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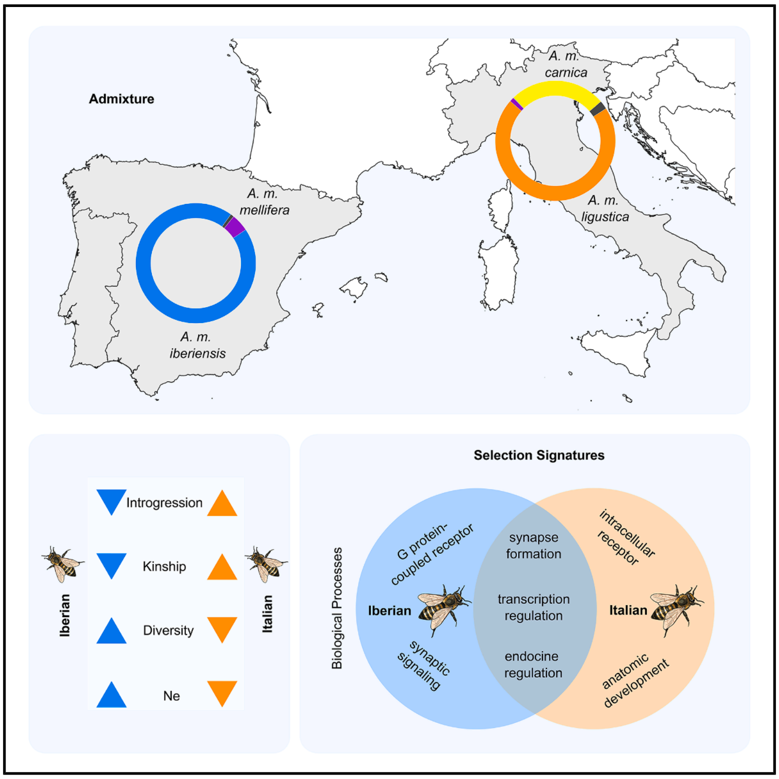
Contrasting whole-genome diversity patterns and adaptation in honeybees from two southern European glacial refugia

Graphical abstract



Highlights

- Higher human-mediated introgression in Italian than in Iberian honeybees
- Lower genetic diversity and Ne in Italian than in Iberian honeybees
- Higher kinship in Italian honeybees than in Iberian honeybees
- Convergent selection signatures in regulatory pathways and synapse formation

Authors

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In brief

Zoology; Entomology; Evolutionary biology

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Contrasting whole-genome diversity patterns and adaptation in honeybees from two southern European glacial refugia

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SUMMARY

Human-driven environmental change makes understanding genetic variation essential for protecting keystone species such as the honeybee, *Apis mellifera*. We compared whole-genome mitonuclear variation in subspecies inhabiting the Iberian and Italian Peninsulas, which have been shaped by distinct glacial histories and modern beekeeping pressures. Italian honeybees showed a stronger anthropogenic imprint, driven largely by recent human-mediated gene flow. Both subspecies showed mitonuclear asymmetry, an approximate south-to-northeast clinal pattern, evidence of ancient or recent admixture with other subspecies and lineages, and genomic signatures of a 20th-century bottleneck. African ancestry was present in both, though ancient and predominantly mitochondrial in Iberia, but recent and predominantly nuclear in Italy. Italian honeybees also had persistently lower historical effective population sizes, lower nucleotide diversity, and higher kinship. Shared and subspecies-specific enriched genes suggest both convergent and unique adaptive responses. These results highlight complex evolutionary dynamics and the significant genetic impact of modern beekeeping.

INTRODUCTION

The southern European peninsulas of Iberia, Italy, and the Balkans served as refugia for many temperate organisms during the Pleistocene glaciations and as reservoirs of genetic diversity for the post-glacial recolonization of Europe.[1,2] Organisms that persisted in these refugia often display intricate patterns of genetic diversity, forged by genetic drift, gene flow, and natural selection over successive cycles of population contraction, expansion, and adaptation to Pleistocene climatic oscillations[3–5] In recent times, however, humans have reshaped these patterns, whether inadvertently or intentionally, by blending, narrowing, selecting, and fragmenting gene pools[6–8] to such an extent that the current geological era has been coined the Anthropocene.[9]

As for many other organisms, the evolutionary history of European honeybees (*Apis mellifera* L.) is closely tied to the southern European peninsulas. In addition to providing shelter during the glaciations, they are home to 6 of the 10 recognized European subspecies: *A. m. iberiensis* and *A. m. mellifera*, both historically associated with Iberia; *A. m. ligustica*, associated with Italy; and *A. m. carnica*, *A. m. macedonica*, and *A. m. cecropia*, associated with the Balkans. These can be grouped into two morphologically and genetically well-differentiated lineages: the western and northern European M-lineage, including *A. m. iberiensis* and A. *m. mellifera*, and the southern and eastern European C-lineage, comprising the remaining four subspecies. A third lineage, the African A-lineage, co-occurs with the M-lineage in Iberia according to mitochondrial DNA variation, suggesting an ancient out-of-Africa expansion and a hybrid origin for *A. m. iberiensis*.[10–13] With climate amelioration at the end of the Last Glacial Maximum, *A. m. mellifera* expanded from its refugia in northern Iberia, colonizing vast areas west of the Alps and the Carpathians, reaching as far north as 60°N in Scandinavia and as far east as the Ural Mountains.[10,14,15] Simultaneously, *A. m. carnica* expanded from the Balkans into the Italian Peninsula and central Europe.[16,17] As these populations expanded their ranges, they came into contact and formed hybrid zones in various regions of Europe, including Iberia, Italy, Poland, and Ukraine.[13,17–19]

Natural patterns of genetic diversity in *A. mellifera* have been disrupted across much of its distributional range by contemporary anthropogenic processes, primarily involving long-distance translocations, artificial selection, and reproductive bottlenecks.[20–22] Manipulation of honeybees dates back at least 3,000 years to the Near East.[23] However, the most significant human-driven genetic changes have occurred over the past 150 years, through long-distance migrations, bringing allopatric subspecies into contact in both native and introduced ranges.[19,24,25] By translocating queens and swarms across vast regions, humans have not only extended the honeybee's natural distribution, from Europe, Africa, and western Asia to the Americas and Australia, but have also reshaped the gene pools of many native populations through introgressive hybridization and, in some cases, even complete replacement of native gene pools, as documented in Germany, Denmark, and Poland.[16,26–29] Other activities driving genetic change involve selective breeding for traits desirable in apiculture, such as reduced swarming, docility, low propolis production, and high honey yield, as well as reproductive bottlenecks associated with the mass rearing of a limited number of selected queen lines, often mated with selected drones via artificial insemination or at isolated mating stations.[30–33] In addition to blurring subspecies boundaries, these processes may disrupt local adaptation by introducing or favoring alleles advantageous for beekeeping but potentially maladaptive in specific environments,[34–36] threatening both the genetic integrity and long-term viability of native populations and subspecies. This concern finds support in recent reciprocal translocation experiments, which have consistently demonstrated that locally adapted colonies outperform introduced colonies across several traits, including survival.[37–40]

Humans have also played a major role in disrupting populations by inadvertently introducing and spreading alien predators, parasites, and pathogens, often hitchhiking in migratory colonies or in commercial queens and swarms, to which honeybees are not adapted. Among these, the ectoparasitic mite *Varroa destructor* (hereafter varroa) is unanimously recognized as the most serious threat to honeybee health and is frequently implicated in colony losses worldwide.[41,42] After shifting from its original host, *Apis cerana*, to *A. mellifera* in the 1950s in eastern Russia,[43,44] varroa rapidly spread across the globe, causing devastating losses in many countries. In Europe, the first confirmed detection was in Bulgaria in 1967,[45] and by the late 1980s, the mite had become well established throughout the southern peninsulas.[46,47] Although varroa outbreaks led to significant colony losses upon arrival in various European countries, many beekeepers responded promptly with chemical treatments.[48] These treatments mitigated immediate damage, but also prevented natural selection from favoring traits associated with varroa resistance.[49] In contrast, in many regions worldwide, untreated honeybee populations of both African and European ancestry have evolved resistance to varroa,[50,51] and this resistance is known to have a complex genetic basis.[52]

The Iberian (*A. m. iberiensis*) and Italian (*A. m. ligustica*) honeybee subspecies offer valuable models for understanding the effects of contemporary anthropogenic processes on genetic diversity patterns. Both are native to refugial peninsulas, exhibit hybrid origins, and were invaded by varroa in the 1980s.[46,47] In Iberia, post-glacial expansions brought divergent gene pools into secondary contact, producing a well-documented northeast-southwest genetic cline.[15] In Italy, the widespread presence of M-lineage haplotypes suggests an ancient expansion through the western Alpine arc, while C-lineage haplotypes characteristic of *A. m. carnica* expanded through the eastern Alpine arc, leading to the formation of a natural hybrid zone in the northeast.[17,33]

Despite these shared features, the two subspecies have been subjected to markedly different anthropogenic influences. Although colony numbers and densities have historically been higher in Iberia,[53,54] management practices involving selective breeding, colony reproduction, and translocation have been more intensive in Italy. This country has a long-standing tradition of queen rearing dating back to the late 1800s, when a small group of beekeepers in the Emilia-Romagna region began large-scale production to meet global demand for *A. m. ligustica* queens.[55] Today, Italy remains the largest queen producer in Europe, with an estimated 700,000 queens reared annually from a limited number of selected matrilines.[33,56] Most queens are sold internally to meet the needs of Italian beekeepers, who regularly requeen their colonies. In contrast, breeding and commercial queen production in Iberia are still at an early stage of development. A breeding program was only recently established in the Basque Country to supply local beekeepers,[57] and there is just one commercial large-scale queen-rearing facility operating in Iberia (A. G. Pajuelo, personal communication). Most Iberian beekeepers continue to rely on splitting (artificial swarming) for colony reproduction, a traditional management practice that favors the native *A. m. iberiensis* and keeps the use of exotic queens at very low levels. Intriguingly, despite the renowned desirable traits of *A. m. ligustica*, the importation of queens and swarms derived from inter-subspecific crosses, especially "Buckfast" bees, and from non-native subspecies such as *A. m. carnica* into Italy has increased in recent years.[58]

Whether these contrasting management practices have left distinct genomic imprints on *A. m. iberiensis* and *A. m. ligustica* remains uncertain. In this study, we analyzed whole-genome data to compare these two refugial honeybee subspecies by addressing the following questions: (i) Have recent anthropogenic activities disrupted the genetic structure of both subspecies? (ii) Is there evidence of recent introductions of non-native queens? (iii) Do the two subspecies differ in genetic diversity and relatedness? (iv) Is there evidence of bottlenecks? (v) Do the bottlenecks coincide with varroa arrival? (vi) Are there detectable signatures of local adaptation, despite the beekeeper-driven pressures? (vii) If yes, (viii) which genes are putatively involved, and is there evidence of adaptive convergence?

