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Persistence and expansion of cryptic endangered red wolf genomic ancestry along the American Gulf coast

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Abstract

Admixture and introgression play a critical role in adaptation and genetic rescue that has only recently gained a deeper appreciation. Here, we explored the geographical and genomic landscape of cryptic ancestry of the endangered red wolf that persists within the genome of a ubiquitous sister taxon, the coyote, all while the red wolf has been extinct in the wild since the early 1980s. We assessed admixture across 120,621 single nucleotide polymorphism (SNP) loci genotyped in 293 canid genomes. We found support for increased red wolf ancestry along a west-to-east gradient across the southern United States associated with historical admixture in the past 100 years. Southwestern Louisiana and southeastern Texas, the geographical zone where the last red wolves were known prior to extinction in the wild, contained the highest and oldest levels of red wolf ancestry. Further, given the paucity of inferences based on chromosome types, we compared patterns of ancestry on the X chromosome and autosomes. We additionally aimed to explore the relationship between admixture timing and recombination rate variation to investigate gene flow events. We found that X-linked regions of low recombination rates were depleted of introgression, relative to the autosomes, consistent with the large X effect and enrichment with loci involved in maintaining reproductive isolation. Recombination rate was positively correlated with red wolf ancestry across coyote genomes, consistent with theoretical predictions. The geographical and genomic extent of cryptic red wolf ancestry can provide novel genomic resources for recovery plans targeting the conservation of the endangered red wolf.

KEYWORDS
admixture, ancestry, RADseq, recombination, red wolf, X chromosome

1 | INTRODUCTION

As the field of evolutionary biology moves towards the web-of-life framework, there is broader recognition regarding the role that introgression and admixture have on adaptation and genetic rescue (Burgarella et al., 2019; Hamilton & Miller, 2016; Hufbauer et al., 2015; Kronenberger et al., 2018; vonHoldt et al., 2018). However, the strength and permeability of reproductive barriers shapes the variation in both ancestry proportions and linked phenotypes that result from introgression (Haasl & Payseur, 2016; Harrison & Larson, 2016). Barrier loci substantially reduce gene flow between species or ecotypes and influence the landscape of introgression across the

genome (Barton & Bengtsson, 1986). In species that are spatially structured, ancestry inferences are thus challenged by geographical heterogeneity, although such clines can be utilized to explore variability in introgression rates along an admixture gradient (Gompert & Buerkle, 2011). Furthermore, recombination rates vary across the genome and can consequently result in the inference of different evolutionary histories (Lotterhos, 2019). For instance, regions of low recombination have more extensive linkage disequilibrium (LD), higher differentiation, reduced nucleotide diversity and possibly more recent coalescence relative to regions with more frequent recombination events (Geraldes et al., 2011; Seixas et al., 2018; Stevison & McGaugh, 2020). However, these regions also experience more effective selection against introgression, possibly due to their linkage to and frequent enrichment with barrier loci that impact hybrid fitness (Martin et al., 2019; Schumer et al., 2018). Theory then predicts that introgression and ancestry tract lengths have a negative relationship with the time since the initial hybridization event (Pool & Nielsen, 2009).

The red wolf (*Canis rufus*) has been absent from the landscape since the early 1980s, yet coyotes (*C. latrans*) in southeastern Texas and southwestern Louisiana continue to harbour red wolf genetic ancestry (Heppenheimer et al., 2020; Heppenheimer, Brzeski, Wooten, et al., 2018; Murphy et al., 2018; Wilson et al., 2012). This geographical region is where the last wild red wolves were captured in the 1970s to initiate a captive breeding programme as part of their Species Survival Plan (SSP) prior to extinction in the wild (Carley, 1975). Hybridization between these canid lineages before their captive founding has been reported (McCarley, 1962; Paradiso & Nowak, 1972; Riley & McBride, 1972), and the persistence of canids resembling red wolf hybrids continued to be documented through the 1990s (Giordano & Pace, 2000). Although initial genetic assessments of canids in Texas did not detect substantial admixture (Hailer and Leonard 2008), more comprehensive genomic surveys suggest that cryptic red wolf ancestry may be widespread in coyotes along the Gulf coast (Heppenheimer et al., 2018a, 2020; Murphy et al., 2018), and evidence of a red wolf Y-chromosome haplotype was reported in neighbouring Louisiana and North Carolina (Wilson et al., 2012). Red wolves remain critically endangered with less than 10 known wild wolves persisting in a reintroduced population in North Carolina and approximately 250 in SSP captive facilities. The species suffers from a reduced effective population size as a consequence of its founding with 14 individuals, concomitant with a conservation concern that wild red wolves interbreed with coyotes (Brzeski et al., 2014; Hinton et al., 2013). There is an urgent need to better understand the timing and extent of historical introgression, and to identify ghost red wolf genetic variants in extant coyotes that could be utilized for the future of red wolf recovery.

Genetic ancestry and timing estimates of gene flow in North American canids have been previously examined (Sinding et al., 2018; vonHoldt, Cahill, et al., 2016; vonHoldt et al., 2016). However, there is a paucity of inferences based on chromosome types (i.e., autosomal vs. sex chromosome) and integration with recombination rates. Given the contrasting patterns of demographic estimates for regions of differing recombination and evolutionary rates, we predicted that such genomic regions may show divergent patterns in ancestry and gene flow. We explore the impact of variable recombination rates and admixture across a dense sampling of canids from the southeastern range of coyotes and red wolves. Here, we genotyped 120,621 genome-wide single nucleotiode polymorphisms (SNPs) in 310 canid genomes that traverse the remnant hybrid zone in southeastern Texas and southwestern Louisiana (Heppenheimer et al., 2020; Heppenheimer, Brzeski, Wooten, et al., 2018; Murphy et al., 2018). Our objective was to determine if the recently discovered cryptic red wolf ancestry has a much broader geographical distribution. We were motivated to describe the extent to which undetected introgressed alleles from an endangered and extirpated species occupy the landscape. We predicted that introgressed red wolf ancestry found in coyotes would be highest in southeastern Texas and southwestern Louisiana, the geographical areas proximal to the source of the SSP red wolf founders (Carley, 1975; McCarley, 1962; McCarley & Carley, 1979; Nowak, 2002; Paradiso, 1968). Furthermore, we compared patterns of ancestry on the X chromosome and autosomes, which has been performed rarely in previous studies of canid phylogeography. We further aimed to explore the relationship between admixture timing and recombination rate variation to identify gene flow events that are historical, contemporary or are signals of incomplete lineage sorting (ILS). Finally, we predicted that younger introgressed fragments have radiated away from the hybrid zone as coyotes with admixed and introgressed genomes disperse.

