A sliver of the past: The decimation of the genetic diversity of the Mexican wolf

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Abstract
The endangered Mexican wolf (Canis lupus baileyi) is known to carry exceedingly low levels of genetic diversity. This could be (i) the result of long-term evolutionary patterns as they exist at the southernmost limit of the species distribution at a relatively reduced effective size, or (ii) due to rapid population decline caused by human persecution over the last century. If the former, purifying selection is expected to have minimized the impact of inbreeding. If the latter, rapid and recent declines in genetic diversity may have resulted in severe fitness consequences. To differentiate these hypotheses, we conducted comparative whole-genome analyses of five historical Mexican wolves (1907–1917) and 18 contemporary Mexican and grey wolves from North America and Eurasia. Based on whole-genome data, historical and modern Mexican wolves together form a discrete unit. Moreover, we found that modern Mexican wolves have reduced genetic diversity and increased inbreeding relative to the historical population, which was widespread across the southwestern United States and not restricted to Mexico as previously assumed. Finally, although Mexican wolves have evolved in sympatry with coyotes (C. latrans), we observed lower introgression between historical Mexican wolves and coyotes than with modern Mexican wolves, despite similarities in body size. Taken together, our data show that recent population declines probably caused the reduced level of genetic diversity, but not the observed differentiation of the Mexican wolves from other North American wolves.

KEYWORDS
bottleneck, carnivore, historical DNA, historical genome, introgression, mitochondria
1 | INTRODUCTION

The Mexican wolf (Canis lupus baileyi) is the subspecies of grey wolf that inhabited Mexico and southwestern United States (Hendricks et al., 2016; Nowak, 1995). The subspecies status was first defined morphologically by their small size and distinct cranial shape (Bogan & Melhop, 1983; Nelson & Goldman, 1929). Subsequent studies of mitochondrial DNA (mtDNA) supported the distinctiveness of the Mexican wolf from other grey wolf populations, and suggested that they represent a separate, earlier colonization of North America by Eurasian wolves (Vilà et al., 1999; Wayne et al., 1992). Similarly, mtDNA data from century-old museum specimens strengthened this distinction of the Mexican wolf lineage by describing additional, related haplotypes (“southern clade” in Leonard et al., 2005). More recently, genomic data further provided significant support for the Mexican wolf as a distinct lineage (Fan et al., 2016; Hailer & Leonard, 2008; vonHoldt et al., 2011).

Despite the plethora of evidence supporting the Mexican wolf as a distinct lineage, gene flow through hybridization followed by introgression has been an important component of the history of all North American Canis populations (Heppenheimer et al., 2020; vonHoldt et al., 2011, 2018). The grey wolves of the Great Lakes region have substantial, ancient introgression from coyotes (C. latrans), and the red wolf (C. rufus) appears to have an extensive and long evolutionary record of gene flow with coyotes (vonHoldt et al., 2011, 2016). A growing body of evidence suggests that genomic admixture and introgression, genome-wide and of fitness-related genes, is the expectation rather than the exception throughout the evolutionary history of the genus Canis (Adams et al., 2003; Anderson et al., 2009; Fan et al., 2016; Hedrick, 2013; vonHoldt et al., 2011, 2016). American canids exhibit a wide range of body size across habitat, longitudinal, and elevational gradients (Mech & Paul, 2008; Monzón et al., 2014; Way, 2007). Many suggest that similarities in body size increase the probability of interbreeding and hybridization, especially in canids (Hinton et al., 2018; Nagel & Schluter, 1998; Stronen et al., 2012). Low population size is another factor that increases the chance of hybridization in both of these cases (Hedrick et al., 2014; Liberg et al., 2005; Räikkönen et al., 2009, 2013).

Although inbreeding depression has been identified in the Mexican wolf, and despite their low population size, no evidence of hybridization with domestic dogs has been documented (Asa et al., 2007; Fitak et al., 2018). Evidence for historical or ancient introgression from coyotes or red wolves into the Mexican wolf genome has been documented from both nuclear genome-wide single nucleotide polymorphism (SNP) data (vonHoldt et al., 2011) and historical mtDNA (Leonard et al., 2005). Here, we use whole genome sequences from historical and contemporary Mexican wolves to characterize (i) the amount and timing of introgression, (ii) the timing of the decline of genetic diversity and change in inbreeding in Mexican wolves, and (iii) the breadth of their geographic distribution. We combined these data with published genomes from other North American canids to test the hypotheses that recent introgression is the result of persecution and declining population size as they faced extinction, and that the observed low levels of genetic diversity are a consequence of historical, human-caused demographic decline. Finally, we put these new data from the old Mexican wolf population into the context of ongoing Mexican wolf conservation efforts.

