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Leonardo Pablo Porrini, Silvina Quintana, Constanza Brasesco, Matias Daniel Maggi, Martín Pablo Porrini, et al.. Current genetic diversity of managed and commercially produced *Apis mellifera* colonies in Argentina inferred by wing geometric morphometrics and COI-COII mtDNA locus. *Apidologie*, 2022, 53 (5), pp.61. 10.1007/s13592-022-00970-1 . hal-04243137

**HAL Id: hal-04243137**

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Submitted on 16 Oct 2023

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# Current genetic diversity of managed and commercially produced *Apis mellifera* colonies in Argentina inferred by wing geometric morphometrics and COI-COII mtDNA locus

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Received 4 July 2022 – Revised 6 September 2022 – Accepted 22 September 2022

**Abstract** – The current genetic diversity of *Apis mellifera* colonies in Argentina was evaluated using geometric morphometric analysis of wing shape in 480 managed colonies collected in different regions and 154 colonies commercially produced by 28 different queen breeders. In addition, mitotype (COI-COII) characterization was conducted on queen breeders' colonies. We found European morphotypes in managed colonies from central and southern regions and greater diversity of *A. mellifera* subspecies compared to the northern region where African morphotype predominates. In queen breeders' colonies, we found a fairly heterogeneous degree of hybridization, similar to managed colonies in the central and southern regions but with a clear predominance of *A. m. ligustica* and *A. m. carnica*, which was consistent with the mitotype analysis. Current genetic diversity in Argentina is wide and comprises mainly seven subspecies belonging to four evolutionary lineages (C, M, A, and O). This probably results from both historical and current importation events, transhumance, and exchange of queens between regions lacking strong geographical barriers, in combination with genetic dominance factors, gene flow, or adaptation to different environmental conditions.

*Apis mellifera* / genetic diversity / breeding lines / geometric morphometrics / mtDNA

## 1. INTRODUCTION

The distribution of honeybee *Apis mellifera* originally occupied Europe, Africa, and Western Asia, and has currently been described 33 subspecies belonging to five evolutionary lineages (A, M,

C, O, Y) based on morphological and nucleotide polymorphism data (Ilyasov et al. 2020). Since the beginning of human management, a wide expansion of its native distribution has occurred, displacing, exchanging, and artificially mixing naturally established honeybee populations (Harpur et al. 2012; Leclercq et al. 2018), with consequences that have not only affected current genetic diversity but have also promoted the transmission of diseases and pathogens (Mutinelli 2011). A great diversity of subspecies of *A. mellifera* has been introduced

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Manuscript editor: Klaus Hartfelder

since colonial times in Argentina, mainly from Europe, including *Apis mellifera mellifera*, *Apis mellifera ligustica*, *Apis mellifera carnica*, *Apis mellifera caucasica*, *Apis mellifera intermissa*, and *Apis mellifera iberiensis* (Bierzuchudek 1979). In 1956, the African subspecies *Apis mellifera scutellata*, began to spread into the American continent from southern Brazil, in an effort to establish honeybee populations better adapted to tropical conditions (Scott Schneider et al. 2004). The Africanization process involved the bidirectional flow of maternal and paternal genes between previously introduced European and Africanized honeybees (Sheppard et al. 1991; Clarke et al. 2001; Pinto et al. 2005).

In Argentina, Africanized honeybees predominate in the northern region and European honeybees in the central and southern regions. In the area between 28° and 35° S, mixed populations derived from Africa and Europe overlap, causing a hybridization zone (Whitfield et al. 2006; Abrahamovich et al. 2007; Agra et al. 2018; Porrini et al. 2020). There are almost 15,000 beekeepers and 3, 5 million beehives mainly found in the central region of the country which are used for honey production (80,000 tons/2018, MAGYP 2019). However, in the last 20 years, honeybee queen production and commercialization have been strongly intensified in the local market as well as for export to other countries (Ferrari et al. 2011), reaching 280,000 queen bees exported in 2019 (MAGYP 2019). In 2017, there were 24 breeding apiaries in the national registry (SENASA 2017; Min Agri 2019), although the current number of queen breeders possibly exceeds 50 apiaries. An important limitation is that most queen breeders in Argentina lack the tools or budget necessary to regularly control genetic diversity and yet annually distribute a large number of queens, multiplied by a small number of mothers. Intentionally or not, this process could reduce genetic variability. In addition to natural selection pressures, humans contribute, deliberately or not, to shaping the genetic diversity of honeybee populations around the world (Ferreira et al. 2020; Oleksa et al. 2021; Carpenter and Harpur 2021).

Human management and massive colony losses have raised concerns about decreased genetic variability, especially in Europe and

North America (Meixner et al. 2010; Cobey et al. 2012; Espregueira Themudo et al. 2020). Globally, hybridization rates are increasing due to honeybee movement and habitat alteration (Allendorf et al. 2001). Introgression can disrupt local adaptation and negatively impact biodiversity (Moritz et al. 2005). However, management increases genetic diversity in honeybee populations (Harpur et al. 2012) and improves productivity, thermoregulation, and overall colony fitness (Taryp et al. 2013; Jones et al. 2004; Graham et al. 2006), giving to admixed genotypes more resistance against parasites and diseases (Desai and Currie 2015; Oldroyd et al. 1992; Mattila and Seeley 2007). It has also been proven that locally adapted colonies have higher survival (Hatjina et al. 2014) and a lower incidence of pathogens (Burnham et al. 2019; Meixner et al. 2014), and therefore, it is essential to conserve the underlying genotypic variation (Frankham et al. 2002).

Introgression may be detrimental to *A. mellifera* subspecies conservation within their native range (Muñoz and De la Rúa 2021; Parejo et al. 2018; Oleksa et al. 2011). However, it occurs intentionally or not in many regions outside this range, resulting in admixed populations or similar commercial stocks (Carpenter and Harpur 2021; Saelao et al. 2020; Harpur et al. 2015; Chapman et al. 2016). Beyond this, queen breeders must ensure to promote genetic diversity since honeybees have a haplodiploid mating system making them more sensitive to inbreeding (Zayed 2009). Currently, many queen breeders often import new genetic material, not only to preserve genetic diversity, but also to increase certain desirable traits (Niño and Jasper 2015). The importation of queen bees affects the genetic structure of local populations, and this fact has been illustrated by the preferential use of some subspecies and hybrids (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasica*, Buckfast) and the accidental development of Africanized populations (*A. m. scutellata*) in America.

Geometric morphometrics analysis of wing shape has been previously applied in the evaluation of genetic structure within *A. mellifera* subspecies as well as in the identification of evolutionary lineages (Francoy et al. 2008; Tofilski 2008; Barour and Baylac 2016), even

obtaining a similar characterization compared to SNP analysis (Minozzi et al. 2021; Henriques et al. 2020). On the other hand, genetic markers such as the intergenic region COI-COII of mtDNA have been widely used to differentiate maternal lineages and to discriminate mitochondrial haplotypes between *A. mellifera* subspecies (Garnery et al. 1992; Franck et al. 2000; Szalanski and Magnus 2010).

