Received: 30 January 2024 | Revised: 4 May 2024 | Accepted: 13 May 2024

DOI: 10.1111/mec.17418

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Maintenance of a narrow hybrid zone between native and introduced red foxes (*Vulpes vulpes*) despite conspecificity and high dispersal capabilities

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Funding information
California Department of Fish and Wildlife, Grant/Award Number: P0780029 and S0810020

Handling Editor: Josephine Pemberton

Abstract

Human-facilitated introductions of nonnative populations can lead to secondary contact between allopatric lineages, resulting in lineage homogenisation or the formation of stable hybrid zones maintained by reproductive barriers. We investigated patterns of gene flow between the native Sacramento Valley red fox (*Vulpes vulpes patwin*) and introduced conspecifics of captive-bred origin in California's Central Valley. Considering their recent divergence (20–70 kya), we hypothesised that any observed barriers to gene flow were primarily driven by pre-zygotic (e.g. behavioural differences) rather than post-zygotic (e.g. reduced hybrid fitness) barriers. We also explored whether nonnative genes could confer higher fitness in the human-dominated landscape resulting in selective introgression into the native population. Genetic analysis of red foxes ($n = 682$) at both mitochondrial (cytochrome b + D-loop) and nuclear (19,051 SNPs) loci revealed narrower cline widths than expected under a simulated model of unrestricted gene flow, consistent with the existence of reproductive barriers. We identified several loci with reduced introgression that were previously linked to behavioural divergence in captive-bred and domestic canids, supporting pre-zygotic, yet possibly hereditary, barriers as a mechanism driving the narrowness and stability of the hybrid zone. Several loci with elevated gene flow from the nonnative into the native population were linked to genes associated with domestication and adaptation to human-dominated landscapes. This study contributes to our understanding of hybridisation dynamics in vertebrates, particularly in the context of species introductions and landscape changes, underscoring the importance of considering how multiple mechanisms may be maintaining lineages at the species and subspecies level.

KEYWORDS
contact zone, dispersal, introgression, red fox, reproductive barriers, *Vulpes vulpes patwin*

1 | INTRODUCTION

Hybrid zones between species or incipient species have long-been known for their value in studying evolutionary processes underlying adaptation and speciation (Barton & Hewitt, 1985; Barton & Gale, 1993; Abbott et al., 2016; Taylor & Larson, 2019; Grummer et al., 2021; Wong et al., 2022). Less well studied are systems composed of more recently divergent populations of conspecifics, yet these systems still fall along the speciation continuum and therefore contain lessons integral to our understanding of the speciation process. Anthropogenic hybrid zones can result from the translocation of wildlife populations or by facilitating secondary contact of previously allopatric lineages due to human disturbances like habitat alteration or climate change (Grabenstein & Taylor, 2018; Ottenburghs, 2021). A unique category within such systems occurs when the translocated population has been domesticated or bred in captivity for multiple generations prior to interbreeding with their wild counterparts (Adavoudi & Pilot, 2021; Randi, 2008; Rhymer & Simberloff, 1996). These zones are becoming more common and are increasingly relevant for biodiversity conservation efforts (Feulner et al., 2013; Howard-McCombe et al., 2021, 2023; Ottenburghs, 2021; Tensen & Fischer, 2023).

Several general principles have been well-established that can help shape predictions when investigating the outcomes of hybridisation between lineages. When considering two previously allopatric populations that have come into contact, the time since divergence generally correlates with the strength of reproductive isolation between lineages due to evolved differences that accumulate while the populations are in allopatry (Edmands, 1999). Thus, hybrid zones that form between distantly diverged lineages (e.g. sister species) tend to result in stable hybrid zones with varying degrees of introgression (i.e. gene flow) into the parent populations, while those formed between more recently diverged lineages (e.g. conspecifics) are more likely to result in lineage swamping or fusion (Barton & Hewitt, 1985; Endler, 1977; Moore, 1977; Wilson, 1965). This is because post-zygotic barriers, such as reduced hybrid fitness or Dobzhansky–Muller incompatibilities (DMIs), typically evolve during extended periods of isolation (Bierne et al., 2011; Edmands, 1999).

Several candidates for post-zygotic reproductive isolation (i.e. 'speciation genes') have been proposed. For example, PRDM9, a zinc finger protein with a Krüppel-associated box domain (KRAB-ZNF), was the first hybrid sterility gene identified in mammals (Mihola et al., 2009), and there is a growing body of literature supporting the role of other KRAB-ZNF genes in reproductive isolation more broadly (Nowick et al., 2012). Similarly, the X and Z chromosomes have been identified as drivers of post-zygotic reproductive isolation in several taxa with XY and ZW sex-determining systems respectively (Good et al., 2008; Janoušek et al., 2012; Payseur et al., 2004; Presgraves, 2018). Alternatively, pre-zygotic barriers such as assortative mating and natal habitat-biased dispersal also have the potential to reinforce stability (Davis & Stamps, 2004) and may not require thousands of generations to evolve. Numerous studies have documented the existence of narrow, stable hybrid zones among lineages at the species level (Barton & Hewitt, 1985; McEntee et al., 2020; Moore, 1977), while fewer studies have reported analogous features between hybridising conspecifics (Aguillon & Rohwer, 2022; Knief et al., 2019; Ruegg, 2008).

Over the past century, human translocations have increasingly led to secondary contact, often between introduced and native populations of the same species, where reproductive barriers might be relatively weak (Grant & Grant, 1992; Larsen et al., 2010; Rhymer & Simberloff, 1996; Seebens et al., 2017). Anthropogenically facilitated hybridisation between closely related taxa may be more likely to result in the genomic swamping or fusion of lineages, leading to breakdown of local adaptations and loss of biodiversity (Adavoudi & Pilot, 2021; Laikre et al., 2010). Hybridisation with nonnative species or subspecies that stem from captive-bred populations can be especially threatening to native populations, as their genetic makeup may have been altered by selection to artificial environments, which can have detrimental effects on wild populations and ecosystems (Frankham, 2008; Gering et al., 2019; Lavretsky et al., 2020; McFarlane & Pemberton, 2019; Rhymer & Simberloff, 1996).

Alternatively, recent studies have shown that secondary contact and hybridisation have played a creative role in the evolutionary history of many extant lineages, facilitating adaptive introgression and generating novel gene combinations for selection to act upon (Edelman & Mallet, 2021; Hedrick, 2013; Taylor & Larson, 2019). Specifically, some studies have identified positive selection of domestication-related traits that appear to have a fitness advantage when introduced into wild populations, and selective introgression of genes related to domesticity or captivity may become more prevalent as landscapes are increasingly modified by humans (Feulner et al., 2013; Fulgione et al., 2016). Thus, even when hybridisation is limited and populations remain largely distinct at the genome-wide level, secondary contact still has the potential to alter the evolutionary trajectory of native populations (Harrison & Larson, 2014; Racimo et al., 2017; Taylor & Larson, 2019).

Here we investigate a hybrid zone between native and nonnative populations of low-elevation red fox (*Vulpes vulpes*) in California. The Sacramento Valley red fox (SVRF, *V. v. patwin*) is endemic to the semi-arid region of California's northern Central Valley (Sacks, Statham, et al., 2010; Figure 1). The only other population of red fox native to California, the Sierra Nevada red fox (*V. v. necator*), has been isolated for at least a century ≥65 km away and >2000 m higher in elevation (Sacks, Statham, et al., 2010). Abutting the SVRF range to the south is a population of nonnative red foxes, found primarily in the San Joaquin valley. Although nonnative red foxes also occur along the Coastal Lowlands, previous studies suggest these are relatively isolated from both the native and nonnative valley populations (Sacks et al., 2016). Additionally, a small hybrid population (native × nonnative) occurs in the town of Cottonwood, which is north of the native range (Sacks, Wittmer, & Statham, 2010). Despite the presence of nonnative red foxes in Cottonwood and the Coastal Lowlands, the primary contact zone between the native and nonnative populations bisects a long linear valley, allowing for the investigation of gene flow across a single, primary contact zone (Sacks et al., 2011).

