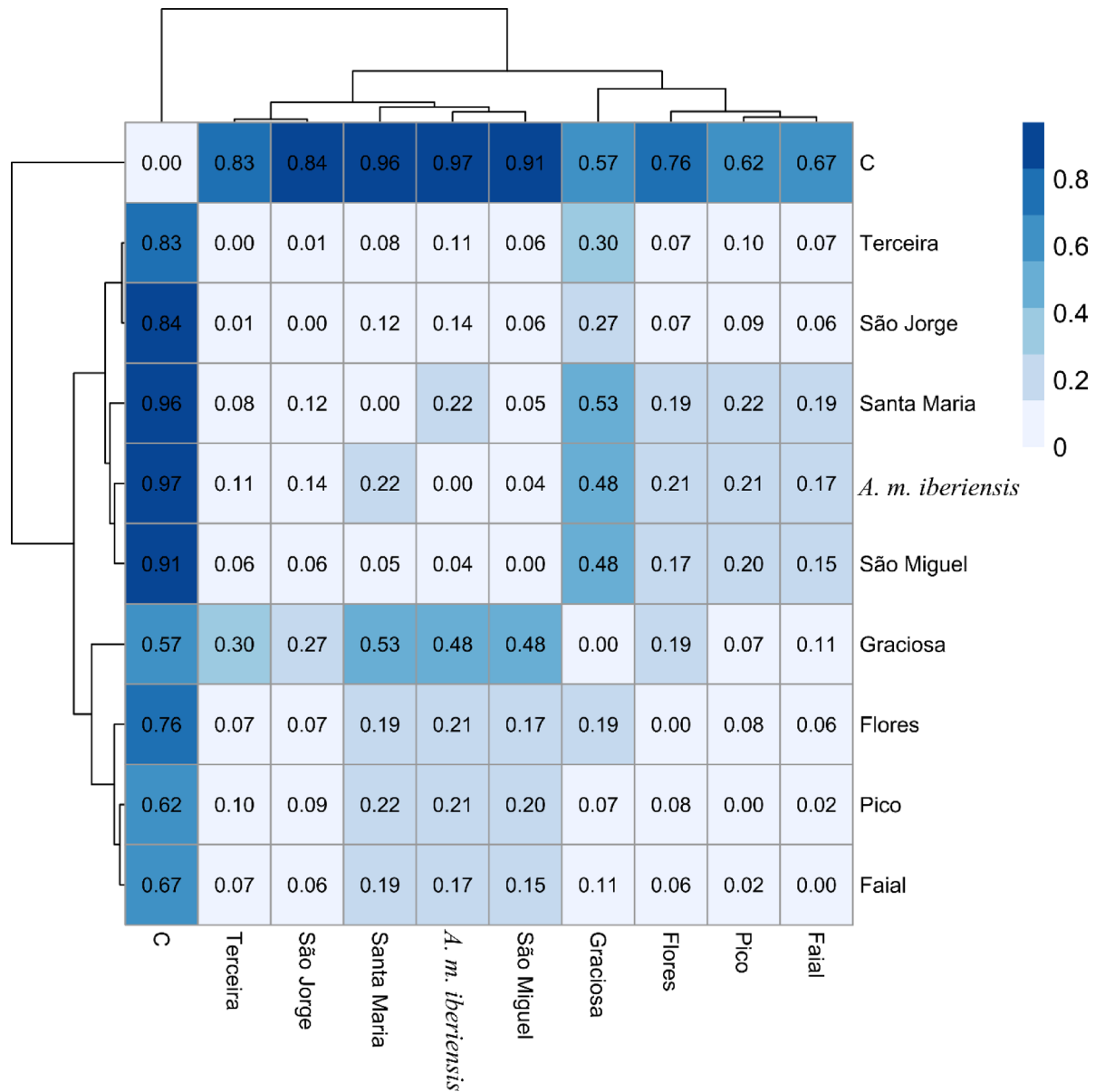
Fig. 2. Clustered heatmap generated with pairwise F_{ST} values. The heatmap was constructed with pairwise F_{ST} values between island populations of the Azores and between islands and the references *A. m. iberiensis* and the C-lineage (C). All values are significant (*p* value < 0.05).

