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The population bottleneck of the Iberian wolf impacted genetic diversity but not admixture with domestic dogs: A temporal genomic approach

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Abstract

After decades of intense persecution, the Iberian wolf subspecies faced a severe bottleneck in the 1970s that considerably reduced its range and population size, nearly leading to its extinction in central and southern Iberian Peninsula. Such population decline could have impacted the genetic diversity of Iberian wolves through different processes, namely genetic drift and dynamics of hybridization with domestic dogs. By contrasting the genomes of 68 contemporary with 54 historical samples spanning the periods before and immediately after the 1970s bottleneck, we found evidence of its impact on genetic diversity and dynamics of wolf–dog hybridization. Our genome-wide assessment revealed that wolves and dogs form two well-differentiated genetic groups in Iberia and that hybridization rates did not increase during the bottleneck. However, an increased number of hybrid individuals was found over time during the population re-expansion, particularly at the edge of the wolf range. We estimated a low percentage of dog ancestry (~1.4%) in historical samples, suggesting that dog introgression was not a key driver for wolf extinction in central and southern Iberia. Our findings also unveil a significant decline in genetic diversity in contemporary samples, with the highest proportion of homozygous segments in the genome being recently inherited. Overall, our study provides unprecedented insight into the impact of a sharp decline on the Iberian wolf genome and refines our understanding of the ecological and evolutionary drivers of wolf–dog hybridization in the wild.

KEYWORDS
anthropogenic hybridization, bottleneck, dog, genomics, Iberian Peninsula, wolf

1 | INTRODUCTION

Large carnivores, such as the grey wolf (*Canis lupus*), have a long history of human persecution and habitat fragmentation across the European continent (Chapron et al., 2014; Ripple et al., 2014). Grey wolves, which were once widely distributed throughout the Holarctic realm (Boitani, 2003), suffered such a dramatic decline that were vanished from most of their western/central range in the last two centuries, remaining only in small scattered populations mainly located in southern European Peninsulas (Blanco et al., 1992; Boitani, 2003; Randi, 2011). From a genetic perspective, such small and isolated populations are expected to experience severe effects of genetic drift and inbreeding, triggering mechanisms for genetic impoverishment that may ultimately drive a population to extinction (Charlesworth & Willis, 2009; Lande, 1994). One such isolated wolf population persists in the Iberian Peninsula in a highly human-dominated landscape (Llaneza et al., 2012; Sazatornil et al., 2016).

The Iberian wolf population, ascribed to its own subspecies *C. l. signatus*, became isolated from other European populations circa 10,000 years ago (ka) (Silva et al., 2020). While more widespread throughout the Iberian Peninsula in the past (Clavero et al., 2022; Nores & López-Bao, 2022), this population mainly ranges now in north-western Iberia (Figure 1a). Systematic persecution, particularly through poisoning campaigns since the first-half of the 19th century (Nores & López-Bao, 2022; Ortiz de Zúñiga & De Herrera, 1832) and intensified in the mid-20th century, resulted in the decline of the Iberian wolf population to its minimum in the 1970s (Garzón, 1979; Petrucci-Fonseca & Álvares, 1997; Sastre et al., 2011; Valverde, 1971). Consequently, the population vanished from most of the central and southern regions of the Iberian Peninsula, remaining only two isolates in those areas: a small population in the south of Douro River in central Portugal, currently persisting; and three nuclei in Sierra Morena, southern Spain, all now extinct (Gómez-Sánchez et al., 2018; López-Bao et al., 2018). Following the 1970s, the persisting population in the north-western region expanded in Spain (Blanco & Cortés, 2009; Chapron et al., 2014; López-Bao et al., 2018), while still showing a notable regression pattern in Portugal, particularly south of the Douro River (Pedra, 2015). Additionally, low levels of genetic diversity in the Iberian wolf have been revealed through genome-wide studies (Fan et al., 2016; Pilot et al., 2014), and this has been associated with the population bottleneck and long-term isolation.

Events of wolf–dog hybridization have also been documented in the Iberian wolf population (Fan et al., 2016; Godinho et al., 2011, 2015; Gómez-Sánchez et al., 2018; Pacheco et al., 2017; Torres et al., 2017). Notably, an in-depth genomic analysis of one of the last wolves from Sierra Morena, road-killed in 2003, revealed massive levels of introgression from the domestic dog (*C. l. familiaris*), suggesting that hybridization events may have contributed to wolf extinction in that area (Gómez-Sánchez et al., 2018). In fact, anthropogenic hybridization between wolves and dogs has often been a topic of scientific debate owing to its potential conservation implications (e.g. Donfrancesco et al., 2019; Hindrikson et al., 2017; Salvatori et al., 2020; Stronen et al., 2022), which include the dilution of genetic distinctiveness of wolves through dog introgression, and disruption of behaviour and local adaptations (Bassi et al., 2017; Caniglia et al., 2014; Popova & Zlatanova, 2019; Salvatori et al., 2019). The occurrence of wolf–dog hybridization correlates with disruption of social structure in wolf packs and it is more frequent in peripheral areas of the wolf range, particularly in fragmented and small populations (Galaverni et al., 2017; Godinho et al., 2011; Hindrikson et al., 2017), which are outnumbered by free-ranging dogs (Pilot et al., 2021; Randi & Lucchini, 2002). Although cases of wolf–dog hybridization have been reported in Europe over the past years using both genetic (autosomal and sex chromosome microsatellites, nuclear genes and mitochondrial DNA; e.g. Godinho et al., 2011; Hindrikson et al., 2012; Kusak et al., 2018; Pacheco et al., 2017; Randi et al., 2014; Vilà et al., 2003) and genomic approaches (thousands of single nucleotide polymorphisms, SNPs, and whole-genome sequences; e.g. Galaverni et al., 2017; Pilot et al., 2018; Smeds et al., 2021), a historical perspective of its extent remains largely unclear. Although Galaverni et al. (2017) have reported increased rates of wolf–dog hybridization in Italy over the last three decades, their estimates of admixture were derived solely from contemporary samples and did not cover the temporal period before the wolf population bottleneck in Italy. A robust assessment of hybridization dynamics over larger temporal periods requires the incorporation of historical data in the analysis.

In this work, we aim to understand the impact, at a genomic level, of the 1970s bottleneck suffered by the Iberian wolf population. We hypothesize that the dramatic decline and population contraction within central and southern regions of Iberia have facilitated an increase in hybridization events and depleted genetic variability, ultimately contributing to wolf extinction. The persistence and ensuing expansion of wolves in the northern range would have allowed the population to maintain stable levels of genetic diversity. To approach this, we analysed thousands of SNPs across contemporary and historical biological Iberian wolf samples representing the periods before and following the bottleneck. We first inferred rates of wolf–dog hybridization within historical and contemporary data, then estimated the percentage of dog introgression in wolf historical samples, and lastly quantified levels of genetic diversity and autozygosity in both sample sets.