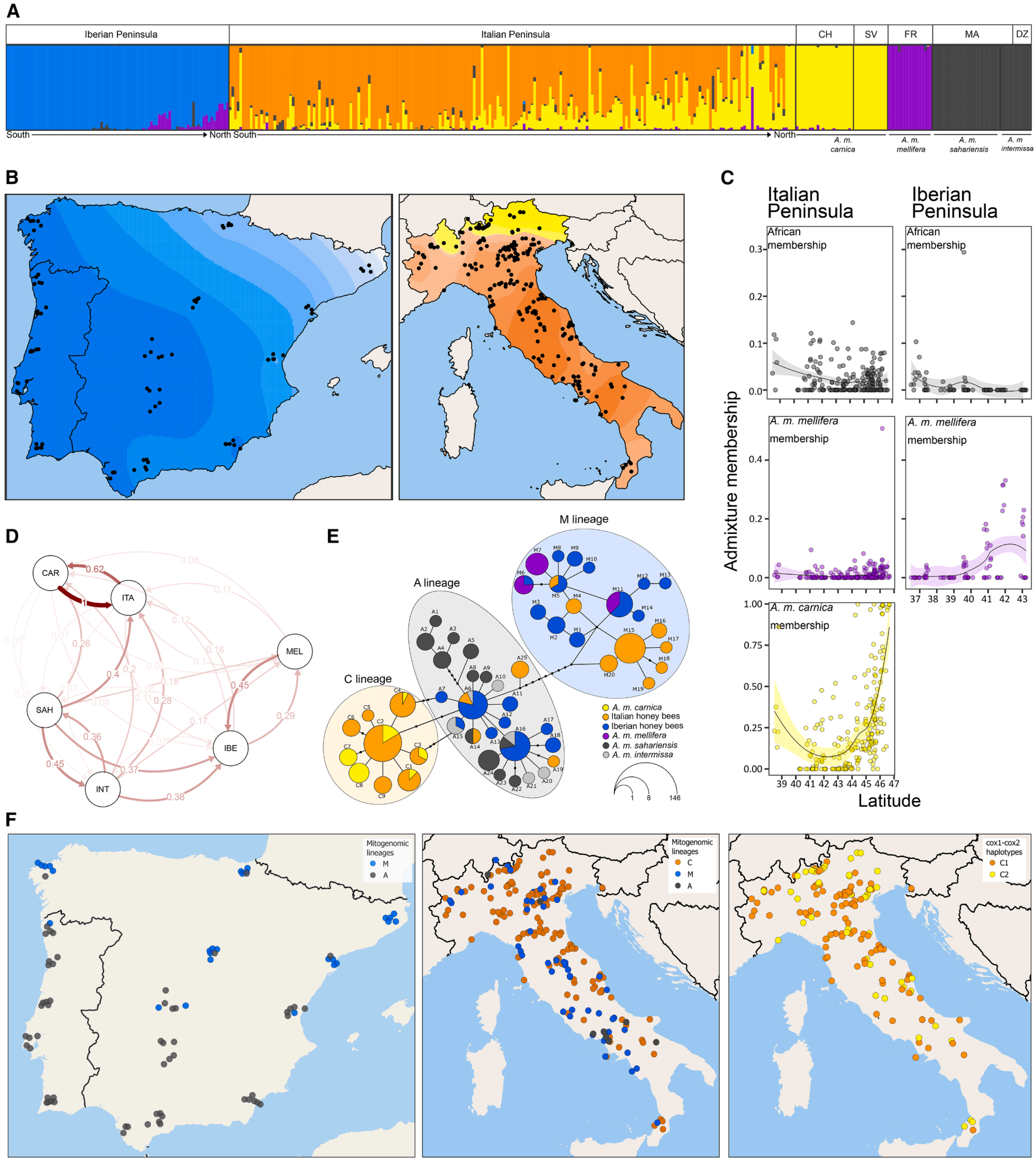

Figure 1. Population structure inferred from whole nuclear and mitochondrial genomes

(A) Population structure estimated by ADMIXTURE analysis at the optimal K = 5. Sampling sites across the Iberian and Italian Peninsulas are ordered from south to north. Each vertical bar represents an individual, with colored segments indicating their proportional ancestry in each cluster.

(B) Color gradients represent ancestry proportions inferred from ADMIXTURE and interpolated by *tess3r*, in which darker colors indicate a stronger assignment to the corresponding cluster, as represented in the ADMIXTURE plot.

(C) Percentage of ancestral membership of non-native subspecies in the Italian Peninsula (left) and in the Iberian Peninsula (right) as a function of latitude; A-lineage *A. m. sahariensis* and *A. m. intermissa* (dark gray), M-lineage *A. m. mellifera* (purple), and C-lineage *A. m. carnica* (yellow line). Colored lines represent LOESS-smoothed trend curves with 95% confidence intervals (shaded areas).

(legend continued on next page)

Determining to what extent contemporary anthropogenic processes have impacted natural patterns of genetic diversity and adaptation potential is important for understanding the long-term consequences of beekeeper-driven evolution on honeybee diversity and informing strategies to mitigate potential detrimental impacts.

RESULTS

Genomic population structure

The structure inferred by ADMIXTURE from the nuclear genomes for the populations sampled in the Iberian and Italian Peninsulas is shown for the optimal K in Figure 1A (see Figure S1 for genome partitioning up to K = 9). Based on the cross-validation error (Figure S2), the optimal model fit for all 391 analyzed genomes was K = 5, corresponding to the native subspecies from both peninsulas as well as the reference subspecies from north Africa (A-lineage *A. m. sahariensis* and *A. m. intermissa),* from France (M-lineage *A. m. mellifera*), and from Slovenia and Switzerland (C-lineage *A. m. carnica*). Both Iberian and Italian individuals exhibited varying levels of mixed ancestry, roughly following a northeast-south cline (Figures 1B, 1C, S3, and S4). However, admixed ancestry was significantly higher in Italian (median = 0.21; IQR = 0.07–0.40) than in Iberian honeybees (median = 0.01; IQR = 0.00–0.07; *p*-value = 1.77×10^{-10}; Wilcoxon rank-sum test).

At K = 5, the Iberian genomes were partitioned into two major components. One component (blue) was dominant, with 81.2% of the individuals exhibiting Q-values >0.90. The other component (purple) was shared with the reference M-lineage *A. m. mellifera* and was present in all individuals from the northeastern side of Iberia, where it reached its highest proportions (Q-value = 0.33; Table S2). Conversely, except for a single individual from northern Italy, which exhibited a Q-value of 0.51 (Figure 1A), M-lineage ancestry was residual in the Italian genomes, with a median of 0.00 (IQR = 0.00–0.01; Figures 1A and 1C and Table S2). This contrasts with the substantial shared ancestry with the neighboring *A. m. carnica* (median = 0.17; IQR = 0.04–0.37), particularly in the northeast, where 15 Italian individuals could not be distinguished from the Slovenian and Swiss reference individuals (Q-values in the yellow cluster >0.90). Substantial admixture was also detected in the southern half of the Italian Peninsula, where 18 individuals had Q-values >0.10 in the yellow cluster (Figures 1B and 1C). Furthermore, a notable African-derived component (Q-value >0.10) was detected in nine individuals, including one resembling an F1 hybrid (Q-value = 0.39). Interestingly, the African contribution (median Q-value = 0.01) to the Italian gene pool exceeded that of the M-lineage (median Q-value = 0.00) and was more prominent in the south, gradually decreasing toward the north (Figure 1C). Moreover, it surpassed the African contribution observed in the Iberian gene pool (median Q-value = 0.00; Figure 1C). Overall, of the 216 Italian individuals, only 68 (31.5%) had Q-values higher than 0.90 in the orange cluster (Figure 1A; Table S2), possibly representing an *A. m. ligustica* component.

The divMigrate analysis revealed two main gene flow trajectories: the first between *A. m. carnica* and Italian honeybees, and the second between *A. m. mellifera* and Iberian honeybees (Figure 1D). The first trajectory represents the strongest and most asymmetric gene flow, with a marked directionality from *A. m. carnica* into Italian honeybees (gene flow 1.00 from *A. m. carnica* into the Italian sample, and 0.62 in the opposite direction). The second trajectory shows a moderate gene flow with slightly higher values from *A. m. mellifera* into Iberian honeybees (gene flow 0.45 from *A. m. mellifera* into the Iberian sample and 0.29 in the opposite direction). Beyond these two trajectories, considerable gene flow from North African subspecies was detected into the Iberian honeybees (0.38 from *A. m. intermissa* and 0.37 from *A. m. sahariensis*) and, to a lesser extent, into the Italian honeybees (0.28 from *A. m. intermissa* and 0.40 from *A. m. sahariensis*).

