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Genetic diversity assessment of Italian honey bee populations for conservation of *Apis mellifera ligustica* biodiversity

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ABSTRACT

The European honey bee (*Apis mellifera*) is a key pollinator of significant ecological and economic importance. However, its genetic biodiversity is increasingly threatened by human practices such as queen trade and migratory beekeeping, which facilitate introgression with non-native subspecies. In this study, we investigated the genetic composition of three *A. m. ligustica* queens from apiaries located in the Conero Park (Marche region, Italy) by performing whole-genome sequencing (WGS) of their drones. For comparison, additional genomes from *A. m. ligustica*, *A. m. carnica*, *A. m. siciliana*, *A. m. mellifera*, and Buckfast bees were included. Principal component and admixture analyses confirmed the assignment of the Conero queens to the *ligustica* lineage while revealing weak introgression signals in one apiary, primarily from *carnica* subspecies. The ongoing introgression suggests a limited introduction of alleles characteristic of other subspecies that are not adapted to the specific territory. Moreover, a set of private single-nucleotide polymorphisms (SNPs) specific to the *ligustica* genetic makeup was identified, representing valuable markers for monitoring and conservation. These findings emphasize the importance of conserving the local gene pool and implementing genetic monitoring strategies to safeguard the adaptive traits and resilience of Italian honey bee populations.

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Introduction

The European honey bee *Apis mellifera* is one of the most ecologically and economically important pollinators for agricultural food crops and flowering plants. Moreover, it is one of the few intensively managed insect species for wax, honey, and pollination (Da Santos et al. 2021; Osterman et al. 2021). Diversification of *A. mellifera* has resulted in the evolution of 31 morphologically and geographically distinct subspecies (Alpatov 1929; Goetze 1930; Tomassone & Fresnaye 1971; Ruttner et al. 1978; Ruttner 1988; Engel 1999; Nielsen et al. 2000; Ivanova et al. 2010; Meixner et al. 2011; Chen et al. 2016; Fontana et al. 2018; Utzeri et al. 2021). These subspecies are grouped into five genetically distinct evolutionary lineages: the A lineage in Africa, the M lineage in Northern Europe and Central Asia, the C lineage in Central and Southern Europe, the O lineage in the Middle East and Western Asia, and the Y lineage in the Arabian Peninsula and Ethiopian highlands (Cridland et al. 2017).

Studies on *A. mellifera* populations are highlighting a reduction in genetic diversity, a trend projected to intensify in the future (Espregueira Themudo et al. 2020; Lin et al. 2025). This erosion of genetic variability has been implicated as a potential factor contributing to the global decline of honey bees (Espregueira Themudo et al. 2020; Panziera et al. 2022). Indeed, honey bee populations are threatened by a combination of stressors including climate change, parasites, pathogens and agricultural activities (Genersch et al. 2010; Alburaki et al.

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2015; Goulson et al. 2015; Sánchez-Bayo et al. 2016; Sánchez-Bayo & Wyckhuys 2019; Jones et al. 2020; Hisamoto et al. 2024; Vommaro & Giglio 2024). In addition, some practices, such as queen trade or migratory beekeeping, have altered the distribution and diversity of local honey bees such that they no longer correspond to the natural state (Kükrer et al. 2021; Panziera et al. 2022).

Many breeders prefer non-native subspecies, for their higher productivity compared to local honey bees. This practice might lead to introgression and hybridization that can influence genetic diversity. Although these processes may enhance genetic diversity by introducing alleles characteristic of other subspecies, introgression can also disrupt adaptive genetic architectures shaped by local selective pressures, thereby undermining local adaptation. In fact, the genotype of local honey bees carries traits selected by natural evolution, which allow them to survive in a specific territory (Yadró Garcia et al. 2025). These traits include, for example, the ability to recognize and synchronize their foraging activity with local floral bloom periods, enabling efficient nectar collection while also ensuring the pollination of plant species that serve as vital ecological substrates for the bees themselves (Henriques et al. 2018). On the other hand, imported bees show worse responses to challenges compared to local bees (Costa et al. 2012; Büchler et al. 2014; Dražić et al. 2014; Hatjina et al. 2014; Wallberg et al. 2014; Kovačić et al. 2020; Schaumann et al. 2024). Therefore, it is critical to preserve the local gene pool of honey bee populations to guarantee tolerance to environmental changes and resistance to diseases. Knowledge about patterns of population structure is necessary for implementing informative management practices aimed at preventing the loss of genetic diversity while maintaining adaptive genetic variation. The introduction of non-autochthonous queens in Italy is increasing, exposing native honey bees to progressive hybridization (Minozzi et al. 2021). It is unclear whether and how these practices affect the existing populations considering also that introgression and admixture can occur very fast in honey bees due to the difficulties in mating control (Plate et al. 2019). Genetics studies are therefore required to monitor the degree of introgression in local populations.

Several genomic resources have recently been developed for monitoring the introgression of honey bee populations, which can be applied to conservation and breeding programmes for monitoring and maintaining the genetic integrity of locally adapted subspecies or populations (Momeni et al. 2021; Henriques et al. 2025). Furthermore, higher-resolution individual genome sequencing will likely enhance our understanding of the importance and consequences of admixture in honey bees.

In this work, we focused on the Conero Regional Park, located in the Marche region of central Italy. This area is far from the distribution ranges of the other subspecies present in Italy (*A. m. siciliana*, *A. m. mellifera*, and *A. m. carnica*) and, as a protected natural park, it is subject to stricter conservation measures. This park was established in 1987 and covers an area of approximately 6011 ha. It includes the limestone promontory of Mount Conero, which rises 572 m above sea level and overlooks the Adriatic Sea. The park is rich in biodiversity, protecting a wide variety of flora and fauna, and it also preserves important cultural and historical treasures within a unique natural landscape. *A. mellifera ligustica* is the native subspecies of the Italian peninsula and belongs to lineage C together with *A. mellifera carnica*, which is diffused in regions of north-eastern Italy (Cridland et al. 2017). In order to reduce genetic erosion resulting from hybridization, the Regional Council, through law no. 2/2023, has protected *Apis mellifera ligustica* by prohibiting the selection and reproduction for commercial purposes of queen bees belonging to other subspecies. Here, by analysing newly generated whole-genome sequencing (WGS) data as well as publicly available data, we screened the genetic makeup in queens sampled in the Conero Park, analysing their drones with the aim to identify introgression in *A. m. ligustica*. Specifically, we performed genotype comparisons with *A. m. ligustica*, *A. m. carnica*, *A. mellifera mellifera*, *A. mellifera siciliana* and the Buckfast breeding line. This study advances knowledge on the genetic composition of honey bees of the Conero Park and provides a reliable basis for the creation of effective management plans to protect the local gene pool of these populations.