2 | MATERIALS AND METHODS

2.1 | Contemporary and historical SNP data set

We genotyped contemporary samples of 61 putative wolves, seven previously identified wolf–dog hybrids (based on genetic assessment using microsatellites; see Table S2 for more information on hybrids, including morphological traits) and 43 dogs (23 village dogs and 20 livestock guarding dogs), all from the Iberian Peninsula (Table S1), for a panel of 170,000 genome-wide SNPs using the Canine HD BeadChip microarray (Illumina, San Diego,

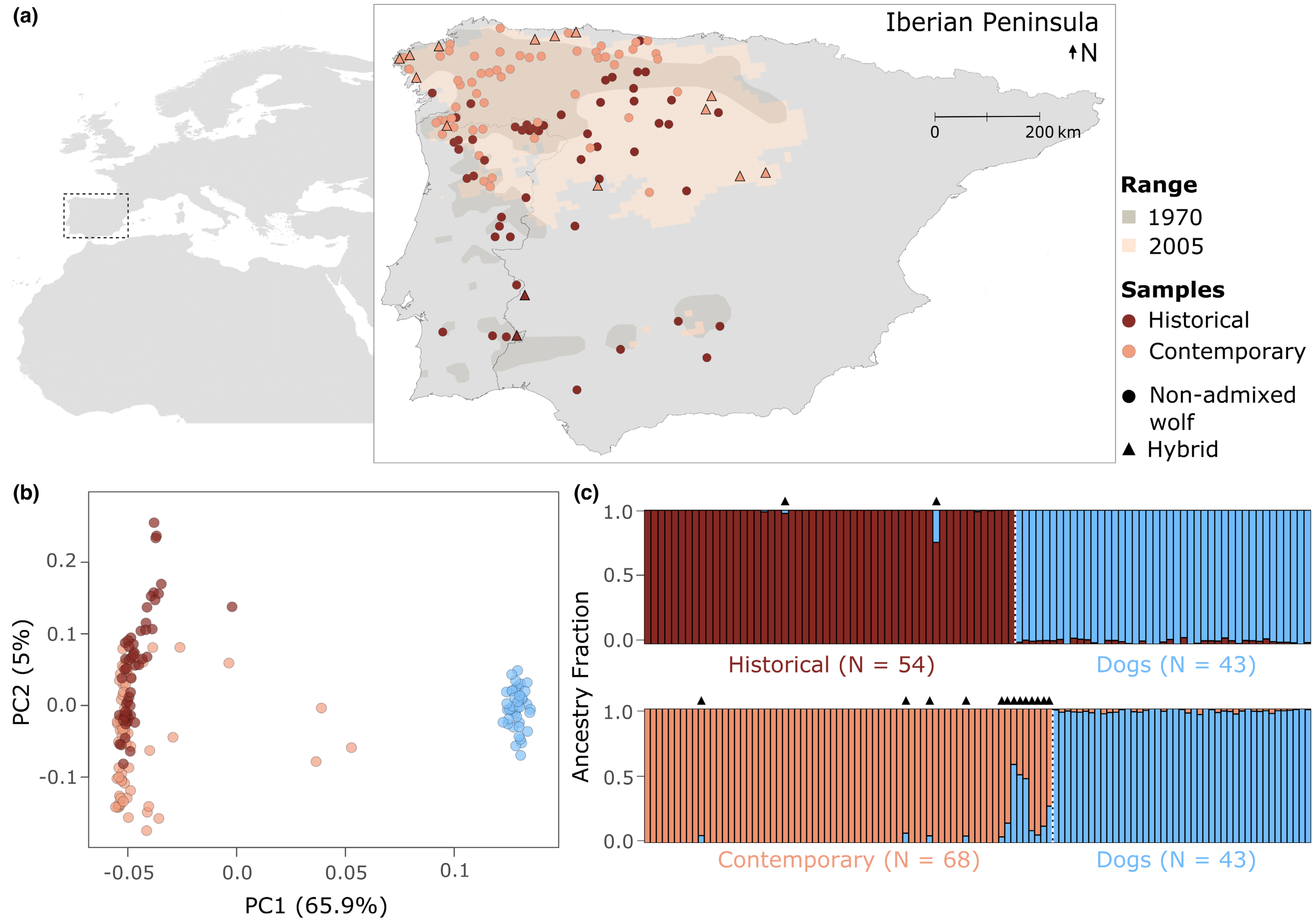

FIGURE 1 Sampling location and admixture levels. (a) Geographical location of the analysed wolf and wolf–dog hybrid samples in the Iberian Peninsula (delimited by the dashed line in the left map). Grey and light orange shades indicate the estimated wolf range in the Iberian Peninsula in the 1970s and in 2005 respectively (extracted from López-Bao et al., 2018 and adapted from Álvares et al., 2005; Petrucci-Fonseca, 1990; Valverde, 1971). Historical ($n=54$) and contemporary ($n=68$) samples are represented in red and orange respectively (the same colour scheme was followed in panels b and c). Circles and triangles denote non-admixed wolves and hybrids respectively. Further details about samples can be assessed in Table S1. (b) Principal component analysis (PCA) for historical and contemporary wolves, wolf–dog hybrids and dogs ($n=43$; represented in blue). Each dot denotes an individual. Principal components 1 and 2 are displayed with the corresponding variance values. (c) Individual ancestry fractions inferred in ADMIXTURE for historical/contemporary wolves, wolf–dog hybrids and dogs, at K=2 clusters. Historical and contemporary samples were analysed independently. Individuals are represented by a single bar and different colours indicate the ancestry proportion attributed to each cluster. White dotted lines separate wolf and dog clusters and triangles represent the identified wolf–dog hybrids.

CA, USA). Wolf samples comprised muscle and blood mainly from road-killed animals and were collected across the full current wolf range in Iberian Peninsula (Figure 1a) mostly between 2005 and 2017 (90%; Table S1). Wolf–dog hybrid samples, comprising muscle and blood tissues, were collected from dead animals between 2004 and 2014 (Table S1). Dog samples derived from muscle, blood and buccal swabs and were donated by local shelters and collaborators. DNA extraction was performed using the QIAGEN DNeasy blood & tissue kit (Qiagen) and the concentration of DNA products was estimated using the Qubit DNA quantification system (Thermo Fisher Scientific) with the Qubit broad range assay reagents, following the manufacturer's protocol. DNA concentration was normalized to 50 ng/μL to be genotyped using the Canine SNP chip. Genotype calling was performed using GenomeStudio software (Illumina) following Illumina's recommendations. Additionally, we leveraged the availability of a comprehensive genome-wide data set of 145 historical putative wolf samples genotyped for 100,000 genome-wide SNPs targeted based on the Canine SNP chip, using an in-solution target capture enrichment approach (Pacheco, Lobo, et al., 2022; Table S1). All individuals in our data set are not directly related to each other (identity-by-descent <0.5, following Galaverni et al. (2017); IBD estimated in PLINK v.1.9 using the *–genome* function following PLINK's guidelines; Purcell et al., 2007).

Only autosomal SNPs with unique map positions in the genome were maintained. Then, we filtered the historical data set to keep only samples with more than 6000 SNPs and for high call rates per sample (>0.85) and per locus (>0.90) using PLINK v.1.9.