(A) Effect of mtDNA	C-lineage	Other lineages	Effect size	Probability
Q-value	0.172 ± 0.012	0.123 ± 0.008	0.049 ± 0.013	100%
(B) Effect of *V. destructor*	*V. destructor* islands	*V. destructor-*free islands	Effect size	Probability
Q-value	0.217 ± 0.012	0.099 ± 0.009	0.118 ± 0.014	100%

Table 2. Posterior distributions generated by Bayesian hierarchical GLMM models. The table presents the mean ± standard deviation levels of introgression (Q-value) from the posterior distributions generated by Bayesian hierarchical GLMM models, accounting for the apiary effect. Effect size represents the mean difference in introgression between mitotypes (A) or between islands with and without *V. destructor* (B). 'Probability' refers to the probability that the effect size > 0.

incomplete and potentially misleading view of introgression signatures. Therefore, mtDNA should not be used in isolation, as is often done in studies of honey bee populations from islands[34,35,57]. More importantly, these findings indicate bidirectional gene flow between the two highly divergent lineages M and C[58] and the absence of strong prezygotic barriers related to asynchrony in mating flight times, as previously reported for *A. m. mellifera* and *A. m. carnica* subspecies[59]. Although some degree of prezygotic isolation cannot be ruled out, the observed asymmetric introgression may also reflect differences in the sizes of resident and migrant populations, as well as mitonuclear incompatibilities. MtDNA can influence nuclear genome evolution following hybridisation[60–62]. Because mitochondrial function depends on proteins encoded by both mtDNA and nuclear DNA, hybridisation may disrupt co-adapted mitonuclear interactions, potentially reducing fitness. Such mismatches have been proposed as a mechanism underlying reduced hybrid viability in crosses between European and Africanized honey bees[63].

The island-level results revealed a significantly heterogeneous introgression landscape in the Azores. Some islands, such as São Miguel and especially Santa Maria, were virtually free of C-lineage alleles and closely related to the original *A. m. iberiensis* introduced in historical times. In contrast, other islands, such as Flores, Faial, Pico, and particularly Graciosa, hosted highly admixed populations, with most or all colonies showing variable signs of introgression. A similar pattern was reported for the Canary Islands, where Tenerife, El Hierro, and La Gomera exhibited high levels of introgression, whereas La Palma and Gran Canaria remained well preserved[33,34,36,38,39].

The C-lineage ancestry of Azorean honey bees dates back to the 1980s when a breeding program aimed to replace the local genetic stock with allegedly more productive and docile queen lines acquired from breeders in France (*A. m. caucasia* by anecdotal reports, but likely *A. m. carnica* by mtDNA and wing shape patterns, and assumed here as the introduced subspecies) and Italy (*A. m. ligustica*)[40]. The queens and drones of *A. m. ligustica* and "*A. m. carnica*" were produced on Santa Maria and Graciosa, respectively. These were crossed on Pico to obtain a superior phenotype by taking advantage of heterosis expressed in the F1 offspring, following brother Adam's experiments[64]. The F1 hybrids were then disseminated primarily on São Miguel and Terceira and, to a lesser extent, on Faial and particularly on São Jorge[65,66]. This C-lineage import and crossbreeding program ran annually from 1985 to 1989. However, beekeepers occasionally imported queens of C-lineage ancestry, both legally and illegally, before and after this period, until the first arrival of *V. destructor* in 2000 prompted the local veterinary authority to implement stringent quarantine measures, prohibiting further import into the Azores (Dr Paula Vieira, pers. comm.). The introduction of *V. destructor* in the Azores poses a significant threat to apiculture, as the mite has been associated with colony losses on a global scale[48,67,68], and the Azores is no exception.

