Large variance in inbreeding within the Iberian wolf population

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ABSTRACT

The gray wolf (*Canis lupus*) population on the Iberian Peninsula was the largest in western and central Europe during most of the 20th century, with its size apparently never under a few hundred individuals. After partial legal protection in the 1970s in Spain, the northwest Iberian population increased to about 300-350 packs and then stabilized. In contrast to many current European wolf populations, which have been connected through gene flow, the Iberian wolf population has been isolated for decades. Here we measured changes on genomic diversity and inbreeding through the last decades in a geographic context. We find that the level of genomic diversity in Iberian wolves is low compared to other Eurasian wolf populations. Despite population expansion in the last 50 years, some modern wolves had very high inbreeding, especially in the recently recolonized and historical edge areas. These individuals contrast with others with low inbreeding within the same population. The high variance in inbreeding despite population expansion seems associated with small-scale fragmentation of the range that is revealed by the genetic similarity between modern and historical samples from close localities despite being separated by decades, remaining differentiated from other individuals that are just over 100 km away, a small distance for a species with great dispersal capacity inhabiting a continuous range. This illustrates that, despite its demographically stable condition, the population would probably benefit from favoring connectivity within the population as well as genetic exchange with other European wolf populations to avoid excessive fragmentation and local inbreeding depression.

GRAPHICAL ABSTRACT

1970

Inbreeding coefficient

0   0.1   0.2
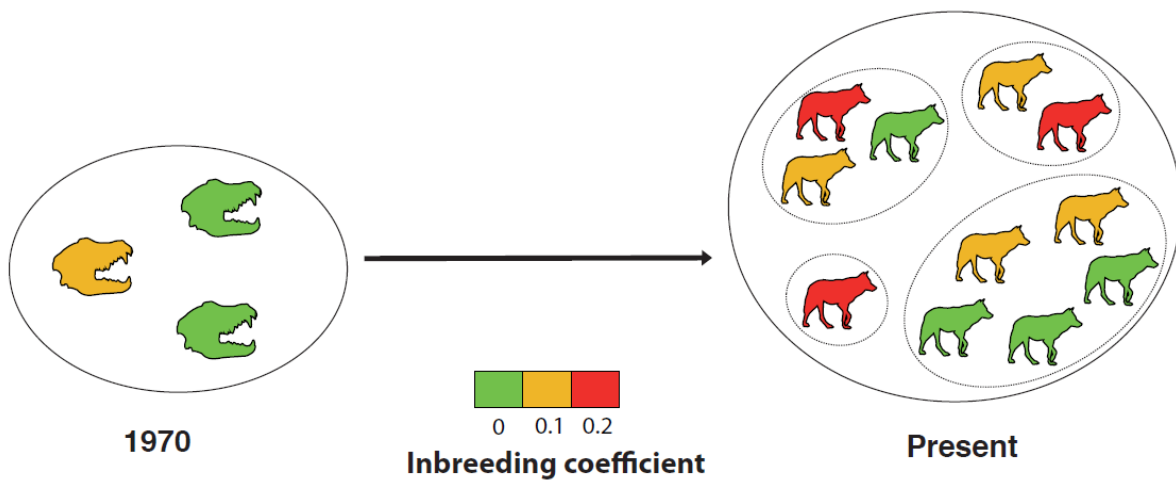
Present

INTRODUCTION

The gray wolf (*Canis lupus* L.) is a large carnivore that has been heavily persecuted worldwide in recent times (Ripple et al., 2014). Wolves are a potential threat to livestock, are seen as a competitor for game species, and sometimes even considered vermin. During past centuries they were eradicated from much of western Europe, Mexico, the contiguous United States, the British Isles, and the Japanese archipelago (Boitani, 2003). In the 1970s, some gray wolf populations were officially declared extinct in the wild, such as the Mexican wolves (*C. l. baileyi*; Hedrick et al., 1997). At that time in Europe, large populations remained only in eastern Europe (Russia and Carpathian region), while smaller, isolated populations persisted on the Iberian, Balkan and Italian Peninsulas (Breitenmoser, 1998). On the Iberian Peninsula, the gray wolf was widely distributed until the mid-19th century (Clavero et al., 2022). The population severely declined during the first half of the 20th century due to direct human persecution, reaching its minimum population size in the 1970s (about 500-700 individuals, Chapron et al., 2014), when partial legal protection was put in place in Spain through a change in legal status from vermin to game species (Blanco et al., 1992; Grande del Brío, 1984; Valverde, 1971). This change favored a partial recovery to the current population size. Thus, unlike many countries in Western Europe, wolves were never totally extirpated on the Iberian Peninsula and the presumed population size was never below hundreds of individuals (Chapron et al., 2014).

Gray wolf populations worldwide have been demographically dynamic during the last decades. In the United States, gray wolves have recovered in some states now forming one continuous wolf population in the north, while the reintroduced Mexican wolf population in the southwest still remains isolated (US Fish and Wildlife Service, 2020). In the Middle East, few reliable data are available but it is assumed that the wolf population has been stable and is mainly maintained in three countries: Turkey, Israel, and Saudi Arabia (Boitani, 2003;