This resulted in 54 historical samples with 5173 SNPs, dating from 1912 to 1991 (distribution: 1910–1950 = 17%; 1960–1980 = 79%; 1991 = 4%) and covering the former wolf range distribution in the Iberian Peninsula ($n = 19$ and $n = 35$ from the contracting population range in central and southern regions and the stable/expanding population range in north-western Iberia, respectively; Figure 1a; Figure S1). The time interval of historical samples comprehends the decades before, during and immediately after the population bottleneck in the 1970s.

The historical and contemporary data sets were then merged using PLINK v.1.9, and we applied the same filters for high call rates and removed loci with minor allele frequency (MAF) below 0.01, resulting in 4470 common SNPs (hereafter 5 K data set; Table S1 and Figure S2). This set of 5 K SNPs allowed to maximize the number of historical samples included in the data set, and still providing enough resolution to differentiate wolf and dog genomes. Additionally, we assembled two other data sets with a reduced sample size but with an increased number of SNPs (i.e. 20,000 and 60,000 SNPs, hereafter 20 and 60 K data sets, respectively; Figure S2) for specific analyses and to exploit the potential effects of using decreased SNP density over the genome (see below). The 20 K data set resulted in 47 historical samples (15 from the contracting and 32 from the stable/expanding population ranges) and all the contemporary samples, having in common 19,754 SNPs (Figure S2), and the 60 K data set resulted in 22 historical samples (10 from the contracting and 12 from the stable/expanding population ranges) and all the contemporary samples, having in common 61,896 SNPs (Figure S2). The 5 K data set was used for all subsequent analyses, unless otherwise stated.

2.2 | Global ancestry estimation and hybrid identification

We assessed genome-wide differentiation (F_{ST}) levels using the Hudson method (Bhatia et al., 2013; Hudson et al., 1992) between Iberian wolves (historical and contemporary) and dogs, as well as between contemporary and historical Iberian wolf samples using PLINK v.2.0 (Alpha 4.4). A principal component analysis (PCA) was performed in PLINK v.1.9 to explore the average genome-wide global clustering across all samples. As uneven sample sizes can create artefact group clustering in PCA (Elhaik, 2022), we also conducted the analysis in a small subset with an equal randomly selected sample size of wolves ($n = 42$; 21 from each historical and contemporary sets) and dogs ($n = 43$) to test for bias. We then estimated individual ancestry proportions (q) at $K = 2$ for historical/contemporary wolves, hybrids and dogs using a maximum likelihood approach implemented in ADMIXTURE v.1.3 (Alexander et al., 2009). ADMIXTURE was run separately for historical and contemporary wolf samples in 2000 iterations using a 10-fold cross-validation procedure (Alexander & Lange, 2011). Ten independent runs were performed, and q values from the run with the highest likelihood were used. For validation purposes, all the analyses described above were also performed using the 20 K data set (Figure S2).

To establish the q thresholds for hybrid identification, we simulated parental and hybrid genotypes in HYBRIDLAB v.1.0 (Nielsen et al., 2006). Genotypes of individuals with q values ≥0.980 (estimated in ADMIXTURE; following Godinho et al., 2015 and Kusak et al., 2018) were selected as references for each parental group (historical/contemporary wolves and dogs) for the training data set to generate 200 of each simulated parental genotype. The number of reference individuals was uniformized across each parental group ($n = 31$) to avoid bias during allele frequencies estimation, as recommended in HYBRIDLAB documentation. Input ped files were converted into genepop format using PGDSpider v.2.1.1.5 (Lischer & Excoffier, 2012). Simulated parental genotypes were then used to simulate 200 of each F_1 and first and second backcross genotypes in wolves (BC_{1W} and BC_{2W}). This procedure was performed independently for historical and contemporary wolf samples. The q values of each individual simulated genotype (parental and distinct hybrid classes) were estimated in ADMIXTURE at $K = 2$ as previously described. The q threshold to classify individuals in our data set as either non-admixed wolf or hybrid was determined based on the lower q value observed among the 200 simulated genotypes of the wolf parental group. Subsequently, admixed individuals were assigned to specific hybrid classes based on the range of $q_{(\text{min–max})}$ values obtained for the 200 simulated genotypes of each F_1, BC_{1W} and BC_{2W} categories.

We used the *f3* test in THREEPOP (TREEMIX v.1.13 package; Pickrell & Pritchard, 2012) to statistically test if the identified hybrid individuals were the product of admixture between wolves and dogs using the 20 K data set (this data set did not exclude any of the hybrids identified among historical samples using the 5 K data set). *f3* statistics is a population-based test and uses allele counts from SNPs as input. We defined three groups to first calculate minor allele frequencies in PLINK v.1.9: parental wolves (historical and contemporary), parental dogs and hybrids (historical and contemporary); and then used the python script *plink2treemix.py* from the TREEMIX package to convert clustered allele frequencies to treemix format as input for the *f3* test. *f3* test was run with the ((A, B), C) phylogenetic configuration, where A is the parental wolves, B the parental dogs and C the hybrids, using blocks of 200 SNPs ($n = 98$). Z-score values below −3 were used as evidence of admixture.

To increase accuracy in establishing the naïve prevalence of wolf–dog hybridization (i.e. proportion of admixed individuals in the sampled population data set; see Santostasi et al., 2019, 2021 for a distinction with modelled estimation of prevalence) in the Iberian Peninsula and minimize sampling bias, we integrated our hybrid identification results with those from Godinho et al. (2011), which represent the sole assessment of wolf–dog hybridization to date that is based on the whole Iberian population. Godinho et al. (2011) used microsatellite data to analyse a total of 204 non-admixed wolves and eight wolf–dog hybrids, of which 27 and three, respectively, were included in our data set. In total, this represents 238 putative non-admixed contemporary Iberian wolf samples (177 and 34 exclusively from Godinho et al. (2011) and this study, respectively, and 27 in common to both studies).

2.3 | Local ancestry analysis across hybrid genomes

To estimate with higher accuracy the proportion of dog ancestry within hybrid genomes, we used the local ancestry method LAMP-ANC v.2.5 (Sankararaman et al., 2008). LAMP-ANC is a non-LD-based method that estimates within windows the most likely ancestry per SNP given the reference allele frequencies. The analysis was conducted with the 20K data set using parental wolves and dogs as reference populations and all identified hybrids as admixed population. We used a recombination rate of 9.7×10^{-9} (Campbell et al., 2016; Wong et al., 2010); a mixture proportion of 0.81:0.19 to the wolf and dog populations, respectively (based on ADMIXTURE estimates); and pruned linkage disequilibrium (LD) for $r^2 > .1$, because LAMP-ANC assumes unlinked markers. We assumed 10 generations since admixture (considering a wolf generation time of 4.5 years; Mech et al., 2016), to maximize the detection power of recent hybridization events (Pilot et al., 2018, 2021).

