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ORIGINAL ARTICLE

Multi-population puma connectivity could restore genomic diversity to at-risk coastal populations in California

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Abstract

Urbanization is decreasing wildlife habitat and connectivity worldwide, including for apex predators, such as the puma (*Puma concolor*). Puma populations along California's central and southern coastal habitats have experienced rapid fragmentation from development, leading to calls for demographic and genetic management. To address urgent conservation genomic concerns, we used double-digest restriction-site associated DNA (ddRAD) sequencing to analyze 16,285 genome-wide single-nucleotide polymorphisms (SNPs) from 401 pumas sampled broadly across the state. Our analyses indicated support for 4–10 geographically nested, broad- to fine-scale genetic clusters. At the broadest scale, the four genetic clusters had high genetic diversity and exhibited low linkage disequilibrium, indicating that pumas have retained genomic diversity statewide. However, multiple lines of evidence indicated substructure, including 10 finer-scale genetic clusters, some of which exhibited fixed alleles and linkage disequilibrium. Fragmented populations along the Southern Coast and Central Coast had particularly low genetic diversity and strong linkage disequilibrium, indicating genetic drift and close inbreeding. Our results demonstrate that genetically at risk populations are typically nested within a broader-scale group of interconnected populations that collectively retain high genetic diversity and heterogenous fixations. Thus, extant variation at the broader scale has potential to restore diversity to local populations if management actions can enhance vital gene flow and recombine locally sequestered genetic diversity. These state- and genome-wide results are critically important for science-based conservation and management practices. Our nested population genomic analysis highlights the information that can be gained from population genomic studies aiming to provide guidance for the conservation of fragmented populations.

KEYWORDS
conservation genetics, mountain lion, nested population structure, population genetics, *Puma concolor*, SNP

1 | INTRODUCTION

Human development is reducing habitats on a global scale, undermining efforts to conserve ecosystem structure and function (Newbold et al., 2016). Reports of fragmented wildlife populations and the increasing need for human housing and associated agriculture and energy have emphasized the necessity for development to avoid impacting the long-term sustainability of wildlife populations (Jordan et al., 2007; Kiesecker et al., 2011; Saha & Paterson, 2008). One of the most developed states in the United States is California, which contains the largest census size with over 39 million people (U.S. Census, 2019). Although the development of California has led to historical extirpations of other apex predators, such as the grizzly bear (*Ursus arctos*; Herrero, 1970) and gray wolf (*Canis lupus*; Schmidt, 1991), the puma (*Puma concolor*; also known as mountain lion and cougar) has maintained a widespread distribution throughout the state (Dellinger, Cristescu, et al., 2020).

The puma is a large-bodied felid that originated in South America, migrated and expanded throughout North America, and experienced a human-induced range restriction to the western United States, with an extant remnant population in Florida (Culver et al., 2000). Currently, approximately half of all apparent puma habitats in California is conserved, and the remainder could be subject to further development (Dellinger et al., 2020). Much of the inland areas of California have continous stretches of protected habitat (Dellinger et al., 2020), supporting puma populations with high genetic diversity and large effective population sizes (Gustafson et al., 2019). However, movement corridors among coastal mountain ranges are increasingly being degraded by human development (Burdett et al., 2010; Suraci et al., 2020; Zeller et al., 2017). Despite the natural long-range dispersal abilities of pumas (Gonzalez-Borrajo et al., 2017), interstate highways limit dispersal *via* avoidance and direct mortality in some urban areas (Riley et al., 2014; Vickers et al., 2015). Although human-caused mortality from vehicle collisions and lethal removal after wildlife–livestock conflicts are concerns (Guerisoli et al., 2021; Torres et al., 1996), a larger concern for long-term population viability is the genetic isolation of pumas within small or shrinking patches of habitat, which has led to high levels of intraspecific competition and mortality (Benson et al., 2020) and low genetic diversity in some areas (Ernest et al., 2014; Gustafson et al., 2019; Riley et al., 2014).

Previous studies have reported that two isolated puma populations in southern California, including the Santa Ana Mountains and the Santa Monica Mountains (Figure 1), had the lowest genetic diversity estimates measured throughout the range of *P. concolor* (Ernest et al., 2014; Riley et al., 2014), apart from the endangered Florida panther (*P. c. coryi*). In both the Santa Ana and Santa Monica Mountains, phenotypic evidence of inbreeding depression has been observed, similar to that of Florida panthers (Ernest et al., 2014; Huffmeyer et al., 2021; Roelke et al., 1993). For both populations, freeway traffic isolates pumas (Ernest et al., 2014; Riley et al., 2014; Vickers et al., 2015), and contemporary gene flow has been severely limited. Detailed pedigree analyses following the immigration of one male into each region showed evidence of natural genetic rescue (Ernest et al., 2014; Gustafson et al., 2017; Riley et al., 2014). Although migrant effects were positive, projection models predict the extirpation of these populations in 50 years without enhanced demographic dispersal and gene flow (Benson et al., 2016, 2019).

Recently published genome-resequencing data that included four pumas from California, two from Santa Monica Mountains, and two from the Central Coast North region in the Santa Cruz Mountains indicated that these individuals had ~20%–40% of their genomes represented as long runs of homozygosity, resulting from recent inbreeding (Saremi et al., 2019). However, these runs of homozygosity were not shared among individuals, and different populations exhibited different homozygous haplotypes, suggesting that genetic restoration (Hedrick, 2005; Tallmon et al., 2004) is possible because genetic variation still exists.

The complex distribution of pumas throughout California along a continuum of high genetic diversity populations occupying abundant habitat to strongly isolated populations displaying evidence of inbreeding depression requires a thorough characterization of statewide genomic diversity to achieve proper conservation. In this study, our objective was to characterize patterns of genomic diversity at varying geographic scales. Such an approach has the potential to aid conservation strategies because it can identify at-risk, low-diversity local populations that would benefit from the restored gene flow within a broader geographic region. We identified 16,285 single-nucleotide polymorphisms (SNPs) from 401 individuals using a double-digest, restriction-site associated DNA sequencing method (ddRAD; Peterson et al., 2012). Specifically, our aims were to determine population genomic structure, genetic diversity, evidence for selection, and linkage disequilibrium.