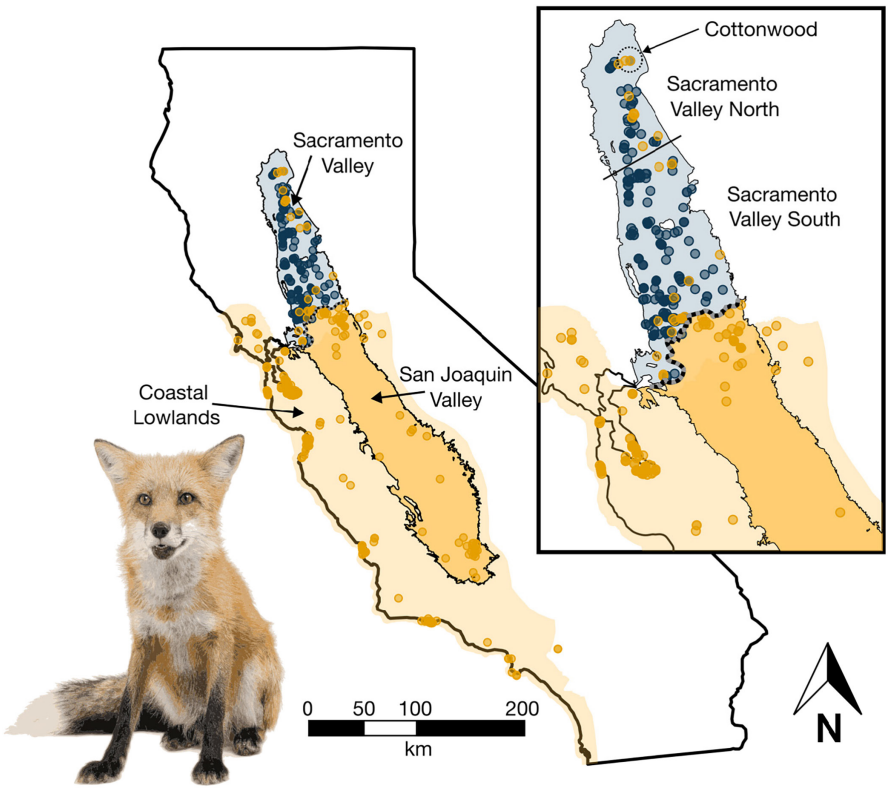

FIGURE 1 Spatial distribution of red fox mitochondrial DNA samples ($n = 682$, including 210 previously unpublished samples) throughout the historical native Sacramento Valley range (blue-shaded) and the nonnative ranges of the San Joaquin Valley (dark-yellow shaded) and Coastal Lowlands (light-yellow shaded). Coloured circles indicate Sacramento Valley red fox (blue; $n = 255$) and nonnative red fox (yellow; $n = 427$) mitochondrial DNA haplotypes inferred from cytochrome *b* and D-loop fragments. The inset highlights the secondary contact zone, with the cline centre denoted by the black and grey dashed line, which is in the southern end of the Sacramento Valley and shows division of the Sacramento Valley into the northern end, which receives introgression from the Cottonwood hybrid zone (dotted circle), and the southern end, which receives introgression from the San Joaquin Valley nonnative population (Sacks et al., 2011; Sacks, Wittmer, & Statham, 2010).

Nonnative red foxes originated from multiple human translocations of fur-farmed foxes in the early 1900s (Lewis et al., 1999; Sacks et al., 2016). Most farmed foxes were originally sourced in the late 1800s from eastern Canadian and Alaskan lineages, which are phylogenetically divergent from the SVRF and their wild predecessors have been geographically isolated from the western red fox lineage for approximately 20–70 ky (Sacks et al., 2018; Statham et al., 2014). These foxes were bred in fur farms for several decades before being released or escaping in California during the mid-1900s (Lewis et al., 1999; Sacks et al., 2016). Although native or nonnative individuals are of similar body mass and cannot be reliably diagnosed based on morphology alone, the SVRF is lankier (i.e. total body length, tail length, ear length and hind foot length) on average than the nonnative red fox (Sacks, Wittmer, & Statham, 2010).

A primary concern surrounding the introduction of nonnative red foxes in California is their potential interbreeding with the SVRF, particularly given the nonnative population's extensive history of captive breeding. Previous studies have shown that mammalian populations subjected to intensive artificial selection can exhibit heritable changes in behaviour and physiology (e.g. increased fecundity, polygyny and tameness) (Fulgione et al., 2016; Kukekova et al., 2018; Lord et al., 2013; Rauw et al., 1998), which has the potential to harm or disrupt the native population if these traits are transferred via gene flow. Alternatively, some of these genetic changes could ultimately prove to be beneficial in the wild, especially because much of the California lowlands have been converted to human-dominated landscapes (e.g. urban, semi-urban and agricultural) (Frayer et al., 1989; Gilmer et al., 1982).

A previous study using mitochondrial DNA and microsatellite data found that these nonnative and native populations had retained their genetic distinctiveness outside of a limited region where they hybridised (Sacks et al., 2011). Given the relatively recent evolutionary divergence between these lineages, their continued separation was notable, particularly in light of the large dispersal distances of red foxes and the limited geographical range of the SVRF (Black et al., 2019; Sacks et al., 2017). One explanation for this observed separation is that time since secondary contact has been insufficient for homogenisation to occur. Alternatively, if there has been sufficient time for secondary contact to homogenise the two populations, then the continued separation must instead be explained by some pre- or post-zygotic reproductive isolating mechanism (Sacks et al., 2011).

To test the two hypotheses, we employed genotyping-by-sequencing (GBS) of thousands of nuclear genomic markers as well as mitochondrial DNA sequencing for both native and nonnative red foxes to clarify population structure and precisely resolve the width of the contact zone. We compared these empirical observations to the expected width under a model of neutral gene flow, which described expected homogenisation dynamics in the absence of reproductive isolating mechanisms. We then used a genomic cline approach to identify specific loci with greatly reduced gene flow relative to the genome-wide average, which could indicate linkage to genes associated with pre- or post-zygotic reproductive barriers. Lastly, we examined evidence for selective introgression from the nonnative into the native population of genes that may have been artificially selected during captive breeding.

2 | MATERIALS AND METHODS

2.1 | Sampling

For mitochondrial sequencing, we obtained both scat ($n=195$) and tissue ($n=490$) samples as well as four hair and swab samples from red foxes in the native SVRF ($n=293$) and nonnative ranges ($n=396$). Samples were collected between 1982 and 2021, and many of them ($n=472$) were first described in previous studies (Table S1). Despite the large temporal sampling window, the patterns of ancestry remained largely stable over time, and we therefore utilised the entire dataset when characterising patterns of ancestry and inferring the width of the hybrid zone (Figure S1). Samples were obtained through non-invasive den and transect surveys, roadkill collection, live captures (following American Society of Mammalogy animal care guidelines and with University of California, Davis Animal Care and Use Committee approval, IACUC No.17860), and nonnative species removal programmes in the case of the introduced red foxes. We selected a geographically representative subset of tissue samples from the native SVRF ($n=69$) and nonnative ranges ($n=62$) for nuclear DNA sequencing.