Cunningham & Wronski, 2010). In Europe, recent socio-ecological changes and legal protection, combined with the high dispersal ability of gray wolves, have allowed them to naturally recolonize parts of their historical range and increase in numbers (Boitani et al., 2022; Chapron et al., 2014; Cimatti et al., 2021). Large, widespread, and well-connected eastern wolf populations have served as a reservoir for recently recolonized areas in Europe, as shown by the ongoing recolonization of the eastern Alps by Dinaric-Balkan wolves (Fabbri et al., 2014; Ražen et al., 2016), the dispersal of wolves from Finland and Russian Karelia to Scandinavia (Flagstad et al., 2003; Vilà et al., 2003), as well as the rapid expansion in the Central European lowlands from the Baltic population (Andersen et al., 2015; Ansorge et al., 2006; Czarnomska et al., 2013; Jarausch et al., 2021; Reinhardt et al., 2019; Szewczyk et al., 2019). On the other hand, on the Iberian Peninsula the wolf population expanded only slightly in distribution in the northwest since the 1970s, while it disappeared in the southern half of its distribution in Spain and Portugal (Alvares, 2011; Junta de Andalucía, 2020; Blanco et al., 1990; MITECO, 2016; Petrucci-Fonseca, 1990), and its population size has remained stable at around 350 packs for the last three decades (Blanco, Cuesta, et al., 1990; MITECO, 2016; Torres & Fonseca, 2016).

Genetic diversity has been shown to be important for the long-term viability of populations (Frankham et al., 2002). Levels of genetic diversity are linked to population size, and demographic declines reduce genetic diversity in wild populations. Loss of genetic diversity and fragmentation are often associated with an average increase in inbreeding, especially in small and isolated wild populations (Frankham et al., 2002). Therefore, large wolf populations, such as the Dinaric-Balkan, have been considered a valuable source of genetic diversity for neighboring populations in Europe (Hindrikson et al., 2017). Since the size of the wolf populations in the Iberian and Italian peninsulas were much smaller, population declines and long-term geographical isolation (Silva et al., 2020) may have caused

more inbreeding in these populations than in north-eastern European wolves (Fabbri et al., 2007; Fan et al., 2016a; Hindrikson et al., 2017; Lucchini et al., 2004; Sastre et al., 2011), although recent research about the extinct French wolf population suggested that this isolation was either incomplete or more recent than previously estimated (Doan et al., 2023). While recent population expansions have resulted in gene flow between the Italian and other central and eastern European wolf populations increasing the genetic diversity of these expanding populations, the Iberian population has thus far remained genetically isolated (Hindrikson et al., 2017).

Thanks to the advent of next-generation sequencing (NGS) technologies and the ongoing development of ancient DNA (aDNA) methodology, it has been possible to assess changes in genetic and genomic diversity through time using museum specimens (Doan et al., 2023; Dufresnes et al., 2018; Salado et al., 2023; Taron et al., 2021) by directly comparing patterns of diversity in historical populations with those of extant populations. It is known that genetic variability has been lost with historically extirpated wolf populations (Doan et al., 2023; Leonard et al., 2005; Taron et al., 2021), and genetic diversity could potentially continue to be lost in demographically stable wolf populations, such as that on the Iberian Peninsula, as has been shown for mitochondrial DNA (mtDNA; Salado et al., 2023). However, the effective population size of mtDNA is smaller than that of nuclear loci, making it more susceptible to losses of diversity. Here we assess changes in genomic diversity and inbreeding in Iberian wolves during the last half century using nuclear genome sequences from historical (from close to the time when the population reached its lowest size) and contemporary samples, considering their geographic context within the population. We compare the patterns of diversity and inbreeding with those revealed in previous studies in other Eurasian populations, and estimate their phylogenetic relationships.

METHODS

Sampling

We initially collected bone or tooth fragments from 32 historical Iberian wolf specimens dating to between *circa* 1970-1984 from the northwestern quadrant of the Iberian Peninsula belonging to the Scientific Collections at Estación Biológica de Doñana (ICTS-EBD, Seville, Spain). This is the area where wolves were not historically eradicated. These samples were subject to an initial screening for the presence of endogenous DNA (Salado et al., 2023) and three samples exhibiting the highest proportion of endogenous DNA and a relatively close geographical proximity to contemporary Iberian wolves available for the study were selected for further analyses (see *Lab processing*; Fig. 1). Modern tissue samples were taken from dead wolves (road-killed, culled, or hunted) dating from 2009-2012 (Table 1). They were collected using sterilized material and preserved in NAP buffer (Camacho-Sanchez et al., 2013) or ethanol, and stored at -20ºC until processing in the lab. No live animals were handled for this project.

We also included in the analyses a total of seventeen previously published whole genomes of modern wolves from northwestern Iberia and other populations: Iberia (n=5), Mexico (n=2), Italy (n=1), Middle East (n=3), Balkans (n=3), Russia (Karelia, n=3) (Fan et al., 2016; Freedman et al., 2014; Gómez-Sánchez et al., 2018; Gopalakrishnan et al., 2018; Ostrander et al., 2019; Smeds et al., 2021; Table S1). Three of the five Iberian wolf genomes used in the analyses were from Portugal, one from Spain and the last one from a Spanish captive breeding population belonging to the European Endangered Species Programmes (EEP, Gómez-Sánchez et al., 2018). We also included one whole genome sequence of a golden jackal (*Canis aureus*, SAMN0336671*3,* Freedman et al., 2014; Table S1) as an outgroup to root the neighbor joining tree (see *Data analyses*). Data from the literature was

processed with the same preprocessing pipeline as the newly generated data from modern

wolves (see *Data analyses*).

Table 1. Sample information of Iberian wolves sequenced in this study. Sample, identification code; Collection #, scientific collection catalog number; Period, time period; Year; Location, approximate geographic coordinates (WGS84).