2 | METHODS

2.1 | Sample collection and DNA extraction

We obtained 354 tissue samples collected by the California Department of Fish and Wildlife between 2011–2017 from pumas either hit by car (~6%), found dead (~2%), poached (<1%), or through depredation permits (>90%), which had never been used in any previous genetic survey. Samples were well-distributed throughout the state, except for smaller populations in smaller mountain ranges. To bolster our sample size in the Los Angeles region of southern California, we added the only remaining DNA extracts (N = 144) from pumas collected between 2002–2015 (Riley et al., 2014; Vickers et al., 2015). After genomic and bioinformatic filtering (described below), we retained 401 out of 498 samples in the final dataset, which spanned the majority of puma habitat in California, excluding desert regions (Figure 1). For samples that lacked a precise GPS location, we used the nearest address or town where they were collected as their GPS point. Samples were stored at −80°C until DNA was extracted using Omega Bio-tek Mag-Bind Blood & Tissue DNA HDQ Kits (Omega Bio-tek, #M6399-01), with a manufacturer-designed protocol for the Kingfisher Duo Prime (ThermoFisher

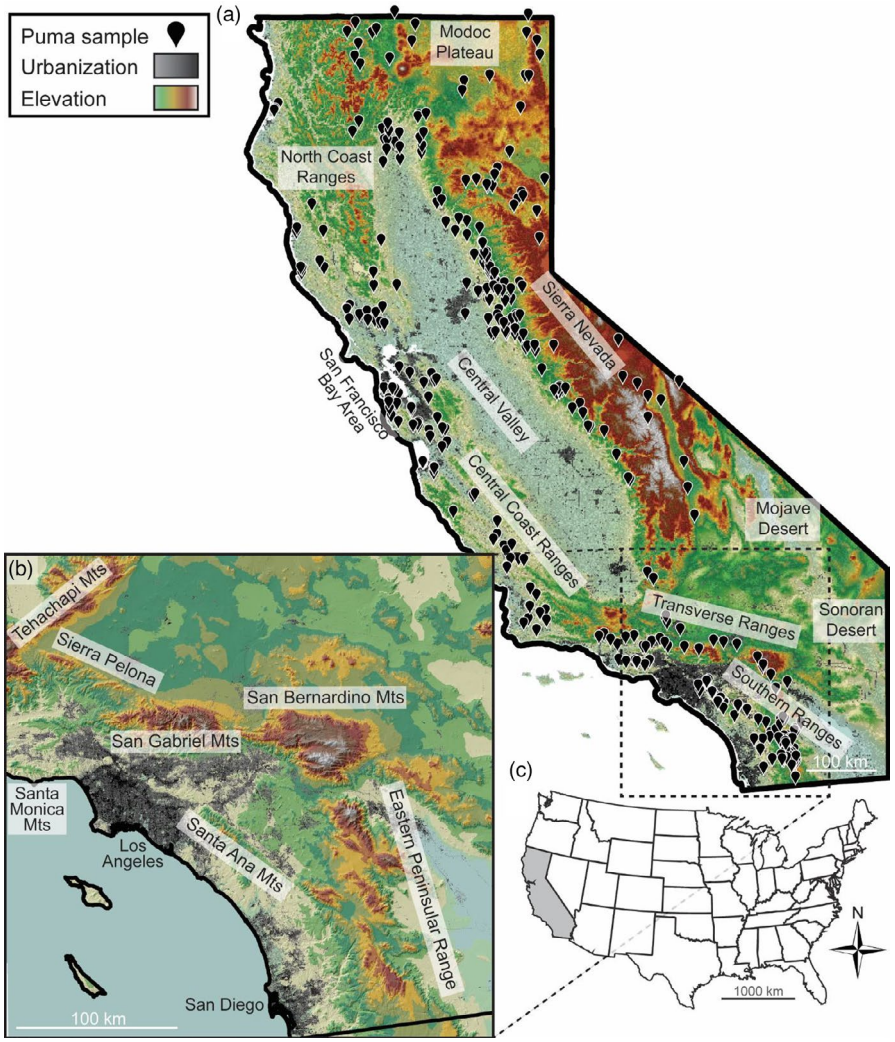

FIGURE 1 Location of 401 sampled pumas used in analyses, including (a) sample distribution across California, (b) geography of the mountain ranges surrounding the Los Angeles and San Diego regions, and (c) inset map showing the location of California in the United States of America

Scientific, #5400110) automated DNA purification system. We measured the concentration of DNA from each sample using a Qubit 3.0 fluorometer (Invitrogen, #Q33216) with Qubit dsDNA high-sensitivity kits (Invitrogen, #Q32854).

2.2 | Double-digest restriction-site associated DNA library preparation and sequencing

We reduced the genome size of our samples and identified single-nucleotide polymorphisms (SNPs) using modifications to the double-digest restriction-site associated DNA sequencing (ddRAD) protocols developed by Peterson et al. (2012). We used a library construction scheme which pooled 48 samples per library based on barcode availability, cost effective multiplexing, and sufficient coverage per individual. For each pooled library, we first normalized DNA concentrations to the sample with the lowest concentration within a library, with the goal to be more than 200 ng DNA starting material in 25 μL elution buffer (>8 ng/μl). The library with the lowest normalized starting concentration for each sample had 17.8 ng/μl DNA, whereas the library with the highest starting material had 51.6 ng/μl DNA. We used digestion enzymes and protocols established with previous puma studies (Trumbo et al., 2019). After DNA was normalized, we double-digested the DNA from each individual using NlaIII (New England BioLabs, #R0125S) and EcoRI (New England BioLabs, #R3101S) restriction enzymes (37°C for 3 h, then held at 4°C) at the manufacturer-recommended enzyme concentrations and used AMPure XP beads (Beckman Coulter, #A63881) at a 1.5X ratio to retain only DNA from the digestion. We omitted the DynaBeads cleanup step used by Peterson et al. (2012) and again used the Qubit to measure DNA concentrations and to guide another round of normalization. After normalization, the library with the lowest per-sample concentration had 2.1 ng/μl (in 29 μl), and the library with the highest per-sample concentration had 8.1 ng/μl.

We then ligated 48 uniquely barcoded P1 adaptors (e.g., P1.1 through P1.48) and two common P2 adapter pairs (i.e., P2.1 and P2.2) to each sample's double-digested fragments using the protocols of Peterson et al. (2012) to identify individual puma samples. Following ligation with individual barcodes, we pooled all 48 samples into a single tube and used AMPure XP beads to clean the library. We used TE buffer (rather than molecular-grade water) as the final step in this cleanup, which is recommended by the manufacturer for running size selection in the Pippin Prep (Sage Science, Beverly, Massachusetts). We selected fragments ranging from 375–475 bp (including 75 bp

of adapters) using 2% dye-free gels run on a Pippin Prep. To minimize random polymerase chain reaction (PCR) duplicate errors, we split the library and ran five high-fidelity Phusion (New England BioLabs, #M0530) PCRs for 12 cycles on a SimpliAmp thermal cycler (ThermoFisher Scientific, #A24811). We then recombined the five PCR products and used an AMPure XP bead cleanup on the amplified library. Sample concentrations after size selection averaged 2.0 ng/μl DNA (range 0.82–3.7) and, after the PCR, averaged 8.2 ng/μl (range 3.6–15.0). We shipped the unfrozen DNA with cold packs to the University of Oregon's Genomics and Cell Characterization Core Facility (https://gc3f.uoregon.edu/) for 150 bp single-end sequencing on an Illumina HiSeq 4000 (Illumina).