2 | METHODS

2.1 | Sample collection and DNA extraction

We obtained 310 blood or tissue samples from state management programmes, government organizations archives, museum archives or previous work (Curtis-Robles et al., 2016). All samples collected have a known US state of origin, and many also have known county (Figure 1a,b; Table S1). Samples were collected between 1987 and 2020. This work was conducted under the approved Princeton University IACUC protocol 1961A-13. Reference populations are based on three previous genome-wide studies that identified populations of little to no admixture, as well as incorporating pre-expansion demographics (Heppenheimer et al., 2020; Heppenheimer, Brzeski, Wooten, et al., 2018; Heppenheimer, Harrigan, et al., 2018). Domestic dogs (*C. familiaris*) from North America were additionally included to address the possibility of recent hybridization, although little evidence suggests they have contributed to current wild canid ancestry (Heppenheimer, Harrigan, et al., 2018; vonHoldt et al., 2011). Reference red wolves were selected as captive individuals that genetically represented the red wolf founders with low pedigree inbreeding values (0.075–0.097). Furthermore, we are cognizant that the demographic histories of the eastern wolf and grey wolves of the Great Lakes region are complex due to admixture

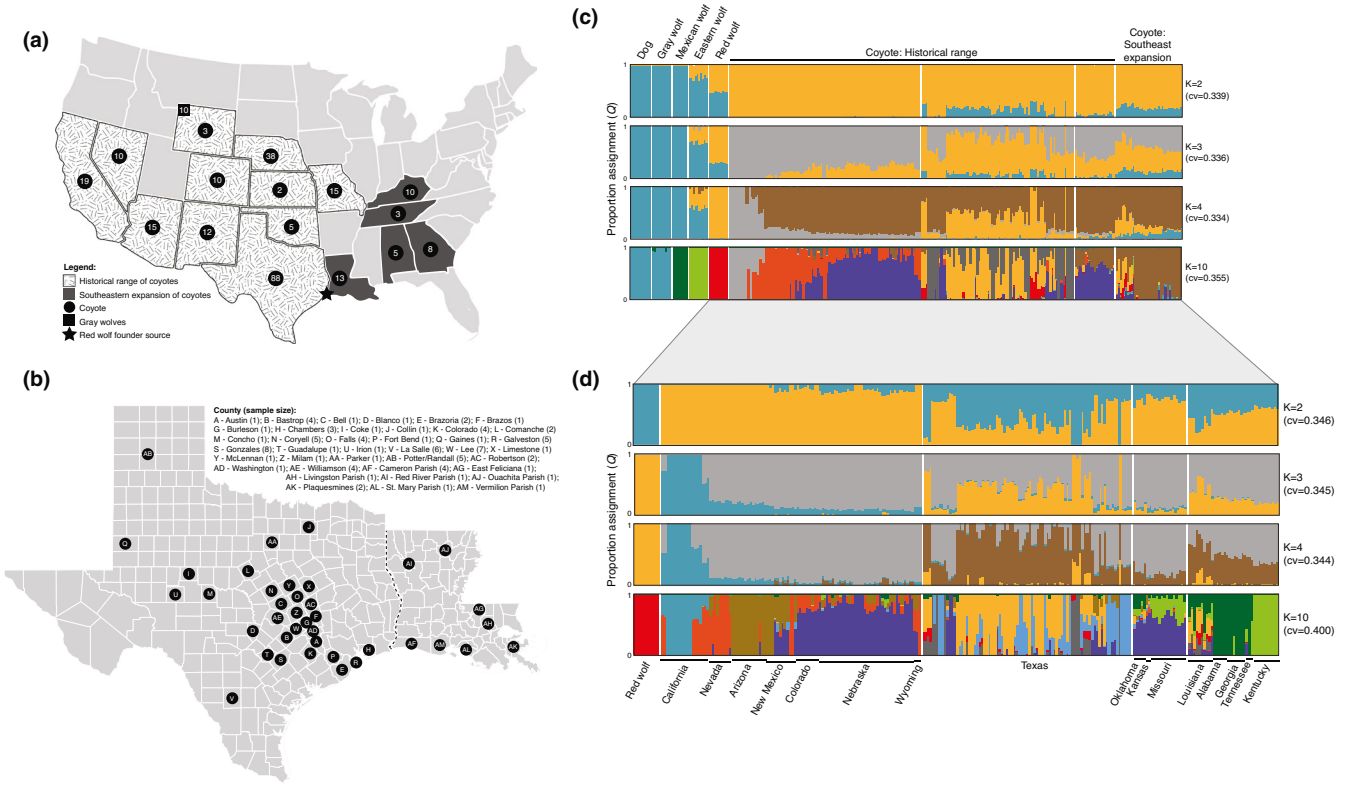

FIGURE 1 Sample map of (a) 310 canids from the lower contiguous USA and (b) from counties in Texas and Louisiana. Shaded states indicate geographic regions included in this study, their broad classification as either part of the historical or southeast expansion range of coyotes, and numbers within represent sample size. Reference genomes not included on the geographical map are domestic dogs ($n = 13$), eastern wolf (Algonquin Provincial Park, $n = 10$), red wolves from the Species Survival Plan captive breeding population ($n = 10$), and Mexican wolves from the captive breeding studbook ($n = 10$) (see Table S1 for more details). Admixture proportions from 83,000 unlinked/neutral SNPs for (c) 293 canids and (d) 254 canids. Solid bars above or below vertical bars indicate the state from which the samples originated

and gene flow, which results in these populations known to carry a detectable, and sometimes substantial, amount of nongrey wolf ancestry (Heppenheimer, Harrigan, et al., 2018). We obtained high-molecular-weight genomic DNA using either the Qiagen DNeasy Blood and Tissue Kit or the BioSprint 96 DNA Blood Kit performed on the Thermo Scientific KingFisher Flex Purification platform and following the manufacturer's blood or tissue protocol for mammals. We quantified DNA concentration using either the PicoGreen or Qubit 2.0 fluorometry systems, and subsequently standardized each sample's DNA concentration to 5 ng μl^{-1}.

2.2 | RAD sequencing and bioinformatic processing

We prepared 205 genomic libraries for restriction-site associated DNA sequencing (RADseq) following a modified protocol (Ali et al., 2015). We digested genomic DNA with *Sbf*I and ligated a unique 8-bp barcoded biotinylated adapter to the resulting fragments. We then pooled equal amounts of 48 DNA samples followed by random shearing to 400 bp in a Covaris LE220. We then enriched the sheared DNA pools for adapter ligated fragments using a Dynabeads M-280 streptavidin binding assay. We prepared these enriched pools using the NEBnext Ultra II DNA Library Prep Kit for Illumina NovaSeq 2 ×150 nt sequencing at Princeton University's Lewis Sigler Genomics Institute core facility. We selected fragments 300–400 bp in size with Agencourt AMPure XP magnetic beads, which were also used for library purification. Sequence data were first processed to identify which read (forward or reverse) contained the unique barcode and the remnant *sbf*I cut site. Using a custom perl script, these reads were then retained in a single file, the matching read pairs that lacked the cut site were curated in a separate file, and all remaining reads were discarded. We then processed these reads in STACKS version 2. We first demultiplexed using *process_radtags* using a 2-bp mismatch for barcode rescue and retained reads with a quality score ≥10. We next removed PCR (polymerase chain reaction) duplicates with the paired-end sequencing filtering option in *clone_filter* and then mapped to the dog genome CanFam3.1 assembly (Lindblad-Toh et al., 2005) using STAMPY version 1.0.21 (Lunter & Goodson, 2011). We additionally filtered mapped reads for a minimum MAPQ of 96 and converted to bam format in SAMTOOLS version 0.1.18 (Li et al., 2019). We included 105 publicly available canid samples already mapped to the same reference genome assembly following the same methods (Table S1).