The aim of this study was to compare genetic diversity in managed and commercial *A. mellifera* colonies from different regions of Argentina using geometric morphometric analysis of wing shape and estimate the current morphotype hybridization through distribution maps of the main *A. mellifera* subspecies previously introduced. In addition, to determine maternal lineage in commercial populations, mitochondrial haplotypes were characterized in colonies from queen breeders of different regions.

## 2. MATERIALS AND METHODS

### 2.1. Sampling

We obtained samples from 480 managed honeybee (*A. mellifera*) colonies from 70 localities in 22 provinces during 2013–2019 (Figure 1; Table S1). In addition, 154 honeybee colonies coming from 28 breeding apiaries (Min Agri 2019) dedicated to the multiplication and commercialization of queens and other live products (queen cells, bee packs, bee nucleus) were collected in different regions during 2018–2020 (Figure 1; Table S2).

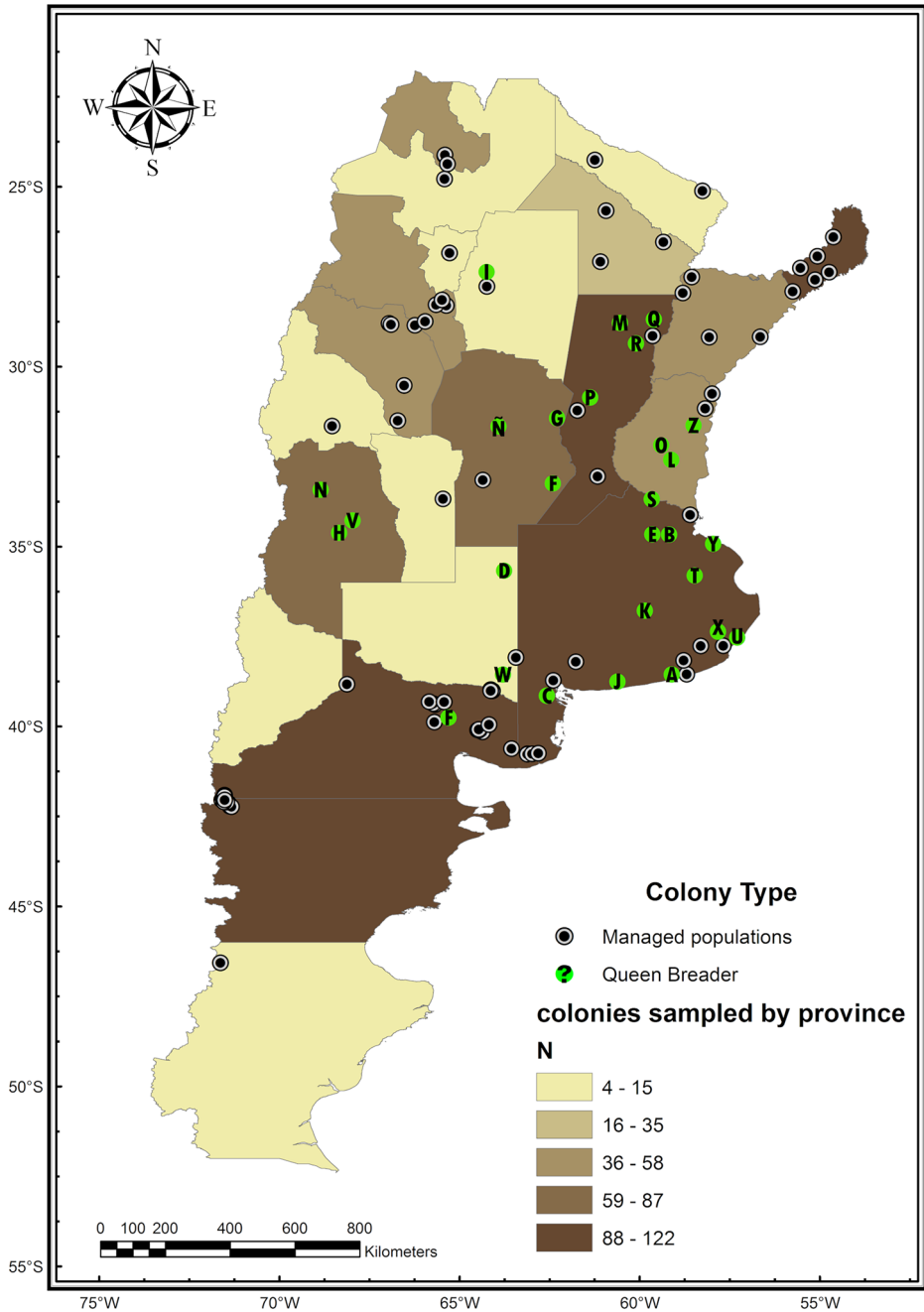
### 2.2. Geometric morphometrics

From the samples obtained, the left forewing of 10 workers per colony was mounted in glass photographic frames and scanned (res. 7200 dpi) with a Plustek Opticfilm 8100 (LaserSoft Imaging, Kiel, Germany). A total of 6340 forewing pictures were successfully obtained. For every wing image, the Cartesian coordinates ( $x$ ,  $y$ ) of 19 homologous landmarks (Francoy et al. 2008) were digitized using tpsDIG v.2.16 and tpsUtil v.1.4 (Rohlf 2010).

Additionally, for each pure subspecies (*A. m. carnica*, *A. m. caucasica*, *A. m. iberiensis*, *A. m. intermissa*, *A. m. ligustica*, *A. m. mellifera*, and *A. m. scutellata*), wing images representing 50 different colonies, obtained from the morphometric Bee Data Bank in Oberursel (Germany), were included in the analysis. The landmark coordinates were processed through MorphoJ software package v.1.06a (Klingenberg 2011). Alignment was performed using Procrustes fit (translation, proportion, and rotation). Based on the spectral decomposition of covariance, principal component analysis (PCA) was performed, and principal components were further analyzed with canonical variate analysis (CVA) to visualize the differences between groups: managed colonies in north region (MCN), managed colonies in central region (MCC), and managed colonies in south region (MCS) and queen breeder's colonies (QB). Statistically significant differences in wing shape were evaluated by the 38 principal components using multivariate analysis of variance (MANOVA) in PAST v4.3 (Hammer et al. 2001). Furthermore, to estimate morphotype distribution and intracolony diversity by region, Mahalanobis distances (MDs) to different *A. mellifera* subspecies were calculated grouping the datasets by province ( $MD_{bp}$ ) or by colony ( $MD_{bc}$ ). Morphometric maps were conducted separately via Esri's ArcMap 10.4.1 (Esri, Redlands, California) using  $MD_{bp}$ . Together in each map,  $MD_{bc}$  obtained from managed colonies in three regions (MCN, MCC, and MCS) or in queen breeder's colonies (QB) were compared by the Box & Whisker Median plots and Kruskal–Wallis tests using STATISTICA 10 (Statsoft 2011). In addition, Spearman's correlation analysis was run to assess the relationship between  $MD_{bc}$  and latitude for each *A. mellifera* subspecies.