2.3 | Bioinformatic SNP filtering

We ran standard quality control analyses using program *FastQC* v0.11.5 (Andrews, 2010). We used the *process_radtags* program in the *Stacks* v2.55 (Catchen et al., 2013) package to de-multiplex the reads based on unique barcodes to assign each sequence to an individual puma sample, to remove sequences with a Phred quality score below 20 (99% accuracy), and to remove Illumina adapter sequences from the data. We then aligned reads for each individual to PumCon1.0—the *Puma concolor* draft reference genome—using program *bwa* (Li & Durbin, 2009). We identified and filtered SNPs with *Samtools* (Li et al., 2009). We discarded loci with a mapping quality score below 20, minimum base quality less than 20, with more than two alleles at a site, and with a maximum depth greater than 100. We skipped indels and used only a random SNP per read to reduce linkage disequilibrium.

Using *vcftools*, we tested the effects of multiple filtering parameters on our dataset, specifically looking at which parameters produced unreliable and inconsistent heterozygosity estimates, inbreeding coefficients, and relatedness values. We retained loci with a minor allele frequency ≥0.05 as lower frequency SNPs could be sequencing error. The relationship between the minimum depth of reads per individual and heterozygosity was asymptotic and plateaued at about 3–4 reads. To be conservative, we selected a minimum depth of four reads per individual to reliably acquire genotypes based on both alleles. We also retained SNPs that had genotypes for at least 50% of the individuals. We iteratively removed samples with more than 50% missing data to maximize the number of SNPs retained in the dataset. Being more conservative with the percent of missing data decreased the number of SNPs in the final dataset but did not affect heterozygosity estimates, inbreeding coefficients, and relatedness values. We scanned for duplicate samples using relatedness values in *vcftools*, but, as expected, found none because most DNA samples were removed from the dead pumas. We also removed two potentially contaminated samples based on negative F statistics in *vcftools*.

In each library of 48 samples, we strategically included puma samples from across a large geographic area so libraries would have no correlation with the spatial location. For example, there was no significant difference between mean sample latitudes ($F_{7,309} = 1.108$, $p = 0.358$) or longitudes ($F_{7,309} = 1.533$, $p = 0.155$) among libraries. However, because the southern California libraries constructed from pre-existing extracts were from a small geographic region, there ended up being some latitudinal ($F_{10,395} = 33.76$, $p < 0.001$) and longitudinal ($F_{10,395} = 33.89$, $p < 0.001$) mean differences between those libraries and the libraries constructed from the new samples. However, as indicated below, there were no detectable biases of the southern California libraries in any analyses.

To test for library-effect biases (i.e., differences among sequencing lanes), we used *BayeScan* to identify outlier SNPs while treating sequencing lanes as "populations" and using a false discovery rate of 0.05 (Foll & Gaggiotti, 2008). There were no outlier loci among any of the libraries, including the southern California libraries. We also assessed bias with various genetic structure analyses. Genotypes resulting from the pre-existing DNA extracts consistently clustered with those genotypes resulting from the new samples collected from southern California. With no apparent library-effect biases, we retained 16,285 biallelic variants (mean ± SD = 12,245 ± 2749) with a mean depth at each locus of 11.7 ± 5.1 and a mean depth per locus per individual of 11.7 ± 7.1.

2.4 | Population structure and outlier loci

We used multiple approaches to identify genetic clusters of individuals, including a linear principal components analysis (PCA) and a spatially explicit population structure analysis in program R (R Core Team, 2020). We ran the PCA using *adegenet* 2.1.1 (Jombart, 2008) and the structure analysis in *tess3r* 1.1.0 (Caye et al., 2016). We used *adegenet*::*colorplot* to present the linear structure identified by the first three principal component axes. In *tess3r*, we ran 20 replicates for each *K* (1–20) at 100,000 iterations each. We kept the most highly supported model (i.e., "best" based on cross-entropy scores) within each of the 20 replicates. To test for evidence of loci under selection, we identified outlier loci among populations (Narum & Hess, 2011) using *BayeScan* and *tess3r* with the Benjamini–Hochberg statistical correction and the recommended α-value of 0.0001.

2.5 | Genetic diversity, effective population size, genetic differentiation, and linkage decay

For each genetic cluster identified in *tess3r*, we calculated observed heterozygosity (H_O), gene diversity (H_S), and allelic richness (A_r) using *hierfstat*::*basic.stats* (Goudet, 2005; Nei, 1987). To test for Wahlund effects within broad-scale clusters, we used t-tests to test for differences between H_O and H_S. We calculated private alleles (A_p) using *poppr*::*private_*alleles (Kamvar et al., 2014). We used *NeEstimator* 2.1 (Do et al., 2014) to estimate effective population size (N_e) using the linkage disequilibrium model, random mating, allele frequencies >0.05, and with a correction factor of 19 haploid chromosomes (Hsu et al., 1963), as recommended by Waples et al. (2016). We used *hierf*

stat::*pairwise.neifst* and *hierfstat*::*pairwise.WCfst* to estimate pairwise genetic differentiation based on F_{ST} according to Nei (1987) or Weir and Cockerham (1984).

We used *Plink* 2.0 (Purcell et al., 2007) to estimate linkage disequilibrium among loci (--ld-window-r2 0 --ld-window 999999 --ld-window-kb 8000). To determine the level of non-random segregation of alleles across the genome, we assessed linkage decay in each genetic cluster by plotting the correlation of loci (R^2) based on genomic distance between SNPs. We correlated loci using binned intervals of 100,000bp from 0 to the maximum scaffold size of PumCon1.0. Meiosis should break up linkage, resulting in low R^2 values. However, populations experiencing strong selection, low mutation, inbreeding, low migration, or strong genetic drift will have higher R^2 values. In short, SNPs that are close together on chromosomes are expected to be correlated (i.e., inherited as chromosomal/haplotype segments), but SNPs far away are expected to assort randomly during recombination. However, if sequences are too similar, which they may be in small and inbred populations, we will not be able to detect events of crossing over despite their occurrence, resulting in higher estimates of linkage disequilibrium, which is still an important indicator of genetic diversity and N_e.

3 | RESULTS

3.1 | Population structure and outlier loci

We recovered 16,285 SNPs that were randomly distributed among 125 draft-genome scaffolds. The first three axes of the PCA accounted for 14.6% of the variance and indicated that there were four broad-scale genetic clusters distributed across California (Figure 2). When each puma was plotted on a map of California (Figure 2a), the four clusters were geographically concordant with the Sierra Nevada (SN), North Coast (NC), Central Coast (CC), and Southern Coast (SC). The first eigenvector separated the negative-valued CC and SC groups from the positive-valued SN and NC (Figure 2b,c). The second eigenvector separated negative-valued CC from positive-valued SC (Figure 2b). Finally, the third eigenvector separated negative-valued NC from all other groups (Figure 2c).