2 | MATERIALS AND METHODS

2.1 | Materials

Bone samples from five historical Mexican wolf skulls collected in the wild between 1907–1917 and deposited in the National Museum of Natural History, Smithsonian Institution, were collected for
genetic analysis (Figure 1, Table S1). The newly obtained data from these samples were analysed together with 21 previously published whole-genome data sets from two modern Mexican wolves, 12 other North American grey wolves, two Eurasian grey wolves, two red wolves, and three coyotes (Figure 1, Table S2). The mitochondrial control region from one of these five historical specimens (USNM 224484) was analysed in a previous study (Leonard et al., 2005).

2.2 | Molecular methods and data preprocessing

Whole genomic DNA was extracted from the historical specimens following (Dabney et al., 2013) using 19–54 mg of bone powder. We used 10 µl of DNA extract from each sample to prepare single-stranded, double-indexed (ssDNA) sequencing libraries (Gansauge & Meyer, 2013) and double-stranded, double-indexed (dsDNA) libraries (Henneberger et al., 2019), with two modifications of the dsDNA protocol. First, we used 10 µl (instead of 20 µl) of extract as template in blunt-end repair reactions. Second, the adapter fill-in reaction was incubated at 37°C for 20 min, followed by 80°C for 20 min and put on hold at 12°C. We applied two library preparation methods to account for variable fragment lengths of DNA extracts (average between 89 bp to 212 bp). The ssDNA protocol was used to capture highly fragmented, damaged DNA fragments from low concentrated extracts (Gansauge & Meyer, 2013) and increase the amount of recovered library molecules (Meyer et al., 2012). By applying both library methods we tried to maximize the amount of available molecules for sequencing. All molecular protocols were conducted in separate facilities that were equipped for proper ancient DNA (aDNA) handling and processing. Negative controls were included in both the extraction and library preparations. All sequencing was performed on an Illumina NextSeq 500 platform, with custom primers for the ssDNA libraries (Gansauge & Meyer, 2013; Paijmans et al., 2017). All libraries underwent test sequencing aiming for roughly one million reads (ssDNA libraries: 1 × 75 bp; dsDNA libraries: paired-end 2 × 75 bp). Since all test runs yielded over 20% endogenous DNA (Table S3), the dsDNA libraries were further sequenced (1 × 75 bp, ~80 million reads per sample).

For the analysis of mitochondrial genomes, all raw reads were preprocessed by trimming Illumina specific adapter sequences using cutadapt (v1.12, Martin, 2011) with default settings except for the options --overlap = 1 and --minimum-length = 30. Overlapping paired-end reads were merged using flash (v1.2.10, Magoč & Salzberg, 2011). For nuclear analysis (new historical raw data and downloaded modern raw data), preprocessing was repeated with the same parameters except for cutadapt (v1.12, Martin, 2011), for which option --overlap = 3 was used instead. Between 90 and 200 million raw reads per sample were generated (Tables S3, S4). Since between 87 and 96 percent of overlapping read pairs (paired-end data) from the historical wolves could be merged, and historical

FIGURE 1 Historic distribution of the grey wolf (Canis lupus) in North America showing currently accepted subspecies. Approximate locations of historical and modern Mexican wolves for which genetic data was previously available and/or is presented here are indicated with a dot. Other canid nuclear genomes used in this study are shown with stars. SB, South Baffin; NB, North Baffin; EI, Ellesmere Island; Q, Qamanirjuaq; S, Saskatchewan; T, Toronto; AC, Atlantic Coast; A, Alaska; PC, Pacific Coast; STLI, St. Lawrence Island
samples are expected to contain fragmented DNA (Sawyer et al., 2012), we only included merged reads in any downstream analyses. For modern data, both merged and unmerged reads were mapped to the reference genome.