Owing to geographical isolation and the limited area of volcanic islands, island populations are typically small and, therefore, more susceptible to genetic changes introduced by recurrent migration events[69,70]. When gene flow ceases, the evolutionary fate of foreign alleles is determined by the interplay between selection and genetic drift, with the outcome influenced by the selection coefficient and population size[71]. Over time, this dynamic may lead to the purging of foreign alleles on some islands while they may become fixed on others. Since the import ban in the early 2000s, these evolutionary forces have solely shaped the genetic structure of honey bee populations in the Azores, resulting in some islands exhibiting low levels of C-lineage introgression, whereas others display high levels.

The weak C-lineage introgression signature detected in the populations of the easternmost islands, São Miguel and Santa Maria, was unexpected. Santa Maria was the stage for producing *A. m. ligustica* queens during the breeding initiative; however, aside from a single instance of C-lineage mitochondrial capture, the introgression signature has been almost entirely erased in less than 30 years. São Miguel was the island most actively involved in disseminating the *A. m. ligustica* × *A. m. carnica* F1 colonies produced on Pico and received mated "*A. m. carnica*" queens directly from Graciosa[66]. Additionally, São Miguel hosts the largest and most active beekeeping industry in the Azores, with several beekeepers favouring an *A. m. ligustica*-like yellow phenotype, allegedly introduced from New Zealand in the early 1980s. While beekeepers have successfully maintained this phenotype[72], genome-wide SNPs do not capture the genetic variation underlying the few genes governing yellow colouration in honey bees[72] and reveal low levels of introgression. Earlier microsatellite surveys also detected low levels of introgression on São Miguel, with a decreasing trend observed between 2001 and 2010[36,37]. However, in contrast to SNPs and microsatellites, the maternal marker revealed that 27% of the extant mitotypes are of C-lineage ancestry[40], aligning more closely with the expectations of beekeepers, who have been selecting for the *A. m. ligustica*-like yellow phenotype. This high frequency of the C-lineage in the 2014 sample is comparable to the 19.3% detected in a fine-resolution sampling conducted in 2021[73] but lower than the 35% and 37% found in 2001 and 2011, respectively[37,40]. This suggests that drift and/or selection have eliminated C-lineage mitotypes over time. Alternatively, the apparent decreasing trend could be due to stochastic sampling variability.

The pronounced asymmetrical mitonuclear introgression patterns observed in São Miguel can be explained by honey bee reproduction dynamics. While beekeepers typically select matrilines through colony splitting or queen rearing, the reproduction of patrilines is markedly different. Unless male-directed gene flow is controlled by instrumental insemination or isolated mating stations, the beekeeper-selected queens fly to drone (male) congregation areas (DCAs) to mate randomly with an average of 12 drones[74,75]. These DCAs attract thousands of drones from neighbouring colonies up to 8 km away[76], representing the population gene pool rather than the beekeepers' preferences.

The other islands with low introgression proportions were Terceira and São Jorge, which is consistent with the absence of C-lineage mitotypes in 2014[40]. Terceira was actively involved in the breeding program, with many F1 swarms distributed to beekeepers in the 1980s[66]. Additionally, during the same period, over 100 honey bee packages of C-lineage ancestry were imported from the USA by the local agriculture authority to restock the heavy losses caused by large-scale spraying with carbaryl to control the dramatic spread of the recently arrived agricultural pest *Popillia japonica*[77]. While genetic drift might have played a significant role in purging

C-lineage alleles, an intriguing, non-mutually exclusive alternative hypothesis is that selection has favoured an *A. m. iberiensis*-derived gene pool in the mite-free islands.