We analysed chromosomal permeability to dog introgression in hybrid genomes using SNP-specific delta (Δ) statistics (Tang et al., 2007), which subtracts locus-specific ancestry from average genome-wide ancestry for each hybrid individual. Values are then average across all hybrid individuals and positive values indicate genomic regions with higher dog ancestry than the genome-wide average and, therefore, can indicate higher permeability to, or retention of, dog introgression. We calculated Δ ancestry across all chromosomes, using local estimates of dog ancestry determined by LAMP, and defined outlier regions as those with Δ scores above two standard deviations from the mean chromosome (mean + 2SD). This analysis only used hybrids resulting from backcrossing with wolves (not F_1). To test for significant differences ($p < .05$) in the average proportion of dog ancestry in backcross hybrid chromosomes, we implemented a generalized linear model (GLM) fitted with a binomial error distribution in R v.3.5.2 (R Development Core Team, 2017).

2.4 | Local ancestry analysis across historical genomes

To assess whether the population decline and concomitant extinction of wolves in central and southern Iberian Peninsula were associated with increased levels of historical dog introgression, LAMP-ANC was used to estimate dog ancestry in historical non-admixed wolves using the 60K data set. Given that we were interested in assessing levels of historical introgression in the wolf genome and not in recent hybridization events, the previously identified hybrids were excluded from this analysis. LAMP-ANC was run considering contemporary wolf samples and dogs as reference populations and the historical Iberian wolves as admixed. An equal sample size of 43 was used for reference wolves and dogs. The parameters were set as previously described, except for the mixture proportion (0.99:0.01 to the wolf and dog populations respectively).

2.5 | Genetic diversity analysis

To explore genetic diversity in historical and contemporary Iberian wolf sample sets (hybrids were excluded), we measured the proportion of heterozygous sites, LD decay curves and autozygosity at both individual and population levels.

We used the 5K data set (without the MAF filter applied; 4613 SNPs) to estimate the proportion of heterozygous sites per individual using the PLINK v.1.9 *–het* function, with an equal sample size of 52 for historical and contemporary sets to avoid bias. We tested for significant differences ($p < .05$) in heterozygosity between historical and contemporary sets using a Mann–Whitney/Wilcoxon rank-sum test in R. We also estimated the distribution of heterozygosity across the genome of historical and contemporary individuals using the *–hardy* function in PLINK v.1.9. For this, we used the 60K data set without the MAF filter applied (63,167 SNPs), which included 22 historical samples. We randomly selected the same number of contemporary samples to avoid bias. Values of heterozygosity were then averaged in non-overlapping windows of 10 SNPs (covering ~350 kb) for plotting.

Estimates of LD decay were calculated independently for historical and contemporary sample sets using the 60K data set without the MAF filter applied, based on pairwise r^2 values between all pairs of SNPs within a 1-Mb window. This analysis was carried out in PLINK v.1.9 using the $–r^2$ function. Values were then averaged in non-overlapping 1 kb windows for plotting.

Autozygosity levels were assessed using two methods: an observation-based model implemented in PLINK v.1.9 and a Hidden Markov Model (HMM)-based approach implemented in *RZooRoH* R package (Bertrand et al., 2019; Druet & Gautier, 2017). PLINK detects continuous homozygous fragments, known as runs of homozygosity (ROHs), using sliding windows with a minimum length defined by the user, whereas *RZooRoH* uses a HMM approach to model the genome as a mosaic of homozygous-by-descent (HBD, obtained from ROHs) and non-HBD segments, without requiring a minimum threshold on ROHs length, which makes it ideal for reduced genomic representation data sets, such as our SNP panel. ROHs distribution can provide reliable information about the population's recent demographic history, with long/short ROHs reflecting recent/distant inbreeding (Bertrand et al., 2019; Palamara et al., 2012). We used the same 60K data set as for heterozygosity and LD analysis to implement both approaches, as ROHs detection can be biased by LD pruning and MAF removal (Meyermans et al., 2020).

To detect ROHs with PLINK, we used the *–homozyg* function with a minimum segment length of 300 kb spanning at least 50 SNPs and allowing for a maximum of one heterozygous SNP (Ceballos et al., 2018; Meyermans et al., 2020). The remaining parameters were maintained with the default values. Inverse cumulative average counts of ROHs per individual were calculated for every 1 Mb non-overlapping windows up to 60 Mb and plotted using *ggplot2* package in R. Then, we ran the *RZooRoH* package using a model with 12 HBD classes (k) and one non-HBD class. Each class is associated with a rate (R_k) corresponding to twice the expected number of generations since the inbreeding event (2^k; in our study R = 2,4,8, …, 4096). Therefore, HBD classes with low R_k correspond to (longer) HDB segments inherited from recent common ancestors. Physical positions (bp) in the map file were converted to genetic distances (cM) based on the average recombination rate per chromosome described for the dog genome (Campbell et al., 2016; Wong et al., 2010). Time to the inbreeding event was estimated considering the wolf generation time of 4.5 years.

3 | RESULTS

3.1 | Levels of admixture and frequency of hybridization

Iberian wolves and dogs are genetically differentiated with $F_{ST}=0.272$ (5 K; $F_{ST}=0.271$ using the 20 K). The PCA analysis is also suggestive of this differentiation, with the first PCA component explaining 65.9% of the variation and separating wolves and dogs (Figure 1b). Historical and contemporary wolf samples clustered together in the PCA, which is compatible with the negligible levels of genome-wide differentiation found between them ($F_{ST}=0.019$; 5 and 20 K). The same clustering patterns were observed in the PCA after sample size correction and using the 20 K data set (Figure S3). The average assignment of Iberian wolves to the corresponding cluster at K = 2, using ADMIXTURE, was $q_W=0.995$ and $q_W=0.993$ for both historical and contemporary samples respectively (Figure 1c). Dogs were assigned to their corresponding cluster with an average ancestry proportion of $q_D=0.982$ (Figure 1c). We observed no differences in individual q values when estimated based on a high number of SNPs ($r^2=.999$, 5 K vs. 20 K data set; Figure S4).

Simulated parental genotypes were assigned to their corresponding cluster at K = 2 with q values of $[0.990\text{–}0.999_{\text{min–max}}]$ for wolves and $[0.960\text{–}0.999_{\text{min–max}}]$ for dogs. Based on the minimum q value observed in simulated parental wolf genotypes, hybrids were defined as individuals with average ancestry proportions below 0.990 (historical and contemporary). Subsequently, hybrids were further assigned to a specific class based on the range of $q_{W\,(\text{min–max})}$ values observed in the simulated genotypes of hybrid classes: $F_1=[0.465\text{–}0.535]$, $BC_{1W}=[0.725\text{–}0.787]$, $BC_{2W}=[0.853\text{–}0.902]$ (Table S3).

