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Neo-sex chromosome evolution and phenotypic differentiation across an elevational gradient in horned larks (*Eremophila alpestris*)

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Abstract

Genetic structure and phenotypic variation among populations are affected by both geographic distance and environmental variation across species' distributions. Understanding the relative contributions of isolation by distance (IBD) and isolation by environment (IBE) is important for elucidating population dynamics across habitats and ecological gradients. In this study, we compared phenotypic and genetic variation among Horned Lark (*Eremophila alpestris*) populations from 10 sites encompassing an elevational gradient from low-elevation desert scrub in Death Valley (285 a.s.l.) to high-elevation meadows in the White Mountains of the Sierra Nevada of California (greater than 3000 m a.s.l.). Using a ddRAD data set of 28,474 SNPs aligned to a high-quality reference genome, we compared genetic structure with elevational, environmental, and spatial distance to quantify how different aspects of the landscape drive genomic and phenotypic differentiation in Horned Larks. We found larger-bodied birds were associated with sites that had less seasonality and higher annual precipitation, and longer spurs occurred in soils with more clay and silt content, less sand, and finer fragments. Larks have large neo-sex chromosomes, and we found that associations with elevation and environmental variation were much stronger among neo-sex chromosomes compared to autosomes. Furthermore, we found that putative chromosomal translocations, fusions, and inversions were associated with elevation and may underlie local adaptation across an elevational gradient in Horned Larks. Our results suggest that genetic variation in Horned Larks is affected more by IBD than IBE, but specific phenotypes and genomic regions—particually on neo-sex chromosomes—bear stronger associations with the environment.

KEYWORDS
Alaudidae, chromosomal inversion, elevation, genome architecture, landscape genomics, neo-sex chromosomes, Sierra Nevada, structural variation

1 | INTRODUCTION

Landscapes affect gene flow among populations, and quantifying population genetic structure and phenotypic variation across variable environments is a longstanding goal in molecular ecology (Richardson et al., 2016). Genetic and phenotypic differences among populations are influenced by various factors, including geographic distance, environmental variation, biotic interactions, and other phenomena that vary over space and time (Endler, 1977; Gould & Johnston, 1972; Zamudio et al., 2016). As geographic distance among populations increases, the accumulation of localized variation results in genetic differentiation through a process known as isolation by distance (IBD; Rousset, 1997; Slatkin, 1993). Similarly, variation in environmental conditions such as temperature and precipitation can facilitate genetic differentiation through adaptation to local conditions, a process known as isolation by environment (IBE; Manthey & Moyle, 2015; Nosil et al., 2002; Wang & Bradburd, 2014). IBD and IBE can act independently or in concert to influence how genetic variation is partitioned among populations (Sexton et al., 2014; Wang & Bradburd, 2014). Molecular ecologists need a better understanding of how and when IBD and IBE shape intraspecific variation to properly contextualize how genotypes and phenotypes vary across landscapes.

IBD is ubiquitous in nature and impacts genetic variation among populations in all species. IBD has been studied both theoretically (Nei, 1972; Wright, 1943) and empirically (Aguillon et al., 2017; Kuchta & Tan, 2004; Sharbel et al., 2000). Associations between geographic distance and genetic variation occur at all scales, from small, localized patterns within ecosystems to larger distributional ranges across continents (Meirmans, 2012). IBD is most prevalent in organisms with limited dispersal (Bohonak, 1999), and has been quantified most frequently using Mantel tests and partial Mantel tests (Rousset, 1997). However, autocorrelation between geographic distance and other factors—such as environmental variation, competition and other selective forces—also impacts spatial partitioning of genetic variation (Bradburd et al., 2013; Meirmans, 2012; Spurgin et al., 2014).

Environmental variation across a species' distribution also affects its population structure. Developments in landscape genetics have shown that environmental variation plays an important role at all spatial scales (Wang & Bradburd, 2014). Environmental heterogeneity—especially if present as a gradient or ecotone—can produce patterns resulting from IBE through local adaptation to changing environmental conditions, and these patterns can be difficult to distinguish from patterns that result from IBD (Forester et al., 2016; Nosil & Sandoval, 2008). While disentangling IBD and IBE can be challenging, advances in the accessibility of fine-scale GIS data and large genomic data sets have facilitated quantitative assessments of IBD and IBE (Van Buskirk & Jansen van Rensburg, 2020; Manthey & Moyle, 2015; Safran et al., 2016; Wang & Bradburd, 2014).