2.3 | Mitochondrial DNA analyses

Preprocessed reads from the historical individuals were mapped to a wolf mitochondrial genome (NC_009686.1) using the Burrows-Wheeler aligner BWA-ALN v0.7.8 (Li & Durbin, 2009) with default settings. Sample Jal550 was additionally mapped against a coyote mitochondrial genome (NC_008093). Since mapping results (more reads mapping more evenly, Table S4) and phylogenetic analysis (see Figure 2) support an affiliation of Jal550 to C. latrans on the mitochondrial level, the read alignment to the coyote mitochondrial reference was used for further analyses. Files were converted to sam format by applying the commands samse (single-end data) or samtools v0.1.19 view and sort functions (Li et al., 2009). Duplicates were removed by considering 5′ mapping coordinates using the PICARD v1.111 MarkDuplicates tool (http://broadinstitute.github.io/picard/). Read alignments were imported to, visualized, and checked in GENEIOUS v10.2.3 (https://www.geneious.com/). Consensus sequences were created applying a minimum read depth of three and a 50% (3 × 50p) consensus threshold to call a nucleotide, otherwise they were called as missing data (“N”).

A multiple sequence alignment (MSA) of 110 canid mitochondrial sequences consisting of ancient (n = 11), historical (n = 5) and modern (n = 94) canid mitochondrial sequences (Table S5) was generated in GENEIOUS using the MUSCLE plugin (Edgar, 2004). For interspecific comparisons, we removed the control region, ambiguous positions, missing data, and gaps (GENEIOUS, MEGA v5) (Tamura et al., 2011). We selected a dhole sequence (Cuon alpinus, NC_034445) as the outgroup. We used JMODELTEST v2.1.10 (Darriba et al., 2012) with default settings and “best” as base tree search option to determine the best fitting maximum-likelihood (ML) tree model. The ML tree was constructed using PHYML v3.0 (Guindon et al., 2010; Guindon & Gascuel, 2003), as implemented in GENEIOUS. We also aligned the consensus sequences of the historical samples to previously published Mexican wolf sequences (KU644661, KU644664, KF661060.1, KF661065.1; Koblmüller et al., 2016; Thalmann et al., 2013). Pairwise differences between Mexican wolf sequences were determined using MEGA v7 with default settings (Kumar et al., 2016). Significance of pairwise differences between groups was assessed with the Welch’s two sample t test. The consensus sequences of all samples were aligned to a fragment of the mtDNA control region I (401–425 bp) that was previously used to investigate the genetic diversity of North American wolves (Leonard et al., 2005). Based on this alignment, a median joining haplotype network was constructed using POPART (Leigh & Bryant, 2015).

2.4 | Nuclear analyses

For nuclear analysis, an African wild dog (Lycaon pictus) genome was used as reference (GCA_001887905.1) to avoid reference ascertainment biases. Historical samples were mapped following the aforementioned methods, while modern samples (Table S2) were mapped using BWA-MEM v0.7.8 (Li & Durbin, 2009) with default settings. Genotype likelihoods estimations (GL) were obtained using ANGS D v0.921 (Korneliusen et al., 2014) with the following flags: -uniqueOnly 1 -remove_bads 1 -doMaf 2 -SNP_pval 1e-6 -minmap 0.1 -dolfl 2 -minMapQ 30 -minQ 30 -rmtrans 1 -docmuts 1 -GL 2 -minInd 25 -doMajorMinor 1 -dohaploCAll 2 -only_proper_pairs 0. We restricted the analysis to autosomes to avoid different rates of evolution and inheritance (Wilson Sayres, 2018). Transitions were excluded because historical DNA usually suffers from cytosine deamination due to post-mortem damage (Briggs et al., 2007). We assessed damage patterns using mapdamage v2.0.7 (Jónsson et al., 2013). All downstream analyses are based on the GL estimations. Individual inbreeding (F) and relatedness (R) between samples were estimated using ngsrelate v2, which uses identity by descent to calculate these metrics and has been shown to be reliable on low-coverage genomes down to 1x (Hanghøj et al., 2019). Population divergence (Dxy) and genetic diversity (α) were calculated from the consensus haploid call using ANGSD with the same arguments as the GL method, additionally with --dohaploCAll 2. We partitioned the resultant haploid output into 1Mb nonoverlapping windows of at least 500 SNPs per window using the poppenWindows.py python script (https://github.com/simonhallmarthin/genomics_general). We composed the following groups of taxa for analysis: all Mexican wolves (modern and historical), modern Mexican wolves (ModMex), historical Mexican wolves (HistMex), grey wolves excluding Mexican wolves (GreyW), coyotes (Coyote), and red wolves (RedW). Significant differences between groups in inbreeding and genetic diversity were assessed with the Welch’s two sample t test.