Geographical patterns were also inferred for mitogenome-wide SNPs and *cox1-cox2* length polymorphisms. The Median-Joining network constructed from 383 mitogenomes revealed three major haplogroups, fully matching lineages M, C, and A, earlier identified by the popular *cox1-cox2* marker (Figure 1E). In contrast, only a partial overlap was observed between the network nodes and the *cox1-cox2* haplotypes (Figure S5), likely reflecting the differing evolutionary dynamics of coding SNPs and the intergenic spacer.[59] Iberian individuals were assigned to lineages A (68.2%) and M (31.8%) in a geographical pattern resembling the cline captured by nuclear variation (Figure 1F). Mitonuclear concordance was less evident in the Italian Peninsula. While the majority (72.1%) of the Italian mitogenomes clustered within the C-lineage haplogroup, M-lineage maternal ancestry was also relatively common (23.6%), as indicated by both maternal markers (Table S3). This contrasts with the residual membership proportions assigned to the M cluster (Figure 1A; Table S2). Notably, neither M- nor C-lineage variation displayed clear phylogeographic structuring, regardless of the marker (Figure 1F). The same applied to the two most abundant C-lineage *cox1-cox2* haplotypes, C1 (47.6%) and C2 (24.5%; Figure 1F; Table S3). The detection of 4.3% African mitogenomes adds further complexity to the Italian maternal gene pool. Most of these were closely related to the African mitogenomes identified in the Iberian Peninsula (Figure 1E). In contrast, the Italian mitogenomes of M-lineage ancestry clustered into a star-shaped sub-haplogroup that was divergent from the Iberian

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(D) Migration network inferred with the *divMigrate* function using Gst estimates in the R package *diveRsity*. Each node represents a population, with closer nodes indicating higher gene flow. Arrow color intensity reflects relative migration rates, with stronger colors indicating higher rates. SAH, *A. m. sahariensis;* INT, *A. m. intermissa;* CAR, *A. m. carnica;* MEL, *A. m. mellifera;* IBE, Iberian; ITA, Italian.

(E) Median-Joining network inferred from mitogenomes, revealing three major clusters corresponding to lineages A (gray), C (orange), and M (blue). Mitogenomic nodes were labeled by a letter corresponding to mitochondrial lineage, followed by sequential numerals.

(F) Geographic distribution of mitochondrial lineages inferred from the median-joining network analysis (maps on the left and center) and of the C1 and C2 haplotypes identified using the *cox1-cox2* marker (map on the right). The maps in (B) and (F) were created using R (v. 4.4.1) with the data from *rnaturalearth* package and QGIS (v.3.28.8) with the Natural Earth Quick Start Kit (https://naciscdn.org/naturalearth/packages/), respectively.

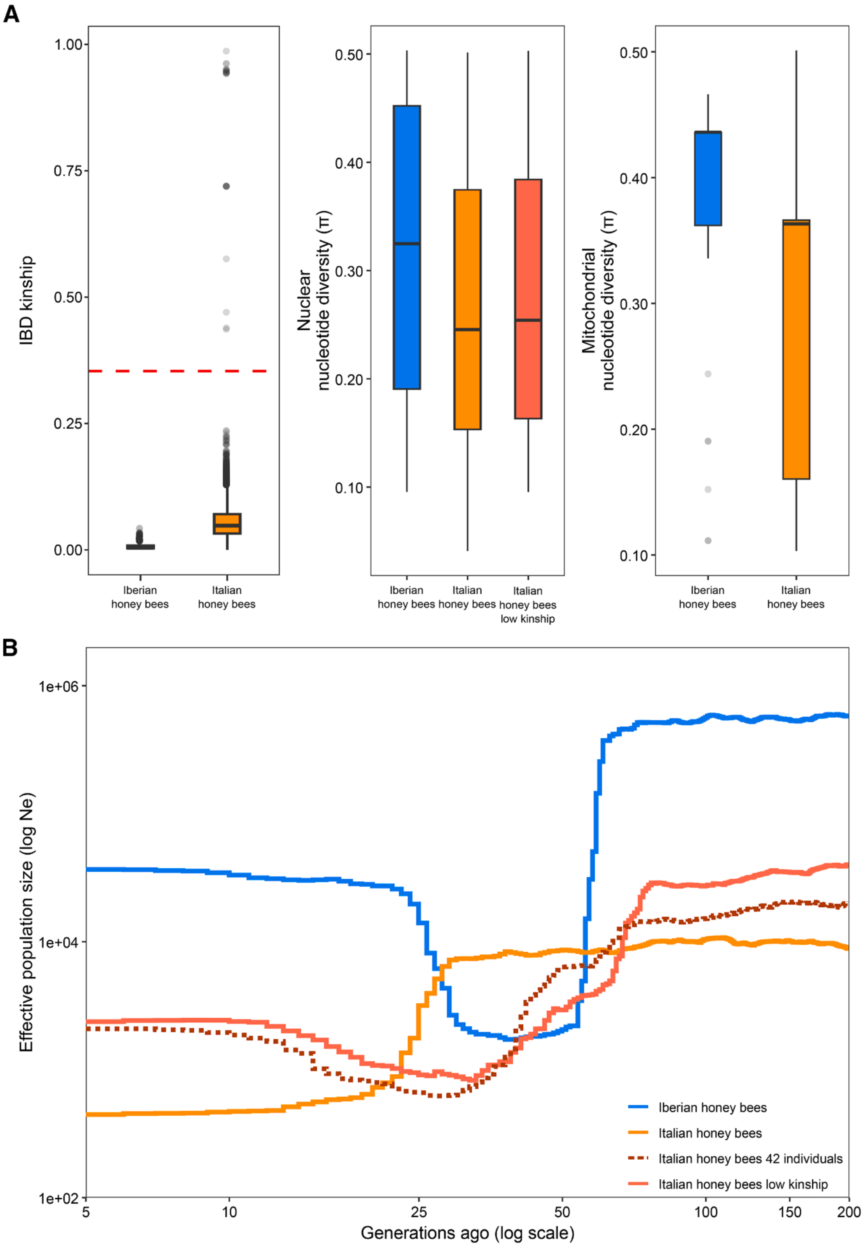

Figure 2. Kinship, genomic diversity, and recent demographic changes

(A) Identity-by-descent (IBD) kinship was estimated for all pairs of individuals within the Iberian and Italian datasets using the hmmIBD software. Boxplots show the median (center line), interquartile range (box), and whiskers extending to 1.5× the interquartile range; points represent outliers. The dashed red line represents the threshold between second- and first-degree kinship ($1/2^{1.5}$). Nucleotide diversity (π) was estimated using the *–site-pi* function in VCFtools for nuclear and mitochondrial genomes of both subspecies, based on the complete datasets and a subset of 201 Italian individuals with kinship below the first-degree threshold as analyzed by hmmIBD.

(B) Linkage disequilibrium (LD)-based effective population size (Ne) over the last 200 generations, as inferred with GONE (40 internal replicates), for the complete Iberian dataset (48 pseudodiploids; solid blue line) and the Italian dataset (108 pseudodiploids; solid orange line). To assess the influence of sample size and elevated kinship on the demographic trajectory of Italian honeybees, two additional datasets were analyzed with GONE: (i) 100 pseudodiploids randomly generated from the 201 remaining Italian individuals, after removing the 15 individuals involved in high-kinship pairs (solid red line), and (ii) 42 randomly generated pseudodiploids (dashed brown line), closely matching the sample size of the Iberian dataset.

M-lineage sub-haplogroup, with the central M15 node corresponding predominantly to the M7 haplotype identified by the *cox1*-*cox2* marker (Figure S3).

Genomic kinship and diversity

Kinship, assessed using identity-by-descent (IBD) implemented in hmmIBD for pairs of individuals, was significantly higher (p-value = 2.2×10^{-16}, Wilcoxon rank-sum test) among the individuals from the Italian Peninsula (median = 0.048; IQR = 0.032–0.071) than among those from the Iberian Peninsula (median = 0.005; IQR = 0.003–0.008; Figure 2A). The highest kinship value was observed in a pair of individuals from the Italian Peninsula that were geographically distant (∼290 km). This pair shared 0.99 of their genomes, far exceeding the theoretical value of 0.75 for full sisters in honeybees. Moreover, 15 Italian individuals were frequently involved in pairs exceeding the 0.3536 ($1/2^{1.5}$) threshold between first- and second-degree kinship,[30] whereas the maximum shared IBD fraction in Iberian pairs was 0.04.

Nucleotide diversity (π) was estimated from nuclear and mitochondrial whole genomes for both Iberian and Italian honeybees (Figure 2A). Genomic diversity was significantly higher in the Iberian Peninsula than in the Italian Peninsula (p-value $<2.2 \times 10^{-16}$, Wilcoxon rank-sum test) in both the nuclear (median = 0.325; IQR = 0.190–0.452 and median = 0.245; IQR = 0.153–0.375, respectively) and mitochondrial compartments (median = 0.436, IQR = 0.362–0.436 and median = 0.363; IQR = 0.160–0.366, respectively). To account for potential bias from highly related individuals, nuclear diversity in the Italian dataset was recalculated after removing one individual from each pair with kinship >0.3536. This adjustment led to a slight increase in nucleotide diversity in Italian honeybees (median = 0.255; IQR = 0.163–0.385).