To tease apart the contributions of geographic distance and environmental variation to genetic differentiation, researchers must employ sampling strategies that traverse environmental gradients and geographic distances appropriate for the dispersal ability of the organism being studied. Several studies of IBD and IBE in non-model organisms have succeeded in demonstrating the relative contributions of geography and environment to genetic differentiation (Nosil et al., 2012; Sexton et al., 2014; Shafer & Wolf, 2013). Such studies have used increasingly large panels of neutral loci spread across the genome to more accurately estimate parameters of genetic differentiation, as different regions of the genome may show signals of IBD and/or IBE (Shafer & Wolf, 2013; Wang & Bradburd, 2014). For example, studies with even a modest data set of mitochondrial DNA and a few nuclear loci have found IBD and IBE to be associated with different proportions of genetic diversity (Wang et al., 2013). Using a much larger, genome-wide data set, Manthey and Moyle (2015) identified multiple loci associated with environmental factors among White-breasted Nuthatches across the Madrean archipelago. These findings indicate that the genomic signature of IBE may be localized on portions of the genome that are independent of IBD signals within the same populations. Using genome-wide data from multiple individuals per population of a widely distributed, well-sampled species is important to properly quantify the relative contributions of IBD and IBE in any system.

The Horned Lark (*Eremophila alpestris*) is a widespread, granivorous songbird that provides an excellent system to study the effects of geographic and environmental variation on population structure. Horned Larks have extensive phenotypic variation across a distribution of open, sparsely vegetated habitats extending from Alaska to Colombia in the Western Hemisphere and from the Siberian Tundra to the Atlas Mountains in the Eastern Hemisphere (Oberholser, 1902). Forty-two subspecies of Horned Larks have been described on the basis of variation in body size and coloration; 26 subspecies occur in North America and 17 in the western United States alone (Beason, 2020; Behle, 1942; Clements et al., 2019; Oberholser, 1902). While most populations of Horned Larks are resident throughout the year (Beason, 2020), there is some evidence of seasonal migration at high latitudes and elevations (Behle, 1942). Dorsal coloration and size in larks are often associated with habitat and soil type (Donald et al., 2017; Mason et al., 2021; Mason & Unitt, 2018), although there is considerable variation among local populations (Niles, 1973; Oberholser, 1902). The extensive phenotypic diversity of Horned Larks across close geographic regions makes this species ideal for studying the role of geographic distance and environment in shaping genetic and morphological variation (Mason et al., 2014).

We collected morphological, ecological, and genetic data to quantify phenotypic and genetic associations among Horned Larks along an elevational transect in eastern California and western Nevada. Our sample localities span a large elevational gradient extending from Death Valley (below 0 m a.s.l.) to the top of the White Mountains (greater than 3000 m a.s.l.), and encompass a diverse range of habitats from lowland desert scrub to alpine fell fields (Table 1). For each of the 10 localities, we obtained morphological data from multiple Horned Lark specimens, extracted environmental data using geographic coordinates representing the collecting locality of each specimen, and sequenced genetic data from thousands of loci using next-generation sequencing methods to evaluate the roles of geographic and environmental variation in

TABLE 1 Locality data for specimens used in this study with habitat and the EPA Level IV ecoregion classification