At the other end of the introgression spectrum are the central islands of Graciosa, Pico, Faial, and the westernmost island of Flores. Graciosa harbours the most homogeneously admixed population and the highest mean introgression proportions in the Azores at the nuclear but not at the mitochondrial level[40]. Elevated C-lineage introgression levels are expected for an island that was a primary site for producing "*A. m. carnica*" queens in the 1980s. However, the significant contribution of *A. m. iberiensis* ancestry to the extant gene pool is intriguing. This is because, as part of the breeding program of Graciosa (but not Santa Maria), every colony on the island was requeened with a mated "*A. m. carnica*" queen. The goal was to eradicate the historically introduced *A. m. iberiensis* and establish a pure foreign queen line on the island[65,66]. Clearly, this effort proved unsuccessful, as evidenced by the genetic data from both nuclear and mitochondrial compartments. At least three non-mutually exclusive hypotheses could explain this result. One possibility is that, after the breeding initiative, beekeepers actively introduced colonies from mite-free islands (trading with mite-infested islands has been interdicted since the early 2000s), where *A. m. iberiensis*-derived alleles and mitotypes are highly prevalent. However, this is unlikely because beekeepers from Graciosa take pride in their honey bees, which are reputed to be gentle and productive. Another possibility is that natural selection favoured genes of *A. m. iberiensis* ancestry, counteracting the strong human-driven selection imposed on the local population in the 1980s. Finally, there may have been a large, undetected feral population of the local breed, which interbred with the domestic "*A. m. carnica*" colonies. This is plausible, as feral populations can reach substantial sizes in mite-free territories[78,79].

Pico, Faial, and Flores exhibit similar patterns of introgression and are the only islands of the Azores colonised by the mite[40]. While Faial and, particularly, Pico were actively involved in the breeding program, Flores was not. This requires an explanation for the origin of C-lineage genes on this remote island. *V. destructor* first arrived in the Azores via Pico in 2000 and reached Flores a year later through illegal queen importations (Dr Paula Vieira, pers. comm.), which, in addition to the mite, most likely brought C-lineage genes to both Pico and Flores. Although the 1-year gap between these invasive events suggests a route to Flores via Pico, phylogeographic patterns of the most important honey bee virus transmitted by the mite, the Deformed wing virus, indicate two independent introductions[15,16]. The third invasion occurred on Faial 8 years later, this time via Pico. Unlike the beekeeper-mediated invasions of Pico and Flores, *V. destructor* likely reached Faial by hitchhiking on swarms travelling by boat from nearby Pico. This natural migration event, which was likely reinforced by beekeepers purchasing colonies from Pico to compensate for losses caused by the mite invasion, led to the homogenisation of both pathogen landscapes and gene pools, as suggested by the genetic proximity of honey bees and viruses between the two geographically close islands[15,16,80]. Notably, a temporal maternal survey revealed that this homogenisation process was unidirectional and occurred within less than 6 years, suggesting a high rate of gene flow from Pico to Faial[40]. In 2011, 3 years after the initial sight of *V. destructor*, Faial was dominated by A-lineage mitotypes (77%), similar to mainland Portugal[45–47]. However, by 2014, the island population had shifted to being dominated by C-lineage mitotypes[40] (75%). A mite-driven evolutionary change of a similar scale but in the opposite direction, from European to African-derived ancestry, was also observed in a feral population in the southern USA shortly after the invasion of *V. destructor*[54,81]. Although we lack nuclear data from the 2011 survey, it is possible that this extraordinary maternal turnover was mirrored by changes in SNPs, as suggested by the symmetrical C-lineage proportions in both the mitochondrial and nuclear compartments observed in 2014. Therefore, we propose that *V. destructor* played a significant role in restructuring the honey bee gene pool of Faial.

Remarkably, Flores was barely targeted by the breeding initiative in the 1980s[66]. Yet, it harbours relatively high introgression levels and is genetically closer to the other mite-infested islands than the mite-free islands. At least three non-mutually exclusive hypotheses could explain this unexpected finding. First, multiple unofficially imported queens likely introduced not only *V. destructor* but also C-lineage genes. As the smallest island with the smallest honey bee domestic population, Flores is more vulnerable to genetic changes introduced through gene flow. Second, after the stringent importation ban in the early 2000s, beekeepers might have purchased swarms to Graciosa or Pico, where C-lineage alleles were likely common at that time. However, this is unlikely as commercial beekeeping is not known on these islands. Finally, *Varroa*-driven selective pressure may favour admixed backgrounds, potentially leading to an increased frequency of C-lineage alleles over time. Resistance to *Varroa* is a complex, multigenic trait involving epistatic interactions[82,83] and is known to vary among honey bee subspecies[84]. While introgression can disrupt co-adapted gene complexes[85–87]—potentially leading to outbreeding depression—it may also facilitate the acquisition of advantageous alleles that enhance resistance to *Varroa*, explaining the differing introgression landscapes observed between islands with and without mite infestation. Adaptively beneficial effects of introgression have been documented in a growing number of organisms[88,89], including recent findings in a wide array of vertebrates where introgressed immune-related genes are thought to enhance defence against pathogens[90]. In honey bees, introgression has been implicated in adaptation to climatic gradients associated with latitude and altitude in neotropical populations[88,89]. Whether admixture increases honey bee fitness under *Varroa* infestation remains an open and compelling question, warranting further investigation.