We completed SNP discovery using all samples with a minimum of 100,000 mapped reads to obtain a catalogue of all polymorphic sites possible. We implemented the *gstacks* and *populations* modules in STACKS version 2 following the recommended pipeline for data mapped to a reference genome (Catchen et al., 2013; Rochette et al., 2019). We increased the minimum significance threshold in *gstacks* to require more stringent confidence needed to identify a polymorphic site using the marukilow model (flags *--vt-alpha* and *--gt-alpha*, $p = .01$). We then selected a subset of individual samples with a minimum of 200,000 mapped reads for comparable geographical and lineage representation in the *populations* module. We reported all SNPs discovered per locus (opted against using the *populations* flag *--write_single_snp*) as ancestry inference is best with high-density data. We established a *gstacks* catalogue of 2,799,665 variants in STACKS version 2 (Catchen et al., 2013; Rochette et al., 2019), of which 94,277 SNPs were retained after filtering for a minimum of 3% minor allele frequency (MAF) and allowed up to 70% genotyping rate per locus (flag *--geno* 0.3) for the initial filtering step in PLINK version 1.90b3i (Chang et al., 2015). To determine if any individual sample was missing a significant proportion of genotypes, we assessed missingness using the PLINK function --missing. We excluded 17 samples that had >40% missing genotype data (Table S1) and repeated the *stacks populations* module for recalling SNP genotype across 293 canids at 2,759,705 SNP loci. This sample set included all reference species and populations in addition to the wide geographical representation across the southern USA. We retained 120,621 SNPs after filtering for 3% MAF and 20% missingness (referred to as the 120K SNP set) with an average density of one SNP every 19 kb. For demographic analyses of neutral aspects of genetic structure and diversity estimates, we constructed a "statistically neutral and unlinked" data set of SNPs by excluding sites within 50-SNP windows that exceeded genotype correlations of 0.5 (with the PLINK argument *--indep-pairwise* 50 5 0.5) and deviated from Hardy–Weinberg equilibrium (HWE) with the argument *--hwe* 0.001. This resulted in a statistically neutral and unlinked data set of 83,851 SNP loci (referred to as the 83K SNP set) for demographic analyses (*n* SNPs autosomes = 83,021 and X chromosome = 830). The X chromosome was analysed independently from the autosomes to ensure the inclusion of the signal driven by the single sex chromosome captured in our sequencing and mapping efforts.

2.3 | Sex inference

To infer bioinformatically the sex of canids, we remapped FASTQ files to the complete CanFam3.1 reference assembly, with the addition of the Y chromosome (GenBank KP091776.1; Li et al., 2013). We estimated the number of reads that covered each nucleotide on the Y chromosome per genome. We expected a distinct difference in the number of Y-linked reads that mapped for males vs. females, with some variation expected for females due to reads that mapped to the Y chromosome within the canine pseudoautosomal region (PAR) on the X chromosome (1 bp to 6.7 Mb; Raudsepp et al., 2012). Several samples had field-based sex reported that were surveyed for accuracy (Table S1). From animals with known sex records, we estimated a 5.2% discordance rate in 96 canids analysed: three females and two males had a genomic sex inference that did not match field-based records (Table S1; Figure S6). For the remaining 42 canids, we inferred 19 females and 23 males.

2.4 | Population structure analysis

To survey genetic clustering, we conducted a principal component analysis (PCA) with FLASHPCA (Abraham & Inouye, 2014). We then employed a maximum-likelihood clustering method to estimate the most likely number of genomic clusters (*K*) in ADMIXTURE version 1.3 (Alexander et al., 2009) with the cross-validation flag to assess inter-*K* likelihoods.

2.5 | Assessment of incomplete lineage sorting and gene flow

Following on from the aforementioned methods in STACKS version 2 for cataloguing and discovering SNP variants, we also independently repeated these methods with a larger data set that included 45 red fox (*Vulpes vulpes*) previously published, with at least 100,000 mapped sequence reads (SRA PRJNA510648) (DeCandia et al., 2019). The red fox was selected as it is the only non-*Canis* data set previously published by the authors constructed with the same protocol and restriction enzyme (DeCandia et al., 2019). We filtered in VCFTOOLS version 0.1.17 (Danecek et al., 2011) to remove loci with >10% missing data across all individuals, singletons and private doubletons, and individuals with >20% missing data. We identified loci that are fixed in all 45 of the red fox genomes, which we considered informative for the ancestral allele state. Any locus lacking data in any of the groups was also excluded from the analysis. For the *ABBA-BABA* inference of ILS or introgression, we implemented an allele-frequency approach for estimating *D* statistics in R using derived allele frequencies across the red fox as an outgroup, the SSP red wolf as P3, reference coyote as P2 and several permutations of coyotes in P1 (following Martin et al., 2013). The autosomes and the X chromosome were analysed as a single data set. We assessed statistical significance through a block jackknife approach with block sizes from 2 to 10 Mb.

2.6 | Inference of canid ancestry

We inferred local ancestry of 138 coyotes with possible red wolf ancestry (Alabama = 5, Georgia = 8, Kansas = 2, Kentucky = 5, Louisiana = 10, Missouri = 11, Nebraska = 5, New Mexico = 1, Oklahoma = 5, Tennessee = 3, Texas = 83) (Table S3) with respect to three reference populations (coyote, red wolf and grey wolf) (defined in Table S1). We implemented a two-layer hidden Markov

model in the program ELAI (Guan, 2014). This approach first evaluates LD within and between the reference canid groups and returns a per-SNP allele dosage score. This score estimates the most likely ancestry and its state. SNPs were automatically excluded if missing from one of the populations. We analysed unphased SNPs that were unfiltered for genotype correlations or HWE. We defined the number of upper-layer clusters (*-C*) to 3 and the lower-layer clusters (*-c*) to 15 (5 × the value of *C*, as recommended). To account for both uncertainty in the precise timing of admixture and increased complexity in admixture scenarios where only a single pulse of admixture is unlikely, we analysed four time points since admixture (*-mg*): 5 , 10 , 15 and 20 generations. We implemented ELAI three times serially for each *-mg* parameter value with 30 EM steps and averaged results over all 12 independent analyses. We considered sites with allele dosage between 0.5 and 1.5 to be heterozygous and sites with allele dosage >1.5 to be homozygous. Sites with multiple and incompatible ancestries (e.g., three heterozygous states) were excluded from the ancestry block analyses. We required that ancestry blocks contain a minimum of two contiguous SNPs with the same ancestry assignment. We also assumed that all ancestry (via allele dosage) outside of the PAR on the X chromosome of males was haploid in ancestry (allele dosage>1).