A spatially explicit population structure analysis indicated that there was broad- to fine-scale nested genetic structure with support for 4–10 genetic clusters (Figure 3). Root mean square error (inset plot in $K = 2$ panel of Figure 3) and cross-entropy scores (inset plot in $K = 3$ panel of Figure 3) provide statistical evidence for nested genetic structure; values begin to curve at $K = 4$, and there is a major increase in variance at $K = 5$, but there is a steady increase in statistical support at higher K values. However, single pumas formed individual clusters at $K > 10$, at which point K lost biological meaning. When K was set to 4, the genetic clusters corresponded to the broad-scale genetic groups identified by the PCA (Figures 2 and 3). Briefly, at $K = 5$, pumas in the Central Coast North (CC-N) emerged; at $K = 6$, the Eastern Sierra Nevada (ESN) cluster separated from the Western Sierra Nevada (WSN); at $K = 7$, the Santa Ana (SA) cluster separated from the Eastern Peninsular Range (EP); at $K = 8$, the San Gabriel–San Bernardino (SGSB) cluster emerged; at $K = 9$, the Klamath–Cascades (KC) cluster emerged; and at $K = 10$, the Central Coast South (CC-S) cluster separated from Central Coast Central (CC-C; Figure 3). We observed no significant evidence for outlier loci using the Benjamini–Hochberg statistical correction in *tess3r* nor *BayeScan* for either $K = 4$ or $K = 10$.

3.2 | Genetic diversity, effective population size, genetic differentiation, and linkage decay

For $K = 4$, calculations of observed heterozygosity (H_O), gene diversity (H_S), polymorphic loci (*Poly*), allelic richness (A_r), and the private alleles (A_p) indicate that the Sierra Nevada cluster had higher

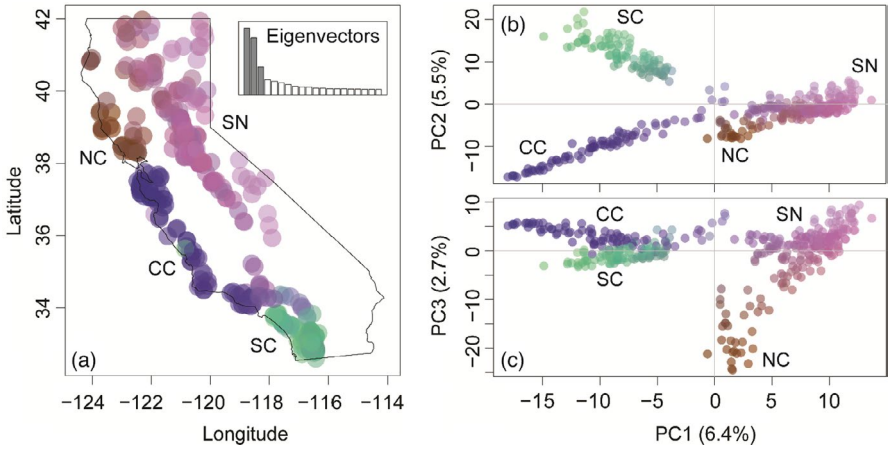

FIGURE 2 Principal component analysis (PCA) of 401 pumas at 16,285 SNPs reveals four genetic clusters. (a) Color plot (R package *adegenet*) of the PCA represents colors corresponding to a combination of the first three eigenvectors. The inset plot shows the proportion of the variance explained by shaded PC eigenvectors 1–3 compared to other eigenvectors. The color values are plotted at sample locations to demonstrate geographic structure. Color plots of (b) PC1 and PC2 and (c) PC1 and PC3 resolved the four broad-scale genetic clusters

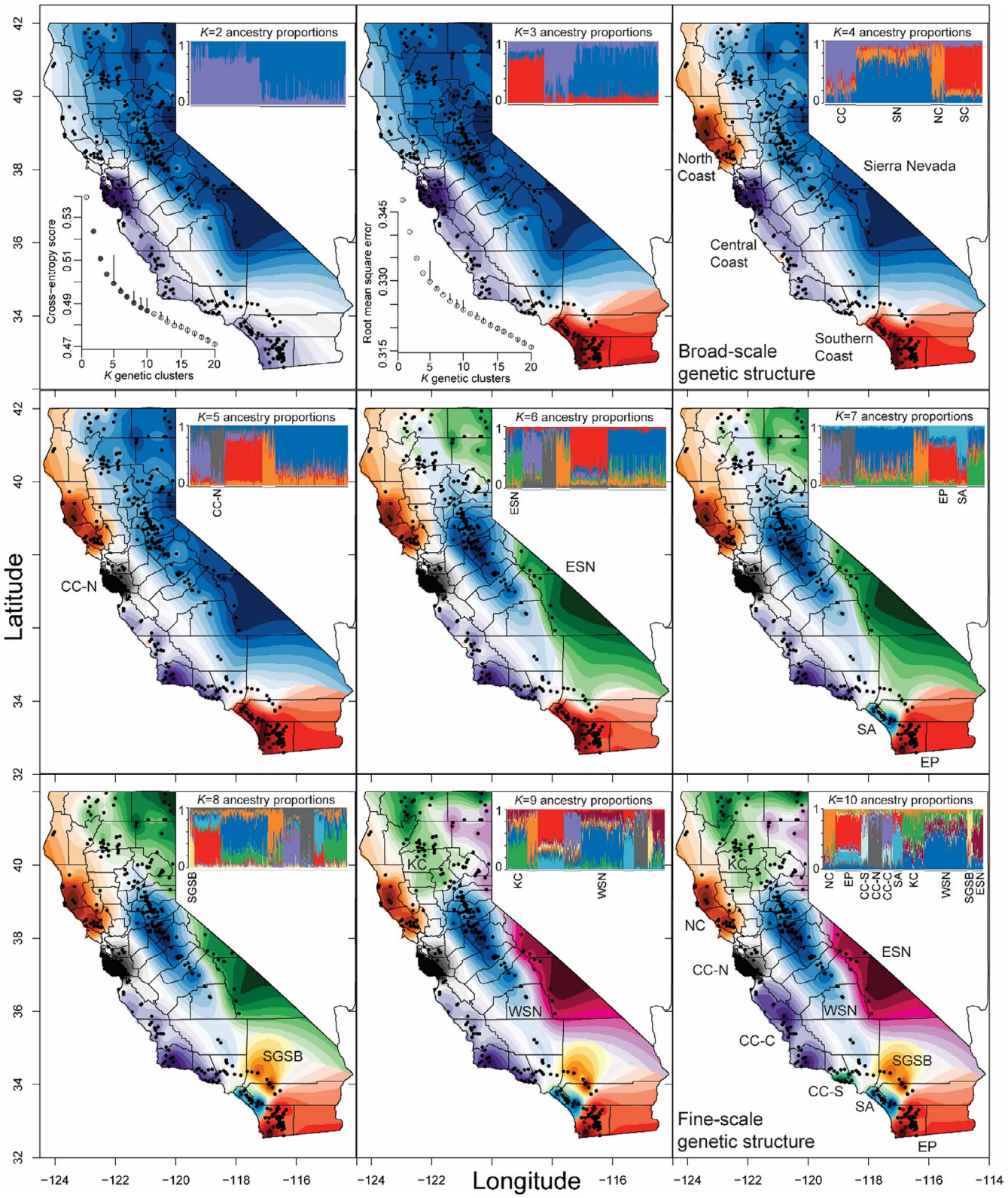

FIGURE 3 Interpolated ancestry proportions from *tess3r*, demonstrating the geographic distribution of biologically meaningful genetic clusters (*K*) ranging from 2–10. The "best" iterations of each *K*, based on the cross-entropy score, is presented (shaded circles of inset plot in *K* = 2 panel). Root mean square error is also presented (inset plot in *K* = 3 panel). Both *tess3r* and the PCA (Figure 2) support *K* = 4 and, therefore, the genetic clusters are labeled. At *K* = 10, nested genetic clusters are labeled consistent with previous microsatellite data (Gustafson et al., 2019). For visualization, at each *K*, the genetic cluster that emerges is labeled. In alphabetical order, acronyms include Central Coast Central (CC-C), Central Coast North (CC-N), Central Coast South (CC-S), Eastern Peninsular Range (EP), Eastern Sierra Nevada (ESN), Klamath–Cascades (KC), North Coast (NC), Santa Ana (SA), San Gabriel–San Bernardino (SGSB), and Western Sierra Nevada (WSN)