We conducted a principal component analysis (PCA) using the above GL beagle file in pcanGSD (Meisner & Albrechtsen, 2018). To examine topological relationships among population groupings, we constructed an unrooted nuclear neighbour-joining (NJ) tree using the R package ape (Paradis & Schliep, 2019) from the distance matrix obtained by using the same flags as above in ANGSD, but adding -doIBS 1 -makeMatrix 1. Individual admixture proportions were also estimated using ngsAdmix (Skotte et al., 2013), specifying K = 2−6. To evaluate convergence in our results for each K, we ran each K multiple times until the log likelihood of consecutive runs were consistent (K = 2 three times, K = 3 five times, K = 4 four times, K = 5 seven times, and K = 6 29 times). Furthermore, we ran evalAdmix to evaluate goodness of fit of the clustering for each K value (Garcia-Erill & Albrechtsen, 2020). We further performed a test for ancient admixture using D-statistics on both individual (-doAbbaba) and population (-doAbbaba2) (Soraggi et al., 2018) levels in ANGSD. The second considers all reads from multiple individuals in each population, outperforming the traditional D-statistics in detecting population-wide admixture events. We grouped individuals into the following
FIGURE 2. Maximum likelihood tree based on a 14,141 bp long alignment of 110 wolf and coyote mitochondrial genomes (Table S5). For collapsed clades, locations and number of samples are given in parentheses. Bootstrap support values are given next to nodes. Outgroup (Cuon alpinus) is not shown. Red text: historical Mexican wolves from this study; green text: modern Mexican wolf sequences; asterisk: ancient samples; light red area: southern clade.
populations: modern Mexican wolves (ModMex, \(n = 2\)), historical Mexican wolves (HistMex, \(n = 5\)), modern coyotes (Coyote, \(n = 3\)), and modern North American grey wolves (GreyW, \(n = 10\)). We tested for both relative levels of introgression between coyotes or grey wolves and modern and historical Mexican wolves (\(D_{\text{HistMex}, \text{ModMex}}\), \(D_{\text{HistMex}, \text{Coyote or GreyW}}\)) as well as within historical samples (\(D_{\text{HistMex1}, \text{HistMex2}}\), \(D_{\text{HistMex}, \text{Coyote or GreyW}}\)). The African wild dog was used as an outgroup in every comparison. The \(D\)-statistic was computed in blocks of 5 Mb and filters -minQ 30 -minMapQ 30 were applied. Significance was evaluated using an m-block jackknife method (Busing et al., 1999). The two-tailed acceptance region for \(z\) score was \((-3, 3)\). A positive \(D\)-value indicates gene flow between \(H_3\) and \(H_2\) taxa, while a negative \(D\)-value shows gene flow between \(H_3\) and \(H_1\). An error correction was applied to the historical samples (HistMex) by using the genome with the highest coverage as a reference in the population level analysis.

### Results

For the historical data, between 2,970 and 18,927 reads per individual successfully mapped to the mitochondrial reference genome (wolf: Jal545, Jal547, Jal548, Jal49; coyote: Jal550), yielding a range of 12x–82x sequence coverage across samples (Table S4). We also obtained 1.1–1.2x average genome-wide coverages across historical Mexican wolves (39,255,346 and 48,562,475 reads per individual) when mapping to the African wild dog reference genome (Table S3). Reads showed slight damage patterns (Figures S1, S2) and short fragment lengths (mean 49–73 bp; Table S3), as expected for historical samples (Sawyer et al., 2012). For analysis of nuclear sequences, we obtained previously published data (21 modern Canis samples, including two modern Mexican wolves and three coyotes) to map to the African wild dog genome. This produced 59,017,254 to 391,455,879 mapped reads per individual, with 2.4x–15.8x average genome-wide coverages across samples (Table S2). We discovered and retained 853,783 nuclear SNPs after filtering for analysis.