Sample	Collection #	Period	Year	Location
H1	EBD 15865M	Historical	c1970	X:-6.54984 Y:43.17738
H2	EBD 7534M	Historical	1984	X:-4.63857 Y:42.10941
H3	EBD 15858M	Historical	1979	X:-6.79625 Y:40.98653
M1	EBD 33175M	Modern	2016	X:-6.61706 Y:43.35659
M2	-	Modern	2012	X:-4.47934 Y:42.13998
M3	-	Modern	2009	X:-5.68397 Y:41.21062
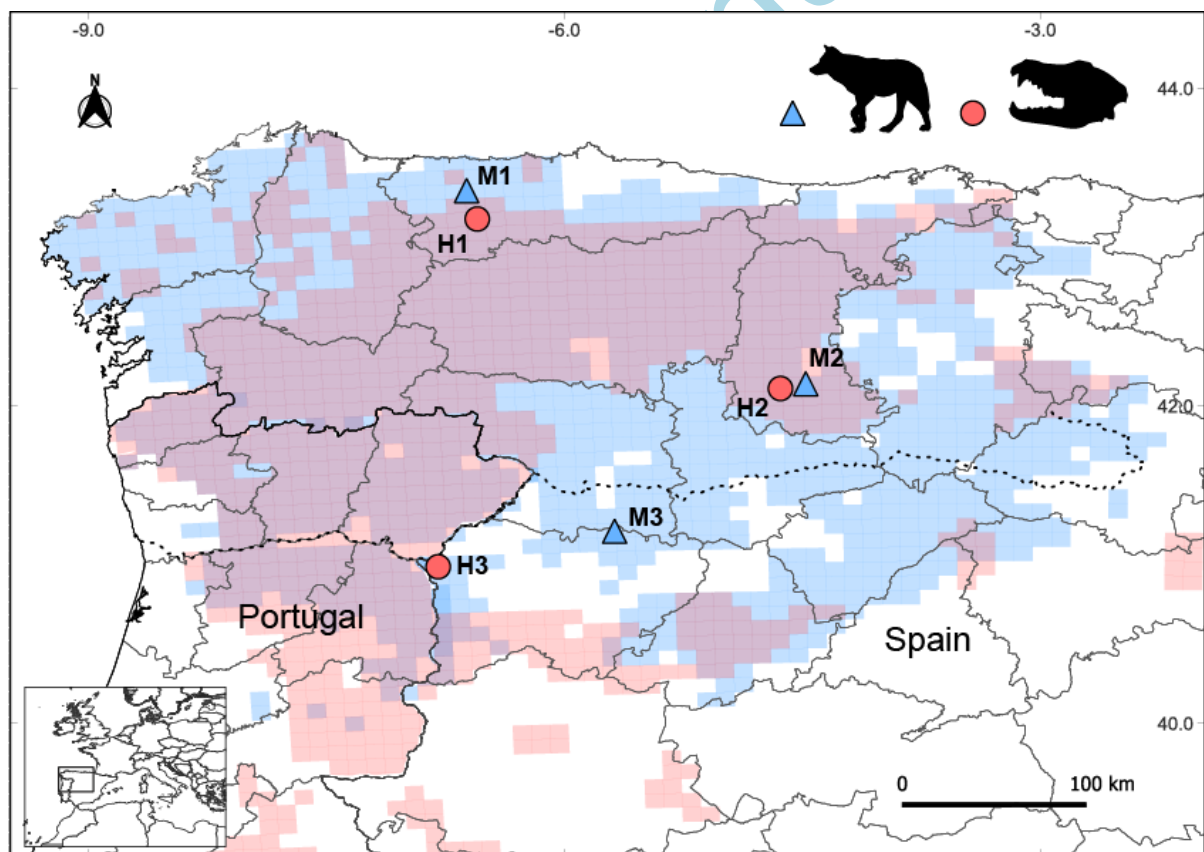
Figure 1. Iberian wolves sample distribution. Modern (blue triangles) and historical (red circles) wolf specimens sequenced, and current (blue; Bencatel, J., Sabino-Marques, H., Álvares, F., Moura, A. E. & Barbosa, 2019; MITECO, 2016)) and past (red; ca. 1970s; Petrucci-Fonseca, 1990; SNPFC, 1968) species distribution on the Iberian Peninsula. Dashed line marks the Douro River, which has been used for delimitation of management units in Spain.

Lab processing

Screening data of historical specimens was done as in Salado et al., (2023). We extracted DNA from historical specimens as in Ford et al., (2020), while a phenol-chloroform DNA extraction and ethanol precipitation was used for modern samples. Modern dual-indexed libraries were prepared following (Neiman et al., 2012). We applied two library preparation methods for historical samples: a double-stranded protocol (dsDNA) for screening a larger set of samples (26 out of 32) in order to identify which samples could offer DNA of high enough quality and abundance, and then a single-stranded protocol (ssDNA) for deeper sequencing (Lan & Lindqvist, 2019). From the first test run, we selected three individuals with relatively close geographical proximity to modern samples (Fig. 1), and whose dsDNA libraries yielded more than 20% endogenous DNA. We estimated the endogenous DNA content as the proportion of filtered reads mapped to the dog reference genome divided by the total reads after a first filtering with Cutadapt (see *Data analyses; Table S1*). We used these samples for ssDNA double-indexed library preparation and further shotgun sequencing. Negative controls were included in both the extraction and library preparation for both modern and historical samples. Sample and library preparation for historical samples were conducted in facilities that were isolated from those used for modern samples and that were properly equipped for ancient DNA handling and processing. We sequenced all the historical libraries on an Illumina NextSeq 500 platform with 75 cycles of single-end sequencing at the University of Potsdam, using custom primers for the ssDNA libraries (Gansauge & Meyer, 2013; Paijmans et al., 2017). Modern libraries were shotgun sequenced (paired-end reads of 150 bp) on an Illumina NovaSeq R at MedGenome Inc. (Foster City, CA, USA), through Genohub Inc. (Austin, TX, USA).