Conclusions

The introduction of commercial queens of C-lineage ancestry and *V. destructor* invasion reshaped the historic honey bee gene pool in the Azores. The long-term evolutionary fate of C-derived mitochondrial and nuclear introgression in Azorean honey bees remains uncertain. If gene flow from external sources and within the archipelago continues to be severely restricted, selection and drift will be the primary forces shaping the genetic structure of these populations. In an *A. m. iberiensis* genetic background, C-lineage alleles may be disfavoured. If negative selection indeed occurs, these C-lineage alleles may gradually be purged from Azorean populations.

Alternatively, if selection favours individuals of mixed ancestry, the C-lineage contribution will persist. However, stochastic processes might override selection, particularly on small islands with small populations, such as Graciosa and Flores.

The SNP panels used in this study consist of widely distributed, unlinked loci previously selected for their strong differentiation between the C-lineage and *A. m. iberiensis*. While these panels are well-suited for accurately estimating ancestry and admixture, they are subject to ascertainment bias, which limits their utility for investigating evolutionary processes beyond gene flow. To overcome this limitation, future studies of Azorean honey bee populations should leverage the decreasing costs of high-throughput sequencing to generate genome-wide data from both nuclear and mitochondrial genomes. Such comprehensive genomic approaches would allow for a more robust disentangling of neutral *versus* selective forces shaping genetic variation in these populations. Additionally, incorporating a temporal dimension will be crucial for understanding the long-term dynamics and evolutionary trajectory of C-lineage ancestry in the Azorean gene pool.

Methods
Study setting
The Azores is the northernmost archipelago of the Macaronesia biogeographic region and has been managed under Portuguese sovereignty since the fifteenth century. The archipelago is located approximately 1360 km from the Portuguese mainland and comprises nine volcanic islands organised by their geographical proximity into three groups: eastern (São Miguel and Santa Maria), central (Faial, Pico, Terceira, São Jorge, and Graciosa), and western (Corvo and Flores). São Miguel and Santa Maria were the first islands discovered and were the first to be colonised by humans and honey bees in the fifteenth century[91,92]. Corvo, the smallest island, recently lost its honey bee population, but it was recolonised with six colonies from Terceira after this study (Paula Vieira, pers. comm.). The total number of registered colonies in the Azores was 5011 in 2014 and 5738 in 2015. São Miguel is the largest island of the archipelago and has the most developed beekeeping sector and the highest number of colonies: 2035 in 2014 and 2345 in 2015. Terceira was the second most populated island, with 972 and 1172 registered colonies in 2014 and 2015, respectively, closely followed by Pico, with 907 and 943. Flores has the least developed beekeeping sector in the Azores, harbouring the smallest population: 120 colonies in 2014 and 121 in 2015 (see census size for every island in Ferreira et al.[40], as provided by the Azorean agriculture authority "Direção Regional de Agricultura dos Açores").

Sampling
A total of 474 colonies were randomly sampled in the summers of 2014 and 2015 from 156 apiaries distributed across the Azores (Fig. 1 and Table S2; Additional file 1). The great majority of the apiaries, which belonged to different beekeepers, were represented by three colonies. The sample size per island was proportional to the population size (see census size as of 2013 in[40]). Adult workers (sterile honey bee females) were collected from the inner frames of the hives and placed in absolute ethanol. The samples were stored at − 20 °C until analysis.