Material and methods

Sampling

During summer 2023, 15 drone larvae were sampled 72 h after hatching (derived from unfertilized eggs) from a single hive in each of three apiaries located in the Conero Park (CON1, CON2, and CON3, Table S1). These haploid males derived from unfertilized eggs through arrhenotokous parthenogenesis and thus

present the genetic material of the queen bee (Rabeling & Kronauer 2013). The sampling strategy was designed to avoid the direct sacrifice of the queen bee and thus compromising the entire hive. To verify whether the collected bees belong to the *ligustica* subspecies, 15 drones of *A. m. ligustica* were collected from Faenza (LIG) (Table S1). To study potential patterns of introgression, 15 drones from each of the other two subspecies present in Italy, *A. m. siciliana* from Palermo (SIC) (Table S1) and *A. m. carnica* from Tolmino (CAR) (Table S1), were included in the analysis (Figure 1). In addition, to increase resolution of the genomic variation of *Apis mellifera*, we performed a more exhaustive analysis by including the genomes of 30 drones downloaded from the Zenodo repository (Zenodo. CERN and OpenAIRE. Zenodo – Research. Shared. <https://zenodo.org>) using the access codes PRJEB16533 and PRJNA311274. These data included five drones belonging to the *carnica* subspecies from Slovenia (SLO) and five from Switzerland (SWCAR), 10 drones of the *ligustica* subspecies from central Italian regions (ITA), five drones of the *mellifera* subspecies from Switzerland (MEL), and five drones of the Buckfast bee from Switzerland (BUCK) (Wragg et al. 2016, 2022; Parejo et al. 2016, 2023) (Figure 1 and Table S2).

DNA isolation and sequencing

For apiaries CON1, CON2, CON3, LIG, CAR, and SIC, total DNA was extracted from 15 drones using the kit “MagaZorb DNA Mini-Prep” (Promega). The workflow was optimized by increasing the amount of starting material to about 40 mg and increasing the sedimentation time with the magnetic rack to 20 min. The extracted genomic DNA was treated with RNase A (Invitrogen) and checked by agarose gel electrophoresis. Subsequently, DNA purification and concentration were performed through precipitation with sodium acetate and ethanol, following the requirements of Next Generation Sequencing (NGS) providers (a main

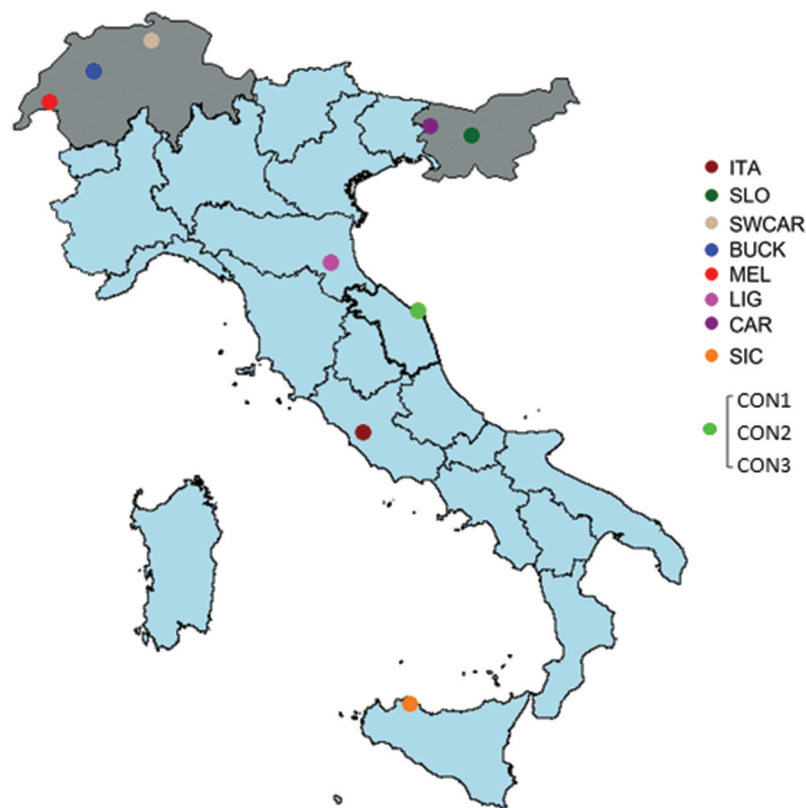


Figure 1. Geographic distribution of *A. mellifera* drone samples analysed. Each coloured dot represents a different drone origin, as indicated in the legend. ITA (Italian *A. mellifera ligustica*), SLO (Slovenian *A. mellifera carnica*), SWCAR (Swiss *A. mellifera carnica*), BUCK (Busckfast), MEL (*A. mellifera mellifera*), LIG (*A. mellifera ligustica* sampled in this work), CAR (*A. mellifera carnica* sampled in this work), SIC (*A. mellifera siciliana* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park).

band of > 5 kb, concentrations between 20 and 50 ng/μL, a minimum of 1 μg DNA in at least 50 μL volume, with 260/280 ratios ≥ 1.7 and 260/230 between 1.8 and 2.2). After resuspension, genomic integrity was further assessed by agarose gel electrophoresis, verifying the presence of a high molecular weight band indicative of intact DNA. Each sample was quantified using the Qubit fluorometer with the Qubit 1X dsDNA HS (Life Technologies) and purity was evaluated by measuring the 260/280 and 260/230 absorbance ratios using Nanodrop spectrophotometer (Thermo Fisher Scientific).