Recent demographic history

Estimation of effective population size (N_e) over the past 200 generations, using the linkage disequilibrium-based approach

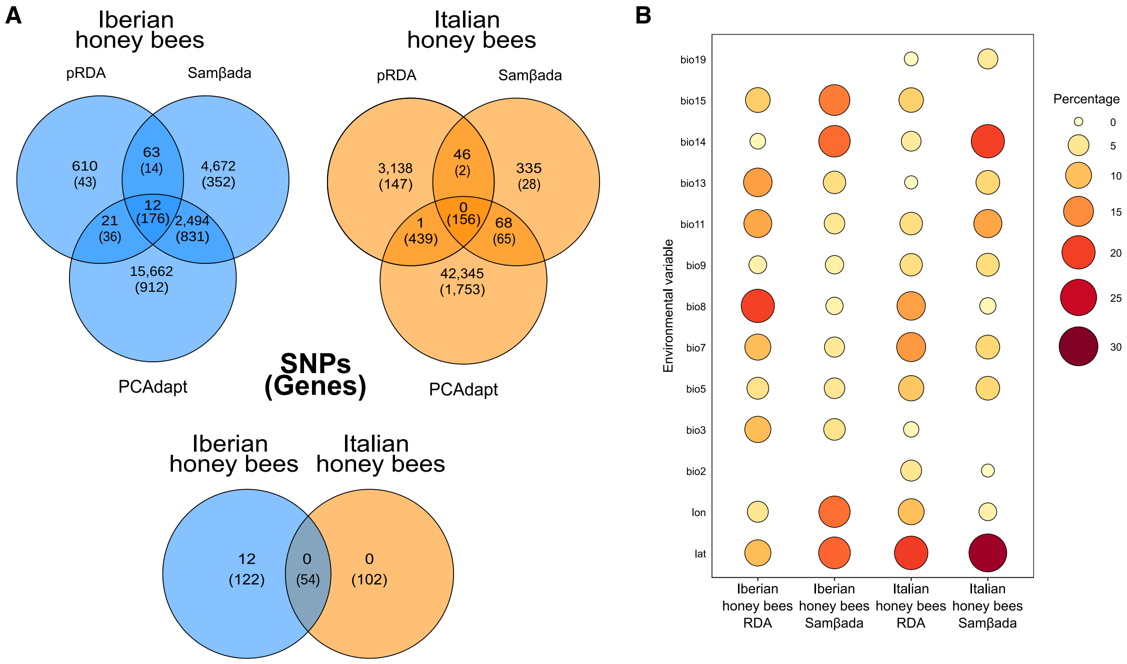

Figure 3. Candidate adaptive SNPs and genes

(A) Venn diagram of the number of outlier SNPs and annotated genes (in parentheses) identified for the Iberian and Italian datasets by Samβada, pRDA, and PCAdapt (top diagrams) and the overlap between both datasets (bottom diagrams).

(B) Proportion of candidate SNPs associated with each environmental variable as detected by Samβada or pRDA in each dataset. lat, latitude; lon, longitude; bio3, isothermality; bio5, max temperature of warmest month; bio7, temperature annual range; bio8, mean temperature of wettest quarter; bio9, mean temperature of driest quarter; bio11, mean temperature of coldest quarter; bio13, precipitation of wettest month; bio14, precipitation of driest month; and bio15, precipitation seasonality.

implemented by GONE, revealed distinct demographic trajectories (Figure 2B). In the Iberian Peninsula, *Ne* remained relatively stable until ∼60 generations ago (median for generations 200 to 60 = 547,626; IQR: 524,970–574,661), when a severe bottleneck occurred, lasting ∼40 generations. The most severe period of this bottleneck occurred between 29 and 53 generations ago (median for generations 53 to 29 = 1,865; IQR: 1,787–2,045). N_e then gradually recovered (median for generations 20 to 1 = 32,103; IQR: 29,968–35,133), reaching 5.86% of its pre-bottleneck levels (Table S4). In the Italian Peninsula, *Ne* was consistently lower than in the Iberian Peninsula and remained relatively stable until a decline began ∼30 generations ago (median for generations 200 to 30 = 9,630; IQR: 8,822–9,937). In contrast to the Iberian honeybees, the N_e of the Italian honeybees did not recover, stabilizing at lower levels (median for generations 19 to 1 = 463; IQR: 438–550). Excluding Italian individuals from pairs with kinship >0.3536 (resulting in a sample size of 201) led to a substantial increase in the *Ne* before the bottleneck (median for generations 200 to 77 = 33,940; IQR: 28,339–36,434) and shifted the bottleneck ∼47 generations earlier. A slight recovery occurred between 30 and 12 generations ago, when *Ne* stabilized (median for generations 12 to 1 = 2,325; IQR: 2,295–2,391). Downsizing the low-kinship Italian dataset to match the size of the Iberian dataset resulted in a trajectory similar to that observed when all low-kinship individuals were included.

Candidate genes underlying genomic adaptation

The genomes of Iberian and Italian individuals were scanned for selection footprints using two genotype-environment association (GEA) methods (Samβada and RDA) and a frequency-based method (PCAdapt). The total number of SNP outliers (q-value <0.05) detected by each method and their overlaps are shown in Figure 3A (see Tables S5, S6, S7, S8, S9, and S10 and Figures S6–S11 for details). The GEA methods revealed different proportions of SNPs: in Iberian honeybees, Samβada detected 7,241 (27.7%) and pRDA 706 (2.7%), while in Italian honeybees, Samβada detected 449 (1.0%) and pRDA 3,185 (6.9%). These were associated with a varying number and proportion of environmental variables (Figure 3B). From an initial set of 94 variables, those with pairwise correlations of $|r| > 0.8$ were excluded, resulting in 11 and 13 retained for selection analysis in the Iberian and Italian datasets, respectively (see Tables S5, S6, S8, and S9). Latitude was among the most frequently associated variables, particularly in Italian honeybees (Samβada: 20.38%; pRDA: 28.05%). Other commonly associated variables across both peninsulas included the temperature-related bio7 (temperature annual range), bio8 (mean temperature of the wettest quarter), and bio11 (mean temperature of the coldest quarter), as well as the precipitation-related bio14 (precipitation of the driest month).

Of the total number of outlier SNPs detected by all three methods (26,136 SNPs in the Iberian and 46,048 in the Italian dataset), PCAdapt contributed the highest proportion: 69.6%

(18,189) in Iberian honeybees and 92.1% (42,414) in Italian honeybees (Figure 3A). When superimposing the detections from all three methods, only 12 SNPs were jointly identified in the Iberian honeybees, and no intersection was detected for Italian ones (Figure 3A). This contrasts with the large number of overlapping genes (176 in Iberian honeybees and 156 in Italian honeybees), consistent with multiple independently detected SNP outliers mapping to the same genes (Tables S5, S6, S7, S8, S9, S10, and S11). Notably, 54 genes were shared between the two honeybee origins, with a median of 107 SNPs per gene (IQR: 50–174). While the vast majority of the candidate SNPs in these 54 genes (7,000, 99.15%) were intronic (Table S11), a small proportion were located within 1 kb upstream of transcription start sites (1, 0.01%) or in coding regions (59, 0.81%), including 55 (0.76%) synonymous and four (0.06%) missense variants, three mapped to gene GB54884 and one to gene GB47036 (Table S11). The 54 genes were associated with several environmental variables, most frequently latitude, longitude, temperature annual range (bio7), and precipitation seasonality (bio15).

To identify overrepresented GO terms within the 54 overlapping genes, a gene enrichment analysis was performed. From these 54 genes, 39 (72.22%) were associated with GO terms, whether enriched or not (Table S11). Two GO terms were significantly (p-value <0.05) enriched: calcium ion binding (GO:0005509) and nervous system development (GO:0007399). The latter term included six genes (GB54884, GB55923, GB55213, GB46325, GB54537 and GB52002), while the former included three (GB55670, GB53701, and GB42603). Although not part of any enriched term within these 54 genes, SNP outliers were also detected in genes involved in endocrine and neuroendocrine regulation (GB42577, GB49696, and GB49166), sensory development (GB43476), circadian regulation (GB45054), thermal stress (GB49567), and transcription regulation (GB48919).

Gene enrichment analysis also revealed shared and unique GO terms between the Iberian and the Italian honeybees. GO terms were identified for 112 of 176 candidate genes in Iberian honeybees (63.64%) and for 107 of 156 in Italian honeybees (68.59%). The individual gene lists for both honeybee origins were significantly (p-value <0.05) enriched for genes involved in biological regulation, response to stimulus, cell communication, and signaling (GO:0065007, GO:0050789, GO:0050896, GO:0023052, respectively). However, biological process GO terms related to synaptic signaling (GO:0007267, GO:0099536, GO:0099537, GO:0007268, GO:0098916), cell surface (GO:0007166), and G protein-coupled receptors (GO:0007186) were exclusively enriched in Iberian honeybees. These included three α subunits of the nicotinic acetylcholine receptors (*nAChRa4*, *nAChRa6*, *nAChRa7*), an allatostatin receptor (GB54178), an epidermal growth factor (EGF) receptor (GB54477), and two octopamine receptors (GB52879, GB49696). In contrast, the biological process GO terms exclusively enriched in Italian honeybees were related to developmental processes (GO:0032502), anatomical structure development (GO:0048856), and intracellular receptor signaling (GO:0030522). The development processes term included the genes GB18813, GB46487, GB52279, GB42603, and GB46128, whereas the intracellular receptor signaling term primarily included genes involved in the downstream ecdysone signaling pathway (GB48059, GB11364). Molecular function GO terms also revealed major differences between the two honeybee origins. In Iberian honeybees, there was significant (p-value <0.05) enrichment in molecular activities related to transmembrane signaling and signal transduction (GO:0007165), particularly those mediated by G protein-coupled receptors (GO:0007186) and gated ion channels (GO:0015276, GO:0022834, GO:0022824, GO:0022835). In contrast, enriched molecular functions in Italian honeybees were related to binding (GO:0005488), especially calcium ion binding (GO:0005509), and to serine peptidase activity (GO:0008236, GO:0017171).