Data analyses

Raw reads from historical and modern samples followed the same preprocessing pipeline and settings as in Salado et al., (2023). In brief, data quality was checked with FastQC (Andrews, 2010). Adapter trimming and quality filtering were performed using Cutadapt v2.10 (Martin, 2011) and Fastp v0.23.1 (Chen et al., 2018) for historical and modern samples, respectively. We mapped reads to the dog reference genome (*Canis familiaris*), including autosomal sequences of CanFam3.1 (Lindblad-Toh et al., 2005), Y chromosome (GenBank KP081776; Li et al., 2013), and mitochondrial genome (NC_002008.4; Kim et al., 1998), to identify nuclear copies of mitochondrial DNA (NUMTs, den Tex et al., 2010). We used BWA-aln and BWA-mem v0.7.17 for mapping historical and modern samples each (Li & Durbin, 2009). We used SAMTOOLS v1.9 to sort, filter mapped reads by quality and calculate mapping statistics (Li et al., 2009). We used PICARD MarkDuplicates v2.26.6 to remove duplicates (http://broadinstitute.github.io/picard, accessed on 07 June 2023). GATK v3.8.1 was used for local realignment around indels (McKenna et al., 2010). Postmortem DNA damage (PMD) of historical samples was evaluated using MapDamage v2.0 (Jónsson et al., 2013).

For downstream analyses, we restricted the data to autosomal chromosomes to avoid different rates of evolution and patterns of inheritance of the sex chromosomes and mitochondrial DNA (Wilson Sayres, 2018). As we obtained low coverage data for historical samples (see Results), so we estimated genotype likelihoods (GL) to account for genotype uncertainty instead of directly calling genotypes. We called the GL using ANGSD v0.935 (Korneliussen et al., 2014) using the GATK algorithm (-GL 2), specified the output as a beagle binary file (-doGlf 3), and applied the following filters: only include reads with a mapping quality greater than 30 (-minMapQ 30), only include bases with base quality greater than 30 (-minQ 30), only include reads that map uniquely to one location (-uniqueOnly 1),

remove nonprimary, failed and duplicate reads (--remove_bads 1), a minimum minor allele frequency of 0.05 (-minMaf 0.05), only call a SNP if the p-value is smaller than 1×10^{-6} (-SNP_pval 1×10^{-6}), infer major and minor alleles from genotype likelihoods (-doMajorMinor 1), only include sites if they were not missing in more than one individual (-minInd 23, 22 or 10, depending on dataset, see below), call allele frequencies based on a known major allele based on the reference genome and an unknown minor allele inferred by the sum of the three possible minor alleles weighted by their probabilities (-doMaf 2), and remove transitions (-rmtrans 1), as post-mortem damage usually causes cytosine deamination in historical DNA (Briggs et al., 2007).

We considered the following wolf populations for analyses: modern Iberian wolves (ModIbe, n=8), historical Iberian wolves (HistIbe, n=3), modern Mexican wolves (Mexico, n=2), modern Middle Eastern wolves (Middle East, n=3), modern Balkan wolves (Balkan, n=3), and modern Russian Karelia wolves (Russia Karelia, n=3). The Italian wolf genome was only included in the dataset for the calculation of individual estimates, but not at the population level, as there was just one individual with whole genome data publicly available from this population. We constructed a rooted neighbor-joining (NJ) tree to assess topological relationships between taxa with the R package ape (Paradis & Schliep, 2019) using the complete dataset (n=23) and including the golden jackal as outgroup as in previous studies (Fan et al., 2016b; Freedman et al., 2014). We built a distance matrix generated in ANGSD (-makeMatrix 1) with the same arguments as above, but counting the different bases at each position (-doCounts 1) and using a consensus base approach (-doIBS 2).

We calculated individual inbreeding (F) and relatedness coefficient (R) using ngsRelate v2 (Hanghøj et al., 2019), which allows the estimation of relatedness among inbred individuals and bases the calculation of these coefficients on linear combinations of the 9 condensed Jacquard coefficients (denoted as J1 to J9, (Jacquard, 1974; Weir et al., 2006) that

derive from identity-by-descent patterns (IBD). According to this method, R is defined as the proportion of homologous alleles IBD (Hedrick & Lacy, 2015) estimated as [R = J1 + J7 +0.75(J3 + J5) + 0.5J8], while per individual inbreeding coefficients are estimated as [F1 = J1+J2+J3+J4] and [F2 = J1+J2+J5+J6] (Jacquard 1974; Vieira et al. 2013). NgsRelate v2 has been shown to provide reliable estimates of R and F on low-coverage genomes down to 1x (Hanghøj et al., 2019) and has been used previously to evaluate and compare inbreeding and relatedness for historical and contemporary data (e.g., Prost et al., 2022; Taron et al., 2021; Westbury et al., 2021). This software uses as input the genotype likelihoods and the population allele frequencies estimated through ANGSD. As allele frequencies may vary depending on the dataset used, we calculated F both for the complete dataset (n=23) and only for the Iberian wolf population, including historical and modern samples (n=11). Relatedness was estimated using only the Iberian dataset. We used 100 bootstrapping replicates as implemented in ngsRelate v2 (command -B 100) for F for each pair of individuals to recover confidence intervals (CI). We estimated the CI using the t.test() function from R package stats (R Core Team, 2020).