Whole-genome sequencing, variant calling and filtering

All the 90 DNA samples extracted from drones of apiaries CON1, CON2, CON3, LIG, CAR, and SIC were sent to Biomarker Technology (BMK) GmbH (<https://international.biocloud.net>) for whole-genome sequencing (WGS), sample QC, Library preparation and Sequencing. WGS was performed using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA) with a paired-end 150 bp sequencing strategy. Library preparation was carried out following the Reseq-M protocol. Approximately 8 Gb of clean data were generated per sample and raw reads were filtered to remove adapter sequences and low-quality bases (Accession number: PRJNA1431762). High-quality reads were then aligned to the *Apis mellifera* reference genome available on National Center for Biotechnology Information (NCBI) (accession number: GCA_003254395.2) using Burrows-Wheeler Aligner-Maximal Exact Matches (BWA-MEM) (Li & Durbin 2009). Subsequently, variant calling was performed using the Genome Analysis Toolkit (GATK, McKenna et al. 2010). Single-nucleotide polymorphism (SNP) datasets were generated by applying minimum depth thresholds of 10 and retaining variants with genotyping rates of at least 80% using VCFTools (Danecek et al. 2011). A minor allele frequency (MAF) filter of 0.0015 was also applied to exclude low-frequency variants. To reduce redundancy caused by linkage disequilibrium (LD), we pruned putatively linked loci with PLINK (Purcell et al. 2007) using an r^2 threshold of 0.5. This procedure ensured the retention of a set of largely independent SNPs suitable for unbiased downstream analyses. The final dataset provided a robust foundation for subsequent investigations of population genetic structure and diversity.

Multivariate analysis of genetic structure

Initially, multivariate analysis of genetic structure was performed considering all 90 drone samples collected from the CON1, CON2, CON3, SIC, CAR and LIG sites. A second analysis was carried out taking into account the additional samples belonging to other *Apis mellifera* subspecies (5 drones from MEL, SLO, SWCAR, BUCK, and 10 from ITA). Since each of these samples represents a single queen genome, as specified in the work of Parejo et al. (2023), while also taking into account the genetic uniformity of our samples, we standardized the dataset by reducing our samples to one per subspecies/site, considering a total of 36 drones.

Both datasets were subject to principal component analysis (PCA) using the adegenet package (Jombart 2008) in Rstudio. The percentage of total genetic variance explained by each principal component was calculated, with the first two components (PC1 and PC2) visualized in a scatter plot. Individuals were plotted using coloured dots based on their population assignment. Centred axes were added to aid in interpretation of clustering patterns. Subsequently, we assessed population structure and individual ancestry by implementing a sparse non-negative matrix factorization analysis (sNMF) with the LEA package (Frichot & François 2015) in Rstudio. These analyses were performed with the ploidy set to 1 (haploid). We ran sNMF with the number of ancestral populations (K) ranging from 2 to 6, and identified the optimal value as the one with the lowest cross-entropy. For each K, the run with the lowest entropy was selected, and the corresponding Q-matrix (admixture proportions) was visualized using custom-coloured bar plots to enhance interpretability. Finally, to reconstruct the phylogenetic relationships among genotypes, a maximum likelihood (ML) approach was employed using haploid variant data in a script using the ape (Paradis & Schliep 2019), phangorn (Schliep 2011), vcfR (Knaus & Grünwald 2017), adegenet and pegas (Paradis 2010) packages in Rstudio. Specifically, genotypes in VCF format were converted into DNA sequences and processed using the Jukes-Cantor model (JC69). Neighbour-joining trees were built and optimized via ML estimation with the phangorn package, accounting for nucleotide frequencies, substitution rates and rate heterogeneity. The final trees were rooted and annotated with population-based colour-coded labels.

Identification and annotation of private and shared SNPs

To identify private SNPs, the R packages *vcfR*, *adegenet*, *poppr* (Kamvar et al. 2014, 2015), *dplyr* (Wickham et al. 2025), and *ggplot2* (Wickham 2016) were used. The VCF files were loaded and converted into *genlight* objects, allowing for efficient management of large SNP datasets. Each individual was associated with its subspecies/sampling site via .txt files containing the names of the samples, creating a *popmap* for subsequent analysis. The data were filtered to include only samples present in both the VCF and the *popmap*. Average allele frequencies were calculated and private SNPs were identified for each population using the “private_alleles” function. A binary-population SNP matrix was constructed to identify SNPs shared between populations and generate heat maps and bar charts representing the distribution of private and shared SNPs. To identify a set of SNPs representative of the *A. m. ligustica* genetic makeup, SNPs specific to specimens of this subspecies were identified by performing comparative analyses against the SNP datasets of the other subspecies.

Results

The primary aim of this work was to assess the degree of introgression in *A. m. ligustica* of the Conero Park, located in the Marche region in Italy, through the analysis of three queens. The experimental design included verification of the subspecies assignment of the selected queens by analysing their male offspring. Drones develop from unfertilized eggs and therefore carry the single haploid genome inherited from the queen, providing direct access to maternal genetic information. WGS of individuals generates high-resolution genetic data compared to pooled sequencing approaches. In addition to 15 drones from each apiary, as reference, the analysis included WGS data from 15 drones for each subspecies present on Italian territory: *A. m. ligustica* (LIG), *A. m. carnica* (CAR), and *A. m. siciliana* (SIC) (Figure 1). A total of 90 individual bees were sequenced with a paired-end strategy, generating 5,375,001,328 clean reads. An average of 96.51% was properly aligned to the honey bee reference genome (accession number: GCA_003254395.2) resulting in 2,103,309 identified SNPs. After applying stringent filtering steps, 195,045 high-quality SNPs represented the final data set (see Supplementary Information Tables S2 and S3).