Among 54 historical samples, two hybrids were detected (3.7% of the historical wolf dataset) with dog ancestry proportions of $q_D=0.026$ and $q_D=0.241$ (Figure 2a; Table S3). These two individuals were originally from southern Iberia, collected sparsely in space and time in Barrancos, district of Beja, Portugal, in 1959, and in Villanueva del Fresno, in the province of Badajoz, Spain, in 1972 (Figure 1a). Within the contemporary sample set, we confirmed the hybrid origin of seven individuals previously identified using

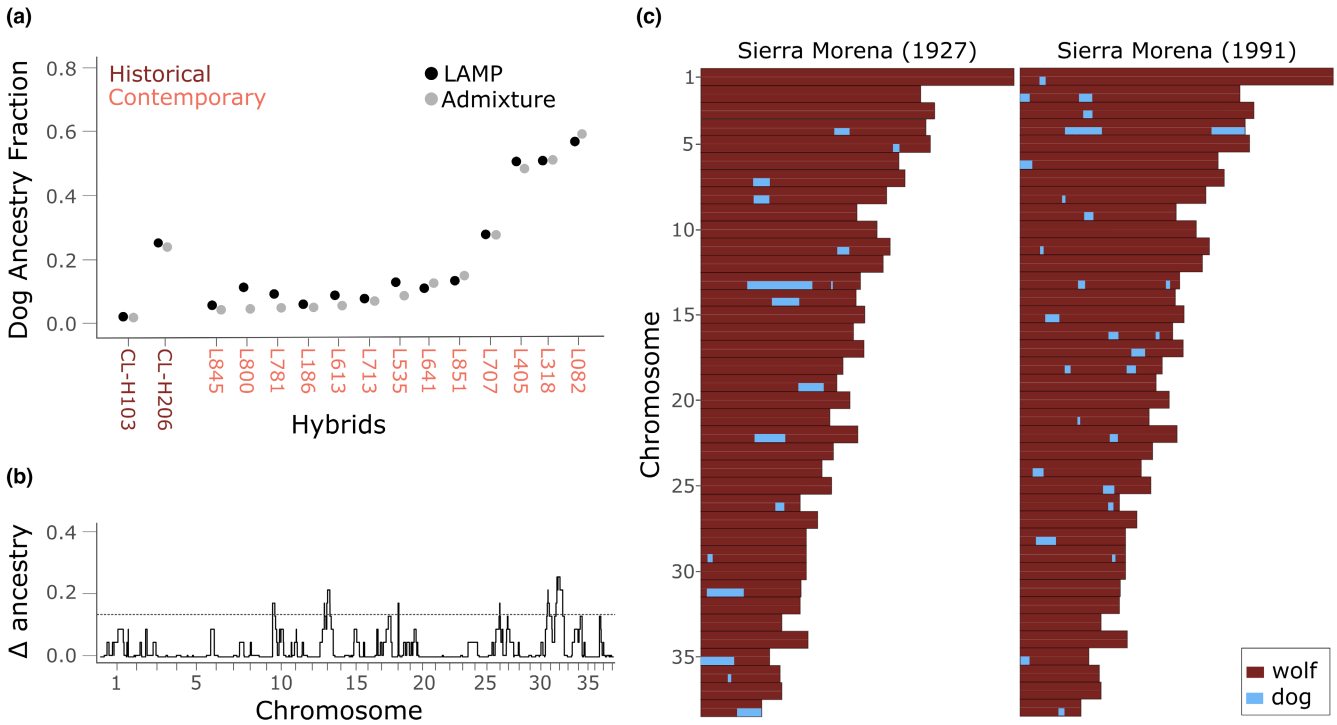

FIGURE 2 Local ancestry analysis and introgression levels. (a) Fraction of the global dog ancestry estimated for the 15 wolf–dog hybrids using LAMP-ANC (black dots) and ADMIXTURE (grey dots). Historical and contemporary hybrid samples are represented in red and orange, respectively, with the corresponding sample ID on the x-axis. (b) Δ ancestry scores indicating the excess of dog ancestry across the hybrid chromosomes (backcrosses only). The dashed line identifies the cut-off used to consider outlier regions (0.14; mean + 2SD). (c) Local ancestry in the genome of two wolves from Sierra Morena collected in 1927 and 1991. Genomic blocks were coloured based on the attributed local ancestry: red for wolf (both chromosomes), blue for dog (both chromosomes) and half red/blue for wolf/dog (one chromosome of each).

microsatellites (L082, L318, L405, L535, L613, L641 and L707) with dog ancestries ranging from $q_D = 0.061–0.584$ (Figure 2a; Table S3); and found six additional hybrid individuals (9.8% of the contemporary wolf data set) with dog ancestries of $q_D = 0.046–0.149$ (Figure 2a; Table S3). Among the 13 contemporary hybrids, we identified three F_1 hybrids, one BC_{1W}, three BC_{2W} and six individuals conforming to older hybridization events (Table S3). Using the original collection year for each hybrid, we estimated that most of these hybridization events likely occurred between 1988 and 2005 (Table S3). All hybrids from the contemporary sampling, except one in Portugal, were found at the edge of the species range in Spain (Figure 1a).

We observed a low error rate associated with the *q* threshold for hybrid identification. If we applied a more conservative *q* threshold (i.e. increased from 0.990 up to 0.998), no wolves in contemporary samples would be erroneously classified as admixed (Type I error), while in historical samples, three wolves would be assigned as hybrids (two with $q_W < 0.991$ and one with $q_W < 0.998$). Conversely, decreasing the *q* threshold from 0.990 up to 0.950 would result in one hybrid in each historical ($q_W > 0.970$) and contemporary ($q_W > 0.950$) samples being erroneously assigned as wolf (Type II error).

Results of the *f3* test confirmed a highly significant admixture between wolves and dogs across the 15 hybrids detected across both historical and contemporary sets ($f3 = -0.015$; Z-score $= -41.916$; Table S4). By combining our results with those reported by Godinho et al. (2011) for the naïve prevalence of contemporary wolf–dog hybridization in the Iberian Peninsula, we accommodate a total of 18 hybrids among 232 analysed individuals (contemporary samples only). This refines the occurrence of wolf–dog hybrids in the Iberian Peninsula to 7.8%.

3.2 | Local ancestry analysis of hybrids

Local ancestry analysis confirmed the extent of dog ancestry estimated by ADMIXTURE in hybrids (Figure 2a). The three F_1 hybrids identified in the contemporary sample set showed the expected pattern of homologous chromosomes having ancestry from each parental species (Figure S5). As expected, the 12 BC_W hybrids had fewer and shorter dog blocks in their genome than F_1 hybrids (average of 17 blocks per individual, $11–28_{\text{min–max}}$, with an average size of 27.769 Mb; average genome size with dog ancestry of 483.651 Mb, corresponding to 19.4%; Figure S5). Among BC_W hybrids, we observed differences in the amount of dog genomic blocks retained per chromosome (Figure S6), with outlier regions for excess of dog ancestry (Δ ancestry statistics) present in chromosomes 10, 13, 18, 26, 31 and 32 (Figure 2b), whereas chromosomes 5, 9, 22 and 36 showed the lowest average proportion of dog ancestry (Figure 2b). The average size of these overrepresented dog genomic regions was 9.181 Mb, with the longest fragment found on chromosome 32 with 23.673 Mb. The differences in the proportion of dog ancestry per chromosome did not present statistical significance ($p > .05$; Table S5). However, this result should be interpreted with caution due to the limited number of backcross hybrids available for analysis.