DISCUSSION

While the evolutionary history of European honeybees is linked to the southern glacial refuges, their modern history has been shaped by human activity, primarily driven by honey production. Anthropogenic processes have been implicated in honeybee hybridization with conspecifics and often in a reduction in genetic diversity. However, the extent of their impact varies across the honeybee range. In this study, we explored the relative roles of historical and human-mediated processes in shaping extant genetic patterns of honeybees from the Iberian and Italian Peninsulas.

Human-driven disruption of population genetic structure was more pronounced in the Italian than in the Iberian Peninsula

Genome-wide SNP data from nuclear and mitochondrial compartments revealed an approximate south-to-northeast clinal structure in honeybees originating from the Iberian and Italian Peninsulas (Figures 1A and 1B). While evolutionary processes driven by Pleistocene climatic oscillations played a central role in shaping the present-day genetic structure of honeybees from both peninsulas,[15,17] accumulating evidence indicates that recent anthropogenic influences are increasingly erasing the genetic imprints of past evolutionary dynamics, more markedly in Italian than in Iberian honeybees.

Clinal variation was first detected in the Iberian Peninsula using morphological,[60] allozyme,[11,61] and mitochondrial markers.[11,14,62,63] More recently, it was confirmed by a reduced SNP panel[15] and by genome-wide mitochondrial and nuclear SNPs (this study and Henriques et al.[59]). Remarkably, despite intensified beekeeping practices over recent decades, the shape of the honeybee cline closely resembles that reported for wild taxa,[64,65] and references therein), suggesting that the genetic patterns shaped by historical processes had persisted with minimal disruption, at least until 2010 when the Iberian sample was collected.

Whether beekeeping activity has since blurred the clinal signal in the Iberian Peninsula is uncertain, but it appears unlikely for two main reasons. First, diversity patterns inferred from morphology,[66] allozymes,[11,61] and mitonuclear SNPs[15] indicate that the long-standing tradition of migratory beekeeping had not led to a homogenization of the Iberian gene pool, despite the seasonal movement of over a million colonies across long distances each year.[15] This may reflect local mating prior to transhumance and/or selection against crosses between resident and migrant colonies. Second, the importation of foreign subspecies and large-scale queen rearing remains uncommon in Iberia (A. G. Pajuelo, personal communication), as most beekeepers prefer the native *A. m. iberiensis* over commercial breeds, relying on traditional reproduction through colony splitting and natural mating in drone congregation areas.

The migration network (Figure 1D) further confirms these results, showing considerable gene inflow primarily from neighboring M-lineage *A. m. mellifera*, probably as a result of the secondary contact zone along the Pyrenees,[15] and, to a lesser extent, from north African subspecies, which is consistent with previous findings.[14,67] Only a negligible signal of gene flow from C-lineage honeybees was detected, reinforcing the view that honeybees in the Iberian Peninsula have remained relatively protected from large-scale introgression due to beekeeping activities.[54]

In contrast to the honeybees from the Iberian Peninsula, our mitochondrial and nuclear data suggest that the Italian gene pool has undergone substantial anthropogenic change (Figure 1), at least since the 1990s, when the first comprehensive genetic survey was undertaken in Italy.[17] Over the past two decades, the frequency of M-lineage variants has markedly declined, from 50.3% to 23.6% in the mitochondrial compartment, as inferred from the *cox1–cox2* marker, and from 20% to 2% in the nuclear compartment, as inferred from microsatellites and genome-wide SNPs, respectively (Franck et al.[17] versus this study). While mitochondrial estimates are directly comparable between these two surveys, nuclear estimates are not, due to the different evolutionary dynamics of SNPs and microsatellites and how they might affect ancestry estimations,[68–70] with the former providing greater accuracy.[71,72] Despite this limitation, the two genomic compartments consistently reflect a recent M-lineage decline, aligning with a recent maternal assessment based on eDNA extracted from honey.[73] This suggests that the M-lineage signature in the genome of the subspecies native to the Italian Peninsula, *A. m. ligustica*, which traces back to the mid-Riss glaciation,[17] is being progressively erased and replaced by other ancestries. Nevertheless, M-lineage haplotypes remain relatively common and widespread, contrasting with the modest contribution of this ancestry to the nuclear gene pool, even in colonies from the westernmost area of the Alpine arc, where a natural hybrid zone had previously been described.[17,74–76] The observed mitonuclear asymmetry, first noted by Franck et al.,[17] is likely of historical origin, as it has also been documented in the *A. m. ligustica* sanctuary on Kangaroo Island, Australia, established in the 19th century with stock imported from Italy.[77,78] Different non-mutually exclusive mechanisms could account for ancient mitonuclear discordance, including incomplete lineage sorting, adaptive introgression of mitochondrial DNA, sex-biased dispersal, and hybrid zone movement (see Toews and Brelsford,[79] and references therein, as well as subsequent studies citing their work). However, recent anthropogenic processes, including the selective breeding of beekeeper-favored C-lineage phenotypes and their large-scale dissemination,[54] have likely exacerbated the asymmetry between the two genomic compartments in recent decades.

Unlike M-lineage haplotypes, A-lineage haplotypes were virtually absent in the 1990s survey, detected in only one southern sample (0.18%; Franck et al.[17]), but now account for 4.3% (Figures 1E and 1F), or even up to 14%, according to the eDNA survey in honey.[73] However, this is likely an overestimate, as eDNA is not a truly quantitative method. Interestingly, signatures of African ancestry were also observed in the nuclear genomes, slightly more pronounced in Italian than in Iberian honeybees (Figure 1D). This was unexpected because African mitochondrial haplotypes remain uncommon in the Italian Peninsula, whereas in the Iberian Peninsula they are predominant due to an ancient expansion from Africa.[80,81] Also surprising is that A-lineage ancestry exceeded M-lineage ancestry in the nuclear genomes of the Italian honeybees, while mitochondrial data show the opposite. Two non-mutually exclusive explanations may account for the African influence currently observed in the Italian Peninsula. First, African ancestry may have recently reached the Peninsula through human-mediated gene flow from Sicily, Malta, or Africa, resulting in a patchy distribution of A-lineage haplotypes. However, this seems unlikely, as A-lineage subspecies are usually perceived to possess undesirable traits (e.g., heightened defensiveness and a strong tendency to swarm) that are avoided by the beekeeping industry. A more plausible scenario is the unintentional introduction of African-derived genes through commercial imports of queens marketed as C-lineage stock from South America, mainly Argentina, where Africanized honeybees are present, or through Buckfast queens from Europe, an artificial composite breed developed using crosses that sometimes include African subspecies.[73,82–85] Second, the low but pervasive African signal in the nuclear gene pool of the Italian honeybees, which follows a south-to-north gradient, could reflect an ongoing expansion from Sicily, home to the African subspecies *A. m. siciliana*, with natural selection favoring African alleles in the dry, warm climate of southern Italy.

The most notable change in the Italian gene pool is the emergence of the *cox1–cox2* C2 haplotype, characteristic of *A. m. carnica*.[17,86] Remarkably, C2 was not detected by Franck et al.[17] in any region of Italy, including the natural contact zone in the northeast, where hybridization between *A. m. ligustica* and *A. m. carnica* had previously been identified, initially using morphometrics and allozymes,[87–89] and, later on, microsatellites.[33] Its undetection in the 1990s survey, even in the hybrid zone,[17] may reflect mitonuclear asymmetry, similar to that observed for the M-lineage, or simply result from stochastic sampling effects. Regardless of the cause, C2 appears to have been rare two decades ago and has since become both common (24.5%) and widespread across the Italian Peninsula (Figure 1F). This remarkable expansion has contributed to the decline of M-lineage haplotypes, particularly the most frequent and *A. m. ligustica*-specific M7 (central node M1 and its adjacent nodes in the MJ-network), which diverged from the concurrently major Iberian M4 about 190,000 years ago.[17] If C-lineage ancestry continues to be favored by Italian beekeepers,[73,90] this ancient mitochondrial variant may become relictual in the *A. m. ligustica* gene pool.

Nuclear introgression from *A. m. carnica* (29%) closely paralleled the frequency of the C2 haplotype (24.4%), consistent with biparental gene flow. Introgression was highest in the northeast, where the two C-lineage subspecies come into natural contact, but it was also widespread across the peninsula. Although honeybees are capable of rapid, long-distance dispersal (e.g., Africanized honeybees colonized the Americas in just a few decades[91]), the observed introgression pattern is more parsimoniously explained by human-mediated gene flow. This likely involved the dissemination of introgressed queens through domestic trade networks and/or the importation of *A. m. carnica* and Buckfast lines, which have increased in recent years.[58] Regardless of the source, the overall trend is clear: the genome of *A. m. ligustica* is undergoing genetic dilution. In Europe, conservation concerns have focused on *A. m. mellifera*, *A. m. siciliana*, and *A. m. ruttneri*, all threatened by introgression primarily from *A. m. ligustica*.[92–94] However, our study reveals that, ironically, even *A. m. ligustica*, long regarded as a cornerstone of global apiculture, is not immune to the erosion of its genetic identity and now warrants targeted conservation efforts.

Historical and contemporary signals of reduced genetic diversity in *A. m. ligustica*

Because of their haplodiploid mating system and social organization, honeybees are particularly sensitive to inbreeding depression. Pathogen load,[95] productivity,[96,97] survivorship,[98] thermoregulation,[99] and homeostasis[100] have all been linked to the intracolonial genetic diversity. Therefore, maintaining a high level of genetic diversity is critical and challenging in any stock improvement program, which especially applies to honeybees.