genetic diversity than the Southern Coast, Central Coast, and North Coast (Table 1). Although significant, the North Coast was the only broad-scale genetic cluster that did not exhibit a strong Wahlund effect (i.e., significantly lower H_O than H_S; SN: $t = -50.6$, $p < 0.001$; SC: $t = -48.2$, $p < 0.001$; CC: $t = -58.5$, $p < 0.001$; NC: $t = -10.6$, $p < 0.001$) or finer-scale substructure. Effective population sizes were not reported for broad-scale clusters because substructure introduced major biases (i.e., near-zero values) into N_e estimates.

Broad-scale genetic clusters were moderately differentiated based on F_{ST} estimates which ranged from ~0.1–0.2 (Table 2). The Sierra Nevada cluster was least differentiated from the others, and the lowest F_{ST} estimates were between the Sierra Nevada and the North Coast clusters. In contrast, the Southern Coast cluster was the most differentiated from the others, and the highest F_{ST} estimates were between the Southern Coast and the North Coast, followed by the Southern Coast and the Central Coast. At the broad scale, the linkage decay plot indicated that linkage disequilibrium (LD) was lowest in the Sierra Nevada and slightly increased in the Central Coast, Southern Coast, and North Coast clusters (Figure 4a). When ignoring population assignments, California pumas ($N = 401$) had an LD R^2 of ~0.3 which decreased rapidly to less than 0.1 at a distance of 0.3 Mbp, then approached 0 at farther distances. Nearly, the same result was observed in the Sierra Nevada. The Central Coast also had a major reduction in LD with distance but did not fall under 0.1 until ~3 Mbp in distance. In contrast, the Southern Coast and North Coast started with an LD R^2 of ~0.4 which remained above 0.1 even at distance of 8 million bp (Figure 4a).

The nested genetic clusters within the Sierra Nevada—including KC, WSN, and ESN—had the highest genetic diversity estimates, as well as the highest estimates of N_e. Only the WSN had an N_e above 50, a threshold commonly considered to be sustainable over the long term (Table 1; Franklin, 1980). Pairwise F_{ST} estimates among nested genetic clusters within the Sierra Nevada suggested weak substructure, with little genetic differentiation (i.e., pairwise $F_{ST} < 0.05$), indicating substantial gene flow throughout this region (Table 2). Within the Sierra Nevada, the ESN showed slightly higher LD than KC or WSN, and all three retained a high proportion of polymorphic loci (i.e., 87–91%).

The nested genetic clusters within the Southern Coast—including EP, SGSB, and the SA—exhibited lower genetic diversity estimates when than the Sierra Nevada, as well as large differences when compared to each other (Table 1). Estimates were generally lowest in SA, whereas EP and SGSB had similar overall estimates. However, both SA and SGSB had extremely low estimates of N_e. Unlike the Sierra Nevada, nested genetic clusters within the Southern Coast had moderate to strong genetic differentiation from one another (pairwise F_{ST} values ~0.1–0.2; Table 2). Except for the moderate differentiation with EP (i.e., pairwise F_{ST} of ~0.1), SA was the most differentiated among the 10 finer-scale genetic clusters (pairwise F_{ST} values range: ~0.2–0.3). The SGSB cluster had relatively lower pairwise F_{ST} estimates with the Sierra Nevada and EP clusters, moderate F_{ST} estimates with CC-C and CC-S, and was more strongly differentiated from the CC-N and NC. The EP cluster showed similar patterns of differentiation but was least differentiated from the geographically

TABLE 1 Heat map of genetic diversity statistics for $K = 4$ broad-scale and $K = 10$ nested fine-scale genetic clusters, including sample size (N), observed heterozygosity (H_O); gene diversity (H_s), proportion of polymorphic loci out of 16,285 (*Poly*), allelic richness corrected for sample size (A_r), private alleles (A_p), and effective population size (N_e)

Genetic diversity	Genetic Cluster									
$K = 4$	SN				SC			CC		NC
N	193				96			79		33
H_O	0.31				0.26			0.24		0.26
H_s	0.34				0.29			0.28		0.27
Poly	0.93				0.79			0.77		0.78
A_r	1.79				1.69			1.67		1.67
A_p	37				34			17		0
$K = 10$	KC	WSN	ESN	EP	SGSB	SA	CC-C	CC-S	CC-N	NC
N	53	110	27	66	13	25	27	17	35	28
H_O	0.32	0.31	0.31	0.27	0.29	0.23	0.27	0.24	0.22	0.25
H_s	0.33	0.33	0.33	0.29	0.30	0.24	0.29	0.27	0.24	0.26
Poly	0.91	0.90	0.87	0.78	0.78	0.64	0.77	0.70	0.63	0.74
A_r	1.33	1.33	1.33	1.29	1.30	1.24	1.29	1.27	1.24	1.26
N_e	28.9	54.4	42.2	14.8	2.3	3.5	26.9	4.1	19.0	14.1

Note: Values for N_e are not presented for the $K = 4$ Sierra Nevada (SN), Southern Coast (SC), Central Coast (CC), or North Coast (NC) because of model assumption violations. There were no private alleles at $K = 10$, including Klamath–Cascades (KC), Western Sierra Nevada (WSN), Eastern Sierra Nevada (ESN), Eastern Peninsular (EP), San Gabriel–San Bernardino (SGSB), Santa Ana (SA), Central Coast Central (CC-C), Central Coast South (CC-S), Central Coast North (CC-N), and North Coast (NC). Heat map colors bound the minimum (white) and maximum (darkest gray) values within rows.