2.2 | DNA extraction and mitochondrial sequencing

We extracted tissue samples using the Qiagen DNEasy Blood and Tissue Kit and scats using the QiaAmp DNA Stool Mini Kit, in both cases following manufacturer's instructions (Qiagen Inc., Valencia, CA). Using previously published primers, we amplified and sequenced samples new to this study at a 354-bp region of the cytochrome *b* gene (RF14724, RF15149; Perrine et al., 2007; Aubry et al., 2009; Sacks, Statham, et al., 2010) and at a 343-bp region of the D-loop (VVDL1, VVDL6; Aubry et al., 2009). We conducted 11 μL polymerase chain reactions (PCR) containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μg/μL of bovine serum albumin, 0.5 μM of each primer, and 1 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 94°C for 10 min, followed by 40 cycles at 94°C for 45 s, 50°C for 45 s and 72°C for 45 s; followed by a 10-min extension period at 72°C. We purified PCR products using exonuclease/shrimp alkaline phosphatase prior to sequencing on a 3730 DNA Analyser (Applied Biosystems) and resulting sequences were aligned and called in Sequencher (v5.4.6, Gene Codes Corporation, Inc.). We compared resulting sequences to a large database of reference samples and classified mitochondrial haplotypes as native or nonnative based on previous work (Aubry et al., 2009; Perrine et al., 2007; Quinn et al., 2019, 2022; Sacks et al., 2011, 2016; Statham et al., 2012).

2.3 | Nuclear DNA library preparation

We used a GBS approach modified from Elshire et al. (2011) to construct reduced representation genomic libraries for a representative subset of red fox samples ($n = 131$). All extracts were normalised to 10 ng/μL prior to library preparation and sequencing. We digested 100 ng of DNA per sample at 37°C for 2 h with a restriction enzyme (NsiI-HF, an equivalent to EcoT22I; New England BioLabs Inc., Ipswich, MA). We then ligated both a common and uniquely barcoded adapter to each DNA sample (95 samples and 1 negative control per run). To minimise unevenness among individual libraries in the final sequencing pool, we pooled ligated samples into eight separate sub-pools (12 samples each) and purified each sub-pool via QiaQuick PCR columns (Qiagen Inc.) prior to the PCR reaction. To increase the complexity of our final library, we conducted four replicate PCR reactions for each of our eight library sub-pools, for a total of 36 reactions. Each 50 μL reaction contained 10 μL of purified adapter ligated DNA, 25 μL of NEB 2× Master Mix, 25 pmol of both forward and reverse PCR Primer, and dH_2O. The PCR conditions were as follows: 5 min at 72°C, 30 s at 98°C, followed by 18 cycles of 10 s at 98°C, 30 s at 65°C and 30 s at 72°C, with a final extension at 72°C for 5 min. We purified PCR products with QiaQuick PCR columns and quantified library concentrations with a Qubit fluorometer (Qiagen Inc.). All libraries were run on 1% agarose gels and a Bioanalyzer (Agilent Technologies, Santa Clara, CA) trace before pooling in equal masses for sequencing (96 libraries/lane) on an Illumina HiSeq4000 (SR100) at the University of California, Davis Genome Center DNA Technologies core.

2.4 | SNP calling and data filtering

We demultiplexed reads and trimmed adapters using GBSX_v1.3.jar (Herten et al., 2015) and then used ngsShort (Chen et al., 2014) to remove any remaining adapters and reads with >50% of bases having a quality score < 2 (–methods lqr -lqs 2 -lq_p 50). We aligned the trimmed reads to the red fox genome (vv2.4; Kukekova et al., 2018; Rando et al., 2018) using BWA-MEM (Li, 2013; Li & Durbin, 2010) and removed reads with mapping quality scores <10 (–q 10) using SAMTOOLS (Li et al., 2009). We used the 'ref_map.pl' program

in Stacks (v2.53) to process the sorted BAM files and call SNPs (Catchen et al., 2011). The 'gstacks' module was run with default SNP model parameters (–model marukilow, –var-alpha 0.05, and –gt-alpha 0.05) for unpaired reads (–unpaired) to create a catalogue of SNPs across our sample set as a single population. We then ran the 'populations' module to filter out monomorphic and low frequency variants (–min_maf 0.01), as well as SNPs with excess heterozygosity likely to reflect paralogs (–max_obs_het 0.60). We randomly selected a single SNP per locus (contig) (–write_random_snp) to reduce physical linkage across markers. We used a stepwise filtering approach in Plink (v1.90; Purcell et al., 2007) to arrive at a dataset composed of SNPs called in ≥80% of individuals and individuals with ≥80% of SNPs called. More specifically, we first removed the lowest quality individuals from the dataset that were missing ≥75% of SNPs (–mind 0.75). We then removed SNPs present in ≤80% of remaining individuals (–geno 0.2). Finally, we removed any additional individuals with <80% of SNPs called (–mind 0.2).

2.5 | Geographical patterns of ancestry

To qualitatively explore the genetic distances among samples in relation to their geographical location (Sacramento Valley vs. nonnative range), we used the GBS dataset to generate a multidimensional scaling (MDS) plot based on pairwise identity-by-state distance (–cluster) in Plink (v1.90; Purcell et al., 2007). Then, to assess population structure and admixture, we used the maximum-likelihood program ADMIXTURE (Alexander et al., 2009) to assign individuals at K = 1–5 clusters and used a 10-fold cross-validation approach to determine K with the highest likelihood. Additionally, we ran 2000 bootstrapped replicates to obtain 95% confidence intervals around our ancestry assignment point estimates. For downstream analyses, we identified 'pure' native and nonnative individuals as those with 95% confidence intervals overlapping 1 (pure native) and 0 (pure nonnative) (McFarlane & Pemberton, 2019). However, several putatively pure individuals had wide confidence intervals suggesting the possibility of up to 12% admixed ancestry in some samples. Because downstream analyses (e.g. Bayesian genomic cline) relied heavily on accurate estimates of pure native and nonnative population allele frequencies, we used an additional filter to more stringently classify pure individuals and only included those for which the lower 95% confidence interval was ≥0.98 (pure native) or upper 95% confidence interval ≤ 0.02 (pure nonnative).

2.6 | Width of hybrid zone

We estimated the width of the hybrid zone between native and nonnative red foxes sampled in the southern Sacramento Valley and San Joaquin Valley and then compared this empirical estimate to expectations based on a null model that assumed no reproductive barriers and neutral diffusion for the duration of time the two populations were in contact at the southern end of the native range. We compared empirical and modelled hybrid zone widths for both mitochondrial and nuclear ancestry. For the empirical estimates, we used only the data from the San Joaquin and southern Sacramento valleys, excluding the Coastal Lowlands, which were previously identified as genetically distinct from foxes found within the San Joaquin valley, presumably due to the mountainous regions of low human density that separate the two (Sacks et al., 2016), We additionally excluded the northern Sacramento Valley, which received introgression from the Cottonwood hybrid population to the north (Sacks, Wittmer, & Statham, 2010). We calculated averages of native ancestry among individuals within geographical bins corresponding to 32-km-wide latitudinal zones along the south–north length of the cline. We then fit equilibrium cline models under a likelihood framework using the Metropolis-Hastings Markov chain Monte Carlo algorithm in the program HZAR (Derryberry et al., 2014). For each marker type, we ran 10 model combinations using the default number (100,000 with 10,000 burn-in) of Markov chain Monte Carlo (MCMC) steps for three iterative cycles and assessed the best fitting model using Akaike information criterion scores corrected for small sample size (AICc). We obtained maximum likelihood estimates for centres, widths, and their corresponding ±2 log likelihood (LL) intervals (approximately 95% credibility intervals).