Several studies support the hypothesis of a recent and sustained decline in *A. mellifera* genetic diversity, particularly among C-lineage subspecies, a process that has been linked to domestication.[22,30,101] Our estimates of diversity, kinship, and recent historical *Ne* trajectories for both Iberian and Italian honeybees further reinforce the previous assertion that the extant genetic patterns of *A. m. ligustica* warrant attention. Italian individuals exhibited high kinship levels, yet this appears to have only a limited impact on nucleotide diversity (π). Indeed, removing individuals more frequently involved in remarkably high-kinship pairs (those contributing most to the overall elevated kinship) resulted in only a slight increase in π. The reduced genetic diversity and elevated relatedness among individuals from the Italian Peninsula underscore the need for precautionary management in Italian beekeeping (Figure 2A).

Historically, the Iberian Peninsula has had a higher number of colonies than the Italian Peninsula,[53] supported by an area approximately 3.9 times larger. GONE analyses partially reflected such a difference between populations through the estimation of historical effective population sizes (*Ne;* Figure 2B). The historically lower *Ne* in the Italian Peninsula may have established a baseline contributing to the lower contemporary diversity values, as reflected in π. In addition to potential natural causes, this long-term reduced genetic diversity might be related to a combination of factors, including the aforementioned characteristics of Italian beekeeping, relatively small and geographically constrained breeding stock compared to the high number of queens produced, and high rates of migratory activities, which might have contributed to homogenizing local diversity.

While the evolution of the number of registered hives can be a poor, completely independent indicator of the *Ne* trajectories, investigating historical episodes of massive colony losses may help clarify potential drivers. Assuming a generation time of one year for the queen and considering that Iberian colonies were sampled in 2010, the bottleneck for Iberian honeybees can be dated between 1950 and 1990. At least the initial period of this event coincides with the entrance of *Acarapis woodi* into the Iberian Peninsula, around 1948, where it caused significant losses.[102] This bottleneck could also be associated with the sharp decline in the number of managed colonies registered during the Spanish Civil War due to the widespread abandonment of beekeeping practices.[103]

For the Italian honeybees, the bottleneck likely began around 1991, as inferred from the full dataset developed from colonies sampled in 2021. A factor possibly accounting for the population decline may be related to the introduction of varroa. First detected in 1981 near the border with Slovenia, it spread during the following years to the rest of the Italian Peninsula, causing colony losses as high as 65% in some apiaries.[47,104] However, *Ne* estimates proved sensitive to the inclusion of high-kinship individuals, indicating that recent beekeeping practices have influenced the demographic inference. When these high-kinship individuals were excluded from the analysis, the bottleneck was traced to approximately 77 generations ago, dating back to 1944. During this period, no reports related to apicultural pathogenic outbreaks are available in Italy. Although there are no results from formal investigations, the presence of *A. woodi* in Italy was reported anecdotally during the 1920s.[105] Yet, it is still possible that a misidentification occurred and that *A. woodi* arrived on the Italian Peninsula at around the same time as on the Iberian Peninsula.[102,105] Another possible cause of the 1940s bottleneck is the decline of Italian colonies that occurred as a consequence of World War II, which nearly halved the number of existing colonies.[106]

If we consider the recent demographic history inferred from low-kinship individuals to be more realistic than that derived from the full Italian dataset, which includes high-kinship individuals, then varroa does not appear to have had a drastic impact on the *Ne* of either subspecies. This is consistent with census size data, which did not report major population declines during the 1980s in either the Italian or Iberian Peninsulas.[53] This may be related to greater preparedness among beekeepers and the availability of chemical treatments, in contrast to earlier decades, when, during the spread of *A. woodi*, such control measures were lacking.

Common paths of environmental adaptation

Among the 54 genes identified as candidates for local adaptation to the climate of both Peninsulas, nervous system development was the only enriched biological process. This GO term includes genes such as the RNA-binding protein *fox-1 homolog 2* (GB55760), the *Dscam3* (GB53701), and *NLG-3*(GB42603). *NLG-3*, a member of the neuroligin protein family, is a member of the neurexin-neuroligin synaptogenic complex, a heterophilic adhesion system that organizes pre- and post-synaptic compartments.[107] What is more relevant is that several previous studies have linked members of this complex to adaptation in several *A. mellifera* subspecies.[108,109] Far beyond basic synapse formation, these complexes are known to influence sensory regulation and memory in honeybees,[110,111] and behavioral plasticity and social behavior in other insects.[112,113]

Although not included in the enriched biological process, endocrine and neuroendocrine regulation emerged as a central axis of environmental adaptation, as evidenced by environmentally associated SNPs in genes coding for key receptors for dopamine (GB42577), octopamine (GB49696), and diuretic hormone (Dh44-R1, GB49166). These molecular pathways govern behaviors intimately tied to environmental response: circadian rhythms,[114,115] locomotor activity,[116] foraging,[117] or memory formation.[118] Especially notable is the octopamine receptor (GB49696), which is located within a genomic region previously linked to altitude adaptation in honeybees.[119]

Candidate SNPs were also detected in genes involved in DNA transcription (GB48919) and mRNA splicing (GB55760), both previously associated with cold adaptation in *Leptinotarsa decemlineata* and *Spodoptera frugiperda*.[120,121] Environmentally associated variants were also detected in the gene encoding the olfaction-related protein alpha-Mannosidase class Ia, whose orthologues in *Bombus vancouverensis* and *Rhizoglypus robini* have been linked to climatic adaptation.[122,123]

When conducting the gene enrichment analysis in the candidate gene sets specific to honeybees from both the Iberian and Italian Peninsulas, biological processes including biological regulation, response to stimulus, cell communication, and signaling (GO:0065007, GO:0050789, GO:0050896, and GO:0023052, respectively) were commonly enriched. The identification of these shared candidate genes and enriched GO terms highlights a clear pattern: despite their distinct genetic background and divergence time, honeybees of both geographical origins rely on common molecular pathways in their adaptation to local environments. This convergence underscores the central role of sensory and regulatory mechanisms in shaping climate adaptation. It also implies that different lineages may have maintained certain functional responses to climate conditions.

Subspecies-specific mechanisms of environmental adaptation

As expected, specifically enriched functions and processes were detected for each geographical origin. In the honeybees from the Iberian Peninsula, two prominent clusters of biological processes were enriched. The first involved synaptic transmission and included three α subunits of the nicotinic acetylcholine receptors (*nAChRa4, nAChRa6, nAChRa7*). Acetylcholine, the most abundant insect neurotransmitter,[124,125] plays a central role in *A. mellifera* physiology, coordinating sensory input, motor control, and responsiveness to water and sugar.[124,126,127] The second group of processes included cell-surface and G-protein coupled receptors, such as an allatostatin (GB54178), and two octopamine receptors (GB49696 and GB52879). Allatostatin signaling is implicated in a broad range of functions, including gut physiology, cardioactivity, food and water intake, carbohydrate sensing, locomotor activity, and sleep.[128] Moreover, specifically in honeybees, it is related to learning and stress response modulation.[129–131] On the other hand, octopamine in honeybees has been associated with behavioral traits including the division of labor, dance communication, and hygienic behavior.[132–134]

Honeybees from the Italian Peninsula also showed exclusively enriched biological processes: intracellular receptor signaling pathway, developmental process, and anatomical structure development. The first term included two ecdysone-related genes: one ecdysone receptor (GB48059) and the ecdysone-related protein 75 (GB11364). Ecdysteroids regulate key developmental transitions such as moulting, metamorphosis, and diapause,[135,136] but they also influence sleep, nutrition, and foraging behavior.[137–140]

The latter two enriched categories are associated with anatomical development and include genes such as *antennapedia* (GB18813) and *short gastrulation* (GB46487). Both genes are related to establishing body plans,[141,142] *antennapedia* specifically for the identity and development of the thoracic segment.[143,144] These two terms also included genes related to neural development, including *NLG-3* (GB52279) and *NRX-1* (GB42603); the roles of neurexin-neuroligin complexes and sensory and behavioral regulation were discussed above. *Sickie* (GB46128), which regulates axonal growth and has been linked to active forgetting in *Drosophila*, was also detected detected.[145,146]

This study uniquely shows how anthropogenic pressures have differentially altered the genetic structure of two divergent honeybee subspecies from Southern European glacial refugia. *A. m. iberiensis* exhibits a genetic pattern that more closely reflects its evolutionary history, due to relatively conservative beekeeping practices in the Iberian Peninsula. In contrast, *A. m. ligustica* exhibits stronger signatures of human-mediated genetic change, as indicated by the recent decrease of the mitochondrial M-lineage proportion and the reduced M-lineage membership, the high frequency and geographical spread of C2 mitotypes and *A. m. carnica* ancestry, the patchy presence of samples with African maternal ancestry, and the latitudinal cline of African-derived nuclear ancestry. These patterns suggest that human-mediated gene flow, likely driven by the introduction of non-native colonies and the diffusion of commercial stocks, is blurring the historical genetic structure.

Compared to *A. m. iberiensis*, *A. m. ligustica* consistently shows lower *Ne* across time and elevated relatedness, accompanied by lower diversity estimates. Bottleneck signatures are evident in both subspecies, coinciding with the occurrence of biotic threats. These findings suggest that long-standing demographic histories, together with pathogen pressures and apicultural practices, have played a central role in shaping the current genetic makeup of both subspecies.

The two subspecies exhibit common signatures of local adaptation, reflected in 54 shared candidate genes primarily associated with nervous system development, sensory processing, and regulatory pathways. This suggests a pattern of adaptive convergence despite their divergent evolutionary histories. Additionally, subspecies-specific analyses revealed exclusive functional enrichments, pointing to distinct mechanisms that evolved in each subspecies to cope with environmental challenges.