### 3.1 Relationship between historical and modern Mexican wolves

Except for differences in length and a small number of uncalled positions, the published modern Mexican wolf mitochondrial genomes (KF661060.1, KF661065.1, KU644661, KU644664; Koblmüller et al., 2016; Thalmann et al., 2013) were all identical to one another and carry the most common historical haplotype, lu33 (Leonard et al., 2005). This haplotype has not been identified outside of Mexican wolves. Pairwise comparisons of historical and modern Mexican wolf mitochondrial genome sequences revealed differences between them (mean = 21.9, SD = 21.2), with slightly greater variation found in historical samples (mean = 37.7, SD = 20.1). However, this difference was not significant (\(p = .138\)) (Table S6). Sample Jal550 was excluded from this analysis due to its introgressed coyote haplotype. Due to the low mapping quality of short aDNA reads in low complexity regions, all historical samples contained uncalled positions within the mitochondrial control region which complicates the comparison to previously published mitochondrial control region sequences (Leonard et al., 2005). After we excluded these uncalled sites, we retained an alignment of 343 bp and lost eight diagnostic positions. The single sample which was also previously sequenced by Leonard et al., (2005) (Jal545; USNM 224484) yielded results consistent with haplotype lu33, as previously found. We discovered two novel Mexican wolf haplotypes that each differed from lu33 by 1 bp (Figure 3). Sample Jal550 (USNM227885) from Texas is consistent with haplotype lu60, which was previously identified in one individual from
Mexico (Ja1474, USNM98313) (Leonard et al., 2005) and is within the diversity of coyotes, as noted above (Figure 2).

We obtained a final alignment of 14,141bp of the five historical Mexican wolf consensus mitochondrial genomes and 105 other canid mitochondrial sequences. jmodeltest identified HKY + I + G as the most suitable substitution model using four substitution categories and estimating the ratio of transitions to transversion, the proportion of invariable sites and the gamma distribution parameter as the best fitting model and parameters according to the Bayesian information criterion.

The topology of the ML phylogeny was qualitatively similar to trees from previous studies (e.g., Koblmüller et al., 2016; Thalmann et al., 2013). Three of the five historical samples (Ja1545, Ja1547, and Ja1549) clustered with modern Mexican wolf samples in the previously identified southern clade (Figure 2). Historical Mexican wolf sample Ja1548 was outside the southern clade, but within the diversity of North American grey wolves with high bootstrap support (100%; Figure 2). The last historical Mexican wolf sample, Ja1550, clustered within the coyote clade (Figures 2, 3), although it did not match any coyote haplotype described to date (Hailer & Leonard, 2008; Koblmüller et al., 2012).

The nuclear NJ tree clustered modern and historical Mexican wolves together, separated from other North American grey wolves, but within the overall grey wolf diversity. Red wolves and coyotes clustered separately, with the grey wolves from Great Lakes and Algonquin Park closer to them than to other North American grey wolves (Figure 2).

In the admixture analysis, modern and historical Mexican wolves clustered together across all K values (Figure 4). Mexican, Eurasian and other North American grey wolves are differentiated from coyotes and red wolves at K = 2. Mexican wolves were differentiated from other grey wolves, red wolves and coyotes at K = 3 and higher. At K = 4 and 5, geographic groups within the grey wolves become apparent, and at K = 6, the red wolves become differentiated from the coyotes. evaladmix found K = 6 the best fit for our data set (Figure 4).