TABLE 2 Heat map of mean pairwise genetic distance values for the broad-scale $K = 4$ and fine-scale $K = 10$ genetic clusters. Weir and Cockerham F_{ST} is presented below the diagonal, and Nei's F_{ST} is presented above the diagonal (WC\Nei)

WC\Nei F_{ST}	Genetic Cluster			
$K = 4$	SN	SC	CC	NC
SN	-	0.133	0.124	0.100
SC	0.129	-	0.173	0.198
CC	0.120	0.173	-	0.156
NC	0.094	0.196	0.156	-

$K = 10$	KC	WSN	ESN	EP	SGSB	SA	CC-C	CC-S	CC-N	NC
KC	-	0.022	0.041	0.141	0.109	0.215	0.117	0.146	0.183	0.093
WSN	0.022	-	0.045	0.149	0.111	0.222	0.121	0.147	0.188	0.126
ESN	0.041	0.045	-	0.163	0.116	0.226	0.168	0.189	0.233	0.183
EP	0.141	0.146	0.166	-	0.130	0.100	0.164	0.196	0.231	0.214
SGSB	0.105	0.106	0.113	0.132	-	0.212	0.140	0.163	0.210	0.205
SA	0.202	0.203	0.221	0.095	0.217	-	0.254	0.287	0.319	0.301
CC-C	0.114	0.116	0.168	0.163	0.141	0.251	-	0.060	0.098	0.164
CC-S	0.137	0.136	0.183	0.192	0.164	0.289	0.059	-	0.148	0.202
CC-N	0.178	0.176	0.237	0.227	0.221	0.320	0.100	0.152	-	0.229
NC	0.090	0.118	0.183	0.211	0.210	0.300	0.164	0.203	0.230	-

Abbreviations: CC, Central Coast; CC-C, Central Coast Central; CC-N, Central Coast North; CC-S, Central Coast South; EP, Eastern Peninsular; ESN, Eastern Sierra Nevada; KC, Klamath–Cascades; NC, North Coast; SA, Santa Ana; SC, Southern Coast; SGSB, San Gabriel–San Bernardino; SN, Sierra Nevada; WSN, Western Sierra Nevada.

All pairwise F_{ST} estimates were significant ($p < 0.001$) based on a bootstrapping analysis using *hierfstat*::*boot.ppfst*.

Heat map colors bound the minimum (white) and maximum (darkest gray) values either below or above the diagonals.

adjacent SA and SGSB clusters. Although EP exhibited LD estimates similar to the Southern Coast as a whole, SGSB and SA started with a high LD R^2 of ~0.5 which decreased to just more than 0.3 at a distance of 0.3 Mbp, then remained high (more than 0.25) at farther distances (Figure 4).

The nested genetic clusters within the Central Coast exhibited the most variation in estimates of genetic diversity (Table 1). The CC-C cluster had the highest diversity within the region, including the largest estimate of N_e. The CC-S cluster had intermediate levels of diversity but exhibited the lowest N_e in the region. The CC-N cluster had as low, or lower, genetic diversity estimates than most of the 10 fine-scale genetic clusters examined overall, but had one of the higher N_e estimates outside of the Sierra Nevada. Differentiation within the Central Coast was moderate overall (pairwise F_{ST} ~ 0.06–0.15) and appeared to correlate with distance (i.e., CC-N more differentiated from CC-S than CC-C; Table 2). Within the Central Coast, CC-C had the lowest LD R^2 values (Figure 4). The CC-N cluster had higher LD values, especially at lower distances between SNPs, and CC-S had among the highest LD R^2 values, comparable to those of SGSB and SA in the Southern Coast.

Finally, the NC had genetic diversity estimates that were lower than those of the Sierra Nevada and comparable to the Southern Coast and Central Coast, with an N_e estimate of 14.1 (Table 1). Overall, the NC showed strong differentiation from the other fine-scale genetic clusters with the exception of KC and WSN for which differentiation was moderate (Table 2). The linkage decay plot indicates that the NC had similar LD R^2 values to that of ESN and EP (Figure 4).

4 | DISCUSSION

Our analyses of genetic diversity and linkage disequilibrium based on 16,285 SNPs from 401 pumas throughout California demonstrated that the complex geography and land use patterns in California result in equally complex patterns of gene flow and population structure. The high-density SNP data provided resolution to detect both four broad-scale genetic clusters with high genetic diversity as well as substructure at a finer scale that we designate as 10 genetic populations with highly variable genetic diversity. Our data further support the notion that puma populations in California form a "horseshoe" network around the Central Valley with San Francisco Bay acting as a barrier to gene flow along the coast (Gustafson et al., 2019). For the Sierra Nevada cluster, the nested finer-scale populations had consistently high genetic variation. However, within the coastal groups, genetic variation within certain fine-scale genetic populations was concerningly low, while others appeared to have retained sufficient variation to be capable of serving as sources of genetic rescue under various management scenarios to restore connectivity. In fact, our linkage decay analysis indicated that populations with low genetic diversity and high linkage disequilibrium may not necessarily share the same fixed loci, consistent with what was suggested by Saremi

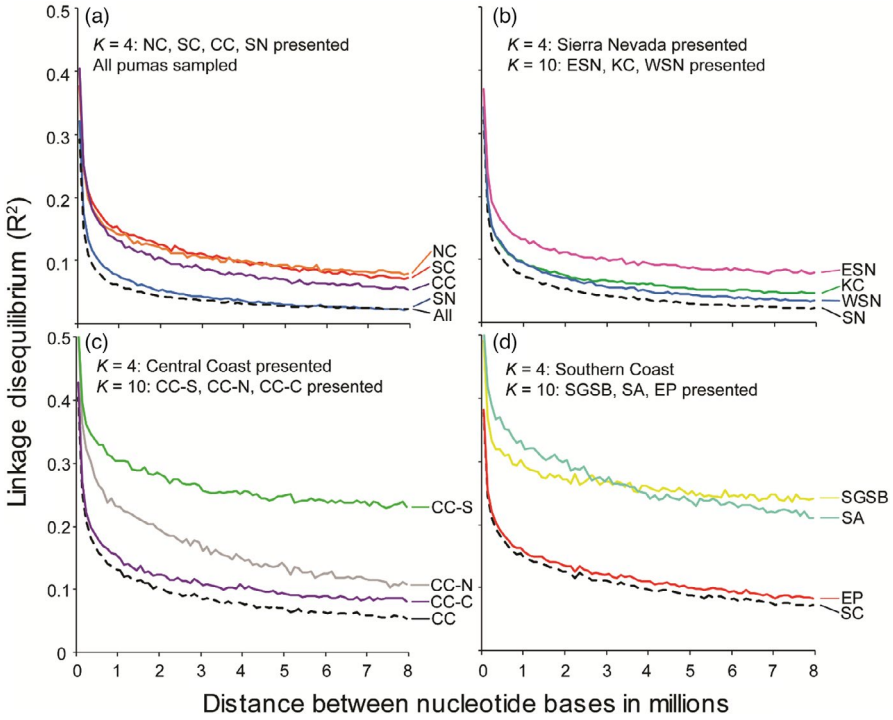

FIGURE 4 Correlation of SNPs with genomic distance, ranging from hundreds to 8 million nucleotides in distance. Based on pairwise estimates from 16,285 SNPs, linkage decay is presented for all 401 pumas sampled in California (All), from the $K = 4$ broad-scale genetic clusters (a: North Coast, NC; Southern Coast, SC; Central Coast, CC; Sierra Nevada, SN) and from the $K = 10$ fine-scale genetic clusters (b–d). Nested and finer-scale clusters are presented within their corresponding broad-scale group. The NC is presented only in the first panel because it did not exhibit substructure. (b) Eastern Sierra Nevada (ESN), Klamath–Cascades (KC), and Western Sierra Nevada (WSN) are nested within SN. (c) Central Coast South (CC-S), Central Coast North (CC-N), and Central Coast Central (CC-C) are nested within CC. (d) San Gabriel–San Bernardino (SGSB), Santa Ana (SA), and Eastern Peninsular Range (EP) are nested within SC. In each figure, the dashed line represents the broadest-scale designation within the group

et al. (2019). Specifically, when individuals from nested populations were combined within the four broader-scale groups, linkage decay values were much lower, indicating variation still exists among populations. Therefore, maintaining and enhancing connectivity within and among broad-scale groups could increase genetic diversity to entire regions and could decrease the apparent effects of genetic drift and inbreeding to some at-risk coastal populations (Ernest et al., 2003, 2014; Gustafson et al., 2017; Riley et al., 2014).