Limitations of the study

Although the overall genetic patterns revealed in this study are robust, one limitation lies in the spatial and temporal scope of the sampling, particularly for the Iberian population. To further strengthen our inferences and rigorously assess the alternative hypotheses outlined in the Discussion, future work will require an improved sampling design that incorporates several key features, including (1) synchronous sampling in both peninsulas to allow a more comparable and up-to-date assessment of the conservation status of the two subspecies; (2) a larger Iberian sample size, relative to the Italian sample, to reflect its broader geographic range and higher census size; (3) expanded sampling coverage in southern Italy to achieve finer resolution of the African component within the gene pool; and (4) additional African reference samples beyond *A. m. intermissa* and *A. m. sahariensis*, such as *A. m. siciliana* and sub-Saharan subspecies, particularly the ancestor of Africanized honeybees, *A. m. scutellata*, to enable a more robust inference of the origin of the African component detected in Italian honeybees and to better understand its potential role in adaptation to rapidly changing environmental conditions.

Another limitation is that the evolutionary inferences drawn here are based on managed honeybee colonies, which are subject to varying levels of human-mediated gene flow, selection, and breeding. These human-mediated processes may alter the trajectory of microevolutionary and adaptive processes relative to those in wild populations. Truly wild colonies are extremely rare in Europe, and identifying and sampling them across their natural range would be logistically very challenging. Consequently, our results should be interpreted within the context of managed systems, and caution is warranted when extrapolating to natural populations.

RESOURCE AVAILABILITY

Lead contact

- Requests for further information and resources should be directed to and will be fulfilled by the lead contact, M. Alice Pinto (apinto@ipb.pt).

Materials availability

- This study did not generate new unique reagents.

Data and code availability

- The raw DNA sequence data generated in this study have been deposited at the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under the SRA study and BioProject accessions PRJNA1305528, PRJNA1305509, and PRJNA1307299, respectively.
- Codes for Genotype-Environment association analyses are available on GitHub (https://github.com/cyadrogarcia/GEA_ligustica_iberiensis) with an archived snapshot on Zenodo (https://doi.org/10.5281/zenodo.16726609).
- Any additional information required to reanalyze the data reported in this article is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

M.A.P., R.M-H., A.N., and D.H. conceptualized the study. M.A.P., A.N., G.C., S.S., and A.A. collected the samples in the Iberian Peninsula, Italy and Morocco. C.A.Y.G. performed the DNA extractions. C.V. and M.Z-M. identified the *cox1-cox2* haplotypes of *A. m. ligustica*. C.A.Y.G. performed all bioinformatic analyses with the assistance of D.H. and J.R. R.M-H., A.N., and M.A.P. obtained the funding. C.A.Y.G. and M.A.P. wrote the article with input and approval from all the authors. All authors participated in the revision of the article.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT to improve the readability and language of the article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Apis mellifera ligustica	This paper	N/A
Apis mellifera sahariensis	This paper	N/A
Critical commercial assays		
Maxwell® RSC PureFood GMO and Authentication Kit	Promega	AS1600
Master Mix For PCR	Bio-Rad	1665009EDU
Deposited data		
Raw data of whole genome sequences of *A. m. mellifera*	Wragg et al.[147] 2022	PRJNA311274
Raw data of whole genome sequences of *A. m. carnica*	Wragg et al.[147] 2022	PRJNA311274
Raw data of whole genome sequences of *A. m. intermissa*	Henriques et al.[148] 2021	
Raw data of whole genome sequences of *A. m. iberiensis*	Henriques et al.[149] 2018	PRJNA1307299
Oligonucleotides		
Primer: COI_Seq-F: ACCACCTC TAGATCATTCACATTT	Alburaki et al.[150] 2023	N/A
Primer: COII_Seq-R: AGGATGGA ACTGTTCATGAATGAA.	Alburaki et al.[150] 2023	N/A
Software and algorithms		
bwa-mem2	Vasimuddin et al.[151] 2019	https://github.com/bwa-mem2/bwa-mem2
samtools 1.15	Danecek et al.[152] 2021	https://samtools.sourceforge.net/
picard 2.26.11	Broad Institute 2019	https://broadinstitute.github.io/picard/
GATK 4.2.5.0	Van der Auwera GA & O'Connor BD[153] 2020	https://gatk.broadinstitute.org/hc/en-us/sections/4418054201755-4-2-5-0
bcftools 1.15	Danecek et al.[152] 2021	https://github.com/samtools/bcftools
vcftools 0.1.16	Danecek et al.[154] 2011	https://vcftools.sourceforge.net/
Plink 1.9	Chang et al.[155] 2015	https://www.cog-genomics.org/plink/
admixture 1.3.0	Alexander et al.[156] 2009	https://dalexander.github.io/admixture/download.html
PONG 1.4.9	Behr et al.[157] 2016	https://github.com/ramachandran-lab/pong
tess3r 1.1.0	Caye et al.[158] 2016	https://github.com/bcm-uga/TESS3_encho_sen
diveRsity	Keenan et al.[159] 2013	https://rpubs.com/kkeenan02/divMigrate
Hapsolutely	Vences et al.[160] 2024	https://github.com/iTaxoTools/Hapsolutely
hmmIBD	Schaffner et al.[161] 2018	https://github.com/glipsnort/hmmIBD
R	R Core Team,[162] 2021	www.R-project.org
GONE	Santiago et al.[163] 2020	github.com/esrud/GONE
Samβada	Duruz et al.[164] 2019	https://github.com/SolangeD/R.SamBada
QGIS 3.38.3		https://qgis.org/
SnpEff 5.1	Cingolani, Platts et al.[165] 2012	https://pcingola.github.io/SnpEff/download/
Vegan	Oksanen et al.[166] 2022	https://vegandevs.github.io/vegan/
Pcadapt	Privé et al.[167] 2020	https://bcm-uga.github.io/pcadapt/
Geneious Prime 2025.2		https://www.geneious.com/updates

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

A total of 216 drones (haploid males), each representing a single apiary and beekeeper, were collected in 2022 from across the Italian Peninsula (Figure 1). The sampled area encompassed *A. m. ligustica's* natural distribution and varying climatic conditions, ranging from the Mediterranean in the south and west of the Apennines to the subtropical in the east of the Apennines and the Po Valley, and up to the coldest areas near the Alps. Apiaries were selected from different beekeepers and spaced at least 7 km apart to minimise the probability of sampling related colonies, as drones can fly up to that distance to drone congregation areas where they mate with queens in flight.[168] Of the 216 beekeepers, 67 (31.0%) practised migratory beekeeping, and 71 (32.9%) requeened colonies with queens purchased from breeders. Samples were stored in absolute ethanol at -20°C until molecular analysis.

In the Iberian Peninsula, 85 drone whole-genome sequences previously sequenced by Henriques et al. (2018) were used in this study. These originated from single beekeepers and apiaries sampled in 2010 from across the Iberian Peninsula (Figure 1). The distance between apiaries exceeded 7 km. The sampled area encompassed *A. m. iberiensis'* natural range and varying environmental conditions: from semi-arid in the southeastern to oceanic in the northwestern side of the Iberian Peninsula. Of the 85 beekeepers, 33 (38.8%) practised migratory beekeeping, and 6 (7.1%) requeened colonies with purchased Iberian queens.

Additionally, 90 drone whole-genome sequences showing high purity in previous admixture analyses were used as references for four subspecies representing different evolutionary lineages. This reference dataset included: 26 A-lineage *A. m. sahariensis* re-sequenced here from drones collected in 2023 from single apiaries in Morocco; 12 A-lineage *A. m. intermissa* from northern Algeria and Morocco[148]; 35 C-lineage *A. m. carnica* genomes from Slovenia and Switzerland[147]; and 17 M-lineage *A. m. mellifera* genomes from the Solliès and Porquerolles conservatories in France.[147] The *A. m. carnica* and *A. m. mellifera* genomes were obtained from the sequencing projects PRJEB16533 and PRJNA311274,[147] available in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra).

METHOD DETAILS

DNA extraction and cox1-cox2 mitochondrial analysis

DNA of the 216 Italian and 26 *A. m. sahariensis* samples was extracted from the thorax of individual drones using the Maxwell® RSC Instrument (Promega, USA) with the Maxwell® RSC PureFood GMO and Authentication kit (Promega, USA). To investigate temporal changes in maternal diversity in the Italian honeybees, mitochondrial DNA was analysed, focusing on the highly polymorphic *cox1-cox2* intergenic region. This length polymorphism marker has been widely used in honeybee studies worldwide for over 30 years.[169] Therefore, DNA extracts of the Italian samples were PCR-amplified for this non-coding region using the COI_Seq-F and COII_Seq-R primers[170] and the PCR conditions described by Alburaki et al.[150] PCR products were Sanger sequenced by Macrogen Europe. Haplotypes were determined using the *in silico Dra*I digestion tool and through alignments with Geneious Prime 2025.0.3. For the Iberian samples, *cox1-cox2* haplotypes were retrieved from Chávez-Galarza et al.[13]

QUANTIFICATION AND STATISTICAL ANALYSIS

Whole-genome re-sequencing and mapping

Whole genome re-sequencing was outsourced to Novogene Europe and performed using the Illumina NovaSeq 6000 platform following a paired-end 150 strategy, which produced a mean coverage of 20X (ranging from 10X to 29X) for the nuclear and 632X (ranging from 12X to 4564X) for the mitochondrial genomes. Sequencing libraries were generated using a customised kit from Novogene based on the NEBNext® Ultra^{TM} II DNA Library Prep Kit. The whole genomes of the Iberian honeybees and the reference subspecies *A. m. intermissa, A. m. carnica*, and *A. m. mellifera* were also re-sequenced using Illumina technology, albeit with earlier models of the sequencing platform.[147,149] Reads of the 391 drone samples representing the Iberian and Italian samples, as well as the four reference subspecies, were mapped against the reference honeybee genome Amel_HAv3.1[171] using the Burrows-Wheeler Aligner.[151]

Variant calling and quality filtering were implemented using the pipeline developed by Wragg et al.[147] PCR duplicates produced during sequencing were marked using Picard (http://broadinstitute.github.io/picard/), and the reads were sorted and indexed using SAMtools (http://samtools.sourceforge.net/). Samples sequenced in different lanes were merged, and a new round of duplicate marking, sorting, and indexing of reads was performed. Variants were called using the Genome Analysis Toolkit.[172] The BAM files were refined with two Base Quality Score Recalibration rounds. Variant calling was performed using GATK's HaplotypeCaller and merged on a per-linkage-group basis by GATK's GenomicsDBImport. Both the nuclear and mitochondrial haploid data were intentionally misspecified as diploid in the SNP calling process, and heterozygous positions were removed as they represented spurious SNPs. Single chromosome VCF files were merged and jointly genotyped with GATK's GenotypeGVCFs. Indels were removed with SelectVariants to retain only SNP variants.