### 2.3. Mitotype characterization

In order to characterize the evolutionary lineage and mitochondrial haplotypes, the COI-COII intergenic region (mtDNA) was amplified from honey bee thoraces from 28 different colonies from queen breeders. Amplifications were carried out in a total volume of 20  $\mu$ l, containing 10  $\mu$ l of 2 $\times$ MyTaq



**Figure 1.** Map indicating the type and number of honeybee colonies sampled in different Argentinean provinces. Collection sites from managed colonies are shown with black circles and queen breeder sites with green circles and different letters.

PCR mix (Bioline, London, UK) master mix, 1  $\mu$ M of each primer (E2: 5'-GGCAGAATAAGTGCA TTG-3' and H2: 5'-CAATATCATTGATGACC-3') (Garnerly et al. 1992), and 1  $\mu$ l of DNA. All reactions were carried out in a Rotor-Gene thermocycler (Qiagen, Hilden, Germany) using EvaGreen (KAPA Fast, Biosystems, Woburn, USA) as fluorescent intercalator. The amplification products were examined by electrophoresis in 1% agarose gels containing TAE buffer (Tris–acetate 0.04 M, 1 mM EDTA, pH 8), ethidium bromide staining, and a molecular weight marker (GeneRuler™ 100pb DNA Ladder, Ferments). After verifying the correct amplification, PCR products were purified using AccuPrep® PCR Purification Kit (Bioneer Co., South Korea), and sequenced by Sanger reaction using the BigDye® Terminator v3.1 Cycle Sequencing kit, ZR-96 DNA Sequencing Clean-up™ in an Applied Biosystems 3500 Genetic Analyzer. Electropherograms obtained were manually checked to correct erroneous readings in BioEdit (Hall 1999) and aligned using ClustalX2 (Larkin et al. 2007). To characterize the obtained sequences, they were compared with previously reported *A. mellifera* haplotype sequences available on GenBank (NCBI: National Center for Biotechnology Information, Bethesda, MD, USA), using BLASTn (Basic Local Alignment Search Tool) with high-quality parameters of similarity (megablast) <http://blast.ncbi.nlm.nih.gov/Blast>. Similarities were calculated by Neighbor joining method, and circular phylogenetic tree was constructed by UPGMA in MEGA7 (Kumar et al. 2016) using the maximum composite likelihood (MCL) approach (Tamura et al. 2004). After processing, 23 of the sequences obtained from queen breeder's colonies were deposited in the GenBank database with the following access numbers: MN026273-95.

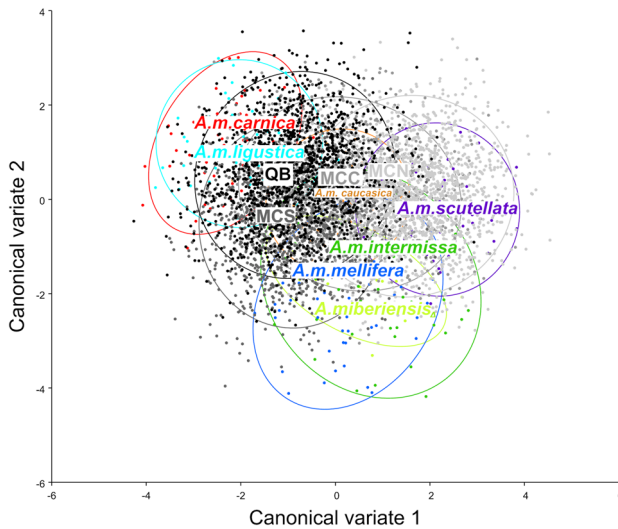
### 3. RESULTS

#### 3.1. Geometric morphometrics

The canonical variate analysis (CVA) of wing shape using origin (MCN, MCC, MCS, QB) as classification criterion revealed that the first three components (CV1 = 58.623%; CV2 = 11.914%; CV3 = 16.615%) explain 81.152% of the total

variation (Figure 2). Wing shape (represented by the 38 principal component scores) differed significantly between groups (MANOVA: Wilks' lambda = 0.00021;  $P < 0.0001$ ). Mahalanobis distances and  $P$ -values from permutation tests were also calculated for groups with different origins. In pairwise comparisons, all groups significantly differed from each other (Table 1). The CVA revealed consistent results but had differences in the magnitude of Mahalanobis distances (MD) obtained when datasets ( $N = 634$ ) were grouped by colony (MD<sub>bc</sub>) to compare morphotype in managed colonies ( $N = 480$ ) from different regions (MCN, MCC, and MCS) with queen breeder's (QB) colonies ( $N = 154$ ) (Figure 3), or were grouped by province (MD<sub>bp</sub>) to build morphotype maps (Figure 4). The African morphotype predominates in managed colonies from the northern region with shorter MD<sub>bc</sub> to *A. m. scutellata* than to *A. m. intermissa* (Figure 3). The closest MD<sub>bp</sub> to *A. m. scutellata* were found in Misiones (2.22), La Rioja (2.62), Jujuy (2.75), Catamarca (2.77), and Chaco (2.87) (Figure 4a). For *A. m. intermissa*, the closest ones were found in Santiago del Estero (3.22), Misiones (3.32), Santa Fe (3.48), La Rioja (3.50), and Catamarca (3.55) (Figure 4b). Both African subspecies showed MD<sub>bc</sub> that increased in managed colonies from central and southern regions, and even more in relation to queen breeder's colonies (Figure 3). Something to highlight regarding the *scutellata*-morphotype is its distribution limited only to the northern region, with MD<sub>bc</sub> that increase significantly at higher latitudes, but with greater magnitude compared to other subspecies. *A. m. iberiensis*, native to the Iberian peninsula and taxonomically located between M and A lineage, presented the highest MD<sub>bp</sub> compared to other *A. mellifera* subspecies being the morphotype with less territorial distribution mainly found in Córdoba (4.44), Santa Fe (4.77), and Corrientes (4.85), (Figure 4c). The smallest MD<sub>bc</sub> were found in managed colonies from the north and central regions with significant increases for the southern region and queen breeder's colonies (Figure 3). *A. m. caucasica* assigned to the O lineage by morphometry and native to the Caucasus valleys revealed shorter MD<sub>bp</sub> than *A. m. iberiensis*, and it