To create the null model, we used the R package HZAM (Irwin, 2020) to simulate genotypes along a one-dimensional (linear) cline that spanned the Sacramento and San Joaquin valleys, with the contact point situated at their formal boundary (Sacks et al., 2011). This approach simulates a cline based on a specified density, mating system, average (and SD) dispersal distance and time since contact. We simulated genotypes for native and nonnative individuals that were dispersed uniformly across their respective ranges assuming a constant population density of one breeding pair per 15 km^2 (Black et al., 2019). We parameterised dispersal distances separately for males and females as 45 km (SD = 80 km) and 8 km (SD = 9.0 km), which allowed for sampling from a lognormal distribution that is more reflective of mammalian dispersal patterns. These dispersal estimates were derived for the Sacramento Valley population using a genetic relatedness approach (Preckler-Quisquater, 2022; Walton et al., 2021) and were similar to those for other lowland red fox populations (Table S2; Phillips et al., 1972; Demaray, 1981; Gosselink et al., 2010; Walton et al., 2021). To test the sensitivity of our results to dispersal distance we additionally simulated cline widths for both mtDNA and nuDNA using dispersal distances that were 0.75×, 0.5×, 0.25×, and 0.1× smaller than the estimates derived for this system. The average collection date for both nuDNA and mtDNA samples was 2011 (Table S1). We therefore ran simulations of neutral gene flow for 18 generations, which assumed a 2-year generation time (Sacks, Statham, et al., 2010) and initiation of secondary contact in 1975 (Gray, 1975). Although contact potentially occurred before 1975, erring on the side of minimising contact time and using a 2-year generation time was conservative. We assumed random mating (Converse, 2013) and ran simulations for each marker type, including a single, maternally inherited locus to represent mitochondrial DNA and 100 biparentally inherited loci to represent nuclear DNA

patterns, and additionally replicated the simulations 100 times for both marker types. The resulting clines were fit using the same approach described for empirical data.

2.7 | Genomic cline analysis

To quantify the variation in locus-specific introgression potentially tied to reproductive isolation or selective introgression, we conducted a Bayesian genomic cline analysis (Gompert & Buerkle, 2011). Bayesian genomic clines measure a genome-wide or locus-specific cline centre (α) and rate (β) based on the relationship between the relative parental ancestry at each locus (φ) and the hybrid index (h), both of which range between 0 (pure nonnative ancestry) and 1 (pure native ancestry) and are expected to be equivalent under a null model. Loci with positive values of α reflect increased introgression of native ancestry (φ) into nonnative genomic backgrounds relative to the hybrid index (h). In contrast, loci with negative values of α reflect increased introgression of nonnative ancestry (φ) into native genomic backgrounds. The β parameter measures the slope of the cline, or the rate of transition from one ancestry type to another, relative to the hybrid index (h). Positive β values can reflect selection against hybrid phenotypes and may correspond to reproductive barrier loci, while negative β values may indicate introgression of alleles that have a selective advantage within one or both populations. We used all individuals sampled within the Sacramento and San Joaquin valleys ($n=83$), that is, excluding those found within the Coastal Lowland region. To reduce computational time, we selected a subset of 3572 ancestry informative markers (AIMs) that had allele frequency differences >0.2 between our stringent set of pure parentals. We calculated the allele frequencies across all AIMs within pure Sacramento Valley ($n=18$) and nonnative ($n=21$) parental datasets. We then generated allele counts for individuals with admixed ancestry ($n=44$) to utilise the genotype-likelihood approach within the *bgc* software (v1.04b; Gompert & Buerkle, 2012). We ran five replicate chains, each for 400,000 MCMC iterations with 200,000 burn-in cycles, thinning the data every 40 steps. We assessed convergence by examining the correlation of parameters across independent runs. We classified α and β outliers as loci with 95% credibility intervals for the posterior probability distribution that excluded zero (Gompert & Buerkle, 2011).

To uncover regions potentially underlying pre- or post-zygotic barriers to introgression, we explored genes within the 1 Mb region surrounding our positive β outliers. Because the red fox genome is not well-annotated, we extracted reads aligning to a 1-Mb region surrounding each locus and realigned them to the dog genome (CanFam 3.1, Lindblad-Toh et al., 2005; UCSC Genome Browser; NCBI Canis lupus familiaris Annotation Release 105 [2019-12-10]). We investigated whether any of the positive β outlier regions were previously associated with domestication and captive breeding in canids, as behavioural differentiation could act as a pre-zygotic barrier limiting gene flow (Axelsson et al., 2013; Hori et al., 2013; Kukekova et al., 2014, 2018; Pendleton et al., 2018; Trut et al., 2009; Wang et al., 2018). While we expected that post-zygotic reproductive barriers were unlikely to be the primary drivers of the continued separation between lineages, we also investigated whether any of the outlier loci were linked to regions previously proposed as candidates for reproductive isolation in mammals (i.e. 'speciation genes'). While largely exploratory, we also assessed whether any of the positive β outlier regions were associated with reproductive function (e.g. spermatogenesis and gamete recognition) more broadly. Additionally, we conducted a gene enrichment analysis using Panther (v16.0) to evaluate whether any associated GO terms were overrepresented (Mi et al., 2021).

We followed the same approach for regions potentially under selective introgression across the contact zone, mapping all loci associated with negative β outliers to the dog genome (CanFam 3.1, Lindblad-Toh et al., 2005; UCSC Genome Browser; NCBI Canis lupus familiaris Annotation Release 105 [2019-12-10]). We similarly compared our list of negative β outlier regions to those that were previously associated with domestication and captive breeding in canids, as these may also be predicted to have a selective advantage in a largely human-dominated landscape (Axelsson et al., 2013; Hori et al., 2013; Kukekova et al., 2014, 2018; Pendleton et al., 2018; Trut et al., 2009; Wang et al., 2018). As above, we assessed whether any of the negative β outlier regions were previously associated with other aspects of behavioural divergence or adaptation to human-dominated landscapes more broadly and conducted a gene enrichment analysis using Panther (v16.0), evaluating whether any associated GO terms were overrepresented (Mi et al., 2021).

3 | RESULTS

We obtained cytochrome *b* mitochondrial sequences for 682 individuals. After SNP calling and filtering, our final GBS dataset contained 107 individuals and 19,051 SNPs. The genome-wide coverage for our final sample set averaged 0.4% of the genome (approximately 1 million bases), and the sequencing depth (±SD) averaged 48× (±37×) across loci for all individuals.

3.1 | Geographical patterns of ancestry

We detected 255 individuals with native SVRF mitochondrial DNA haplotypes (D, D2; GenBank Accession Nos: EF064209, GU004541) and 427 individuals with nonnative mitochondrial DNA haplotypes (E, F, G, N, O; GenBank Accession Nos: EF064210-EF064212, EF064218, EF064219; Sacks et al., 2011, 2016). We observed no new (previously unreported) haplotypes. All native haplotypes were found within the historical range of the SVRF. In addition to the nonnative ranges (San Joaquin Valley and the Coastal Lowlands), nonnative haplotypes were detected in the historical SVRF range, primarily in the far-north or far-south end (Figure 1).

Based on 19,051 loci in the GBS dataset ($n=107$ individuals), the first axis in the multidimensional scaling plot highlighted the

genetic structure within the nonnative red foxes, more specifically distinguishing between the nonnative red foxes in the San Joaquin Valley and those in the Coastal Lowlands. The second axis differentiated between the native and nonnative red foxes. Notably, several individuals ($n=18$) from the native SVRF range showed intermediate placement on the MDS plot, suggesting signatures of admixed ancestry. In contrast, individuals from the nonnative range showed strong consistent clustering away from the native cluster, consistent with little or no native ancestry (Figure 2). The ADMIXTURE results identified $K=2$ with the highest likelihood (Figure 3a,b), followed closely by $K=3$ (Figure S2). The ADMIXTURE analysis revealed similar population structuring in the nonnative population at $K=3$ to that shown in the MDS plot as well as in previous studies using microsatellite data (Figure S2; Sacks et al., 2016). While no pure nonnative individuals were detected in the Sacramento Valley, there was more nonnative ancestry found within primarily native individuals in the Sacramento Valley region than there was native ancestry in nonnative individuals from the San Joaquin Valley (Figure 3c).