To evaluate genetic structure at the individual level, a PCA was performed (Figure 2). The results revealed a strong clustering of individuals within each apiary, with the exception of one Sicilian drone, two LIG drones and two CON3 drones. Overall, as the majority of drones from each apiary clustered together, they can

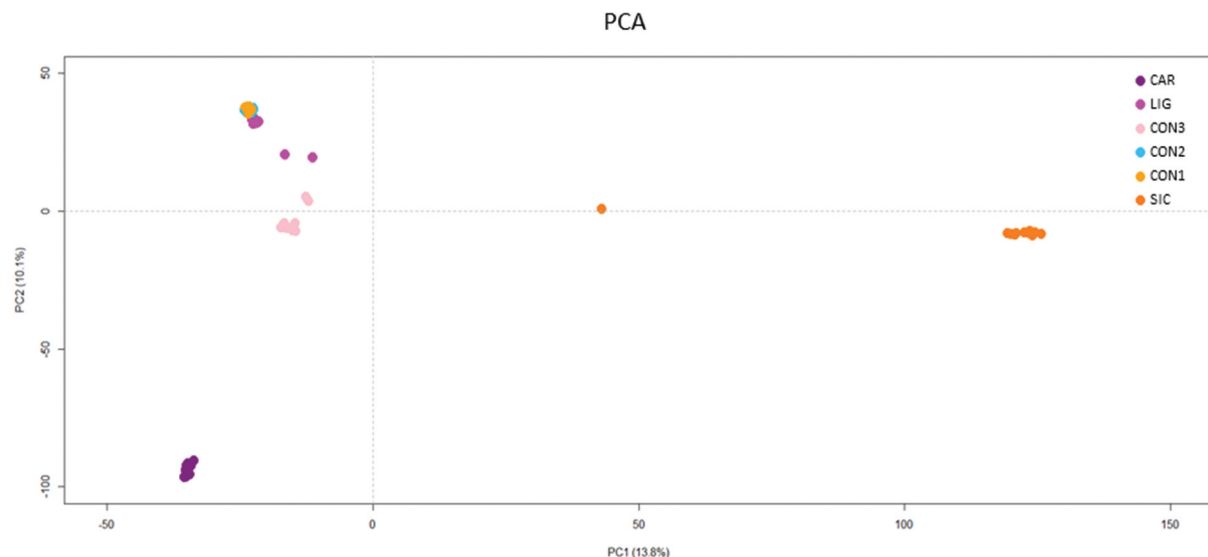


Figure 2. Principal component analysis (PCA) of Italian drones showing genetic differentiation among subspecies/sites. Each point represents an individual drone, coloured according to its subspecies/site (CAR, LIG, CON1, CON2, CON3, and SIC). CAR (*A. mellifera carnica* sampled in this work), LIG (*A. mellifera ligustica* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park), SIC (*A. mellifera siciliana* sampled in this work).

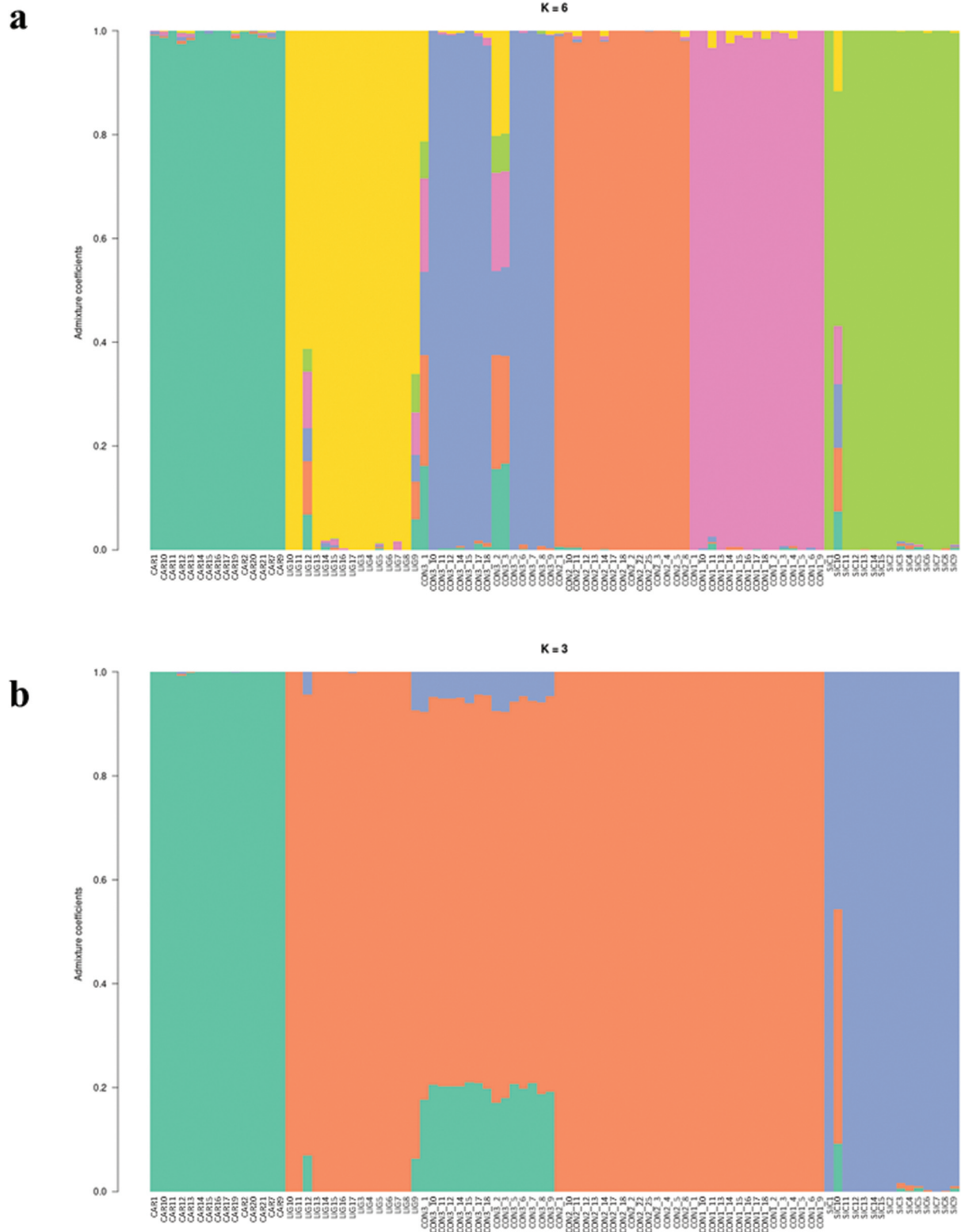


Figure 3. Ancestry of *A. mellifera* Italian drones. Admixture bar plot for K=6 (a) and for K=3 (b) displays individual ancestry proportions across the sampled drones. Each vertical bar represents one individual, and colours indicate the proportion of ancestry from each inferred genetic cluster. CAR (*A. mellifera carnica* sampled in this work), LIG (*A. mellifera ligustica* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park), SIC (*A. mellifera siciliana* sampled in this work).

reasonably be regarded as reliable representatives of their respective queen's genome. Moreover, PC1 clearly demonstrated the distinct positioning of *A. m. siciliana* relative to the other analysed subspecies. PC2 resolved the partitioning between *A. m. carnica* and the other samples. Two of the three sampled apiaries from the Conero Park (CON1 and CON2) clustered in high proximity with our *A. m. ligustica* sample, while the third (CON3) clustered separately, with lower PC2 values.