2.7 | Estimating the timing of admixture

We counted the number of ancestry block identity switches per individual genome, with a focus on the genome-wide ancestry estimates of red wolf and grey wolf. Given the reduced representation focus on *Sbf*1 cut sites and size selection step, the resulting blocks are probably inflated in size with a paucity of small block sizes. Although this design is incredibly useful for rapid genotyping of thousands of SNP loci across hundreds of genomes, the estimates are probably skewed towards more recent admixture events given the density of markers. Following Johnson et al. (2011), we then used the below equation to estimate the number of generations since admixture for diploid genomes:

$$B = (2*2*0.01)*T*L*z(1 - z).$$

where B is the estimated number of ancestry switches, T is the number of generations since admixture, L is the total genome length (2,085 centimorgans [cM] for autosomes and 111 cM for the X chromosome; Wong et al., 2010), and z is the genome-wide ancestry proportion of either red wolf or grey wolf specific to autosomes or X chromosome.

2.8 | Recombination rate integration

To determine how recombination variation along the genome influences the patterns of admixture and structure, we integrated recombination rates from the autosomes and X chromosome (Wong et al., 2010), with all positions from CanFam2 lifted over to the CanFam 3.1 reference genome assembly. The Wong et al. (2010) map contains 1496 framework markers and ~1500 additional markers that are "map validators," for a total of 3075 markers that provide a mapping resolution of <1 marker per 1 Mb. Following Li et al. (2019), we implemented a two-step approach for assigning recombination rates and smoothed averages. We first estimated recombination rates (cM Mb^{-1}) for each marker within 100-kb nonoverlapping windows (step = 100 kb) and used the female-based estimates for the X chromosome. To mitigate finer-scale variation in recombination rates and focus on broader scale rates, we smoothed recombination rate averages across 2-Mb sliding windows. These resulting windows were then partitioned into either low- or high-recombination-rate windows if their smoothed average was <0.5 or >2 cM Mb^{-1}, respectively. From this, we obtained 1093 autosomal and 75 X-linked windows of high recombination rates, and 10,770 autosomal and 500 X-linked windows of low recombination rates. Each SNP locus contained within a high- or low-recombination window were parsed into separate data sets for analysis using the *intersect* function flag *-loj* of BEDTOOLS version 2.28.0 Similarly, as described above, we estimated admixture timing for high- and low-recombination blocks with their respective total centimorgan lengths analysed (autosomes: low = 820 cM, high = 697 cM; X chromosome: low = 8 cM, high = 37 cM).

3 | RESULTS

3.1 | Coyotes show a west-to-east geographical gradient of probable red wolf assignments

We obtained 120,621 SNPs from RADseq data genotyped in 293 canids sampled across the mid-to-lower latitudes of the USA (Figure 1a,b). Coyotes were classified as either being sampled in their pre-1900 historical range (Arizona, California, Colorado, Kansas, Missouri, Nebraska, New Mexico, Nevada, Oklahoma, Texas and Wyoming) or their post-1900 southeastern expansion (Alabama, Georgia, Kentucky, Louisiana and Tennessee) (Table S1). We included representatives from North American grey wolves (*Canis lupus*, including Mexican wolves *C. lupus baileyi*), eastern wolves (*C. lycaon*) from Algonquin Provincial Park, SSP captive red wolves and domestic dogs (Table S1).

The PCA of 83,851 unlinked neutral SNPs revealed a gradient in the first two PCs for coyotes, polarized by red wolves and California coyotes that explained 3.5% of the variation on PC1 (Figure S1). This pattern was present albeit weaker in spatial distinction for the X chromosome PCA. Maximum-likelihood admixture analyses with nine partitions (K = 2–10) also supported a west-to-east geographical gradient of red wolf assignments across coyotes of the southern USA (Figure 1c). With the inclusion of five reference groups (dogs, grey wolf, Mexican wolf, eastern wolf and red wolf), coyotes displayed increased red wolf assignments in Texas (K = 4 average Q Texas = 0.42, min = 0.02, max = 0.99) and across the southeast expansion (Alabama = 0.16, Georgia = 0.14, Louisiana = 0.37, Tennessee = 0.10, Kentucky = 0.02) relative to coyotes of the

TABLE 1 *D*-statistic testing for variable P1 and P2 groups of coyotes against P3 (reference red wolves), with the outgroup of red fox. Standard errors (*SE*) and significance values were evaluated using a jackknife resampling method with a block size range from 2 to 10 Mb. No results were obtained for 2–4 Mb block sizes

P1 =	Historical range	Reference	Reference
P2 =	Southeastern expansion	Texas	Louisiana
D statistic	0.156	0.352	0.369
5-Mb block (N_{blocks} = 484) *SE* (*p*-value)	0.01 (2.43×10^{-30})	0.01 (1.85×10^{-129})	0.02 (1.31×10^{-49})
6-Mb block (N_{blocks} = 405) *SE* (*p*-value)	0.01 (2.06×10^{-28})	0.02 (8.48×10^{-118})	0.03 (3.01×10^{-47})
7-Mb block (N_{blocks} = 350) *SE* (*p*-value)	0.01 (2.68×10^{-28})	0.01 (4.91×10^{-127})	0.03 (1.35×10^{-47})
8-Mb block (N_{blocks} = 309) *SE* (*p*-value)	0.01 (7.85×10^{-26})	0.02 (8.72×10^{-120})	0.03 (1.21×10^{-43})
9-Mb block (N_{blocks} = 277) *SE* (*p*-value)	0.01 (3.41×10^{-28})	0.02 (1.21×10^{-114})	0.03 (4.44×10^{-44})
10-Mb block (N_{blocks} = 254) *SE* (*p*-value)	0.01 (2.57×10^{-27})	0.01 (4.91×10^{-126})	0.03 (1.58×10^{-47})

west (California = 0.00, Nevada = 0.00, Arizona = 0.00, New Mexico = 0.01) (Figure 1c; Figure S2; Table S2A). At higher partitions, red wolf assignments showed a pattern of geographical restriction to the southeastern states (Table S2A). Given the negligible differences among $K = 2$–4, we report the same trends are observed for red wolf assignments across coyotes at lower K values ($K = 2$ average Q Texas =0.57, the southeast expansion $Q = 0.48$ and western coyotes $Q = 0.02$; $K = 3$ Texas = 0.42, the southeast expansion $Q = 0.31$ and western coyotes $Q = 0.00$) (Table S2C). Although the best fit partition was $K = 2$ for 830 neutral and unlinked X-linked SNPs, red wolves appeared as a distinct genetic cluster at $K = 4$ (Figure S3); as such, we found a similar trend of increased red wolf assignments in coyotes with eastward geography (Figures S2 and S3; Table S2B).