Population divergence (D_{XY}), population differentiation (F_{ST}), and nucleotide diversity (π) were calculated per population. Since the estimates are sensitive to sample size, the same sample size (n=3) was used in all cases except for the modern Mexican wolves, because only two genomes are available from this population. We used the three modern Iberian wolves from this study as representation of the contemporary Iberian wolf population (M1, M2, M3 as ModIbe) and the three historial for the historical Iberian wolf population (H1, H2, H3 as HistIbe) for this analysis. For the calculation of these population estimates, we used as input the consensus pseudo-haploid sequences generated from genotype likelihoods in ANGSD with the same flags as above, with the addition of -doHaploCall 2. D_{XY}, F_{ST} and π were estimated in 1 Mbp non-overlapping windows that included at least 500

SNPs per window as in Taron et al., (2021) using the Python script popgenWindows.py (https://github.com/simonhmartin/genomics_general; accessed on 19 May 2023). Significance of differences between populations in nucleotide diversity and inbreeding were assessed with Welch's two-sample t-test in R v3.6.3 (R Core Team, 2020), which is appropriate for two samples with unequal variances and sample sizes. We measured dispersion of π through the interquartile range (IQR) of boxplots, by subtracting the upper to the lower quartile (Q_3 - Q_1).

RESULTS

We sequenced the genome of six Iberian wolves with average coverage ranging between 1.4-2.2x (49,912,651 to 75,378,570 mapped reads per individual) and 8.5-9.5x (140,761,788 to 158,046,772 mapped reads per individual) for historical and modern samples, respectively (Table S1). Reads for historical samples showed very slight DNA damage patterns at the 5' and 3' ends (Fig. S1). For seventeen publicly available genomes from different modern populations, the average coverage ranged from 3.2 - 41.9x. Genomes with coverage higher than 20x (from Iran, Spain, Portugal and Russia) were downsampled to moderate coverage (15x) to avoid biases in downstream analyses (Table S1). We used more than 2.2×10^6 filtered polymorphic sites for analyses, with some variation depending on the individuals included in the dataset for each analysis (see Methods and Results below).

Relationship between historical and modern Iberian wolves

The NJ tree, based on 2,210,636 polymorphic sites, showed that modern and historical Iberian wolves clustered together, separated from other populations that similarly clustered according to geographical origin (Fig. 2). Additionally, historical and modern Iberian wolves showed the lowest population differentiation (F_{ST}) and divergence (D_{XY}) among all comparisons between population pairs (Fig. S2), confirming a close relationship.

Iberian gray wolves in our dataset did not show relatedness among any pair of individuals. Pairs of geographically matched historical and modern samples clustered together in the NJ tree (Fig. 2). H1 and M1, originating very close from each other, clustered together despite being sampled more than 40 years apart. Similarly, H2 and M2, sampled 28 years apart, also formed a cluster including the captive sample from unknown origin. H3, sampled at the border with Portugal, clustered with three samples from Portugal originating from a different study.
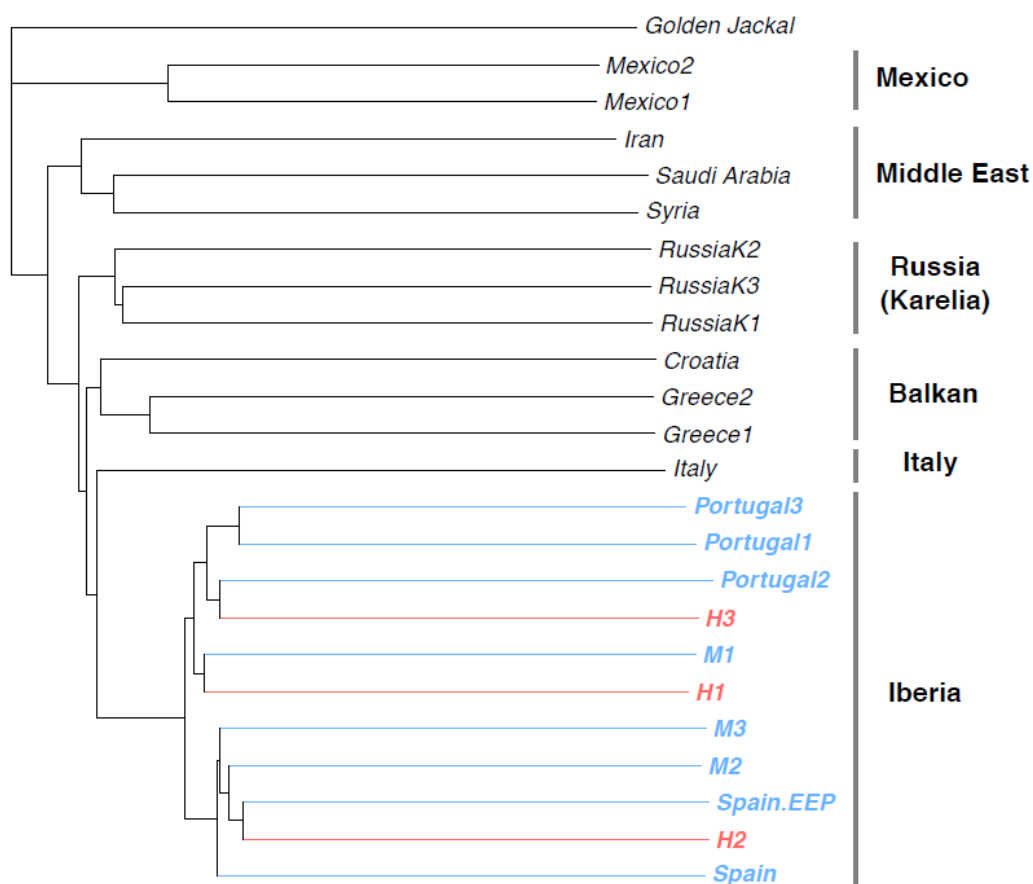

Figure 2. Rooted neighbor-joining (NJ) tree of the historical and modern gray wolf individuals. Based on 2,210,636 polymorphic sites. Iberian wolves are colored, historical (H) samples in red, and modern (M) samples in blue. Other gray wolves are shown in black. A golden jackal was used as an outgroup.