To test the admixture between samples of apiaries of interest and those used as references, the degree of admixture was evaluated (Figure 3). Ten runs of ADMIXTURE were carried out by varying K from 2 to 6 and we found that K = 6 had the lowest cross-validation error value on average over all runs, thus representing the best fitting value. This analysis highlighted clusters related to the three reference subspecies (*carnica*, *siciliana*, and *ligustica*) and three Conero apiaries (Figure 3(a)), probably also capturing family structure given that drones within the same location were derived from the same queen. The analyses carried out with K = 3 revealed three groups corresponding to the three subspecies. Interestingly, this revealed that the specimens of the Conero apiaries were mostly of *A. m. ligustica* ancestry (Figure 3(b)). In particular, the admixture bar plot suggested CON1 and CON2 to be pure *A. m. ligustica* specimens, while drones from CON3 were characterized by a low degree of introgression, consistent with the PCA results. This finding was also in line with the tree topology obtained by the ML method, displaying the lack of clusterization only for the CON3 drones (see Supplementary Information Figure S1).

To get a deeper insight into the genetic composition of the apiaries of interest, we expanded our analysis by adding data related to 30 drones belonging to the *carnica* subspecies from Slovenia (SLO) and Switzerland (SWCAR), the *mellifera* subspecies from Switzerland (MEL), the *ligustica* subspecies from central Italy (ITA), and Buckfast bees from Switzerland (BUCK) (Wragg et al. 2016, Knaus and Grünwald 2017). Since each of these samples represents a single queen genome and taking into account the genetic uniformity of our samples, we standardized the dataset by reducing our samples to one per subspecies/site, considering a total of 36 drones. This resulted in 1,083,225,212 clean reads with an average of 96.66% that properly aligned to the reference genome of the honey bee. 2,572,396 raw SNPs were filtered to retain 12,301 high-quality SNPs (see Supplementary Information Tables S2 and S3). According to PC1, four *A. m. mellifera*

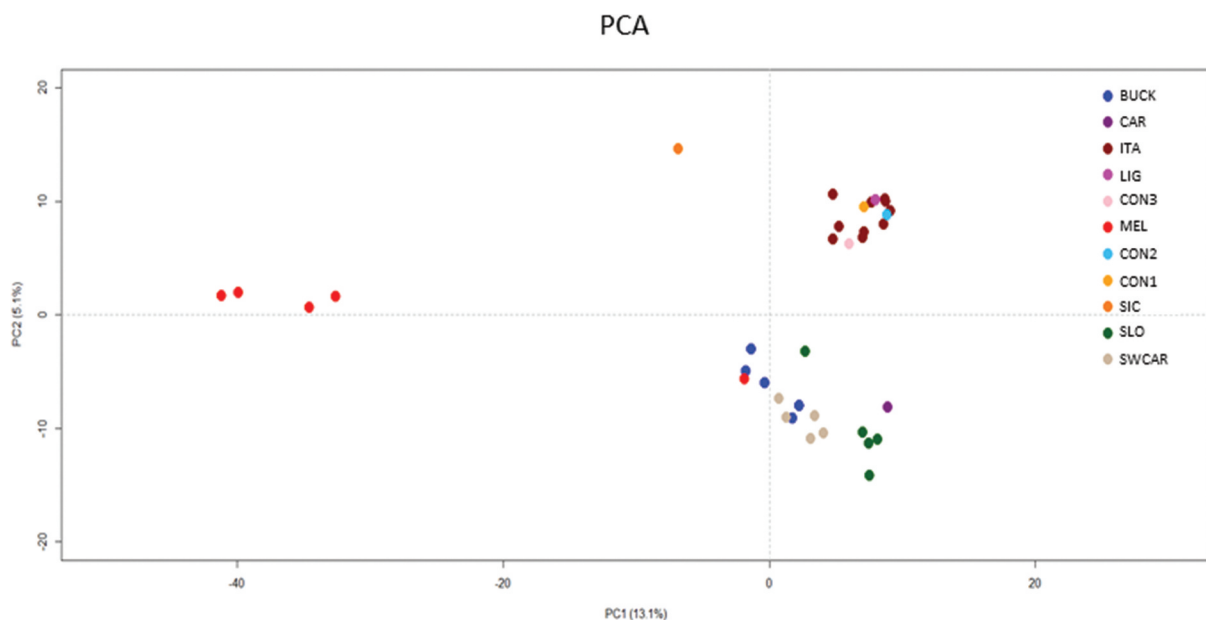


Figure 4. Principal component analysis (PCA) of *Apis mellifera* drones showing genetic differentiation among subspecies/sites. Each point represents an individual drone, coloured according to its subspecies/site (BUCK, CAR, ITA, LIG, CON3, MEL, CON1, CON2, SIC, SLO, and SWCAR). BUCK (Buckfast), CAR (*A. mellifera carnica* sampled in this work), ITA (Italian *A. mellifera ligustica*), LIG (*A. mellifera ligustica* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park), MEL (*A. mellifera mellifera*), SIC (*A. mellifera siciliana* sampled in this work), SLO (Slovenian *A. mellifera carnica*), SWCAR (Swiss *A. mellifera carnica*).