3.2 | Hybrid zone width

The empirical cline widths (±2 log-likelihood units) were significantly narrower than the expected cline widths both for mitochondrial DNA (empirical width = 31 km [21–45 km]; expected width = 206 km) and nuclear DNA (empirical width = 112 km [53–255 km]; expected width = 318 km) under a simulated neutral model of gene flow, assuming average dispersal distances of 45 km (SD = 80 km) for males and 8 km (SD = 9 km) for females (Figure 4a–c). Cline widths were also significantly narrower for mitochondrial DNA than for nuclear DNA, as expected given the male-biased dispersal pattern and the maternal inheritance of the mitochondrial DNA. These assessments were robust to imprecision of our estimate of average dispersal distance in the population. For example, the average dispersal distances would have had to have been <5 km for males and <1 km for females to account for the narrowness of the mitochondrial cline (Figure S3), which are well below any previously documented average dispersal distances for red foxes (Table S2).

3.3 | Genomic cline analysis

Estimates of genomic cline centre (α) and rate (β) parameters were largely concordant across replicate MCMC chains with average r^2 values of 0.997 and 0.985 respectively. We detected similar numbers of positive ($n=253$) and negative ($n=263$) α outlier loci according to 95% credibility intervals, and the magnitude was not significantly different ($\bar{\alpha}_{POS}=0.74\pm0.01$, $\bar{\alpha}_{NEG}=-0.76\pm0.01$). We also detected similar numbers of positive ($n=10$) and negative ($n=9$) β outlier regions, signalling potential involvement

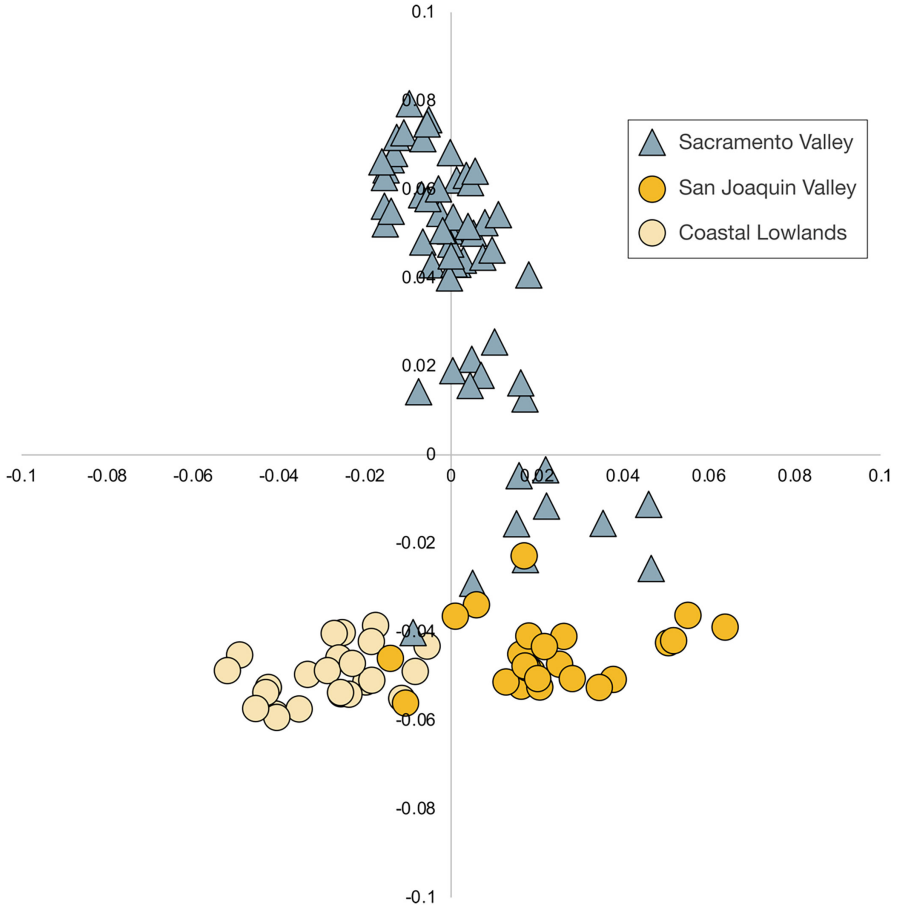

FIGURE 2 Multidimensional scaling plot based on 19,051 autosomal genotyping-by-sequencing (GBS) loci showing relationships among red foxes sampled within the range of the native Sacramento Valley red fox (blue triangles; $n=59$) and the range of the nonnative red fox (yellow circles; $n=48$), including the San Joaquin Valley (dark yellow; $n=24$) and the Coastal Lowlands (light yellow; $n=24$). Clustering patterns suggest that some red foxes ($n=18$) in the Sacramento Valley exhibited intermediate levels of placement indicating nonnative admixture, while those from the nonnative ranges exhibited little to no native admixture. Additionally, red foxes sampled from the nonnative ranges of the San Joaquin Valley and the Coastal Lowlands tended to cluster apart supporting previously described population structure within the nonnative range.