The small sample size of the *Canis* reference groups probably limits the ability of this -ikelihood method to discern subtle differences in assignment. Thus, we excluded domestic dogs, eastern wolves, grey wolves and Mexican wolves for a finer-scale assessment of red wolf assignments among the 244 coyotes (254 canids sample set) across the southern USA. The PCA supported the same polarization of red wolves and western California coyotes on PC1 (2.2% variation) (Figure S1). Admixture analysis also provided the same west-to-east gradient of increasing red wolf assignments (best-fit $K = 4$ average Q Texas = 0.06, Alabama = 0.03, Georgia = 0.02, Louisiana = 0.13, Tennessee = 0.04, Kentucky = 0.03) compared to western populations (California = 0.00, Nevada = 0.00, Arizona = 0.00, New Mexico = 0.00) (Figure 1d; Figure S2; Table S2C). Higher red wolf assignments were found in Louisiana and 11 counties in southeastern Texas (Figure S4). We focused on seven Texas counties that had a minimum of 5% probable assignment to the red wolf cluster ($K = 4$ Q Austin = 0.05, Brazoria = 0.21, Brazos = 0.05, Chambers = 0.26, Colorado = 0.12, Fort Bend = 0.21, Galveston = 0.43). Analysis of X-linked SNPs supported a similar pattern, albeit with greater variation in assignment values (Figures S2 and S3; Table S2D).

3.2 | Differentiating incomplete lineage sorting and gene flow

We explored the degree that ILS relative to gene flow could explain the above genetic structure patterns using the *D* statistic (Durand et al., 2011). We expanded the data set to include data from an outgroup population consisting of data from 45 red foxes (*Vulpes vulpes*) (DeCandia et al., 2019) and repeated the SNP discovery pipeline. We catalogued 5,354,281 SNPs across 293 canids and 45 red foxes to identify derived alleles that were fixed in the red foxes as the ancestral homozygous genotype. After filtering for missing data, we retained 922,115 loci and 333 individuals; two Nebraska coyotes and three domestic dogs were excluded due to an excess of missing data. We identified a further subset of 168,004 autosomal and 1,494 X-linked loci where all 45 red foxes were fixed for a single ancestral allele, with corresponding data for the various coyote group compositions. The captive SSP red wolves (P3) were included to explore the putative source of red wolf ancestry in coyotes (P2) with respect to a reference set of coyotes as P1. We permuted the P1 and P2 groups as follows: P1 = coyotes from within their historical range excluding Texas, or a reference set of coyotes identified at $K = 4$ from ADMIXTURE as an individual that had an autosomal red wolf proportion $Q < 0.02$; P2 = coyotes from Texas or Louisiana (Table S2A). We found overwhelming evidence that allele sharing between coyotes and red wolves was due to gene flow and introgression rather than ILS ($D > 0.16$, $p < 10^{-26}$) (Table 1; Figure S5).

3.3 | Red wolf ancestry proportions are highest in southern coyote genomes

We inferred ancestry using the program ELAI at 120K SNP loci for 138 coyotes with respect to three reference populations: grey wolves (including Mexican wolves), captive SSP red wolves, and

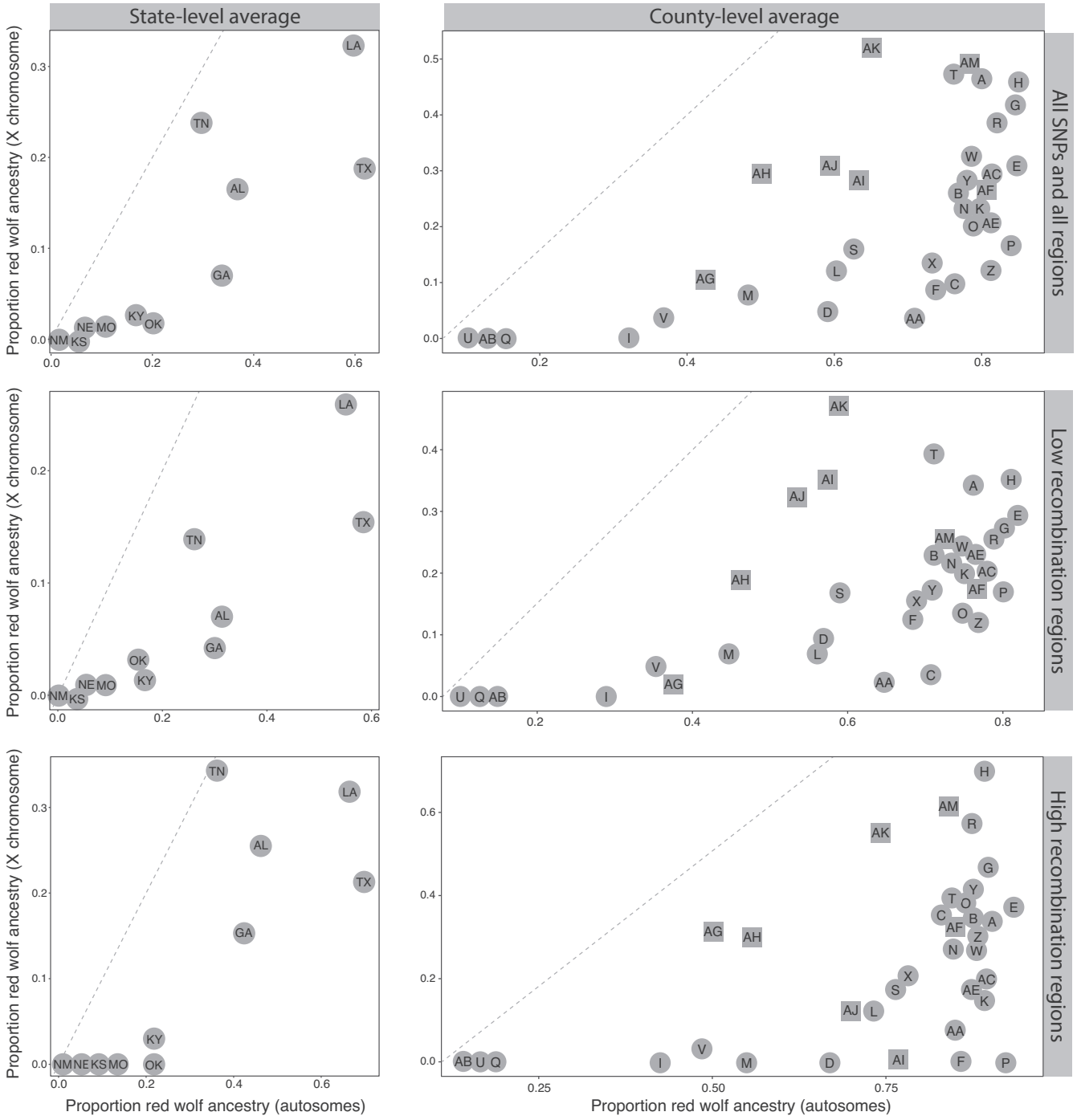

FIGURE 2 The average proportion of red wolf ancestry at the state and county levels (squares: Louisiana parishes; circles: Texas counties) for 138 canids assigned using three reference populations for the 120K SNP set partitioned for all SNPs (top), low-recombination regions (middle), and high-recombination regions (bottom). See Table S3 for individual ancestry assignments to each of the reference populations. County abbreviations are as described in Figure 1(b). State abbreviations- AL, Alabama; GA, Georgia; KS, Kansas; KY, Kentucky; LA, Louisiana; MO, Missouri; NE, Nebraska; NM, New Mexico; OK, Oklahoma; TN, Tennessee; TX, Texas