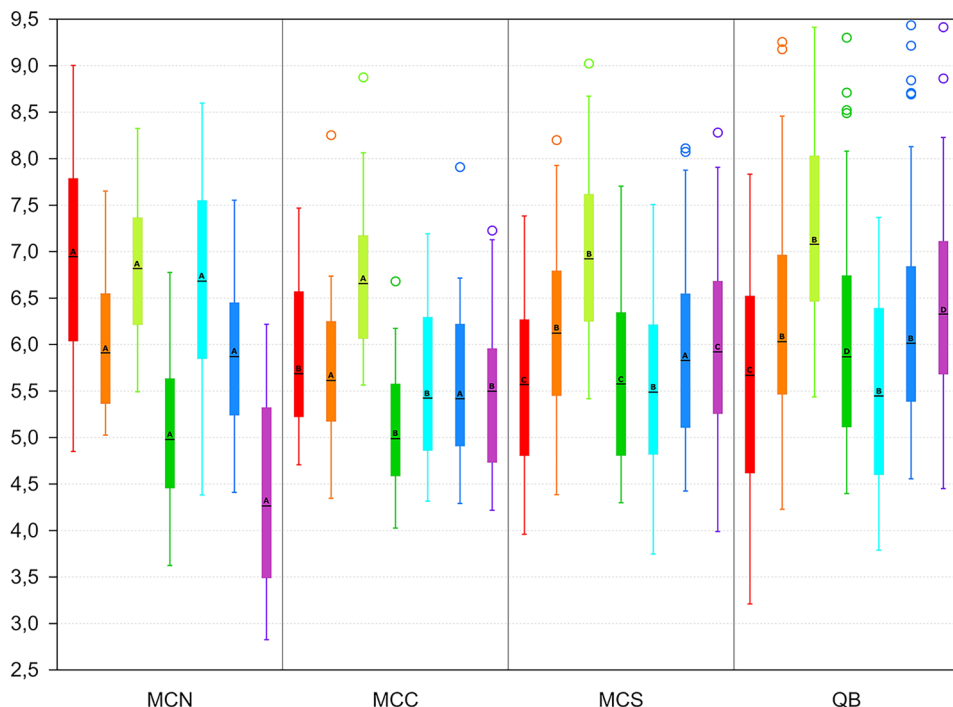
**Figure 2.** Canonical variate analysis (CVA). The first two canonical variates illustrate wing shape variation in different groups (managed colonies in north region MCN = light gray; managed colonies in central region MCC = grey; managed colonies in south region MCS = dark gray; Queen breeder's colonies QB = black), in comparison to reference samples of *A. mellifera* subspecies (*A. m. carnica* = red; *A. m. caucasica* = orange; *A. m. iberiensis* = light green; *A. m. intermissa* = green; *A. m. ligustica* = turquoise; *A. m. mellifera* = blue and *A. m. scutellata* = purple). Each marker (points) represents the mean scores of each colony. The ellipses represent 95% confidence intervals around the centroid of each data cluster.

is distributed mainly on the central region in Entre Ríos (4.08), Córdoba (4.14), San Luis (4.15), and Corrientes (4.17) (Figure 3d).

Although there are short  $MD_{bc}$  in managed colonies from the south region and queen breeder's colonies, these increase significantly when being compared with managed colonies in both the north and central regions (Figure 3). The European dark honey bee (M lineage: *A. m. mellifera*) showed a wide territorial distribution with closer  $MD_{bp}$  in Rio Negro (3.25), Entre Ríos (3.37), Santiago del Estero (3.49), Santa Fé (3.57), Salta (3.58), and Tucumán (3.59), (Figure 4e). When analyzing  $MD_{bc}$  in managed colonies, no significant differences were observed between regions. However, they increased significantly compared to queen breeder's colonies (Figure 3). Although the European morphotype is represented by at least 4 different *A. mellifera* subspecies analyzed here, two of them presented distances that decrease significantly between regions with increasing latitude. The Italian honey bee (C lineage: *A. m. ligustica*) mainly occupies the central

and southern regions with closer  $MD_{bp}$  in Mendoza (3.23), Buenos Aires (3.27), La Pampa (3.28), Rio Negro (3.31), and Santa Cruz (3.42) (Figure 4f). When comparing managed colonies, a significant decrease in  $MD_{bc}$  between the north and central or south regions was observed. However, no significant differences were observed between managed colonies from these regions and queen breeder's colonies (Figure 3). A similar scenario occurs with the Carniolan honey bee (C lineage: *A. m. carnica*) which is distributed with close  $MD_{bp}$  in Rio Negro (3.27), Buenos Aires (3.47), and Chubut (3.57), (Figure 4g), and as latitude increases,  $MD_{bc}$  in managed colonies decrease significantly between the north and central and even south regions, but no significant differences were observed comparing south region with queen breeder's colonies (Figure 3).

By linearly correlating  $MD_{bc}$  and latitude, significant  $P$ -values ( $< 0.05$ ) were obtained for six *A. mellifera* subspecies (Figure 5). Strong negative correlations were observed for *A. m. carnica* ( $r_s = -0.4967$ ) and *A. m.*



**Figure 3.** Mahalanobis distances (MDs) obtained from canonical variate analysis (CVA) of wing shape using datasets grouped by colony. The graph is separated into four blocks comparing managed colonies in the north region (MCN), managed colonies in the central region (MCC), managed colonies in the south region (MCS), and queen breeder's colonies (QB). Box and Whisker plots represent values of MD to different *A. mellifera* subspecies (*A. m. scutellata* = purple; *A. m. intermissa* = dark green; *A. m. iberiensis* = light green; *A. m. caucasica* = orange; *A. m. mellifera* = blue; *A. m. ligustica* = turquoise; *A. m. carnica* = red). The black script markers indicate medians, boxes indicate 25th and 75th percentiles, whiskers indicate non-outlier range, and circles indicate outliers. The same letters next to scripts indicate no significant difference in a Kruskal Wallis test.