Quality filters on SNPs

The first set of filters, which addressed technical concerns related to sequencing and alignment processing, was applied to the nuclear and mitochondrial genomes following the specifications: QD (Quality by Depth) < 2.0, FS (Fisher Strand bias) > 60.0, and MQ

(Mapping Quality) < 40.0 using GATK's VariantFiltration.[173] A second filtering step in VCFtools (Danecek et al. 2011) was used to remove non-biallelic SNPs, variants with low sequencing depth, and variants with low quality (–max-alleles 2, –min-meanDP 2,–maxDP 60, –minQ 50). For mitochondrial genomes, –maxDP was set to 9000, to allow higher read depths. Finally, BCFtools[154] was used to remove variants with missing genotypes exceeding 10% and heterozygous positions.

Three PLINK bed files were generated for the nuclear genomes: one containing only Iberian honeybees, another containing only Italian honeybees, and the third containing all 391 whole genomes. The files were filtered to exclude SNPs with a minor allele frequency below 0.05 and a genotyping rate below 0.3 using PLINK version 1.9.[155] For the bed file containing the 391 whole genomes, linkage-desiquilibrium (LD) pruning was performed with the PLINK function indep-pairwise with a window of 50 SNPs, 5 SNPs overlap between windows, and a r^2 threshold of 0.5. The LD-pruned dataset was used to assess population structure. A fourth PLINK bed file containing mitochondrial variants was also generated. The number of SNPs retained after each of these filtering steps is reported in the Table S1.

Genomic population structure

Population structure analyses were conducted using the LD-pruned nuclear dataset containing the 391 individuals following the maximum-likelihood model-based algorithm implemented in ADMIXTURE.[156] ADMIXTURE analysis was performed with K values ranging from 1 to 9 and 40 iterations with different random seeds each time. The most probable number of K-given populations was estimated by choosing the K with the lowest cross-validation error. ADMIXTURE iterations for each K were aligned and grouped in clustering modes using PONG.[157] Ancestry proportions obtained by ADMIXTURE for the optimal K were geographically interpolated using *tess3r* version 1.1.0 with the default parameters.[158]

To investigate the direction and magnitude of gene flow between populations, the function *divMigrate* from the R package diveRsity[159] was applied to the nuclear SNP dataset. The analysis was run with Gst migration statistic, a filter threshold of 0.05, and 100 bootstrap iterations. For the mitogenome SNP dataset, a haplotype genealogy graph was generated for 383 samples (eight Italian individuals were removed due to substantial missing data) using Median-Joining network analysis[174] in Hapsolutely version 0.2.3.[160]

Genomic diversity and kinship

Genomic diversity was estimated for each Iberian and Italian individual from genome-wide nuclear and mitochondrial SNPs using nucleotide diversity (π) per site in VCFtools. Additionally, kinship analysis was performed for the nuclear compartment to assess connectivity between all individuals within the Iberian and Italian datasets. To that end, additional filters were applied to the dataset using PLINK as follows: call rate > 90%, minor allele count ≥ 2, and pairwise linkage disequilibrium (r^2) < 0.6.[30] Pairwise kinship of all individuals within the Iberian and Italian datasets was then assessed using hmmIBD software,[161] with the parameters *chrom* and *rec_rate* set to 16 and 2.6×10^{-7}, respectively. The value of *rec_rate* corresponds to the per-nucleotide recombination probability, derived from an average recombination rate of 26 cM/Mb Mb.[175] To assess the effect of high kinship on diversity estimates, π was recalculated after excluding one individual from each pair with kinship >0.3536, following the first-degree kinship correction for haplodiploid systems ($1/2^{1.5}$). When deciding which individuals to exclude, priority was given to those involved in multiple pairs with high kinship. When involved in only a single pair, the excluded individual was randomly selected.

Descriptive statistics were presented as the median with the interquartile range (IQR). Wilcoxon rank sum test and graphical representations were performed in RStudio version 4.3.1.[162]

Inference of recent changes in effective population size

Inferences of N_e changes in the last 200 generations were performed using an LD-based approach implemented by the software GONE.[163] Since GONE does not include a haploid mode, *n/2* fully independent hypothetical queens with known phase were simulated by randomly pairing the *n* haploid drone individuals within the Iberian and Italian datasets. As there was an odd number of individuals in the Iberian dataset (N = 85), one of them was randomly excluded, and a total of 42 pseudodiploids were generated. Conversely, all 216 Italian individuals were included in the analysis, allowing the generation of 108 pseudodiploids. GONE was run independently for each dataset in the "known phase, diploid" mode, using a recombination rate of 26 cM/Mb[175] and 40 internal replicates. To account for haplodiploidy in honeybees, N_e was rescaled to 4/3 N_e individuals.[30] Additionally, the maximum value of the recombination rate (*hc*) to be analysed was set to 0.01 to reduce artefacts created by population structure and recent migrants,[163,176] which is common in honeybees. Additional analyses were performed in GONE to assess the effects of unequal sample sizes and kinship on temporal changes in N_e. To that end, a new Italian dataset containing 201 individuals was generated by removing those more frequently involved in high kinship pairs (>0.3536) and used to generate 100 pseudiploids. Additionally, this 201 dataset was downsized to 84 randomly chosen individuals to match the sample size of the Iberian dataset.

Detection of selection footprints

The 85 Iberian and 216 Italian whole genomes were scanned for selection footprints using Samβada,[177] partial redundancy analysis, pRDA,[178] and PCAdapt.[179] Samβada is a spatial genotype-environment association (GEA) method that uses univariate logistic regression models to identify locus-environment associations. Genome scans were performed using the package *R.SamBad*.[164] pRDA is a GEA multivariate ordination method that examines how groups of loci covary in response to multivariate environmental data. Genome scans were performed using the function *rda* implemented in the R package *vegan*.[166] The first three axes of a

Principal Component Analysis were used as conditionals to correct for population structure. Finally, PCAdapt is a frequency-based method that takes into consideration population structure when detecting SNP outliers. Genome scans were performed using the R package *pcadapt*,[167] with the K parameter set to two in the case of the Iberian dataset and to four in the case of the Italian dataset, following the proportion of variance explained by each Principal Component. From the associated p-values obtained for each SNP as a result of the test, q-values were estimated using the R package *qvalue*.[180]

The significance levels of the three selection methods were adjusted using the false discovery rate (FDR) procedure.[181],[182] Before applying FDR in Samβada, the uniform distribution of the p-values was verified, and, if needed, the empirical null hypothesis was applied to recalibrate the distribution.[183] For the pRDA method, FDR and recalibration were conducted using the function *rdadapt.R* (https://github.com/Capblancq/RDA-landscape-genomics/tree/main/src). SNPs with FDR <0.05 were considered candidates for adaptation.

Environmental variables

To identify the genes putatively involved in adaptation to climate, the GEA methods Samβada and pRDA require environmental data as input for the analysis. To that end, seven environmental and 19 bioclimatic variables were retrieved for each Iberian and Italian individual from publicly available databases (WorldClim and Climate Research Unit), based on the geographical coordinates of the sampling locations. These variables included cloud coverage (cld), maximum temperature (tmx), mean temperature (tmp), minimum temperature (tmn), precipitation (pre), solar radiation (srd), altitude (alt), and the WorldClim bioclimatic 1-19 variables. To avoid redundancy, reduce overfitting, and improve model stability, collinearity among climatic variables was assessed using the R function *cor*. In the case of variables correlated at $|r| > 0.8$, only one was retained for further analysis. When deciding which variables to retain, priority was given to WorldClim Bioclimatic variables as they represent annual trends, seasonality, and extreme or limiting environmental pressures.

Annotation of the SNP outliers and ontology of candidate genes

Annotation, annotation impact, and SNP functional state information were obtained for the candidate SNPs (q-values <0.05) identified in the Iberian and Italian datasets using SnpEff version 5.1[165] and the reference genome Amel_HAv3.1. Only SNPs annotated as missense, exonic, intronic, UTR variants, or located within 1 kb upstream or downstream of genes and their corresponding annotated genes were retained. Genes were considered robust candidates if they were simultaneously detected by Samβada, pRDA, and PCAdapt.[184] For these genes, protein features and Gene Ontology (GO) terms were retrieved from Entrez Gene of NCBI and *Drosophila melanogaster* orthologs from FlyBase version FB2024_03.[185] To determine whether the list of annotated genes was overrepresented in specific molecular functions, biological processes or cellular components, a Gene Ontology (GO)-term enrichment analysis was conducted using g:Profiler,[186] applying the g:SCS multiple testing correction method with a significance threshold of 0.05. Additionally, the intersection of the robust candidate genes between the Iberian and Italian datasets was analyzed to identify shared functions putatively related to local adaptation.