106 reference coyotes with negligible ($Q < 0.02$) autosomal red wolf proportion at $K = 4$ (Tables S2A and S3). Average red wolf ancestry proportions were highest on coyote autosomes collected from Texas (average = 62.4%, range = 8%–85%) and Louisiana (average = 61.4%, range = 47%–81%). Louisiana coyotes carried the highest average proportions of X-linked red wolf ancestry at 41% with variation across parishes (range = 26%–67%) (Figure 2; Table S3;Figure S7). At the chromosomal level, there were few regions of the genome where red wolf ancestry levels were markedly different between the Texas and Louisiana coyotes (Figure S8). Substantial red wolf ancestry was identified in coyotes from Alabama (autosomal, X: 41%, 24%), Georgia (39%, 12%), Kentucky (23%, 4%), Missouri (15%, 4%),

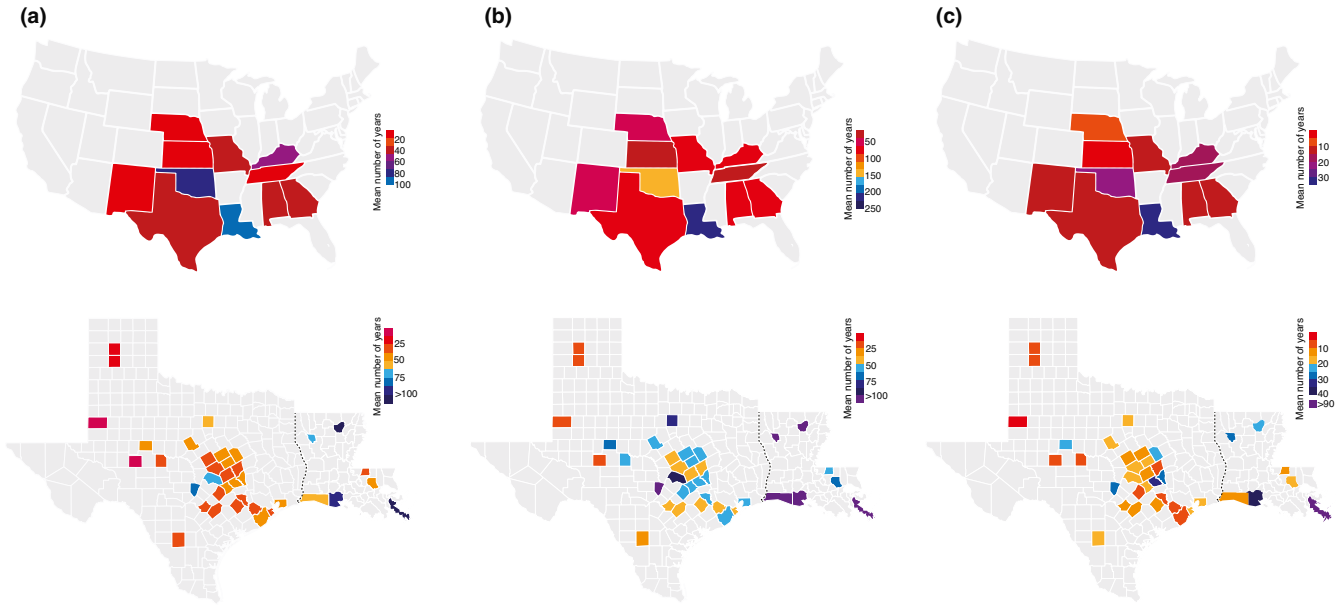

FIGURE 3 Average number of years since appearance of red wolf alleles across the autosomes for each state (top) and county (bottom) for 138 canids with inferred ancestry across (a) the entire genome (120K SNPs), (b) autosomal regions of low or (c) high recombination rates

Oklahoma (23%, 5%) and Tennessee (34%, 28%), concordant with records of the red wolf historical range in these same states. We found a significant enrichment of red wolf ancestry on the autosomes relative to the X chromosome (one-tailed *t* test of unequal variance, $p = 1.639 \times 10^{-20}$). We documented the longest tracks of homozygous red wolf ancestry in coyotes from Alabama (autosomal, X: 3.8 Mb, 3.9 Mb), with longer red wolf ancestry tracks found among autosomes in Georgia (986 kb), Louisiana (403 kb) and Texas (359 kb) (Table S3). We also detected grey wolf ancestry across these coyotes (average autosomal = 5.8%, X = <1%); however, due to small sample sizes, we could not determine if this was driven by Mexican wolf introgression or allele sharing with the grey wolf ancestor (Table S3).

An analysis of coyotes with county-level information for coyotes collected in Texas (74/83 individuals) and Louisiana (9/10 individuals) revealed the highest autosomal red wolf ancestry was found within counties or parishes where trapping efforts to establish the SSP red wolf founders originated: Jefferson and Chambers counties in Texas, and Cameron and Calcasieu parishes in Louisiana (Texas: Chambers = 84%, Brazoria = 83; Galveston = 82%, Fort Bend = 82%; Louisiana: Cameron = 81%, Vermilion = 78%) (Table S3; Figure S9). We found similarly high levels of X-linked red wolf ancestry in Texas counties where some of the SSP red wolf founders were trapped: Chambers (59%), Austin (57%) and Galveston (55%), and Louisiana (Plaquemines = 63%, Vermilion = 52%). A noticeable gradient was observed with increasing red wolf ancestry from northwest to southeast Texas (Figure S9), with the trend much more pronounced on the autosomes relative to the X chromosome (Figure 2).

The distribution of red wolf ancestry switches and block sizes allowed us to estimate the timing at which red wolf alleles appeared in the ancestral genomes either through direct gene flow events or, more likely, through the dispersal of red wolf alleles through dispersing admixed coyotes. We used the average of two generation time estimates, the commonly estimated value of 4 years per generation and an estimate of 2 years per generation to account for scenarios in which a fraction of canids breed in their first year of life (Albers et al., 2016; Hedrick et al., 2014; Kilgo et al., 2017; Mech et al., 2016; Miller et al., 2003; Sacks, 2005; vonHoldt et al., 2008). Autosomal red wolf admixture in coyotes was oldest in Louisiana (97 years ago [ya]), whereas Tennessee coyotes carried the oldest X-linked red wolf ancestry (114 ya) (Table S4A; Figure 3). Coyotes within the historical range of the red wolf (Alabama, Georgia, Kentucky, Louisiana, Missouri, Oklahoma, Tennessee, Texas) acquired their red wolf ancestry on average 50 ya (autosomal = 53 ya, X = 54 ya), which was significantly older than the red wolf ancestry found within coyotes of their own historical range (Kansas, Nebraska, New Mexico: autosomal = 13 ya, X = none, one-tailed *t* test $p = .0008$). County-level time estimates further revealed the oldest autosomal red wolf admixture events within Louisiana where the last wild red wolves were documented (Plaquemines = 234 ya, Ouachita = 121 ya, Vermilion = 98 ya), relative to counties in western or southern Texas (e.g., Gaines = 12 ya, Irion = 10 ya, La Salle = 26 ya, Potter/Randall = 15 ya) (Table S4A). The X chromosome revealed much older red wolf ancestry in southeastern Texas (Austin = 143 ya, Bastrop = 132 ya, Chambers = 117 ya, Williamson = 947 ya) compared to Louisiana, which had much less X-linked red wolf ancestry detected (Table S4A).