Changes in genetic diversity and inbreeding in Iberian wolves through time

Iberian wolves (both modern and historical) had significantly lower levels of genomic diversity (average $\pi = 0.244$) than other Eurasian wolves across 1 Mbp windows ($\pi = 0.274$, t = 35.314, df = 7417.8, p = 2.2 x 10^{-16}). Despite small sample sizes, genomic diversity was

similar between historical (π=0.246, n=3) and modern Iberian wolves (π=0.243, n=3, t = 2.002, df = 4193.4, p = 0.045). The nucleotide diversity on 1 Mbp non-overlapping windows distributed across the genome of modern Iberian wolves showed a greater spread (IQR=0.058) than that of historical Iberian wolves (IQR=0.047), with a slightly lower 25th percentile (Q_1=0.218 vs. Q_1=0.226, respectively) (Fig. 3). This pattern of high dispersion of genomic diversity values was also found in a much more pronounced form in endangered Mexican wolves (IQR=0.095, Q_1=0.187). Other Eurasian wolf populations showed higher values of genomic diversity with lower dispersion in comparison to Iberian and Mexican wolves (IQR-Middle East=0.036, IQR-Balkan=0.038, IQR-Russia =0.039).
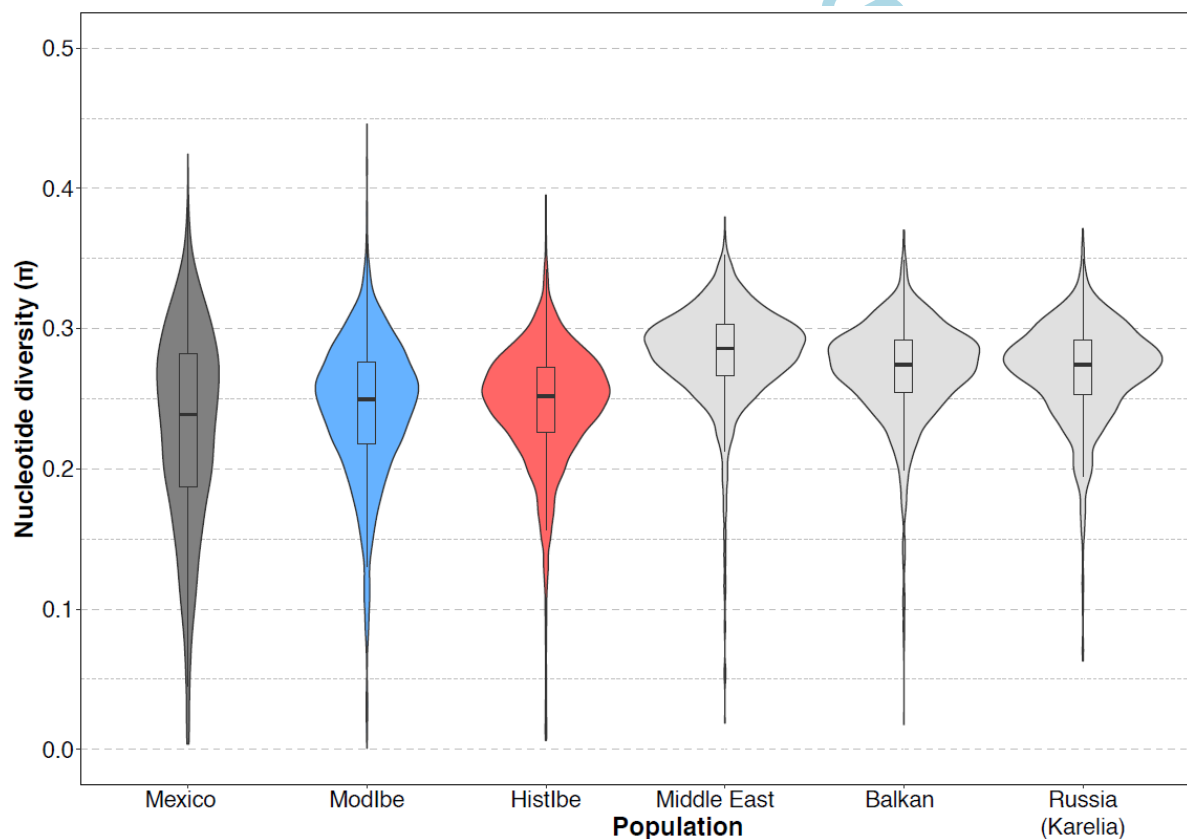
Figure 3. Genome-wide nucleotide diversity (π) in gray wolf populations. Diversity across non-overlapping windows of 1 Mbp with at least 500 SNPs across autosomes (Nwind, total number of windows). Based on a total of 2,504,288 sites. Populations: two modern Mexican wolves (Nwind=1,986), three modern Iberian wolves (ModIbe, blue, Nwind=2,156), three historical Iberian wolves (HistIbe, red, Nwind=2,104), three Middle Eastern wolves (Nwind=2,156), three Balkan wolves (Nwind=2,157), three Russian wolves (Nwind=2,157). In boxplots, the thicker horizontal line indicates median; boxes encompass first (Q_1) to third (Q_3) quartiles; whiskers indicate a distance of 1.5 times the IQR above Q_3 and below Q_1.