### 3.2 | Ancient introgression into Mexican wolves

Although one mitochondrial haplotype shows that there must have been introgression from coyotes at some point, no recent nuclear introgression is discernible in the admixture results (Figure 4). The PCA also did not suggest clustering between the Mexican wolves and the coyotes; even sample Ja1550, which contains the coyote mitochondrial haplotype, was clustered with historical Mexican wolves (Figure 5). The first PC differentiated grey wolves from coyote genomic variation (50.99% of the variation in the data explained by PC1), while PC2 (8.3%) and PC3 (4.95%) differentiated diversity within grey wolves (Figure 5, Figure S5). Further, PC2 appeared polarized by grey wolves and Mexican wolves (modern and historical), with two Eurasian grey wolves (Spain and Iran) included. PC4 (3.78%) differentiated red wolves from grey wolves and coyotes’ genomic variation (Figure S5). Given that PC1 captured coyote genetic variation in North American canids and that red wolves, eastern wolves, and Great Lakes grey wolves were positively loaded on this axis, they were excluded from the population level D-statistics analysis (Figures 4, 5). We detected significant gene flow between coyotes and modern Mexican wolves relative to historical Mexican wolves (D = 0.154; p < .001 in [HistMex, ModMex; Coyote]) at both population (Table S7) and individual levels (Figure 6, Table S8). Although D-statistics also detected different levels of introgression with coyotes within historical Mexican wolves, especially in samples Ja1550 and Ja1547 (Figure 6, Table S9), this gene flow was lower than for modern Mexican wolves. We also found more introgression between coyotes and modern Mexican wolves relative to other North American wolves (Table S7).

Similarly, we also detected gene flow between modern Mexican wolves and other North American wolves relative to the historical Mexican wolves at both population (D = 0.139; p < .001 in [HistMex, ModMex; GreyW]), Table S7) and individual levels (Figure 6, Table S10). Although D-statistics also detected different levels of introgression with North American wolves within historical Mexican wolves (Table S11), suggesting a long term pattern, this gene flow was again lower than with modern Mexican wolves (Figure 6, Table S10). Introgression from North American grey wolves was also identified for Ja1548 in the admixture results (Figure 4).

### 3.3 | Changes of genetic diversity and inbreeding in Mexican wolves through time

We discovered two new "southern clade" mitochondrial haplotypes (Figures 2, 3). This finding markedly increases the known historical mitochondrial diversity of Mexican wolves.

One of the modern Mexican wolves had the highest inbreeding coefficient of any canid in this study (F = 0.365), while the other modern Mexican wolf had the third highest inbreeding coefficient when excluding coyotes (F = 0.226; Table S12). Most grey wolves from Alaska and Canada had a very low inbreeding coefficient (F = 0.005–0.03; Table S12), although endangered and island populations had elevated levels of inbreeding estimates (F = 0.043–0.179). While the level of inbreeding found in the historical Mexican wolves was larger than that found in North American grey wolf populations (F = 0.041–0.172), it was substantially lower than it is today (F = 0.226–0.365). Furthermore, we found the difference between modern (F = 0.295) and historical Mexican wolves (F = 0.125) to be significant (t = -135.7, df = 32.792, p < 2.2 × 10^-16). Similarly, relatedness was high between the two modern Mexican wolves (R = 0.24) and very low on average across grey wolves (Table S13). We noted a large variance in relatedness between historical and modern Mexican wolves (R = 0.0–0.24).

Mexican wolves (modern and historical) had significantly higher levels of genomic diversity (π = 0.278) than coyotes (π = 0.229, t = 14.4. df = 1050.3, p = 2.2 × 10^-16) or red wolves
and significantly lower than other North American grey wolves ($\pi = 0.299$, $t = -6.0$, $df = 1041.7$, $p = 2.3 \times 10^{-9}$). Although sample sizes are small, we found that historical Mexican wolves ($n = 5$) have higher diversity ($\pi = 0.275$) than modern individuals ($n = 2$, $\pi = 0.257$, $t = 4.15$, $df = 969.0$, $p = 3.6 \times 10^{-5}$) (Figure 7a). We also found the levels of genetic divergence between Mexican wolves (modern +historical) and coyotes ($D_{xy} = 0.371$, $SD = 0.07$) similar but slightly greater than that between coyote and grey wolf divergence ($D_{xy} = 0.365$, $SD = 0.08$). Mexican wolves and grey wolves had low levels of divergence ($D_{xy} = 0.314$, $SD = 0.06$), but the lowest was between red wolf and coyote ($D_{xy} = 0.281$, $SD = 0.06$). When we analysed historical and modern Mexican wolves separately, we found that they had about the same divergence with coyotes (modern-coyote $D_{xy} = 0.373$, $SD = 0.08$; historical-coyote $D_{xy} = 0.370$, $SD = 0.07$). The divergence between historical and modern Mexican wolves was low ($D_{xy} = 0.283$, $SD = 0.05$), and about the same as the divergence between red wolves and coyotes ($D_{xy} = 0.281$, $SD = 0.06$) (Figure 7b).