3.3 | Proportion of dog introgression in historical wolves

Historical non-admixed wolf samples from the contracting and stable/expanding population ranges had low levels of dog introgression in their genome (average of 2% and 1%, and maximum values of 4% and 3%, respectively; estimated using the 60K data set; Figures S7, S8 and Table S6). A higher number of dog blocks was detected within the genome of individuals from the contracting range area than those from the stable/expanding area (average of 17 and 4 blocks per individual, respectively); but the average size per block was almost identical (5.083 and 6.756 Mb, respectively). However, given the low sample size in both groups ($n = 10$ and $n = 12$, from the contracting and stable/expanding population ranges, respectively), we cannot rule out an effect of stochasticity in these differences. Interestingly, the genome of four wolves from Sierra Morena captured in 1927 (CL-H116), 1964 (CL-H182), 1970 (CL-H117) and 1991 (CL-H261) carried a low amount of dog ancestry (4%, 3%, 3% and 2%, respectively; Figure 2c; Figure S7), suggesting no increase or retention of specific dog genomic blocks in their genome before and immediately after the population bottleneck.

3.4 | Patterns of genetic diversity

Historical Iberian wolves showed significantly higher heterozygosity than contemporary ones ($het_{HIST} = 0.195$ and $het_{CONT} = 0.173$, $p = 3.65e\text{-}07$; Figure 3a). Despite a decreasing diversity trend around 1970, when the population reached its minimum, no significant linear reduction in heterozygosity was observed across time in historical samples ($r^2 = -.16$; Figure S9A). Still, a closer inspection of heterozygosity in two geographical regions where wolves persisted after the 1970s bottleneck—south of Douro River (central Portugal), where the population contracted its range; and Castilla y León (north-western Spain), where the population has been stable/expanding—indicated a considerable loss of diversity in the former (heterozygosity loss of 0.04 and 0.02, respectively; Table S7). However, the sample size difference ($n = 6$ for south of Douro River; $n = 23$ for Castilla y León) hindered further statistical testing. The lowest heterozygosity levels among historical wolves were recorded in a sample from Sierra Morena collected in 1970 ($het = 0.136$). Nonetheless, a wolf from the same area collected in 1991 showed high levels of heterozygosity ($het = 0.209$).

The distribution of heterozygosity across the genome of historical and contemporary individuals, estimated using the 60K data set, confirmed an overall genome-wide reduction of diversity in the latter (Figure 3b). We found a high positive correlation ($r^2 = .98$; Figure S9b) between estimates of heterozygosity using the 5 and 60K SNP panels.

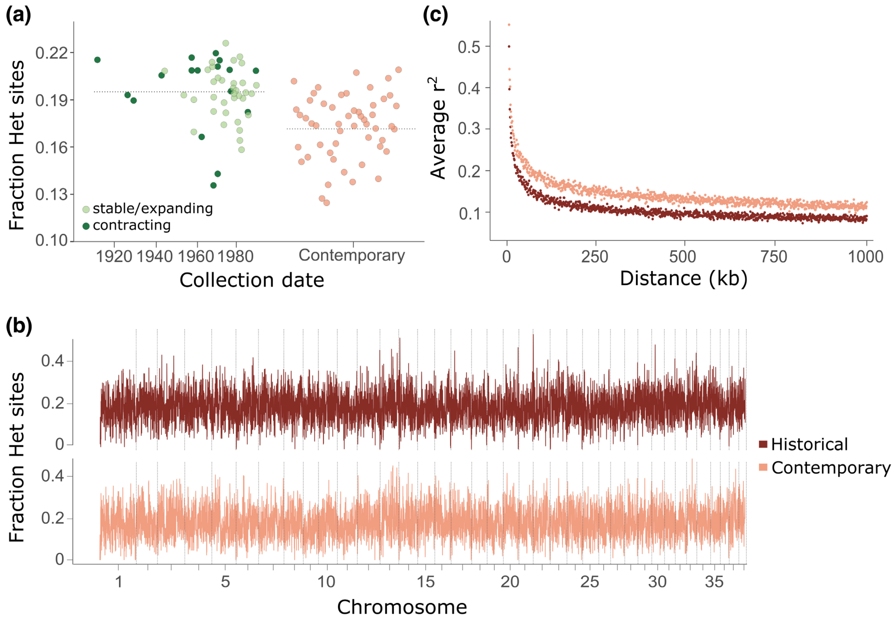

FIGURE 3 Genetic diversity in historical and contemporary samples. (a) Proportion of heterozygous sites present in 52 non-admixed wolves from the historical (green) and contemporary (orange) sample sets across time (year of the original collection). Historical samples from the contracting ($n = 17$) and stable/expanding ($n = 35$) ranges are represented in dark and light green respectively. Each dot denotes an individual and black dashed lines represent the average heterozygosity values. (b) Distribution of heterozygosity across the genome of historical (red) and contemporary (orange) individuals ($n = 22$, in each) in non-overlapping windows of 10 SNPs (~350 kb). (c) Decay of linkage disequilibrium (LD) across sample sets. Each dot represents the averaged pairwise r^2 values between pairs of SNPs in non-overlapping 1 kb windows.

LD levels were higher in contemporary wolves, with r^2 values only decaying below 0.5 at ~2 kb, contrasting with the ~1 kb in historical samples (Figure 3c). The *RZooRoH* approach revealed that historical wolves showed limited autozygosity in comparison to contemporary individuals, with 18.71% and 27.47% of the genome in homozygosity, respectively (Figure 4b; Table S8). This was also associated with shorter HBD segments (Figure S10). The most frequent rate in the genome of historical wolves was $R_k = 16$ (6.10%; Figure 4a), indicating HBD segments inherited from distant ancestors (eight generations ago). Conversely, contemporary wolves exhibited higher rates (most frequent $R_k = 8$; 8.09%: Figure 4a), associated with more recent inbreeding events (four generations ago). Nine contemporary wolves had total autozygosity above 30%, while the same value was only observed for two historical wolves (Figure 4b; Table S8). The wolf from Sierra Morena collected in 1970 had the highest level of autozygosity (42.34%) recorded among historical samples.

The PLINK approach supported the previous findings, detecting a high number of ROHs in contemporary than in historical wolves (average number per individual of 72.86 vs. 55.58, respectively; Figure S11a; Table S9). ROHs were also longer in contemporary samples (average size per segment of 7.28 Mb vs. 6.02 Mb in the historical sample set). Nevertheless, the vast majority of ROHs were shorter than 10 Mb within both historical (83.84%) and contemporary (80.47%) sample sets, suggesting no strong inbreeding in the Iberian wolf. The wolf from Sierra Morena collected in 1970 had the longest homozygous segments (13.9 Mb) in historical samples (Figure S11b). No distinctive pattern was found for inbreeding among historical samples concerning areas of population range contraction versus stable/expanding (Figure 4b; Figure S11b).