When we analyzed inbreeding values using the complete dataset including genomes from other populations, based on 2,239,303 polymorphic sites, the endangered Mexican wolves had the highest inbreeding (F=0.611 and 0.499; Fig. 4; Table S2). Among Eurasian wolves, the Italian wolf (F=0.451) had the highest inbreeding coefficient followed by the modern Iberian wolf M3 (F=0.362). Another three of the modern Iberian wolves had inbreeding values around 0.30, and four had values close to or below 0.1 (including the individual from the captive breeding programme, Spain.EEP). For geographically matched historical and modern samples H1-M1 and H2-M2, the modern sample had higher inbreeding than the historical sample. Despite small sample size, we obtained a significant difference in inbreeding levels between historical and modern Iberian wolves (mean ± stdev (HistIbe)=0.115 ± 0.091, mean ± stdev (ModIbe) = 0.182 ± 0.134, t = -5.1136, df = 192.87, p <0.001). We obtained a large variance in the level of inbreeding in modern Iberian gray wolves, with a standard deviation for F (Fstdev) for Iberian wolves of 0.134, similar only to Middle East wolves (Fstdev = 0.127), but much higher than for Russian (Fstdev = 0.028) or Balkan wolves (Fstdev= 0.038).

The absolute values of inbreeding differed when we used the Iberian data set alone but the relative position of Iberian individuals to each other was maintained (Fig. S3; Table S3).
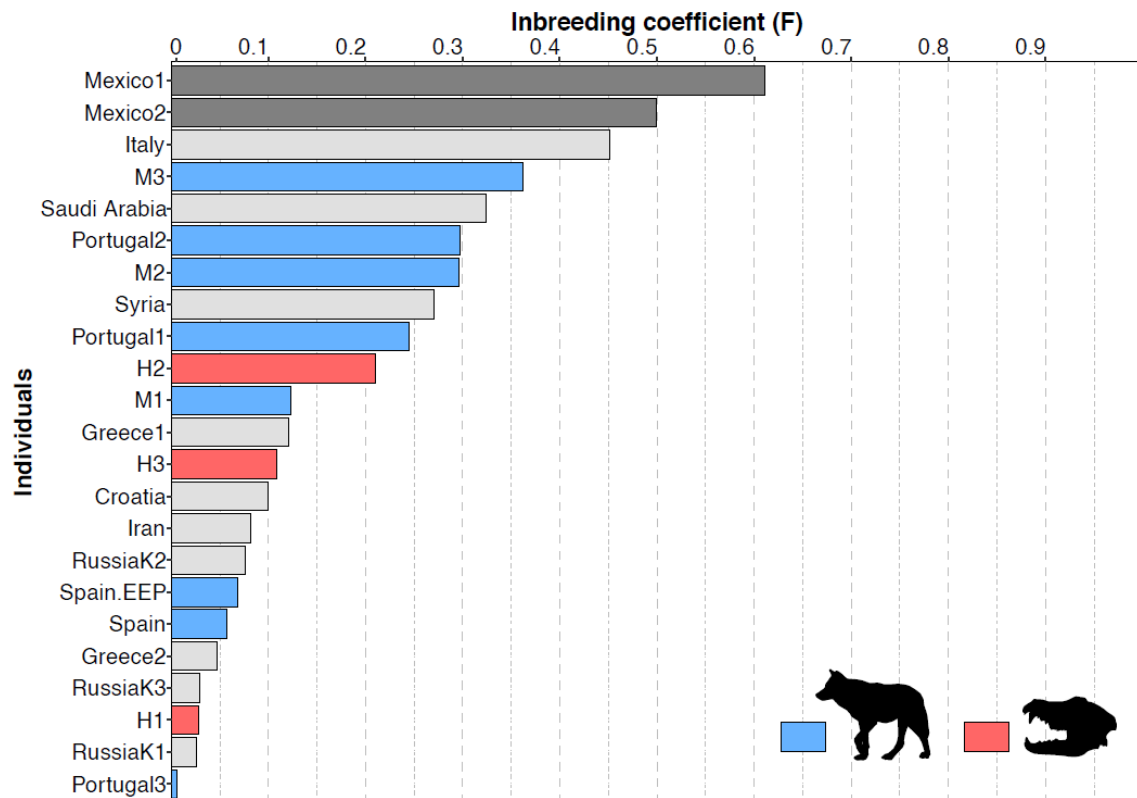
Figure 4. Inbreeding coefficient (F) estimated from wolf genome sequences. Complete dataset (n = 23) with wolf genomes from several wolf populations (based on 2,239,303 polymorphic sites). Individuals are listed from highest to lowest inbreeding. Iberian wolves are colored, historical (H) samples in red, and modern (M) in blue. Other gray wolves are shown in gray.

DISCUSSION

During the last two centuries, the Iberian gray wolf population retreated from being widespread across the peninsula to remaining almost exclusively in the north-western quadrant, a range reduction of 68% (Clavero et al., 2022). However, unlike in other European countries, wolves were not completely eradicated on the Iberian Peninsula and, presumably, the minimum population size in the mid-20th century was about 500-700 individuals (Chapron et al., 2014), the largest population in central and western Europe. The population increased after partial legal protection in the 1970s (Blanco et al., 1992; Grande del Brío, 1984; Valverde, 1971) and has been stable for the last 30 years, at around 350 packs or about 2,000-2,500 individuals (MITECO, 2016; Torres & Fonseca, 2016). Despite this demographically healthy population condition, population genetic estimates using a small

panel of microsatellites suggested an effective population size of about just 50 individuals (Sastre et al., 2011), and previous analyses showed a loss of mitochondrial DNA diversity during population expansion (Salado et al., 2023), and a reduction in genome-wide diversity due to the 1970s population bottleneck (Lobo et al., 2023).