**FIGURE 4** Admixture proportions using ngsadmixture.x, specifying $K$ values of 2 to 6. Historical Mexican wolves are Jal545, Jal547, Jal548, Jal549, Jal550; modern Mexican wolves are Mexico 1 and Mexico 2; modern Eurasian and North American grey wolves are named by their geographic origin. SB, South Baffin; NB, North Baffin; EI, Ellesmere Island; Q, Qamanirjuaq; S, Saskatchewan; T, Toronto; AC, Atlantic Coast; A, Alaska; PC, Pacific Coast; STLI, St. Lawrence Island. Coyotes are also named by their geographic origin.

4 | **DISCUSSION**

In the course of less than a century, the distribution of Mexican wolves dramatically declined from once being widespread across much of Mexico and the American southwest to extinct in the wild (Fredrickson & Hedrick, 2002; Hedrick & Fredrickson, 2010). Here, we add to previous studies of historical genetic data from Mexican wolves (Hailer & Leonard, 2008; Hendricks et al., 2016; Leonard et al., 2005) and find additional historical mitochondrial diversity (Figure 3, Table S6). We also report whole genome sequences of five historical Mexican wolves, which had higher genomic diversity and lower levels of inbreeding than the current captive and reintroduced Mexican wolves (Figure 7). These new data suggest that the low levels of genomic diversity are due to a recent population decline with increased inbreeding rather than a long-term evolutionary history of low genetic diversity. Purifying selection can circumvent inbreeding depression in lineages with low, stable effective sizes despite very low genetic diversity (Robinson et al., 2018). However, drift is strong in populations that have experienced rapid declines in effective size,
undermining the ability of purifying selection to purge deleterious alleles linked to depressed fitness (Hedrick & Garcia-Dorado, 2016). The Mexican wolves fall in the latter category— a rapid decline of effective population size and genetic diversity.

Another risk faced by small, disturbed, and edge populations of grey wolves is an increased risk of interspecific hybridization (Leonard et al., 2014; Stephens et al., 1999). Introgression of genetic material may provide short term advantages, but it is not always enough to rescue a population. For example, the now extinct grey wolf population that inhabited the Sierra Morena in southern Spain hybridized with dogs, which allowed it to survive for some more generations of inbreeding leading up to their extinction about 20 years ago (Gómez-Sánchez et al., 2018). Introgression can also have direct negative effects such as swamping, a serious threat to the red wolf (Heppenheimer et al., 2020; Miller et al., 2003). Endangered populations which have hybridized may also face persecution from managers (e.g., Vilà et al., 2003).

Mexican wolves have evolved in sympathy with coyotes throughout their history, and have coexisted with dogs for thousands of years, including during times of population decline. Mexican wolves are the smallest wolves in North America, and thus the most similar in size to coyotes and dogs, further increasing the probability of hybridization (e.g., Hinton et al., 2018). Despite the opportunity and conditions for interspecific hybridization, no evidence of dog introgression has been identified in the Mexican wolf genome (Fitak et al., 2018). Introgression of mitochondrial DNA from coyotes has been identified in the historical population (Haier & Leonard, 2008; Leonard et al., 2005; this study).

The single coyote-like mitochondrial haplotype previously identified in a single historical Mexican wolf was here discovered in a geographically disparate second individual, despite the small number of Mexican wolves investigated (Figure 2). The animals in which the coyote-like mitochondrial haplotype was identified were collected in 1899 and 1907 (Jal550), and the genome of Jal550 did not show any evidence of coyote ancestry at K = 3–6 (Figure 4) indicating that the introgression event was relatively ancient. Although recent coyote ancestry was not identified in historical Mexican wolves in the admixture analyses, it was identified in the D-statistics, also suggesting an ancient rather than recent gene flow event(s) (Figure 6, Table S7).