The support for four broad-scale genetic groups from SNPs is different than previous studies using microsatellites (Ernest et al., 2003; Gustafson et al., 2019), indicating the importance of using genomic methods in the study of broader-scale wildlife conservation genetics. Our data further support the claim that the Sierra Nevada region is a major refugium of puma genetic diversity in California (Gustafson et al., 2019). Therefore, it is important to protect the Sierra Nevada group from habitat degradation and foster conservation actions that can enhance gene flow with the North Coast, Central Coast, and Southern Coast clusters as well as with the Great Basin to the east (Gustafson et al., 2019). The broad-scale Southern Coast group is least connected to the other genetic clusters in the state but had higher genetic diversity and more private alleles than the Central Coast or North Coast. This indicates that the Southern Coast group retains unique genomic variations that must be conserved in order to maximize genetic diversity among pumas in California. Furthermore, our finding of greater genetic diversity at lower latitudes is consistent with a previous study of gene flow among puma populations across southwestern North America, which found both higher microsatellite allelic diversity and a greater number of private alleles among pumas in southern Arizona and New Mexico (McRae et al., 2005). Those authors suggested that the pattern was consistent with recolonization of North America following a late-Pleistocene extinction (Culver et al., 2000); range expansion from the south was accompanied by decreasing diversity in more northern populations because of serial founder events. Our finding of high genetic diversity in the Southern Coast group suggests the genetic legacy of recolonization is generally consistent across the contemporary range of pumas in North America.

Although the four major genetic clusters are highly consistent among our structure analysis and PCA, there was also statistical support for substructure (i.e., *tess3r* results and moderate to high pairwise F_{ST} values within and among the broad-scale groups), indicating 10 genetic populations at a finer scale. Generally, the 10 genetic populations identified with SNPs correspond strongly to those identified in previous studies using microsatellite markers and different samples (Ernest et al., 2003; Gustafson et al., 2019). However, the northern-most Klamath–Cascade population was not observed

previously with microsatellites (Gustafson et al., 2019). This is likely because there were very few pumas available for analysis in the Klamath or Cascade Mountains during the 2019 microsatellite study. It is also possible that 42 microsatellites may not have been sufficient to detect the low genetic differentiation ($F_{ST} = 0.022$) observed between the Klamath–Cascade and Western Sierra Nevada populations. The 10 populations varied considerably in genetic diversity estimates (H_O range 0.22–0.32; H_S range 0.24–0.33; *Poly* range: 0.63–0.91; A_r range: 1.24–1.33), effective population sizes (N_e range 2.3–54.4), and genetic differentiation (F_{ST} range: 0.22–0.32) as discussed below.

A major difference between this and previous studies is the observation that pumas in the Central Coast North population have genetic diversity estimates as low as those in the Santa Ana and Central Coast South populations, which are highly isolated by urbanization and transportation infrastructure and exhibit evidence of inbreeding depression (Benson et al., 2020; Ernest et al., 2014; Gustafson et al., 2017; Riley et al., 2014; Vickers et al., 2015). Our results are consistent with those of Saremi et al. (2019), which indicated that inbreeding metrics between pumas from the Santa Monica Mountains (in Central Coast South) and pumas from the Santa Cruz Mountains (in Central Coast North) were similar. Interestingly, N_e for the Central Coast North was much higher than that in both the Santa Ana and Central Coast South populations. These observations are consistent with a large breeding population experiencing genetic drift due to dispersal barriers to the north (i.e., San Francisco Bay) and gene flow occurring only with the Central Coast Central population to the south. This pattern could also be driven by carrying capacity processes associated with habitat limitations (Dellinger et al., 2020). If dispersal is limited by continued development southeast of the Central Coast North population, rapid genetic drift and inbreeding may ensue (Mills & Allendorf, 1996; Wang, 2004), and local extinctions may occur as predicted in the Central Coast South and Santa Ana populations (Benson et al., 2016, 2019). Thus, puma population viability will be facilitated when land management agencies and land developers in the region work proactively to preserve or enhance wildlife corridors.

Notably, the San Gabriel–San Bernardino population had the lowest N_e, but had intermediate levels of genetic diversity. Occasional migrants could alter N_e estimates and temporarily inflate estimates of heterozygosity (Gustafson et al., 2017). We suggest this could also be the result of metapopulation dynamics—that is, a small local population with frequent turnover located at the intersection of dispersal corridors for the Sierra Nevada, Central Coast, and Southern Coast groups. Although the genetics of this population are complex and somewhat uncertain, this region is of critical importance for maintaining state-wide puma gene flow. Enhancing connectivity through the Transverse Ranges (including the Tehachapi Mountains, Sierra Pelona, San Gabriel Mountains, and San Bernardino Mountains; Figure 1b) is a critical conservation priority in order to maintain gene flow between the Southern Coast populations and the Sierra Nevada or Central Coast groups.

The three populations with the lowest N_e, including the San Gabriel–San Bernardino, Santa Ana, and Central Coast South populations, have the smallest available amount of habitat (Dellinger et al., 2020) and had the highest linkage disequilibrium throughout their genomes. As we observed, there was great variation among populations in the decay curves, with the Central Coast North population having the next highest linkage disequilibrium after these three populations. Given the genetic diversity, N_e, and linkage data, the San Gabriel–San Bernardino and Central Coast North populations may be approaching levels of genetic drift and inbreeding similar to the well-monitored and genetically depauperate Santa Ana and Central Coast South populations (Ernest et al., 2014; Gustafson et al., 2017; Riley et al., 2014).

Populations with intermediate genetic diversity include the North Coast, Central Coast Central, and Eastern Peninsular Range. Measures of genetic diversity were lower than expected for the North Coast population, given that there are no obvious anthropogenic barriers to gene flow with the Klamath–Cascade, Western Sierra Nevada, or pumas from Oregon (Gustafson et al., 2019). However, the majority of our samples from this genetic cluster came from just north of San Francisco Bay, an area of substantial human density and restricted gene flow on three sides. Thus, our results may not be truly representative of this region as a whole and may represent the most isolated pumas on a "peninsula" of habitat. Future studies would benefit from increased sampling throughout this genetic cluster, north to (and including) Oregon. Nonetheless, pumas and other animals would benefit if decisions for future development between the North Coast and Sierra Nevada consider the future connectivity of private timber land holdings along the coast with the inland national forests.