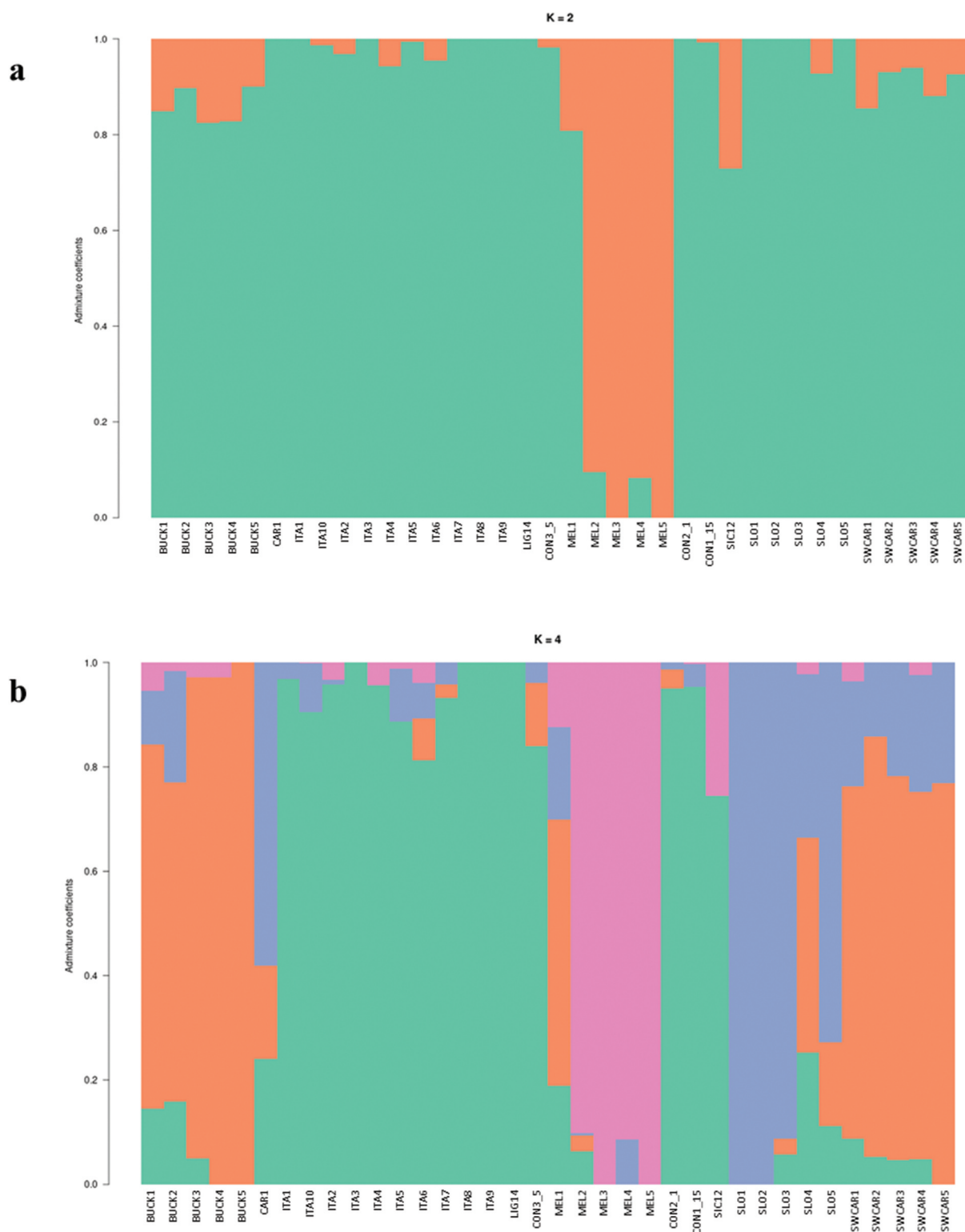


Figure 5. Ancestry of *A. mellifera* Italian drones. Admixture bar plot for $K=2$ (a) and for $K=4$ (b) displays individual ancestry proportions across the sampled drones. Each vertical bar represents one individual, and colours indicate the proportion of ancestry from each inferred genetic cluster. BUCK (Busckfast), CAR (*A. mellifera carnica* sampled in this work), ITA (Italian *A. mellifera ligustica*), LIG (*A. mellifera ligustica* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park), MEL (*A. mellifera mellifera*), SIC (*A. mellifera siciliana* sampled in this work), SLO (Slovenian *A. mellifera carnica*), SWCAR (Swiss *A. mellifera carnica*).