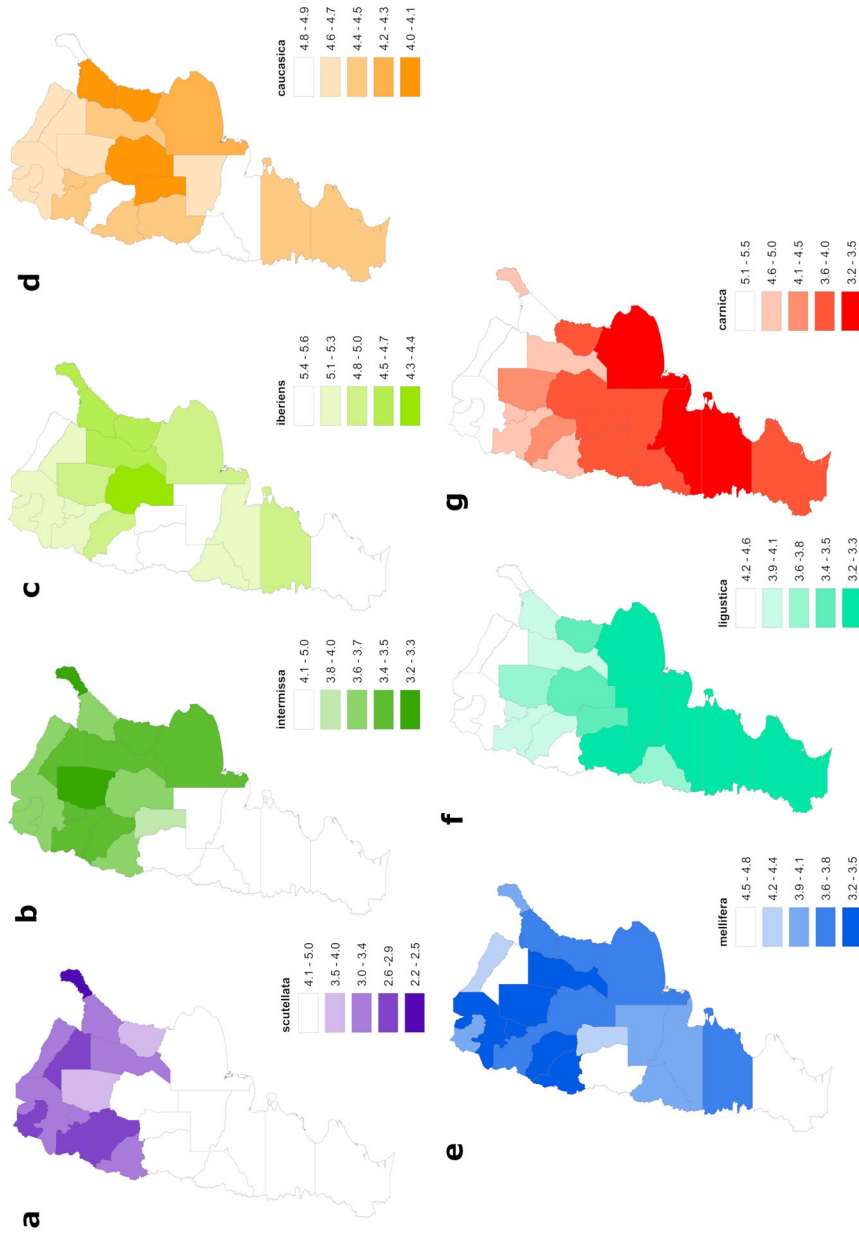
*ligustica* ( $r_s = -0.4510$ ), showing closer  $MD_{bc}$  with increasing latitude. Strong and moderate positive correlations were observed for *A. m. scutellata* ( $r_s = 0.59708$ ) and *A. m. intermissa* ( $r_s = 0.30171$ ), while for *A. m. caucasica* ( $r_s = 0.12379$ ) and *A. m. iberiensis* ( $r_s = 0.13375$ ), the weakest positive correlations were observed, showing closer  $MD_{bc}$  as latitude decreases. No correlations were observed for *A. m. mellifera*.

### 3.2. Mitotype characterization

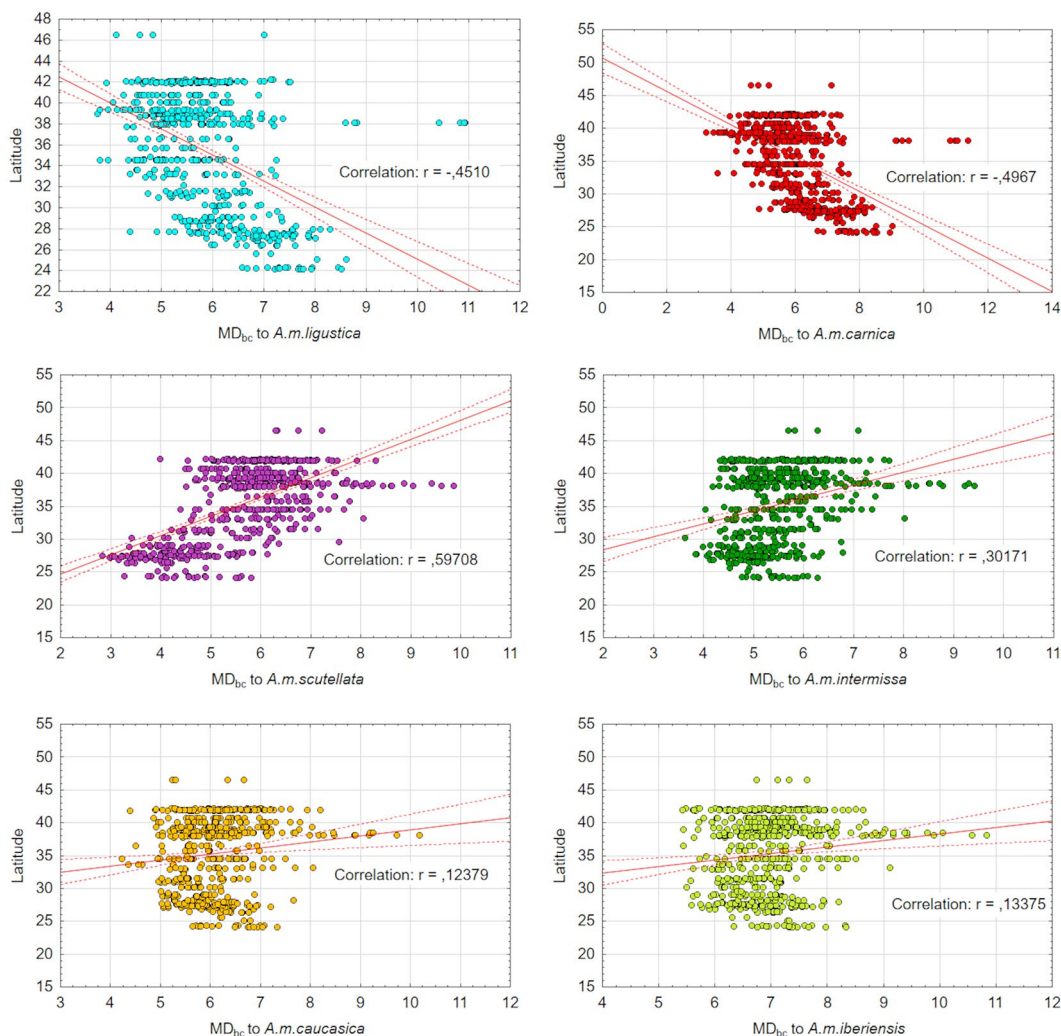
Sequence analysis of the intergenic (COI-COII) region of mitochondrial DNA allowed us to classify queen breeder colonies as belonging

to lineage C (Eastern Europe). The sequences were deposited in GenBank (access no. MN026273-95). The comparison of sequences obtained with the Blast tool yielded a similarity percentage greater than 99% with at least 6 sequences previously deposited (FJ824582.1, MF428426.1, MH341407.1, MH939340.1, JF723976.1, MH939345. 1). These sequences belong to three different haplotypes: C1, characteristic of the *A. m. ligustica*, and C2d and C2j, characteristic of *A. m. carnica* (Table II). In silico DraI restriction analysis revealed the absence of P-type regions (P0, P, or P1) characteristic of lineages A and M. A single Q-type region belonging to lineage C was identified in all samples, with three restriction sites for the