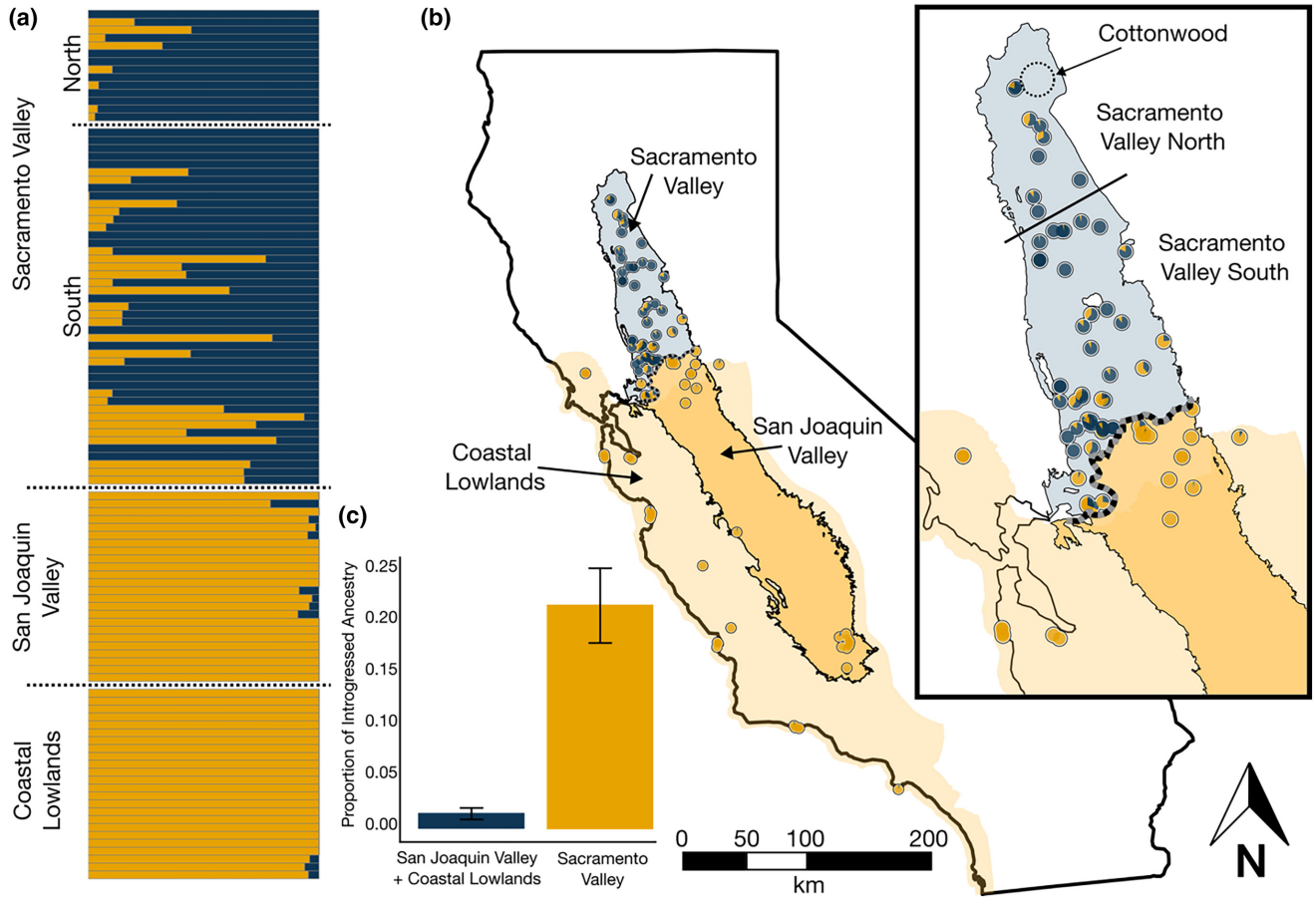

FIGURE 3 Admixture analysis at $K=2$ representing native (blue) and nonnative (yellow) ancestry of 107 red foxes sampled throughout the Sacramento Valley ($n=59$), San Joaquin Valley ($n=24$) and Coastal Lowlands ($n=24$) based on 19,051 autosomal genotyping-by-sequencing (GBS) loci: (a) bar chart showing the nuclear ancestral composition of each individual where individuals are shown in order of proximity to the contact zone centre (dashed black line); (b) map of samples, displayed as pie charts of their ancestral composition, in the native Sacramento Valley range (blue shaded) and nonnative ranges of the San Joaquin Valley (dark-yellow) and Coastal Lowlands (yellow-shaded) and (c) bar graph comparing the mean proportion of foreign ancestry across the focal contact zone between the Sacramento Valley and San Joaquin Valley, illustrating higher nonnative ancestry in the native Sacramento Valley range than native ancestry in the nonnative ranges of the San Joaquin Valley or Coastal Lowlands. The inset highlights the secondary contact zone, with the cline centre denoted by the black and grey dashed line, which is in the southern end of the Sacramento Valley and shows division of the Sacramento Valley into the northern end, which receives introgression from the Cottonwood hybrid zone (dotted circle), and the southern end, which receives introgression from the San Joaquin Valley nonnative population (Sacks et al., 2011; Sacks, Wittmer, & Statham, 2010).

in maintaining barriers to gene flow or selective introgression respectively.

We identified 90 genes within 500 kb of the 10 positive β outlier regions (i.e. reduced introgression between populations) that were previously annotated in the dog genome (Table S3). We identified three outliers near genes (MYCBPAP, Vulp_V002774 and Vulp_V002775) that were previously linked to differential expression patterns and high genetic differentiation between red foxes selectively bred for tame versus aggressive behaviour (Kukekova et al., 2018; Wang et al., 2018). We did not find evidence of positive β outlier regions that encompassed KRAB-ZNF genes and none of the outliers were housed on the X-chromosome. Additionally, we found no evidence of enrichment for specific functional categories dominating these outliers. However, the reduced introgression of three genomic regions were near genes that have been previously associated with reproductive fitness more broadly, including embryonic development (STIL) and spermatogenesis (TSSK2, SPATA20 and MYCBPAP) (David et al., 2014; Furusawa et al., 2001; Li et al., 2011).

We identified 37 previously annotated genes within 500 kb of the nine negative β outlier regions (i.e. elevated levels of introgression between lineages) (Table S4). We found no evidence of enrichment for specific functional categories. However, three regions were located within or adjacent to genes that have been previously associated with neural crest function and social behaviour, including DLG2, NCAM2 and KIF5C (Gong et al., 2022; Reggiani et al., 2017; Wang et al., 2013; Winther et al., 2012). All three of these regions were introgressed from the nonnative into the native Sacramento Valley population (i.e. negative α -values).

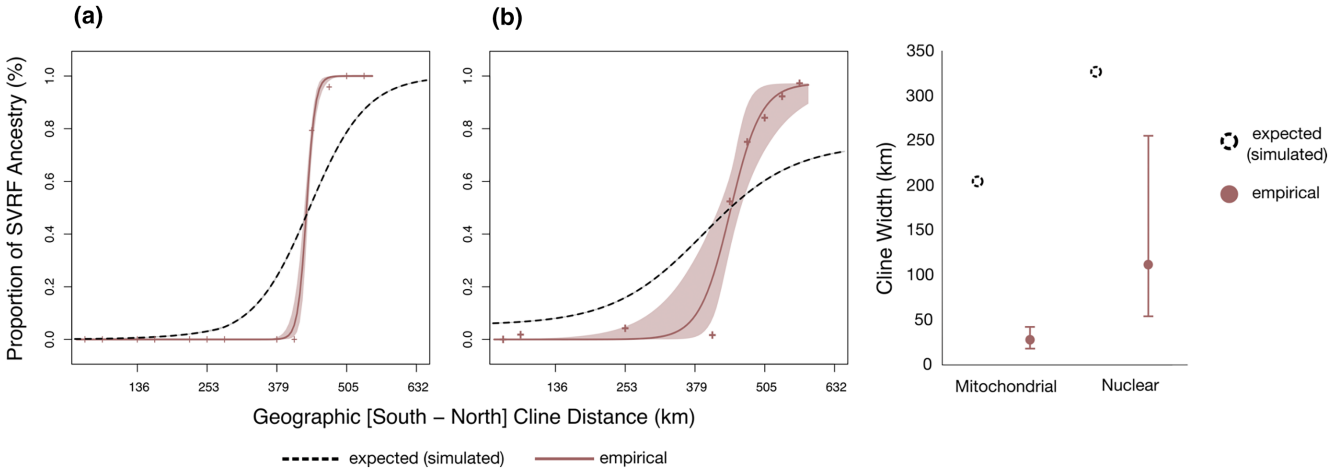

FIGURE 4 Comparison of empirical (red solid lines) and expected (black dashed lines) clines after 18 generations of secondary contact between Sacramento Valley native red foxes and San Joaquin Valley nonnative red foxes based on (a) mitochondrial DNA from cytochrome *b* and D loop fragments and (b) nuclear DNA from 19,051 autosomal genotyping-by-sequencing (GBS) loci. Empirical clines were fit to the average estimated proportions of native ancestry at $K=2$ among individuals sampled within nine 32-km-wide zones (red crosses at zone midpoints) spanning the entire length of the San Joaquin Valley and southern end of the Sacramento Valley. Expected clines assumed average dispersal distances of 45 km (SD = 80 km) for males and 8 km (SD = 9 km) for females and were fit to 100 independent simulations. (c) Empirical cline widths (±2 log-likelihood units) were significantly narrower than the expected cline widths both for mitochondrial DNA (empirical width = 31 km [21–45 km]; expected width = 206 km) and nuclear DNA (empirical width = 112 km [53–255 km]; expected width = 318 km) under a simulated neutral model of gene flow.

4 | DISCUSSION

We confirmed that introgression across a zone of secondary contact between the native Sacramento Valley red fox and a recently introduced nonnative red fox population was lower than expected given recent evolutionary divergence, high dispersal capabilities and at least 18 generations (and possibly many more) of contact. The widths of the hybrid zone estimated from mitochondrial DNA and nuclear DNA were both significantly narrower than expected in the absence of reproductive barriers. Additionally, due to the small extent of the native range relative to the dispersal distances of red foxes, the null (neutral) model of gene flow predicted that we would observe no remaining individuals with pure SVRF ancestry. Contrary to this expectation, however, 37% of individuals sampled within the Sacramento Valley retained statistically pure native ancestry, and we found much higher levels of overall native ancestry in the historical range than expected according to the null model, indicating that some type of barrier to gene flow must be operating. While the conclusions derived from the null model assumed an accurate characterisation of dispersal distances for our system, we tested the effects of shorter dispersal distances and found that for dispersal alone to explain the observed patterns, the distances would have had to have been substantially shorter than anything previously observed for red foxes across a variety of systems (Table S2; Figure S3). Therefore, we feel confident in rejecting a simple model of unobstructed gene flow between the native and introduced red fox populations.