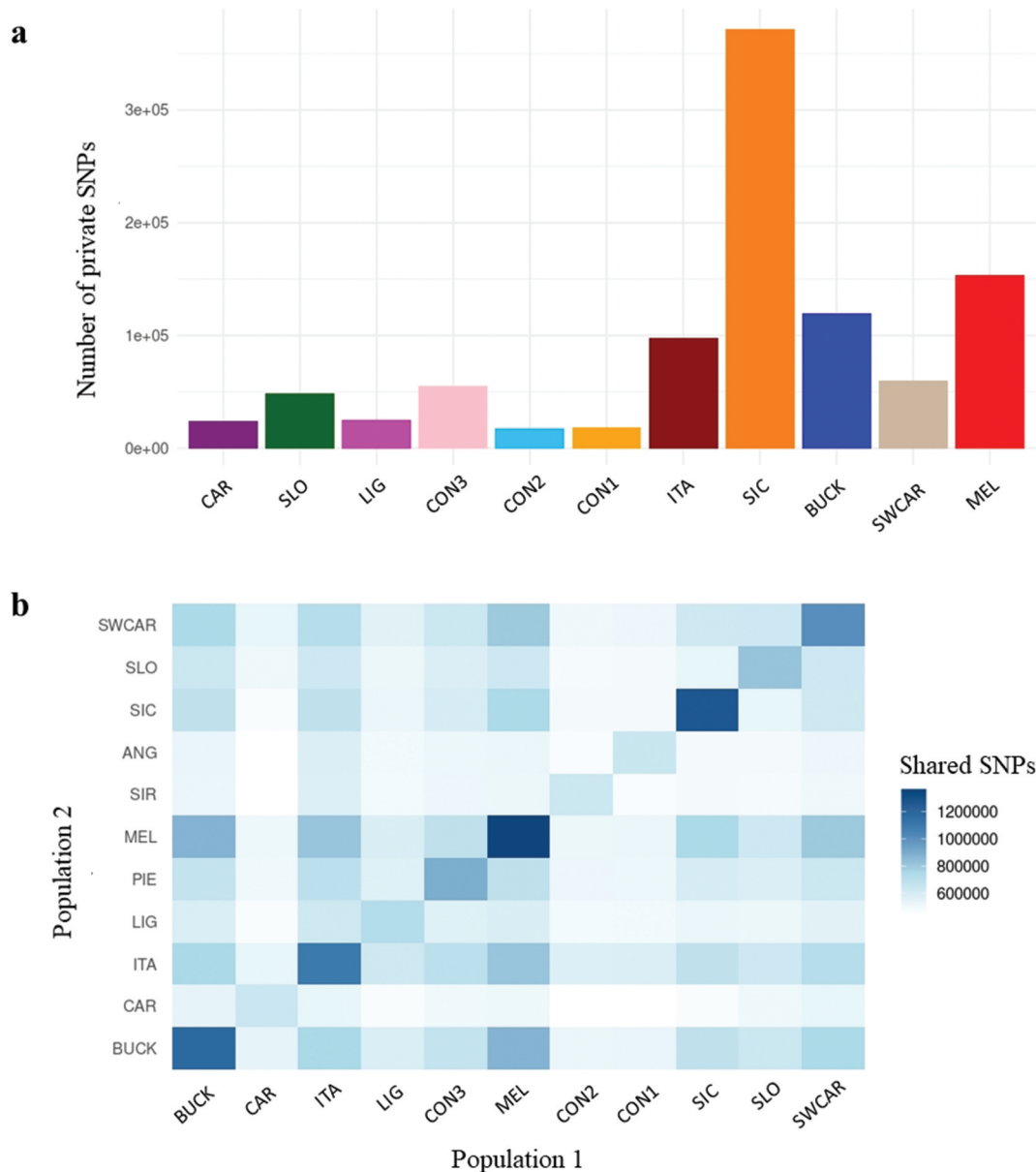


Figure 6. Genetic variation across honeybee subspecies/sites. (a) Bar plot showing the number of private SNPs detected in each subspecies/site. Colours indicate group identity. (b) Heatmap representing the number of SNPs shared between subspecies/sites. The colour gradient reflects the extent of shared genetic variation, with darker shades indicating a higher number of shared SNPs. BUCK (Busckfast), CAR (*A. mellifera carnica* sampled in this work), ITA (Italian *A. mellifera ligustica*), LIG (*A. mellifera ligustica* sampled in this work), CON1/2/3 (*A. mellifera ligustica* sampled in the Conero Park), MEL (*A. mellifera mellifera*), SIC (*A. mellifera siciliana* sampled in this work), SLO (Slovenian *A. mellifera carnica*), SWCAR (Swiss *A. mellifera carnica*).

specimens (MEL) were clearly separated from the others and the fifth individual grouped with the Buckfast (BUCK) and the *carnica* subspecies (SWCAR, SLO, and CAR) (Figure 4). This cluster was distinct from that including drones of ITA, LIG, CON3, CON2, and CON1. Moreover, the PCA analysis revealed a distinction of the Sicilian sample (SIC) from the others (Figure 4). This pattern was in agreement with data obtained from admixture analysis related to the lowest cross-entropy value ($K=2$) (Figure 5(a)). Indeed, the four *A. m. mellifera* specimens showed a more homogeneous genetic composition compared to MEL1 that presented a more similar pattern to drones of BUCK, SWCAR, SLO4, and SIC12. In the tree, the specimens belonging to the *ligustica* subspecies formed a cluster and CON3_5 had an external position (see Supplementary Information Figure S2). At $K=4$ (Figure 5(b)) the bar plot obtained by the admixture analyses

confirmed that within the *mellifera* subspecies MEL1 had a genetic composition more similar to BUCK, SWCAR, and SLO4, while MEL3 and MEL5 were admixed. Regarding the *carnica* subspecies, only specimens SLO1 and SLO2 presented a homogeneous genetic composition. Similarly, for the *ligustica* subspecies, the admixture analysis at $K = 4$ confirmed the absence of introgression for specimens ITA3, ITA8, ITA9, and LIG14. Regarding the drones of the Conero Park, CON3 presented the highest degree of introgression mainly attributable to the *carnica* subspecies. Sample SIC12 showed a genetic composition that was a mix between the *ligustica* and *mellifera* subspecies.

Analysis of private SNP distribution revealed that SIC harboured the highest number of private variants (Figure 6(a)). Within the Conero populations, CON1 and CON2 exhibited very few private SNPs, whereas CON3 showed a comparatively higher number. In addition, CON1 and CON2 shared fewer SNPs with other populations than CON3 (Figure 6(b)). In order to identify SNPs representative of the genetic distinctiveness of *A. m. ligustica* subspecies useful for screening purposes, 281,059 private SNPs were identified. For the analysis, only *ligustica* specimens without evidence of introgression were considered (CON1, CON2, ITA3, ITA8, ITA9, and LIG).

Discussion

The genetic makeup of Italian honey bee populations is a matter of growing attention since human practices like queen trade and migratory beekeeping have exposed native bees to possible hybridization. Moreover, over the last 40 years, the aggressive spread of parasites and the massive use of pesticides in agriculture have led beekeepers to introduce other *A. mellifera* strains considered better in terms of honey productivity and resistance to pathogens, namely the Buckfast (Kulhanek et al. 2017). Therefore, locally adapted honey bee populations, or ecotypes, are increasingly threatened by admixture and crossbreeding with non-native subspecies and genetic pools. As a result, important local adaptation traits may be lost, posing a serious risk to the long-term sustainability of beekeeping and pollination services. Indeed, the decline of local genetic traits is also recognized as a contributing factor to the global reduction in honey bee populations (Espregueira Themudo et al. 2020; Panziera et al. 2022). Consequently, several countries, including Italy, have initiated conservation programmes aimed at preserving the genetic integrity of native honey bee populations. These monitoring and conservation efforts must be grounded in a thorough understanding of the genetic composition of the populations under study. Indeed, investigating the genetic structure and diversity of populations is fundamental to understanding biodiversity and ensuring the effective conservation of species (Chiocchio et al. 2025).