DNA extraction and genotyping
Total DNA was extracted from the mesosoma of one worker per colony via Ron's Tissue DNA Mini Kit (Bioron) following the manufacturer's instructions. An additional step involving RNA degradation (RNase A) was included to ensure the purity of the DNA samples. The DNA extracts were diluted to a concentration of 10–15 ng/μL and then sent to *Instituto Gulbenkian de Ciência* (Portugal) for genotyping of 79 SNPs (Table S3; Additional file 1) via two-best ancestry-informative SNP assays (M3: 40 SNPs; M4: 39 SNPs) previously developed by Henriques[50]. The 79 diagnostic SNPs are unlinked and well distributed across the 16 honey bee chromosomes, covering 63 genes and 16 intergenic regions (see Henriques[50] for further details on the design of the SNP panels). These SNP assays were designed using the F_{ST} differentiation metric ($F_{ST} = 1$), calculated from whole haploid genomes, to accurately estimate C-lineage introgression into *A. m. iberiensis* (Table S1; Additional file 1). The Agena BioScience iPLEX chemistry and the MassARRAY® MALDI-TOF platform[93] were employed for genotyping 474 samples (Table S3; Additional file 1). Samples and genotypes with 30% missing data were discarded (Tables S1 and S2; Additional file 1).

Introgression and differentiation analysis
The proportion of C-lineage introgression into *A. m. iberiensis* (Q-value) was estimated for each of the 474 workers using the software ADMIXTURE[94], and the reference populations (29 *A. m. iberiensis*, five *A. m. carnica*, and 10 *A. m. ligustica* individuals) of Henriques et al.[50]. The ADMIXTURE analysis was conducted for K = 2, employing 10,000 iterations across 20 independent runs. Convergence between iterations was assessed by comparing log-likelihood scores (LLS), with the default termination criteria set to stop when LLS increased by < 0.0001 between iterations. Q-plots were visualised and summarised using CLUMPAK[95]. A C-lineage Q-value threshold of ≤ 0.05 was applied to identify purebred *A. m. iberiensis* individuals.

Differentiation between island populations and the reference populations was assessed by pairwise F_{ST} values estimated using Arlequin 3.5.2.2[96], with departures from zero tested by 10,000 permutations. The F_{ST} matrix was used to generate a clustered heatmap in the R package pheatmap (version 1.0.12).

Comparison between SNPs and mtDNA
To model the relationship between SNP-based introgression, mtDNA (mitotypes), and *V. destructor*, a General Linear Mixed Model (GLMM) framework using a Gamma distribution (Fig. S1; Additional file 2) was employed. The mitotype data (obtained from Ferreira et al.[40]) were transformed into a binary variable, with the C-lineage coded as '1' and all the other lineages as '0'. The islands with *V. destructor* (mite-infested) were coded as '1' (Pico, Faial, Flores), and those without *V. destructor* (mite-free) were coded as '0' (Santa Maria, São Miguel, Terceira,

São Jorge, Graciosa). The mitotype and *V. destructor* were treated as fixed effects, whereas the apiary was included as a group-level random effect on the intercept. This modelling was conducted within a Bayesian framework using JAGS (57), interfaced through with R[97]. A Bayesian approach was used because it allows probabilities of the direction and magnitude of effect sizes to be directly calculated. Minimally informative priors were used, and the Markov chain Monte Carlo (MCMC) chains were sampled for 100,000 iterations after confirming chain convergence through visual inspection of stability and mixing along with Heidelberger and Welch diagnostic (Figs. S2 and S3, Tables S4 and S5; Additional file 2). Model fit was evaluated using posterior predictive checks (see further details in the Additional file 2).

Data availability
All the data generated or analysed during this study are included in this published article and its supplementary information files.

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MAP and DH conceived the ideas and designed the experiment. The analyses were performed by DH with the assistance of ARL and ML. MAP and DH wrote the manuscript with the assistance of ARL. All the authors critically reviewed the manuscript for important intellectual content.

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Declarations

Competing interests

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