At this short time scale, we observed very high variance in inbreeding (F) in the modern Iberian wolves. Some individual wolves were not inbred, and some had levels above that of full sibling matings. The individuals with very high levels of inbreeding were not randomly distributed. One individual, the one with the highest inbreeding, was a modern wolf sampled south of Douro river (M3). This part of the distribution was recently recolonized and the wolves south of the river have been subject to special protection in Spain (Annex II and IV of the EU's Habitats Directive; Quevedo et al., 2019) because of their presumed isolation. As shown in the NJ tree (Fig. 2) , the most related individual to this sample is M2, another modern wolf from an upstream locality, where water flow may have been more reduced; other neighboring populations south of the river went extinct in the late 1980s (Blanco, Cuesta, et al., 1990). Low genetic diversity on the colonization front due to limited availability of potential partners (founder effect) is expected. If the river is a partial barrier, it could exacerbate or further slow the arrival of additional individuals from the core area of the distribution, extending the founder effects through time, and thus could drive high levels of inbreeding there.

A modern individual north of the Douro river (M2) also had very high levels of inbreeding. That individual originated from a locality that was at the edge of the historical minimum distribution, although it is now well inside of the distribution (Fig. 1). The observation of this inbred individual in the primary distribution could indicate fragmentation in this part of the range. The comparison of historical and modern wolves from neighboring localities (M1 and H1, M2 and H2, H3 and modern Portuguese wolves) showed that modern

wolves are most similar to the animals that lived in the region several decades ago. This was unexpected considering that this population is continuous and that wolves have been easily recolonizing isolated areas in Europe, hundreds of kilometers away from the closest wolf populations (e.g., Andersen et al., 2015; Flagstad et al., 2003; Ražen et al., 2016; Vilà et al., 2003). Population structure associated with ecology has been described as a general pattern in wolves at a large geographic scale (Geffen et al., 2004; Leonard, 2014; Pilot et al., 2006, 2012), and previous studies already suggested that subpopulations existed within the Iberian wolf population (Salado et al., 2023; Silva et al., 2018) but there is no apparent underlying ecological driver in this case. Our results suggest that the fragmentation is not just isolation by distance since it has resulted in increasing inbreeding at a very local scale.

Fine scale structure or fragmentation of the population can put it at increased risk of inbreeding at local scales caused by subpopulation fluctuations. Wolf population management in Spain depends on regional autonomous governments, and the five regional administrations that include the wolf range have been putting in place disparate management strategies (Ordiz et al., 2022; Quevedo et al., 2019). Also, within an autonomous region, strategies can geographically vary (for example in Castilla y León, north and south of Douro river). This fragmented management has been seen as one of the main concerns with respect to the conservation of wolves in Spain (Boitani et al., 2022; Quevedo et al., 2019). At the same time, wolves coexist with relatively large human populations which can result in locally high rates of hunting and poaching. These anthropogenic factors likely have resulted in local population fluctuations that could favor local increases in inbreeding.

We also show that levels of inbreeding (F) have increased in magnitude and variance through time. This change from lower to higher levels of inbreeding happened as the population size stabilized at a larger size. Even substantially lower levels of inbreeding have been documented to negatively impact fitness in other wolf populations (vonHoldt et al.,

2023). A reduction in fitness (or phenotype value) due to inbreeding is called inbreeding depression (Allendorf et al., 2022). Inbreeding depression has been documented in the Mexican wolves (Asa et al., 2007; Fredrickson et al., 2007), and a few other small or isolated wolf populations (e.g., Isle Royale: Räikkönen et al., (2009); Robinson et al., (2019); Scandinavia: Åkesson et al., 2016; Liberg et al., 2005; Räikkönen et al., 2006, 2013; Smeds & Ellegren, 2022). Some consequences of inbreeding depression in other wolf populations include reduced litter size and/or juvenile survival (Liberg et al., 2005), lower breeding success (Åkesson et al., 2016), and morphological anomalies (Räikkönen et al., 2009, 2013). Appropriate data to determine if a population is or is not suffering from inbreeding depression has not been collected for very many populations. The high levels of inbreeding that we observe suggest that inbreeding depression could locally be an issue in modern Iberian wolves if the fragmentation continues in the population. Inbreeding depression has not been documented or suggested in Iberian wolves (Lobo et al., 2023), but it is possible that this may simply be due to a lack of data. Relevant data that could be collected include morphological data, behavioral data, and reproductive success since these traits have been shown to be impacted by inbreeding depression in other wolf populations.

In conclusion, our data show that the apparently large Iberian wolf population hides worrisome levels of fragmentation and local inbreeding that should be taken into account when planning future conservation and management strategies. One element should be the coordination of conservation and management plans to avoid actions that could provoke population fluctuations and further fragmentation, thus favoring inbreeding. As far as possible, conservation actions should facilitate increases in local effective population sizes. In addition, the removal of potential dispersal barriers and the promotion of connectivity between population fragments are essential to minimize inbreeding. Our results suggest that

the situation could be of special concern for wolves' south of the Douro river, and additional conservation and facilitation of the arrival of wolves from the north may be of particular importance. The collection of data on morphology, behavior, reproductive success and presence of pathogens, as well as continuous genetic monitoring, could ensure that the health and long term conservation status of the population are better understood. Facilitating gene flow with other European wolf populations could rapidly increase genetic diversity and decrease short term risk of inbreeding depression.

DATA AVAILABILITY

Raw DNA sequence reads of whole-genomes generated in this study were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under Accession Project no. PRJNA1029167. Data used from previous studies is available under project numbers: PRJNA494815, PRJNA255370, PRJNA494815, PRJEB39198, PRJNA274504 and PRJNA64812.

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