**Figure 4.** Distribution map for different *A. mellifera* subspecies. Mahalanobis distances (MDs) obtained from canonical variate analysis (CVA) of wing shape grouping all datasets by province were used to color each province with different intensity, which increases with decreasing MD **a** *A. m. scutellata* (purple), **b** *A.m. intermissa* (dark green), **c** *A.m. iberiensis* (light green), **d** *A.m. caucasica* (orange), **e** *A.m. mellifera* (blue), **f** *A.m. ligustica* (turquoise), **g** *A.m. carnica* (red).



**Figure 5.** Significant Spearman's coefficient of rank correlation for six subspecies of *A. mellifera*. Mahalanobis distances obtained from CVA grouping datasets by colony ( $MD_{bc}$ ) were plotted separately in relation to latitude using different color marks for different subspecies. In addition, the Spearman ( $r_s$ ) values are indicated.

DraI (TTTAAA) enzyme located at positions 44, 84, and 148 and three polymorphic sites at positions 205, 275, and 413 (Figure 6).

#### 4. DISCUSSION

Based on geometric morphometrics and mitochondrial DNA analysis, our comprehensive study provides novel insights into the current genetic

diversity in Argentina, which will serve as background for future population studies. The CVA carried out in different regions allowed us to understand the latitudinal variation of *A. mellifera* morphotype in both managed and queen breeder colonies. In Argentina, the morphotype present in managed colonies is as diverse as the origin of *A. mellifera* subspecies introduced over time. The genetic structure becomes more complex if we consider the population admixture that occurred during

the Africanization process. Africanized populations in America have retained a predominantly African morphotype (Clarke et al. 2001) with a stronger influence of African over European genes on wing venation patterns (Francoy et al. 2012). This genetic dominance, added to competitive advantages in tropical regions, has possibly caused a greater flow of African genes towards European populations. This asymmetric gene flow did not necessarily lead to the loss of European genetic markers; but it possibly contributed to the preservation of an African phenotype (Guzmán-Novoa et al. 2011).

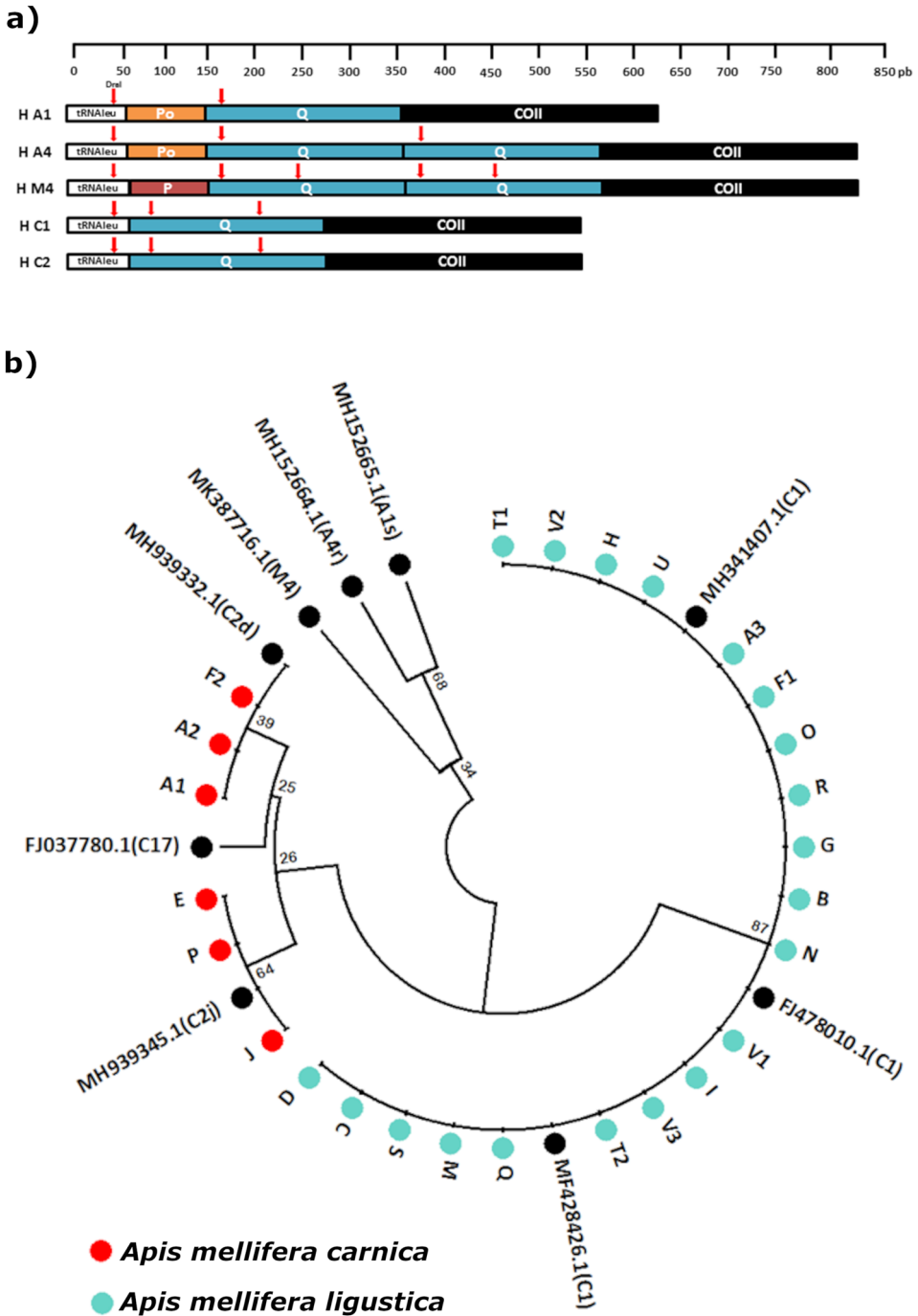
Although we can affirm that *A. mellifera* morphotype in Argentina varies latitudinally, not all *A. mellifera* subspecies are correlated in the same way or with the same intensity. As previously established (Whitfield et al. 2006; Agra et al. 2018; Porrini et al. 2020), the north region is dominated by Africanized populations with high similarity to the subspecies *A. m. scutellata* for which a strong correlation with latitude is observed, but also to a lesser degree with *A. m. intermissa* with a weak correlation. Transition zones began to be delimited through mtDNA analysis and morphometry (Sheppard et al. 1991), and it was possible to determine ALBA haplotype in 25% of Africanized colonies in Argentina (Sheppard et al. 1999). This haplotype is only found in *A. m. intermissa* colonies from North Africa (Morocco) but not in sub-Saharan colonies of *A. m. scutellata*, and was possibly introduced from the Iberian Peninsula. Consistent with these findings, our analysis showed a north–south clinal distribution of *A. m. intermissa* that extends even further than *A. m. scutellata*; nevertheless, for *A. m. iberiensis*, less similarity was found, and more heterogeneous distributions were obtained. A recent study using (mtDNA) sequence data of Iberian honeybee (Chávez-Galarza et al. 2017) revealed a great diversity of haplotypes belonging to the M, but mainly to A lineage. Three sublineages were described, and among them, two ( $A_{II}$  and  $A_{III}$ ) contained *A. m. intermissa*. Considering the hybrid origin of *A. m. iberiensis* (Henriques et al. 2020), it is possible that these two sublineages were the ones introduced in Argentina by early Spanish or Portuguese settlers. Even they could