4 | DISCUSSION

4.1 | Dynamics of wolf–dog hybridization across a population bottleneck

Genome-wide analysis of historical Iberian wolf samples showed that the rate of wolf–dog hybridization (3.7%) did not increase during the most dramatic population decline in the mid-20th century, contrary to what we have hypothesized. Only two hybrids were identified in the historical data set, which were originally collected in different decades (1959 and 1972) and distinct regions in southern Iberia, indicating a non-recurrent event nor confined to a period of substantial population decline.

The number of wolf–dog hybrids was considerably higher among the contemporary sampling. For this data set, we identified 13 hybrids, confirming seven previously recognized (Godinho et al., 2011) and six additional individuals. Almost all hybridization cases (92.3%) were observed in the edges of the wolf range, similar to previous reports in Iberia (Godinho et al., 2011, 2018; Pacheco et al., 2017) and consistent with other European populations (Andersone et al., 2002; Leonard et al., 2013; Verardi et al., 2006). Such geographic pattern is generally associated with lower wolf densities towards the

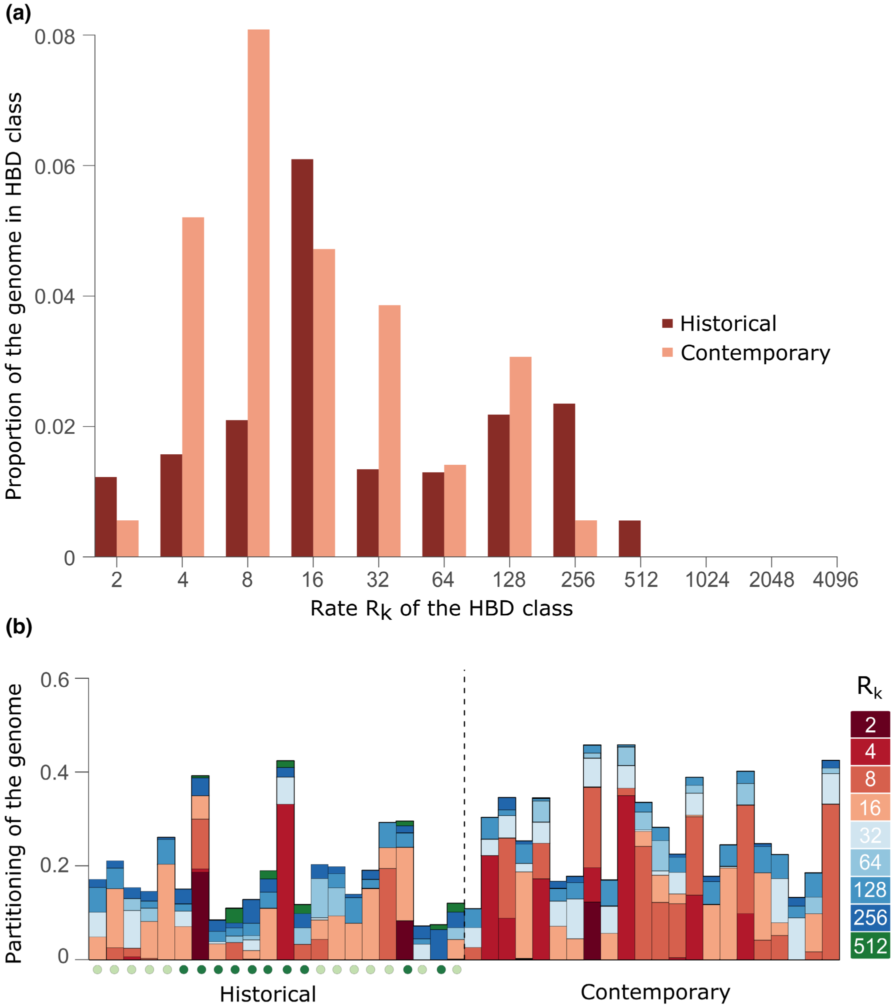

FIGURE 4 Autozygosity and partitioning of the genome in different homozygous-by-descent (HBD) classes. (a) Proportion of the genome in the 12 different HBD classes represented by different rates (R_k) in the historical (red) and contemporary (orange) samples ($n=22$ individuals, in each). Values of R_k correspond to twice the number of generations since the inbreeding event (e.g. an $R_k=4$ indicates HBD segments inherited from ancestors two generations ago). (b) Partitioning of the genome in distinct HBD classes (represented by R_k; see colour scheme) per individual (represented by single bars). The sum of HBD classes corresponds to the total amount of autozygosity in the genome of each individual. Dark and light green dots in the historical sampling identify individuals from the contracting and stable/expanding range areas respectively.

range edge, where individuals may fail to find suitable wolf mates or the social cohesion of wolf packs may be more susceptible to disruption due to anthropogenic disturbance, such as poaching and hunting, compared to core areas where pack structure is more stable (Jedrzejewski et al., 2005; Rutledge et al., 2012; Salvatori et al., 2020). This may lead to increased opportunities for encounters and hybridization with dogs, which is particularly notable in areas with higher levels of anthropogenic disturbance, where the presence of free-ranging dogs is more prevalent (Llaneza et al., 2012; Marino et al., 2016).

From a temporal perspective, most hybridization events in our sampling occurred after 1995 when the wolf population was already recovering and expanding. This is consistent with the dynamics of wolf–dog hybridization documented in Italy, where increased rates of hybridization were recorded two decades after a massive bottleneck in the 1970s followed by population recovery (Galaverni et al., 2017). By increasing the resolution over the genome and integrating our results with a previous genetic assessment of wolf–dog hybridization across the entire Iberia, we could provide a clear refinement of the naïve prevalence of contemporary hybridization that raised from 4% (Godinho et al., 2011) to ~8%. Still, despite our effort to even sampling bias, this may not necessarily indicate an increase in hybridization in relation to historical times due to an unbalanced effort towards hybridization cases in the contemporary sampling. Future assessments of the naïve prevalence of contemporary Iberian wolf–dog hybridization may be facilitated by the use of non-invasive sampling (i.e. scats), which allows for large-scale sampling and comprehensive coverage of the distribution range, while also prioritizing animal welfare. Previous research using non-invasive samples has demonstrated its effectiveness in estimating the naïve prevalence of wolf–dog hybridization in the Iberian Peninsula (Pacheco et al., 2017).

Only five of the 15 hybrids identified had dog content above 20%, confirming low levels of dog introgression and supporting the high genetic differentiation between Iberian wolves and dogs. Uneven distribution of dog genomic blocks across the 38 autosomes of backcross hybrids suggests different capacities to retain or purge introgressed alleles, as previously seen in several mammals, including the grey wolf (Frantz et al., 2015; Geraldes et al., 2006; Good et al., 2010; Pilot et al., 2021; Turner & Harr, 2014), birds (Carling & Brumfield, 2008; Runemark et al., 2018) and fishes (Schumer et al., 2018). Interestingly, the chromosomes of wolf–dog hybrids with significant excess of dog genomic content partially overlap with those previously found for Italian wolf–dog hybrids (Galaverni et al., 2017), suggesting either more permeable regions to introgression or regions with high ancestral shared variation. We found no direct correlation between the proportion of dog ancestry and chromosomal rates of recombination in the dog genetic map (Campbell et al., 2016), or with reported levels of genome-wide genetic differentiation between wolves and dogs (Pendleton et al., 2018) that could explain these results. Therefore, this suggests that admixture proportions between wolves and dogs are more likely due to a high permeability to introgression and low genomic barriers between them, than to differential rates of recombination or ancestral polymorphism (Pilot et al., 2021). Consequently, the dog genomic content found introgressed in the wolf genome is likely to have persisted in regions not linked to deleterious effects, which ultimately would limit the amount of variation in genes that play fundamental roles in wolves.