3.4 | Recombination rates and ancestry

We partitioned the 120K SNP set into high- and low-recombination regions (<0.5 or >2 cM Mb^{-1}, respectively; Wong et al., 2010) to

explore the impact of recombinational variation on admixture and demographic inference. We annotated 13,903 and 47,779 autosomal SNP loci as within regions of high and low recombination rates, respectively. Similarly, 430 and 1,297 X-linked SNPs were in regions of high and low recombination. Red wolf ancestry proportions were highest in coyotes from Texas and Louisiana across regions (autosomal, X average proportions: Texas = 64.3%, 18.5%; Louisiana = 60.9%, 28.8%) (Table S5; Figure S10). There was a clear enrichment in red wolf ancestry in coyote autosomal regions with the highest recombination rates and an enrichment of coyote ancestry in low recombining autosomal regions (Figure S10). The X chromosome showed a similar trend with greater variance. Homozygous red wolf ancestry blocks were largest in autosomal regions of low recombination (average high = 157 kb, low = 779 kb) (Table S5). When we surveyed the X chromosome, regions of high recombination rates carried the longest tracks of homozygous red wolf ancestry (average high = 230 kb, low = 78 kb), with males carrying longer blocks than females (average high female = 64 kb, male = 92 kb, $p > .05$) (Table S5). Coyotes from Alabama carried the longest tracks of homozygous red wolf ancestry regardless of recombination rate (low, autosome = 1.2 Mb, X = 617 kb; high, autosome = 718 kb, X = 2.7 Mb), followed by Tennessee, Louisiana, Texas and Georgia.

We found significantly older red wolf ancestry within low recombining chromosomal regions of coyotes within the historical range of the coyote (autosomal = 92 ya, X = 673 ya) compared to recently expanded southeastern populations (autosomal = 24 ya, X = no data; one-tailed *t* test $p = .0037$) (Table S4B,C; Figure 2b,c). We found similar significant trends within chromosomal regions of high recombination rates (Historical: autosomal = 18 ya, X = 32 ya; Expansion: autosomal = 6 ya, X = no data, $p = .0146$), which followed the expectations that low-recombination regions carry signals of older demography where regions of high recombination probably display increased introgression. Louisiana coyote autosomes carried the oldest red wolf ancestry (Low = 202 ya, High = 35 ya), whereas Kansas carried the most recent (Low = 11 ya, High = 2 ya). Oldest red wolf autosomal ancestries were located within Louisiana parishes (Low = 67–549 ya, High = 13–98 ya), with Texas coyotes carrying the oldest X-linked red wolf ancestries (Low = 42–1,203 ya, High = 18–108 ya).

4 | DISCUSSION

In this study, we demonstrate the persistence of red wolf genetic ancestry in admixed coyotes of the southern USA and show that red wolf ancestry has remained relatively spatially restricted to the last regions that red wolves thrived. Although coyotes are a genetically diverse lineage with an apparent lack of local structure (Heppenheimer, Brzeski, Hinton, et al., 2018; Heppenheimer, Cosio, et al., 2018), we found a strong increasing east-to-west geographical gradient of red wolf ancestry present in coyote genomes. We found significant evidence of red wolf introgression outside the historical range of coyote, as well as in coyotes from Louisiana and Texas, corroborating past findings (e.g., Heppenheimer et al., 2020; Heppenheimer, Brzeski, Wooten, et al., 2018; Murphy et al., 2018; Wilson et al., 2012). This ancestry appears to derive predominantly from introgression rather than ILS, with the highest amounts of autosomal red wolf ancestry (~60%) found in coyotes from southeastern Texas and southwestern Louisiana. We estimated that this ancestry was acquired up to 97 years ago through genetic exchange between coyotes and historical red wolf populations. Some of the most recent fragments of red wolf ancestry were found in coyotes of Kansas (2–11 ya), which are most parsimoniously explained by the movement of red wolf ancestry through dispersal of admixed coyotes, especially given the declaration of the red wolf as extinct in the wild by 1980 (reviewed by Phillips et al., 2003). However, we noted a depletion of red wolf ancestry on the coyote X chromosome relative to the autosomes, which carried the lowest proportions observed (~14%), with the oldest red wolf content in a Tennessee coyote estimated to have been acquired 114 ya. Although we estimated the number of generations since red wolf ancestry fragments were acquired in various coyote genomes and found them to span the dates prior to and concomitant with red wolf extirpation, we cannot conclusively rule out the possibility of coyote introgression into the population that sourced the founders of the red wolf breeding programme.

These results advance our understanding of canid lineage evolution and hybridization and have important implications for red wolf conservation. First, we present evidence that genetic exchange in the past century facilitated the introgression of red wolf alleles into the coyote populations of the Gulf coast, and that red wolf ancestry has remained restricted to this region until fairly recently. This time frame is consistent with the loss of the wild red wolf in the last century, when the detected geographical hot spot for cryptic red wolf ancestry was found in Louisiana and Texas, the last footholds of wild red wolves (Carley, 1975; McCarley, 1962; McCarley & Carley, 1979; Paradiso, 1968; Paradiso & Nowak, 1972; Riley & McBride, 1972). Concurrently with the decline of red wolves in this region due to habitat loss and persecution, coyotes expanded out of their historical range and encountered red wolves along their southeastern expansion front (Heppenheimer, Cosio, et al., 2018; Hody & Kays, 2018; Nowak, 1979; Parker, 1995; Riley & McBride, 1972). Coyotes are a highly mobile species, with individuals often dispersing >100 km (Harrison, 1992; Sacks et al., 2005). However, we found that red wolf introgression has only recently expanded north and westward in Texas. This observation does not appear to be attributed to a tension zone model, in which a hybrid zone is maintained through the balance of ongoing hybridization and counter-selection within the zone (Barton & Hewitt, 1985). Specifically, our data indicate that the zone appears to be expanding rather than being constrained.

Here, we present genetic evidence that the red wolf is indeed a distinct species, supported through the identification of putative barrier or resistance loci, primarily on the X chromosome, but also on centromeric regions of some autosomes, a characteristic typically found in distinct species (Haasl & Payseur, 2016; Harrison & Larson, 2016). Although the red wolf has been considered a distinct species due to their unique ecology, morphology and behaviour

(Waples et al., 2018), genetic and genomic distinctions have proved less well defined (e.g., vonHoldt, Cahill, et al., 2016; vonHoldt et al., 2011). We found low miscibility of the red wolf and coyote genomes in genomic regions known to disproportionately harbour reproductive isolation loci, as evidenced by the reduced red wolf ancestry on the coyote X chromosome which we hypothesize is due to effects of interference across large regions with extremely reduced recombination rates, and parallels results in other mammalian systems (Carneiro et al., 2010; Li et al., 2019). Previous work has documented size-assortative mating with respect to red wolf and coyote hybridization events (Hinton et al., 2017) and suggests sex-biased hybridization where female red wolves more often mate with male coyotes. Furthermore, we described a steep geographical cline of red wolf ancestry in the geographical region where the last wild red wolves were captured in the 1970s. The expansion of red wolf ancestry into coyote genomes has remained spatially constrained around these habitats. We thus suggest that these genomes have failed to "congeal" due to its reduced capacity for introgression, in which the entire X chromosome for example may act as a single super-locus (Bierne et al., 2011; Turner, 1967). We propose that this lack of permeability has played a role in the divergence between coyotes and red wolves (Waples et al., 2018).