have arrived from Brazil together with *A. m. scutellata* at the beginning of the Africanization process (Collet et al. 2006; Whitfield et al. 2006).

In 2007, Abrahamovich et al. found two African haplotypes (A8, A11) belonging to  $A_{II}$  lineage and three (M2, M3, M6) belonging to M lineage in managed colonies from Buenos Aires. In 2018, Agra et al. found feral colonies with mitochondrial haplotypes A1 and A30 belonging to  $A_I$  and  $A_{III}$  sublineage and managed colonies with M4 haplotype characteristic of *A. m. mellifera*. Our morphotype analysis revealed that this is the subspecies within the M lineage most widely distributed in Argentina, showing similar close distances for all three regions, even in queen breeder colonies. Regarding *A. m. caucasica*, there are no previous studies that confirm its presence in Argentina; however, there are queen breeders that produce hybrids between *A. m. ligustica* and *A. m. caucasica* (Min.Agri.BA., 2019), and possibly, there have been unrecorded introductions. Apparently, these hybrids would be more useful in areas with high temperatures and short nectar flows due to their longevity and gloss length. Both M (*A. m. mellifera*) and O (*A. m. caucasica*) lineages lacked a pronounced latitudinal variation in managed colonies, showing equal similarity in queen breeder colonies.

In managed colonies from the central and southern regions, European morphotype clearly predominates, with C lineage (*A. m. ligustica*; *A. m. carnica*) showing a strong north–south clinal distribution inversely proportional to that observed for A lineage (*A. m. scutellata*; *A. m. intermissa*). In queen breeder colonies, we found the closest wing shape proximity with *A. m. ligustica* and *A. m. carnica*. In agreement with these results, mitotype analysis revealed three mitochondrial haplotypes (C1 and C2d and C2j) characteristic of these two subspecies.

Our results are consistent with two previous studies, the first established as most frequent (92%) C1 haplotype in Buenos Aires province (Abramovich et al. 2007), the second in which 396 colonies from different provinces were analyzed (Agra et al. 2018), showing only two haplotypes belonging to C lineage (C1, C2j). The preference of beekeepers to manage *A. m.*



**Figure 6.** **a** Arrangement of the P and Q regions and restriction sites of the *Dral* enzyme in different lineages and mitochondrial haplotypes of *A. mellifera*. **b** Circular phylogeny analysis by UPGMA method. Evolutionary distances were calculated using the composite maximum likelihood method and are in the units of the number of base substitutions per site. C1 haplotypes are shown in turquoise, C2 haplotypes in red, and reference sequences in black.

**Table 1** Canonical variate analysis (CVA) of wing shape using colony origin as a classification criterion. The double entry table shows Mahalanobis distances among groups (managed colonies in north region MCN; managed colonies in central region MCC; managed colonies in south region MCS; Queen breeder colonies QBC; *A. m. carnica*, *A. m. caucasica*, *A. m. iberiensis*, *A. m. intermissa*, *A. m. ligustica*, *A. m. mellifera*, and *A. m. scutellata*) and *P*-values from permutation tests (10,000 permutation rounds)

	MCN	MCC	MCS	QBC	<i>A. m. carnica</i>	<i>A. m. caucasica</i>	<i>A. m. iberiensis</i>	<i>A. m. intermissa</i>	<i>A. m. ligustica</i>	<i>A. m. mellifera</i>	<i>A. m. scutellata</i>
MC (North)	–	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
MC (Central)	<b>1.4137</b>	–	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
MC (South)	<b>2.1627</b>	<b>1.5408</b>	–	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001
QBC	<b>2.5402</b>	<b>1.3184</b>	<b>1.2009</b>	–	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
<i>A. m. carnica</i>	4.6146	<b>3.7039</b>	<b>3.1813</b>	<b>3.0180</b>	–	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
<i>A. m. caucasica</i>	4.3154	4.0643	4.3939	4.2497	5.7306	–	<.0001	<.0001	<.0001	<.0001	<.0001
<i>A. m. iberiensis</i>	4.7890	4.5427	4.9050	4.8431	6.3654	5.6711	–	<.0001	<.0001	<.0001	<.0001
<i>A. m. intermissa</i>	<b>3.1977</b>	<b>3.9214</b>	4.4041	5.6486	5.3124	5.1516	4.1812	–	<.0001	<.0001	<.0001
<i>A. m. ligustica</i>	4.4048	<b>3.6170</b>	<b>3.1227</b>	<b>3.0794</b>	2.5838	5.7761	5.7144	5.2655	–	<.0001	<.0001
<i>A. m. mellifera</i>	3.6643	<b>3.5165</b>	3.7641	3.8256	5.7805	5.3352	3.8345	3.2178	5.4636	–	<.0001
<i>A. m. scutellata</i>	<b>2.2823</b>	<b>3.8092</b>	4.1415	4.5782	5.0318	4.5822	4.9136	3.5307	5.0404	4.1505	–

**Table II** Characterization of maternal lineage (A, C, M, O, Y) and mitochondrial haplotype indicating the characteristic subspecies in queen breeder's colonies from different provinces of Argentina. The access number to the sequence deposited in the GenBank database is detailed for each sample