4.2 | Dog introgression as a conservation threat to wolf populations

We did not find evidence of high levels of dog ancestry in any of the historical non-admixed wolves, suggesting that dog introgression was not a primary driver of wolf extinction from different parts of the Iberian Peninsula in the past centuries. The average dog ancestry proportion of 1.4% in historical wolves is consistent with previous findings in contemporary non-admixed Iberian wolves (Lobo, 2023) and other Eurasian populations (Fan et al., 2016; Pilot et al., 2021). The introgression patterns of few and short dog blocks in the wolf genome from central and southern Iberia were similar to those observed in individuals from the north-western area, and are compatible with historical admixture signatures rather than recent gene flow.

The limited occurrence of dog blocks in the genome of four Sierra Morena wolves suggests that hybridization was not frequent in this region at least until the 1990s. Moreover, we did not detect any increase or retention of dog genomic content between 1927 and 1991 that could be linked to the decline of the wolf population in Sierra Morena. Instead, population collapse likely resulted from a synergic intensification of legal and illegal pressure on wolves in the area. On the one hand, several provinces around Sierra Morena have been particularly affected by increased hunting pressure since the mid-20th century through legal campaigns (Corbelle-Rico & Rico-Boquete, 2008). On the other hand, a remarkable increment in the game hunting business and associated intensive game ranching in this area during the 1970s may have facilitated the resurgence of illegal persecution due to perceived competition for game species in private hunting areas (Blanco et al., 1992; López-Bao et al., 2015). Therefore, the fact that the genome of one of the last wolves from this population (from 2003) had around a third of dog ancestry (Gómez-Sánchez et al., 2018) is probably due to contemporary hybridization and high inbreeding in a very small population, rather than continuous interbreeding with dogs in previous generations. Although our analysis of four wolf genomes may not be entirely representative of the population, we would still expect to find consistent evidence of hybridization if it was frequent at the time, even with a limited representation of the genome. While ancient signatures of hybridization might have been missed, the SNP panels that we used should successfully identify introgressed dog content of few generations (e.g. Galaverni et al., 2017; Pilot et al., 2018). Additionally, our study demonstrates that genome-wide ancestry proportions between wolves and dogs can be accurately estimated using the 5 K SNP panel we assembled, as it showed high concordance with the 20 and 60 K data sets.

4.3 | The loss of genetic diversity

The decline of the Iberian wolf population in the mid-20th century caused a significant reduction in the genetic diversity exhibited today by contemporary wolves. Such genetic diversity declines after a population bottleneck are well described among vertebrates (Charlesworth & Willis, 2009; Keller & Waller, 2002; Lande, 1993; Leonard, 2008). However, despite a continuum of population decline described for the Iberian wolf since the 19th century (Nores & López-Bao, 2022; Rico & Torrente, 2000), the trend is not significantly perceived in heterozygosity levels within historical samples, likely due to the resilience of heterozygosity over a few generations during population bottlenecks (Cabe, 1998; Luikart & Cornuet, 1998; Nei et al., 1975). Nevertheless, these results support the hypothesis of a recent and human-induced loss of genetic diversity in Iberian wolves, rather than an ancient demographic cause. This is particularly relevant given the long-term isolation of Iberian wolves from other European populations (Silva et al., 2020). The recent loss of diversity is consistent with previous evidence based on mitogenomes of Iberian wolves (Salado et al., 2023) and other central European populations (Dufresnes et al., 2018), indicating that the most substantial decline in mitochondrial diversity occurred in recent times, largely due to the systematic and intense persecution of wolves since the 19th century (Boitani, 1995). This view is also supported by the longer HBD segments found in the genome of contemporary Iberian wolves that originated from inbreeding events occurring four or two generations ago, corresponding to the post-bottleneck period. Despite this, the average levels of homozygosity found in contemporary Iberian wolves are much lower than those reported for wolf populations presenting signs of genomic erosion associated with high inbreeding (e.g. grey wolves from Southeast Alaska; estimates based on a similar number of SNPs; Pacheco, Stronen et al., 2022), suggesting that Iberian wolves are not facing a risk of inbreeding depression.

Regarding the Sierra Morena population, despite the lowest genetic diversity found in a wolf collected in 1970, an individual collected in 1991 showed high heterozygosity and few ROHs in its genome. Previous studies estimating the wolf range in the Iberian Peninsula in the past decades suggested that the Sierra Morena population split into small nuclei around 1970 (Valverde, 1971), disappearing subsequently over the next decades (Blanco et al., 1990; López-Bao et al., 2015). Thus, we presume that these samples were from wolves in distinct nuclei, which may account for the different patterns of genetic diversity observed. The genome of the wolf sampled in 2003 exhibited genomic signatures of high inbreeding and long ROHs, which are consistent with a severe population decline.

5 | CONCLUSIONS

Monitoring hybridization and genetic diversity before and after a population bottleneck is crucial to understanding how human-mediated disturbance (a severe human-mediated bottleneck) affects the genomic composition of a species (Frei et al., 2022). Our findings suggest that wolf–dog hybridization in the Iberia Peninsula did not increase during the 1970s bottleneck and do not support dog introgression as a driver of wolf extinction. Furthermore, we show that despite the detection of multiple hybridization events over a large temporal period, Iberian wolves and dogs are two well-defined genetic entities, confirming that levels of gene flow are limited. However, hybridization appears to be more frequent in the edges of the wolf range, and its impact on species conservation requires further investigation. Our results also revealed a significant loss of genetic diversity in contemporary wolves as a consequence of the 1970s bottleneck. Overall, our findings may contribute to predict evolutionary responses of other large carnivores that have experienced or are facing severe declines due to human-induced environmental changes.

AUTHOR CONTRIBUTIONS

R.G. designed and coordinated the study; D.L. performed data analysis; J.V.L.B. helped with the interpretation and critical analysis of the results; D.L. and R.G. wrote the manuscript with input from J.V.L.B.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at 10.17605/OSF.IO/N3CDR.

DATA AVAILABILITY STATEMENT

Data accessibility statement: Individual SNP genotype data of the contemporary and historical samples (5, 20 and 60K data sets) and HYBRIDLAB simulated genotypes (parental and hybrid classes) are available on the OSF repository at: https://doi.org/10.17605/OSF.IO/N3CDR.

Benefit-sharing statement: A research collaboration was established with the museums that provided access to their Iberian wolf natural history collections, which are all acknowledged. Contemporary samples were obtained through already well-established collaboration with governmental agencies in both Portugal and Spain. Additional benefits from this research accrue from the sharing of SNP genotype data on public databases, as described above.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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