The Central Coast Central population has ample habitat for maintaining a breeding population (Dellinger et al., 2020). Given the apparent absence of gene flow across the Central Valley, this population may be the only consistent source of migrants for the Central Coast North and Central Coast South, which have concerningly low levels of genetic diversity and evidence of inbreeding. Thus, we consider the Central Coast Central population to be essential for the long-term viability of both adjacent populations and urge that habitat in this region is not fragmented further.

Despite having less than half of the overall habitat of the Central Coast Central population (Dellinger et al., 2020), the Eastern Peninsular Range population has roughly similar genetic diversity estimates, but a much lower N_e. Dispersal in and out of the Eastern Peninsular Range is extremely limited, and the degree to which pumas disperse across the border between USA and Mexico remains unknown (Gustafson et al., 2019). Given that the Eastern Peninsular Range is the only population known to exchange individuals with the Santa Ana population, management actions which enhance gene flow between these areas remain critical to the recovery of pumas in the Santa Ana Mountains.

Our linkage decay analysis suggests that in the Central Coast South, San Gabriel–San Bernardino, Santa Ana, and perhaps the Central Coast North populations, pumas may have long runs of

homozygosity that are identical by descent. This is consistent with the genome resequencing results of Saremi et al. (2019) in the Santa Cruz (i.e., Central Coast North) and Santa Monica Mountains (i.e., Central Coast South), which suggested that close and recent inbreeding led to runs of homozygosity. Although Saremi et al. (2019) sequenced individuals only from California populations known to have low genetic diversity, our linkage decay results from populations throughout the state indicate that the genome-level problems of inbreeding are not universal throughout California. Instead, the Klamath–Cascades, Western Sierra Nevada, Eastern Sierra Nevada, Central Coast Central, and the Eastern Peninsular Range populations all have low linkage disequilibrium throughout the genome. Additionally, when the inbred populations are analyzed with their broad-scale group, linkage decay curves demonstrated the potential for gene flow with adjacent populations to reduce linkage to negligible levels. We observed up to 30–37% of the SNPs as fixed in the Central Coast South, Santa Ana, and Central Coast North populations. Our linkage decay curves and the resequencing results of Saremi et al. (2019) demonstrate that fixed regions of the genome often differ among populations. Thus, genetic restoration is possible even among genetically depauperate populations. When considering that genetic diversity is much higher in several California puma populations than in those heavily studied along urban coasts, there is high potential for the long-term persistence of pumas throughout the majority of the state.

Genetic restoration or rescue has been successfully demonstrated for isolated, large-felid populations, such as the African lion (*Panthera leo*; Miller et al., 2020) and Florida panther (*P. concolor*; Ralls et al., 2018). There have also been calls for genetic rescue of other large felids, such as isolated populations of tigers (*Panthera tigris*; Armstrong et al., 2021) and leopards (*Panthera pardus*; Perez et al., 2006). Thus, it is becoming increasingly evident that large-bodied cats and other apex predators will need habitat and connectivity for long-term evolutionary survival. Natural events of genetic restoration among fragmented populations of pumas in California (Ernest et al., 2014; Gustafson et al., 2017; Riley et al., 2014), combined with our linkage decay analysis, indicates that pumas and other apex predators may need to be managed in a metapopulation framework that incorporates genomic data (Farquharson et al., 2021).

We tested for outlier loci using multiple methods (Narum & Hess, 2011), but found no evidence of local adaptation when $K = 4$ or $K = 10$. Detection of outlier loci with RAD-seq is limited by the reduced representation of the genome, yet it has often been shown to be an effective approach (Catchen et al., 2017). Pumas are long-distance dispersers (Hawley et al., 2016; Sweanor et al., 2000) and inhabit all major mountain ranges in California (Dellinger, Gustafson, et al., 2020), suggesting that local adaptation may be unlikely. Our results provide preliminary evidence that outbreeding depression resulting from potential active genetic management may be of minimal concern (Frankham et al., 2011). Recent modeling (Kyriazis et al., 2021) does suggest, however, that attempts to maximize genetic diversity in a population can introduce hidden deleterious recessive mutations, enhancing extinction risk. The modeling of Kyriazis et al. (2021) has faced criticisms (García-Dorado & Caballero, 2021), however, and Ralls et al. (2020) argue that the benefits of increasing genetic diversity outweigh the risks. Thus, managers could consider actions (e.g., wildlife overpasses/underpasses, translocation of individuals between populations, etc.) to improve viability of some coastal populations, as was empirically demonstrated to have shifted the trajectory of Florida panther population from extinction (Ralls et al., 2018). However, we suggest whole-genome resequencing methods better suited for detecting selection (Fuentes-Pardo & Ruzzante, 2017) be implemented before such efforts, especially over long distances. Managers would also need to consider other risks as well, such as the movement of pathogens or the ethical implications of moving large carnivores (Bevins et al., 2012). Wildlife managers will have to weigh these concerns against their obligation to minimize the risks of extirpation, such as those predicted for the Santa Ana and Central Coast South populations (Benson et al., 2019), and shown here to be a concern in the Central Coast North population as well. Should connectivity be re-established, then these factors, as well as possible local adaptation, should be weighed carefully. It is our opinion that current efforts to construct or improve wildlife crossing structures that can facilitate natural movement among coastal populations should be considered the primary management strategy for conserving viable puma populations in that region.

5 | CONCLUSION

Our population genomic analyses provide decision makers a contemporary and thorough evaluation of the genetic diversity, effective population sizes, and connectivity of puma populations throughout California. These state- and genome-wide results are critically important for conservation and management practices in California, especially considering the increasing demand for development and the current political climate surrounding the petition to list pumas in Southern and Central California as threatened under the California Endangered Species Act (Yap et al., 2019). In brief, puma populations are widespread throughout the mountains of California. Populations range from major genetic sources to populations with issues of low genetic diversity and inbreeding. Multiple lines of evidence suggest that inbred populations do not share the same runs of homozygosity and, therefore, genetic diversity could be restored through enhanced gene flow. Current challenges to puma populations are highly regional and should be addressed by focusing on how natural geography and human development impacts puma habitat and movements locally. Attention is understandably given to those populations that are highly imperiled, but it is important to note that California has several thriving populations throughout the state which represent an important resource for any genetic management strategy. Protecting tracts of contiguous habitat to preserve large populations will provide greater protection for the species as a whole. Specifically, further fragmentation of habitat in the Sierra Nevada group could be catastrophic to population viability of pumas in

the state because it serves as a genetic refugium. Protecting, enhancing, and creating movement corridors to allow state-wide "stepping-stone" connectivity at broad and fine scales will allow for the migrants needed to counteract the local extirpations faced by some coastal populations.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Individual genotype data and associated location data are provided on Open Science Framework https://doi.org/10.17605/OSF.IO/HUF4K.

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