Colony	Province	Lineage	GenBank Acc. Numb	Subspecies	Haplotype
A1	Buenos Aires	C	MN026287	<i>A. m. carnica</i>	C2d
A2	Buenos Aires	C	MN026287	<i>A. m. carnica</i>	C2d
A3	Buenos Aires	C	MN026280	<i>A. m. ligustica</i>	C1
B	Buenos Aires	C	MN026274	<i>A. m. ligustica</i>	C1
C	Buenos Aires	C	MN026275	<i>A. m. ligustica</i>	C1
D	La Pampa	C	MN026288	<i>A. m. ligustica</i>	C1
E	Buenos Aires	C	MN026279	<i>A. m. carnica</i>	C2j
F1	Rio Negro	C	MN026276	<i>A. m. ligustica</i>	C1
F2	Rio Negro	C	MN026289	<i>A. m. carnica</i>	C2d
G	Rio Negro	C	MN026284	<i>A. m. ligustica</i>	C1
H	Mendoza	C	MN026290	<i>A. m. ligustica</i>	C1
I	Sgo. Del Est	C	MN026291	<i>A. m. ligustica</i>	C1
J	Buenos Aires	C	MN026292	<i>A. m. carnica</i>	C2j
M	Santa Fe	C	MN026285	<i>A. m. ligustica</i>	C1
N	Mendoza	C	MN026282	<i>A. m. ligustica</i>	C1
Ñ	Cordoba	C	MN026293	<i>A. m. ligustica</i>	C1
O	Entre Rios	C	MN026286	<i>A. m. ligustica</i>	C1
P	Santa Fe	C	MN026292	<i>A. m. carnica</i>	C2j
Q	Santa Fe	C	MN026283	<i>A. m. ligustica</i>	C1
R	Santa Fe	C	MN026294	<i>A. m. ligustica</i>	C1
T1	Buenos Aires	C	MN026278	<i>A. m. ligustica</i>	C1
T2	Buenos Aires	C	MN026295	<i>A. m. ligustica</i>	C1
U	Buenos Aires	C	MN026277	<i>A. m. ligustica</i>	C1
V1	Mendoza	C	MN026273	<i>A. m. ligustica</i>	C1
V2	Mendoza	C	MN026273	<i>A. m. ligustica</i>	C1
V3	Mendoza	C	MN026280	<i>A. m. ligustica</i>	C1
W	La Pampa	C	MN026281	<i>A. m. ligustica</i>	C1

*ligustica*, *A. m. carnica*, or even hybrid Buckfast is commonly reported in countries that exceed the native range of *A. mellifera* and could be due to some particular characteristics such as gentleness, productivity, prolificacy, resilience in environmental conditions, or low swarming tendency (Jensen et al. 2005; Moritz et al. 2005).

However, morphotype analysis also revealed a slighter similarity with other European subspecies (*A. m. mellifera*; *A. m. caucasica*). This may be due to the fact that wing venation patterns present a biparental inheritance, contrary to the

mtDNA, and maternal inheritance; it seems to influence more in descendants from interracial hybrids (Węgrzynowicz et al. 2019). Molecular methods based on the analysis of mtDNA sequences are more useful for phylogenetic studies but do not show much efficiency in subspecies characterization (Meixner et al. 2013; Oleksa and Tofilski 2015). The new techniques of gDNA sequencing or SNP analysis (Momeni et al. 2021; Whitfield et al. 2006) are more exhaustive but are not yet easily available for use by beekeepers or queen breeders.



Geometric morphometric analysis of wing shape can be used for a quick and economic characterization of *A. mellifera* subspecies in order to have a better criterion in selective breeding, and its effectiveness has even been proven using automatic image recognition and machine learning applied to honey bee wings (Da Silva et al. 2015; De Nart et al. 2022). Commercial honeybee populations are selected, multiplied, and distributed to and from different regions annually; therefore, environmental factors are not the only parameters that affect morphotype or genetic stock hybridization.

The results presented here show that the current genetic diversity in *A. mellifera* colonies in Argentina is wide and comprises mainly seven different subspecies belonging to four evolutionary lineages (C, M, A, O). The genetic structure of *A. mellifera* in Argentina reflects its evolutionary history but also specific adaptations to different biogeographical regions which lack strong geographical barriers (Arana et al. 2017). Morphotype differences found probably respond to variations in gene flow between populations related to environmental factors such as climate, resource availability, and nesting sites, but mainly to anthropic factors such as agricultural practices including transhumance, honeybee queen's importation, and live beekeeping material (queens, queen cells, bee packs, bee nucleus) commercialization in the local market.

In Argentina, as in the rest of the world, honeybees live predominantly in managed colonies, and this has significantly impacted their genetic structure and admixture promoting the horizontal transmission of diseases (Mutinelli 2011). As future perspectives, it is considered necessary to continue evaluating genetic diversity in queen breeders' colonies and studying through field trials, if the increase in intracolony genetic diversity improves productivity or response to different stressors. We also consider that monitoring and evaluation of productive parameters carried out by queen breeders are essential to increase colony performance. This will allow us to understand how different traits are inherited in hybridized populations from different biogeographical regions and how management practices contribute to the long-term success of

honeybee colonies. Being able to identify subspecies of *A. mellifera* and characterize the colonies in an agile and simple way considering the current hybridization is crucial for selection and multiplication, as well as for managing honeybee colonies in an increasingly dynamic environment. The joint application of morphometric and molecular methods to estimate genetic diversity in hybridized *Apis mellifera* colonies is the basis of the success of such an objective, and this is demonstrated by the results of the present study.

## SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1007/s13592-022-00970-1>.

## ACKNOWLEDGEMENTS

We thank all the beekeepers and queen breeders who collected and forwarded samples from different localities as well as the organizers of the queen breeder meetings 2018, 2019, and 2020 held in “cabaña apícola Pedro J Bover” Gral. Belgrano Buenos Aires. We appreciate the contribution of the anonymous reviewers and editorial team who helped improve this manuscript.

## AUTHOR CONTRIBUTION

Leonardo Porrini was partially in charge of obtaining samples and performing mitochondrial sequence analysis and performing all morphometric analyses, including sample processing, imaging, and statistical data analysis, and writing the manuscript. All the authors provided critical comments and helped shape the research; Silvina Quintana directed mitotype analysis. Constanza Brasesco assisted on DNA extraction and sequencing for mitotype analysis. Matias Maggi, Martín Porrini, and Melisa Garrido participated in sample collection. Pedro Fernandez collaborated on morphotype analysis, and Martin Eguaras was in charge of obtaining the financing.

## FUNDING

This research was supported by the CIAS and UNMdP (EXA 2016–2017, PID 035/2016 and Project PIT-AP-BA 2016 Comision de investigaciones científicas (CIC) and was partially financed through a technology linkage project called “Puesta en valor del sector apícola” (resolución SPU 2373/16).

## DATA AVAILABILITY

The sequences obtained and analyzed during the current study were deposited in GenBank (access no. MN026273-95). The geometric morphometric wing shape datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

## CODE AVAILABILITY

Not applicable.

## DECLARATIONS

**Ethics approval** No approval of the Research Ethics Committee was required to achieve the goals of this study, as the experimental work involved unregulated invertebrate species (*Apis mellifera*).

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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