Rather than reflecting one population of randomly interbreeding conspecifics, our findings were superficially more akin to those describing contact zones in much more evolutionarily divergent populations or species. Previous research comparing observed patterns of cline width relative to dispersal distances across a diverse set of taxa with those expected under different selection regimes found that most clines align with either neutral expectations or low-to-moderate levels of selection (Barton & Hewitt, 1985). Given the time since initial contact and typical red fox dispersal distances, the cline-width observed in our study matched the expectations of a tension zone model maintained by a selection coefficient (s) of ≥0.056 (i.e. cline width = dispersal distance /√s; Barton & Gale, 1993). Similarly, a recent meta-analysis that examined hybrid zones across 131 pairs of hybridising taxa found that the median cline width across all systems was 18.6× greater than dispersal length (McEntee et al., 2020). In contrast, the cline widths observed across the red fox secondary contact zone in our study ranged from <1× (mitochondrial) to <2.5× (nuclear) the average dispersal distance of males. The mitochondrial cline width was also <4× the dispersal distance of females. When placed within the context of previously described hybrid zones, the observed red fox cline widths are therefore similar to those expected under a scenario of moderate selection (i.e. against hybrids) (Figure 5). Importantly, however, these equivalents do not implicate selection as the mechanism maintaining the narrow contact zone in our system. Rather, they indicate that some mechanism must be actively limiting gene flow.

4.1 | The potential role of pre-zygotic reproductive barriers in limiting gene flow

We presume that pre-zygotic reproductive barriers are more likely than post-zygotic mechanisms given the recent divergence of the

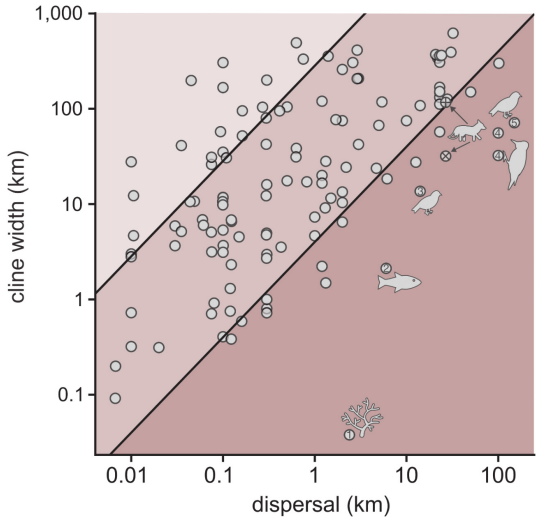

FIGURE 5 Relationship between cline width and average dispersal distance for a diverse set of taxa, modified from McEntee et al. (2020). The region between the upper and lower black lines (medium pink) shows the cline widths that would be maintained by a tension zone model with selection coefficients between 0.00001 and 0.05 respectively (Barton & Gale, 1993). Dots located above the upper diagonal line (light pink) are indicative of neutral clines or clines under extremely low levels of selection ($s < 0.00001$), while those below the lower diagonal line (dark pink) reflect clines widths influenced by moderate selection against hybrids ($s > 0.05$). The nuclear (⊕; 111.7 km) and mitochondrial (⊗; 31.3 km) cline widths are shown relative to the average dispersal distance ($\overline{\sigma} = 26.4$ km) for the red fox population described in this study. Based on the patterns observed for the nuclear cline, these results are consistent with a tension zone model and supports either moderate levels of selection against hybrids ($s \approx 0.05$) or some other force limiting gene flow. We have highlighted several notable secondary contact zones (see circles 1–5) that exhibit patterns consistent with moderate to strong selection against hybridisation (1. *Eunicea flexuosa* ssp. 2. *Cottus* spp. 3. *Sphyrapicus* spp. 4. *Sphyrapicus* spp. 5. *Catharus ustulatus* ssp.; see references in McEntee et al., 2020).

native and nonnative populations. In captivity, foxes in the fur farm industry were subjected to strong selection for increased fecundity, polygyny and tameness, all of which may have modified their social and cultural systems (Belyaev, 1979; Kukekova et al., 2018; Trut et al., 2009). Such distortions could, in turn, translate to increased levels of positive assortative mating. Based on the fine-scale variation in clinal patterns across the genome evident from our Bayesian genomic cline, we found no evidence of enrichment for any functional categories linked to behaviour or any category that could be linked to pre-zygotic barriers specifically. However, several outlier loci were associated with genomic regions shown previously to affect behaviour in canids. For example, the MYCBPAP gene was found to be differentially expressed between red foxes that were selectively bred for tame versus aggressive behaviour (Wang et al., 2018). Additionally, a large outlier region we identified in foxes that maps onto dog chromosome 9 housed several genes (NXPH3, SPOP, SLC35B1, FAM117A, KAT7 and TAC4) that were previously found to be under selection in village dogs when using wolves as an ancestral reference population (Pendleton et al., 2018). Two other outlier regions we identified in foxes in our study (Vulp_V002774 and Vulp_V002775) were shown previously to be differentiated between tame and aggressive captive foxes in a different study (Kukekova et al., 2018). These findingssuggest the possibility that selective breeding in of the captive ancestors of the nonnative population caused behavioural or physiological changes that resulted in pre-zygotic behavioural barriers with native red foxes.

Behaviours, such as assortative mating or natal habitat-biased dispersal, may also have learned components. Red foxes are a highly social canid, with a flexible mating system (Converse, 2013; Dorning & Harris, 2019). Social learning is a key component to the development of cultural behavioural traits, which can lead to assortative mating (Whitehead et al., 2019). Additionally, while cultural traits alone may not leave genomic signatures, there is growing evidence of gene-culture coevolution in which culturally learned behaviours impact genetic evolution by relaxing or intensifying selection (Whitehead et al., 2019).

Natal habitat-biased dispersal, a process where individuals cue on features in their natal habitat to seek as dispersal targets (e.g. Sacks et al., 2004), might also contribute to segregation of native and nonnative red fox populations. Specifically, conspecific scents or other cues indicating conspecific presence have been identified as powerful cues for natal habitat-biased dispersal in some systems (Stamps, 1991). Thus, if there are significant cultural differences between native and nonnative red fox populations, they may not recognise one another as conspecifics, which could limit dispersal across the contact zone. Such a mechanism also could potentially explain the asymmetrical pattern we observed, whereby gene flow is almost entirely in the direction of nonnative to native populations. That is, it seems plausible that the descendants of foxes bred for success in a captive environment would be less choosy than native red foxes about where they disperse.

Related to natal habitat-biased dispersal, it is also possible that a landscape barrier near the contact zone limits dispersal across it and does so more for native than nonnative foxes. The Sacramento-San Joaquin River Delta, along with the city of Sacramento, an urban metropolis with a human population density > 2000 individuals per km^2 could serve as such a barrier (U.S. Census Bureau, 2010; Figure S4). Although both native and nonnative red foxes currently inhabit heavily human-altered landscapes, the nonnative foxes may have a higher tolerance for human presence. The native Sacramento Valley red foxes remain confined to the lowest elevations of the valley bottom and are typically associated with grasslands and dryland agriculture (Black et al., 2019). Since their establishment in California, nonnative red foxes have expanded to inhabit a wide range of habitat types throughout California's lowlands including densely populated urban centres, coastal and wetland regions, as well foothill habitat (Lewis et al., 1999).