One possible explanation of the divergent mitochondrial lineage in Mexican wolves based on modern data is that Mexican wolves represent one end of a grey wolf continuum across North America explained with simple isolation by distance in which the middle part is missing in the analyses due to their extirpation (Leonard et al., 2005). If this was the case, there should be a gradient of Mexican wolf to other North American wolf ancestry from south to north. This was not observed in the historical mitochondrial DNA results, and we do not see it here with nuclear data. Even from the northern edge of the distribution the Mexican wolves have nearly all Mexican wolf ancestry. The single individual with some other (probably more recent) North American wolf ancestry (Jal548; Figure 4) is the exception that proves the rule by illustrating that recent admixture can be identified with our dataset but was not found in the other individuals. We did find evidence for ancient introgression events in other Mexican wolf individuals suggesting that gene flow may have occurred more readily in the past when populations were larger and perhaps more connected. Therefore, our results suggest that their distinction is driven by distinct habitats as compared to other North American grey wolves. Several populations of North American wolves show strong signals of local adaptation and divergent evolution tightly correlated with environment (Carmichael et al., 2007, 2008; Koblmüller et al., 2009; Leonard, 2014; Muñoz-Fuentes et al., 2010; Musiani et al., 2007; Schweizer et al., 2016; Zhang et al., 2014). We therefore suggest that Mexican wolves represent a distinct grey wolf lineage through the process of local adaptation.

As a locally adapted taxon associated with a habitat, habitat may be a useful proxy in determining the appropriate distribution for the Mexican wolf, as was done by Hendricks et al., (2019). They predicted that the range of the Mexican wolf probably included Mexico, New Mexico, Arizona, western Texas, and southern California (wide

FIGURE 5 Principal component analysis (PCA) of 26 Canid genomes (Tables S2 and S3) based on autosomes, only transversions. Modern Eurasian and North American grey wolves are labeled by their geographic origin. SB, South Baffin; NB, North Baffin; EI, Ellesmere Island; Q, Qamanirjuaq; S, Saskatchewan; T, Toronto; AC, Atlantic Coast; A, Alaska; PC, Pacific Coast; STLI, St. Lawrence Island. Coyotes are also named by their geographic origin. The variation in the data explained by each PC is indicated in parentheses.
Hendricks et al., (2019) supported this extended range with genetic analysis of a historical grey wolf from southern California, which was revealed to be a Mexican wolf. Our genomic data from the other edges of the geographic extremes of the Mexican wolf range also support the wide distribution of the Mexican wolf (Figure 1). As this habitat shifts with ongoing climate warming, the area appropriate for Mexican wolves is also predicted to shift northwards.

Genetic rescue policies have been implemented to support the survival of the Mexican wolf in the past, and they appear to have been initially successful (Fredrickson et al., 2007; Kalinowski et al., 1999). However, genetic rescue is a short term fix to buy time to address other threats to the taxon (Hedrick & Fredrickson, 2010). In the case of the Mexican wolf, this time may have been squandered as the population size in the wild is still vastly below the size necessary to buffer against demographic uncertainty and for selection to efficiently act (Frankham, 1996; Tanaka, 2000; Wright, 1931). Genetic rescue is expected to be less successful after the first time, if the same donor population is used (Hedrick & Garcia-Dorado, 2016; Hedrick et al., 2014). The results presented here suggest that, if the population size of Mexican wolves was large enough to avoid the risk of swamping, other sources of donor populations could be considered to increase genetic diversity (following appropriate guidelines, such as Hedrick & Fredrickson, 2010). If it
is not possible for the population size to increase dramatically with the genetic diversity currently present, other tools to reinstate lost genetic diversity in the Mexican wolf, such as the incorporation of frozen sperm from earlier generations or the insertion of diversity observed in genes of historical animals using synthetic biology tools (Piaggio et al., 2017), may require consideration. In the long term, population size must increase for the risk of extinction to subside to acceptable levels.

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AUTHOR CONTRIBUTIONS
This project was conceived by Jennifer A. Leonard and Michael Hofreiter, and data collection was funded by Michael Hofreiter. The laboratory work was done by Ulrike H. Taron, Susanne Butschkau and Johanna L. A Paijmans. Analyses were done by Ulrike H. Taron, Isabel Salado, Mariana Escobar-Rodriguez, Michael V. Westbury and Bridgett M. vonHoldt. The manuscript was written by Jennifer A. Leonard, Ulrike H. Taron and Isabel Salado, and was edited and approved by all authors.

DATA AVAILABILITY STATEMENT
Raw data has been made available on GenBank through the SRA database with the project accession number PRJNA719803.

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Additional supporting information may be found online in the Supporting Information section.