In Italy, *A. m. ligustica* has adapted to the temperate climate and the rich floristic biodiversity of the area, establishing a fundamental relationship with it (Henriques et al. 2018). Other subspecies are also present in the Italian territory, such as *A. m. siciliana* in Sicily, *A. m. mellifera* along the western Ligurian coast, and *A. m. carnica* on the north-eastern side of the Alps. The aim of this study was to assess the presence of genetic introgression from other subspecies or hybrids in *A. m. ligustica* queens. Therefore, we analysed drones of three queens from Conero Park, located in the Marche region of Italy, which is far from the natural distribution areas of other *A. mellifera* subspecies, thus limiting natural introgression. The use of drones as representatives of the queen genotype represents a non-invasive and ethically sustainable approach, as it preserves queen bees, individuals that are limited in number and of high value to beekeepers, while still providing direct access to maternal genetic information (Wragg et al. 2022; Lin et al. 2025). We employed a WGS approach to obtain a high density of markers, favoured due to the limited size of the honey bee genome (226.5 Mb) (Wallberg et al. 2019). Our analyses clearly showed a separation between the *carnica* drones (CAR), the *siciliana* drones (SIC) and a cluster including *ligustica* (LIG) and CON1, CON2, and CON3 samples from Conero, suggesting that the queens of the three surveyed apiaries were genetically close to the *ligustica* subspecies. Moreover, a strong clustering of individuals by apiary was highlighted, indicating low within-apiary genetic diversity, and the few cases of drones that did not cluster with those from their respective apiary (see LIG, CON3, and SIC) could possibly reflect hybridization events that occurred in previous generations. A slight separation of CON3 from CON1, CON2, and LIG drones was consistent with its higher level of introgression from *carnica*.

To further investigate the genetic makeup of the Conero Park apiaries, additional reference data were included: 30 drones from *A. m. carnica* (Slovenia and Switzerland), *A. m. mellifera* (Switzerland), *A. m. ligustica* (central Italy), and Buckfast bees from Switzerland, based on datasets from Wragg et al. (2016, 2022). In the PCA analysis, the separation of the *mellifera* samples from the others was expected, since they belong to the M group (Cridland et al. 2017). In the second clade, constituted by *ligustica*, Buckfast, and *carnica* specimens, the clusterization of *ligustica* and *carnica* specimens was explained considering their evolutionary origin identified in the C group (Cridland et al. 2017). Moreover, the closest proximity between Buckfast and *carnica* specimens reflected the introgressed genome from the Eastern European progenitor population (the C group) (Harpur et al. 2012; Parejo et al. 2016; Minozzi et al. 2021). The admixture analysis also confirmed the highly admixed composition of the Buckfast genome that is the result of different breeds (Harpur et al. 2012). The bar plot revealed that this genetic composition was present in all the specimens belonging to the *carnica* subspecies from Switzerland, in two specimens from Slovenia (SLO4 and SLO5), in the *carnica* specimen sampled in this study (CAR1), and in one specimen belonging to the *mellifera* subspecies (MEL1). This result was in agreement with the clustering of these samples observed in the PCA plot. The presence of a *ligustica* genetic component in natural populations of *A. m. mellifera* and *A. m. carnica* has been reported in previous works, likely resulting from the geographic proximity of their respective distribution areas (Muñoz et al. 2009, 2013; Muñoz & de la Rúa 2012, 2021; Lukic et al. 2024). Natural populations of *A. m. mellifera* were historically confined to areas along the Alpine arc in France, Switzerland, and Liguria, while *A. m. carnica* was found near the Austrian and Slovenian borders, extending into Friuli-Venezia Giulia and Veneto (Utzeri et al. 2021). Alternatively, the genetic composition observed in these samples might also be due to introgression from Buckfast bees.

The PCA analysis revealed the distinct position of the SIC sample reflecting its belonging to the evolutionary A group (Cridland et al. 2017), which is consistent with the high number of private SNPs detected. The admixture analysis evidenced the presence of both *mellifera* and *ligustica* genetic components in its genome. In other studies using mtDNA analyses, mitotypes attributable to groups C and M have also been reported in Sicilian samples, although group A was predominant (Muñoz et al. 2014; Utzeri et al. 2021).

Results indicated the three specimens from the Conero Park belong to the *ligustica* group. However, the CON3 specimen has experienced more extensive genetic exchanges compared to CON1 and CON2. Indeed, a certain degree of introgression from *carnica* was detected in this sample. Considering that the Alps represented a natural barrier for the introduction of this subspecies in Italy, it was clear that this was the result of the importation of non-*ligustica* bees (Franck et al. 2000).

Overall, our findings confirmed introgression not only in samples from the known natural hybridization zones in Northern Italy and adjacent areas, but also in honey bee colonies distributed across the Italian territory, likely as a result of beekeeping practices – in line also with previous studies (Dall’olio et al. 2007; Minozzi et al. 2021). Moreover, we identified a set of private SNPs representative of the genetic makeup of the *ligustica* subspecies, which can be employed as genetic markers for population identification, conservation efforts, and introgression monitoring. These *ligustica*-specific variants may also provide insight into local adaptation processes and contribute to preserving the genetic integrity of these distinct groups.

In conclusion, our results showed that bees sampled in the Conero Park largely retain their *A. m. ligustica* identity, although some signals of introgression were detected. These findings highlight the importance of accounting for genetic factors and population structure in honey bee breeding and management. They also reinforce the need for genetic monitoring to prevent potential admixture with non-native strains and to avoid the loss of local adaptation.

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Author contributions

CRedit: **F. Carducci**: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing; **E. Carotti**: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing; **C. Spinsante**: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing; **S. Casavecchia**: Funding acquisition, Writing – original draft, Writing – review & editing; **D. L. J. Vendrami**: Data curation, Writing – original draft, Writing – review & editing; **A. Canapa**: Supervision, Writing – original draft, Writing – review & editing; **M. Barucca**: Supervision, Writing – original draft, Writing – review & editing; **M. A. Biscotti**: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study were deposited in NCBI under the accession number PRJNA1431762.

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