4.2 | Exploration of post-zygotic reproductive barriers in limiting gene flow

Because post-zygotic isolating mechanisms are typically the result of genetic incompatibilities that have accumulated during long periods of isolation, often involving complex genetic interactions (e.g. Dobzhansky–Muller incompatibilities), we expected they would be less likely to explain the lower-than-expected gene flow we observed between the recently diverged (~20–70 kya) native and nonnative red fox populations. Nevertheless, we identified several outlier regions that were associated with reproductive incompatibilities previously described in the literature some of which seemed particularly amenable to adaptive explanations. The STIL gene plays an important role in embryonic development as well as cellular growth and proliferation, and embryonic lethality was observed in $STIL^{-/-}$ mouse embryos (David et al., 2014). The TSSK2 gene is one of the testis-specific serine/threonine kinases which are expressed in spermatids and are essential for male fertility (Li et al., 2011). Additionally, TSSK2 was identified as a candidate sterility gene within a house mouse hybrid zone in Europe (Turner & Harr, 2014). The SPATA20 gene (spermatogenesis associated protein 20) also has a known role in male reproduction and was identified as a candidate gene associated with a sterility hotspot in a study on hybrid dysfunction in the house mouse (Turner et al., 2014). The MYCBPAP gene similarly plays a role in spermatogenesis and an alteration of its expression may therefore have an impact on sperm function (Furusawa et al., 2001). Although we did not find a statistically significant enrichment for reproductive genes as a whole, this may not be surprising if a small number of genes was responsible for creating a post-zygotic reproductive barrier. Nevertheless, our findings here were suggestive and therefore represent hypotheses to be further investigated.

4.3 | Selective introgression across the contact zone

Given the long history of captive breeding prior to the release of nonnative red foxes in California, one of the primary concerns with the observed gene flow between native and nonnative foxes is that artificially selected genes originating in the nonnative population could be selectively introgressed into the native population with unknown ecological and evolutionary consequences. We identified several directionally introgressed (nonnative to native) regions associated with neural crest function and social behaviour that were previously described in other studies of vertebrate domestication and adaptation to human dominated landscapes. We briefly outline the most notable outlier regions associated with domestication and habituation to human-dominated landscapes, all of which showed elevated rates of gene flow from the nonnative into the native population.

One outlier region was located within the open reading frame of the DLG2 (Discs Large MAGUK Scaffold Protein 2) gene, which plays an important role in complex cognitive and learning tasks (Grant, 2016; Nithianantharajah et al., 2013). The DLG2 gene has been linked to differential fear responses to humans (Bélteky et al., 2018), neurodevelopmental disorders associated with hyper-domestication (Reggiani et al., 2017; Šimić et al., 2020; Yoo et al., 2020), and selection to urban environments (Salmón et al., 2021). The DLG2 gene has been identified as a candidate gene under selection across European red fox populations, although selection to human-dominated landscapes was not specifically explored (Roberts, 2019). Another outlier region was located within the open reading frame of the NCAM2 (neural cell adhesion molecule 2) gene, which encodes a protein belonging to the immunoglobin superfamily and has been proposed to contribute to neurodevelopmental disorders in humans including autism and schizophrenia (Winther et al., 2012). The NCAM2 gene is primarily expressed in the cerebral cortex, the hippocampus, and the olfactory system (Parcerisas et al., 2021) and is putatively under positive selection in domesticated dogs (Wang et al., 2013). Finally, we detected an outlier near the KIF5C (kinesin family member 5C) gene that was linked to behavioural differences between wild and domestic pigs in Europe, specifically increased vigilance, and social sensitivity in wild populations (Gong et al., 2022).

It seems plausible that selective introgression of genes associated with domestication could correspond to fitness advantages in the human-dominated Sacramento Valley environment. While we observed some evidence for selective introgression of genes previously linked to social behaviour and domestication, additional research investigating the genetic variation of reference fur farm foxes (i.e. the source populations for the present day nonnative red foxes in California) as well as their progenitors (i.e. wild eastern and Alaskan red foxes) may help to elucidate whether these variants were specifically amplified in the ancestral nonnative population during the history of captive breeding.

5 | CONCLUSION

Our study revealed that recently diverged conspecifics in secondary contact—particularly those between feral nonnative individuals and their native wild counterparts—can exhibit hybridisation patterns similar to those observed in much more distantly related populations or species. Despite the uncertainty surrounding the specific mechanisms maintaining the seemingly stable admixture zone, the narrowness of the cline must be explained by some form of reproductive barrier, whether pre- or post-zygotic. While pre-zygotic barriers were the a-priori more likely explanation, given the recent divergence of these conspecifics, we also identified several candidate regions previously linked to reproductive incompatibilities that could potentially reduce hybrid fitness and contribute to a narrower zone of introgression. These findings warrant further investigation of the conventional assumption that genic reproductive barriers necessarily require hundreds of thousands of years to evolve.

The limited gene flow between two populations with such recent shared evolutionary history was notable and additionally provided an opportunity to investigate selective introgression. The behaviourally linked genes we detected with elevated levels of gene flow from the nonnative to the native population suggest that genes selected for

during captivity in fur farms could offer a fitness advantage to wild red foxes in urban environments. If indeed these alleles were beneficial to Sacramento Valley red foxes in the short-term, the long-term consequences are unclear, and could mirror the patterns observed in fisheries, where native populations are regularly threatened due to hybridisation with captive bred stock. Notably, as we used a reduced representation GBS approach, we only captured the variation present at a subset (~0.4%) of the genome, and it is possible that other loci involved in pre- or post-zygotic reproductive isolation or selective introgression could be more clearly elucidated through whole-genome sequencing.

Our research underscores the dynamic and rapid nature of evolutionary processes in response to anthropogenic influence and highlights the importance of incorporating genetic insights into broader conservation strategies. These findings not only contribute to our theoretical understanding of hybrid zones and speciation, but additionally serve as a crucial reminder of the nuanced and often unpredictable ways in which human activities can impact natural systems and evolutionary processes.

AUTHOR CONTRIBUTIONS

SPQ designed the research with input from BNS and conducted research activities and data analyses. CBQ modified the spatially explicit simulations of secondary contact to include sex-biased dispersal and genetic markers with distinct inheritance patterns. SPQ wrote the initial manuscript and all authors contributed to the writing of subsequent drafts.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers and the handling editor for helpful comments on an earlier draft of this paper. We also thank Mammalian Ecology and Conservation Unit alumni, Dr Thomas Batter and Dr Kathleen Black for their contributions to field and lab work, as well as many students, volunteers, state, county, and private wildlife personnel who assisted with sample collection and processing. Critical laboratory support was provided by S. Vanderzwan, Z. Lounsberry, and the University of California, Davis Veterinary Genetics Center Laboratory staff. Funding was provided by the California Department of Fish and Wildlife (Agreements Nos. P0780029, S0810020) the University of California, Davis, Mammalian Ecology and Conservation Unit along with the UC Davis Graduate Group in Ecology Fellowship, the Kit Rice Ecology Fellowship, and the William and Linda Sullivan Environmental Science Scholarship.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://doi.org/10.5061/dryad.tx95x6b50.

DATA AVAILABILITY STATEMENT

Tissue and scat samples are archived at the University of California, Davis Mammalian Ecology and Conservation Unit with sample numbers provided in the Supporting Information. Raw sequence reads associated with GBS Data were accessioned in the NCBI SRA under BioProject No. PRJNA1069152 (Preckler-Quisquater & Sacks, 2024). Relevant scripts and datasets to reproduce analyses can be accessed in the associated Dryad repository (https://doi.org/10.5061/dryad.tx95x6b50).

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SUPPORTING INFORMATION

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How to cite this article: Preckler-Quisquater, S., Quinn, C. B., & Sacks, B. N. (2024). Maintenance of a narrow hybrid zone between native and introduced red foxes (*Vulpes vulpes*) despite conspecificity and high dispersal capabilities. *Molecular Ecology*, *33*, e17418. https://doi.org/10.1111/mec.17418