The hemizygous nature of sex chromosomes results in their reduced effective population size (N_e), accelerating the rate of evolution which can lead to the accumulation of loci involved with reproductive isolation (Goldberg & Rosenberg, 2015; Kitano et al., 2009; Van Belleghem et al., 2018). Divergent evolutionary histories are common when inferred from sex chromosomes relative to the autosomes. This has been attributed to enhanced introgression barriers, which are suspected to result from the greater accumulation of incompatibilities that ultimately impact reproduction (the "large X-effect") (Coyne & Orr, 1989; Garrigan et al., 2012; Martin et al., 2013; Masly & Presgraves, 2007; Sankararaman et al., 2014). As expected, we found that red wolf ancestry is highest (and younger) in regions of high recombination rates, consistent with theoretical expectations (Geraldes et al., 2011; Schumer et al., 2018; Seixas et al., 2018; Stevison & McGaugh, 2020). Empirical evidence supports these predictions, showing the X chromosome generally carries a reduced level of introgression (Fontaine et al., 2015; Fraïsse & Sachdeva, 2021; Larson et al., 2014; Li et al., 2019; Payseur & Nachman, 2005). This differential degree of X-linked ancestry is derived from the theory that in a population with equal males and females, two-thirds of the X chromosomes are found in the females whereas one-third are in males (or the Z chromosome in the ZW systems). However, this linear assumption is violated when contributions are unequal across the sexes in an ongoing-admixture model (Goldberg & Rosenberg, 2015).

Similar introgression patterns have been well described in the genome of modern-day humans. Although humans and Neanderthals recently diverged (~400,000–470,000 ya, Harris & Nielsen, 2016), the modern-day human X chromosome is depleted of Neanderthal ancestry (Dutheil et al., 2015; Sankararaman et al., 2014). The inference has been interpreted with respect to putative sterility and other reproductive barriers that prevent introgression, although this has been disputed given that other phenomena could explain these patterns, such as sex-biased hybridization, stronger selection on the hemizygous nature of the sex chromosome and differences in recombination rates (Hammer et al., 2010; Harris & Nielsen, 2016; Juric et al., 2016; Veeramah et al., 2014). Long-term persistence of exogenous ancestry is expected to be shaped by the strength of selection and fitness consequences (Harris & Nielsen, 2016). Reports of reduced X-linked ancestry in humans suggest a role for recessive deleterious mutations as an isolating mechanism, in addition to strong selective sweeps (Dutheil et al., 2015; Harris & Nielsen, 2016).

We have provided evidence that the coyote is an excellent model system in which one can explore the dynamics and consequences of "hybridization load" in admixed individuals (Bierne et al., 2002; Harris & Nielsen, 2016; Juric et al., 2016; Kays et al., 2010; Monzón et al., 2014; vonHoldt et al., 2011). The process of multigenerational admixture can produce a diversity of phenotypes due to recombinant genotypes, with an expectation that some of those phenotypes have higher relative fitness as they are shaped by natural selection (Barton, 1979). For example, the human genome contains several archaic hominid introgressed regions with known fitness impacts, often an aspect of the yet nascent field of personalized medicine (Dolgova & Lao, 2018; Durvasula & Sankararaman, 2020; Rotival & Quintana-Murci, 2020). The impact of specific introgressed regions and variants in coyote remain unknown, but theory suggests that fitness consequences of admixed genomes occur predominantly through the increased genetic load of weakly deleterious alleles that entered the genome from the parental species with a smaller effective size (i.e., red wolf). Several studies have uncovered genomic evidence of adaptive introgression in wild and domesticated species (e.g., Barbato et al., 2017; Burgarella et al., 2019; Figueiró et al., 2017; Jones et al., 2018; Oziolor et al., 2019; Schweizer et al., 2018; Sotola et al., 2019; Suarez-Gonzalez et al., 2016). Such an effort would probably inform both evolutionary theory as well as conservation efforts of red wolves, with respect to fitness considerations and species persistence.

These findings have several implications for the future of red wolves. First, we have provided evidence that coyotes continue to be a significant reservoir of ghost and SSP-lineage red wolf ancestry, a lineage that continues to become more inbred through time. Such introgression appears to have been a frequent event for southern coyotes during the past century. Future full genome sequencing efforts will allow us to explore the timing and genomic impact of introgression more deeply, including the identification of selection for and against red wolf alleles in specific genomic regions. Furthermore, identifying and dating ancestry hotspots in the historical red wolf range can help locate geographical regions that are suitable for retaining red wolf characteristics and assist site selection for future red wolf reintroduction and recovery efforts. The longer-term viability of the red wolf relies heavily upon the potential for the SSP to incorporate novel and variable red wolf genomic content. We promote a species conservation design similar to that of de-introgression (Amador et al., 2014). Here, our suggestion is to enrich the SSP

population for red wolf ghost genetics by careful breeding plans that incorporate controlled interbreeding of red wolves with coyotes that have high red wolf genomic content. Such a mechanism promotes the immediate transfer of the ghost genotypes once thought to be extinct in an effort to recover the native genetic background of the red wolf. Controlled breeding strategies for de-introgression or de-extinction that use hybrids have been proposed in other systems (Quinzin et al., 2019), as has the use of genome-editing tools (Johnson et al., 2016); these bold conservation efforts may become necessary in an era of global change. While de-introgression and genome-editing has the potential to revive management for species like the red wolf, conservation practitioners must be cautious in avoiding negative impacts such as inbreeding that can occur during ancestry-informed selective breeding or outbreeding depression that may be associated with introducing new gene variants into a population. Thus, a de-introgression plan for the red wolf requires extensive screening of individuals for ancestry inference, sampling historical red wolf specimens to better characterize red wolf genetics prior to the 1970 s, a carefully controlled breeding design, and subsequent promotion to the SSP breeding programme. Although de-introgression is likely to be more successful when exogenous ancestry is limited and recent (Amador et al., 2013), the approach used here would provide a means for implementing an innovative recovery plan for the imperiled red wolf.

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AUTHOR CONTRIBUTIONS

B.V. and W.M. designed the study; B.V., K.B., C.J.S., L.Y.R., S.R.F. and A.S. provided sample collections; A.L. assisted in genomic DNA purification; W.M. provided the dog Y chromosome sequence; B.V. conducted the analyses; W.M. and M.A. provided analytical feedback; all authors contributed towards the manuscript preparation.

DATA AVAILABILITY STATEMENT

RADseq BAM files sequenced in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession no. PRJNA684924. See Table S1 for references to additional public data included in this study.

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