Locality	Coordinates	Elevation (m)	Number of individuals	Habitat	US EPA level IV name
California: Mono County, White Mountains	37.55323 N, 118.22479 W	3540	17 (m = 8; f = 9)	Alpine	Sierra Nevada-Influenced High Elevation Mountains
California: Mono County, Sweetwater Mountains	38.45854 N, 119.30416 W	3290	7 (m = 3; f = 4)	Alpine	Sierra Nevada-Influenced High Elevation Mountains
Nevada: Lyon County, Bald Mountain	38.53440 N, 119.11500 W	2806	8 (m = 5; f = 3)	Montane Brushland	Sierra Nevada-Influenced High Elevation Mountains
California: Inyo County, Inyo Mountains	37.05265 N, 118.04860 W	2341	12 (m = 5; f = 7)	Montane Brushland	Sierra Nevada-Influenced Ranges
Nevada: Mineral County, Basalt	38.01263 N, 118.27870 W	1915	12 (m = 8; f = 4)	High elevation desert	Tonopah Sagebrush Foothills
Nevada: Esmeralda County, Clayton Valley	37.74361 N, 117.59000 W	1303	10 (m = 6; f = 4)	Mid-elevation desert	Lahontan and Tonopah Playas
Nevada: Nye County, Sarcobatus Flat	37.04450 N, 116.86269 W	1240	19 (m = 9; f = 10)	Low elevation desert	Tonopah Basin
California: Inyo County, Rose Valley	36.04457 N, 117.91611 W	1052	20 (m = 12; f = 8)	Low elevation desert	Western Mojave Basins
California: Kern County, Hanning Flat	35.6886 N, 118.36096 W	844	18 (m = 9; f = 9)	Low elevation desert	Tehachapi Foothills
California: San Bernardino County, NW side of Soda Lake	35.18161 N, 116.10509 W	285	17 (m = 9; f = 8)	Low elevation desert	Death Valley/Mojave Central Trough

Note: Voucher data available in Table S1.

shaping genotypic and phenotypic variation across an elevational transect.

Another goal of our study was to assess how genetic differentiation is distributed across the genome. Karyotypic studies have shown that larks have exceptionally large sex chromosomes (Bulatova, 1973). In Horned Larks, at least three autosomes may have fused with the sex chromosomes (Dierickx et al., 2020; Sigeman et al., 2019) to form neo-sex chromosomes (Pala et al., 2012). Sex chromosomes have played a major role in local adaptation, divergence, and speciation in many avian systems, especially in the case of selection acting on the larger Z-chromosome (Bourgeois et al., 2020; Irwin, 2018; Pala et al., 2012). Structural rearrangements of chromosomes, often termed supergenes due to their tight linkage and suppressed recombination, underlie adaptive evolution in many systems and have gained increasing attention as drivers of lineage diversification alongside the advent of improved sequencing and bioinformatics methods (Weissensteiner et al., 2020; Wellenreuther et al., 2019). Specifically, chromosomal inversions and translocations have been implicated in divergence and selection in several avian systems (Faria & Navarro, 2010; Hoffmann & Rieseberg, 2008; Hooper et al., 2019; Hooper & Price, 2017; Rieseberg, 2001; Wilson & Makova, 2009). Thus, we also assessed whether structural rearrangements may be involved in driving genomic variation in Horned Larks across the elevational gradient we studied.

2 | MATERIALS AND METHODS

2.1 | Data collection

2.1.1 | Specimen collection

Horned Lark specimens and tissue samples were collected between June 1977 and May 1989 across ten sampling locations in eastern California and western Nevada that ranged in elevation from 285 to 3540 m ($n = 140$, Figure 1a; Table 1; Table S1). Sample sizes per locality varied from seven to 20 individuals. Three additional individuals of *E. a. peregrina* from Embalse de la Copa, Boyaca Dept., Colombia were sampled for blood and included as an outgroup. All specimens and tissues from the US are housed in the Museum of Vertebrate Zoology, Berkeley, California.

2.1.2 | Morphological measurements

We compiled a data set of the following eight morphological characters measured from each specimen: wing chord length, tail length, bill length, bill depth, bill width, tarsus length, middle toe length, and hallux length. Measurements were taken by Ned K. Johnson using dial calipers as part of a larger unpublished study of Horned Lark variation in the western US (see Johnson, 1980 for methodological details; Supplementary Table S1).

2.1.3 | Genetic data

DNA was isolated from frozen tissues using the Qiagen DNeasy Kit (Valencia), and concentrations were evaluated using a Qubit 2.0 fluorometer (Life Technologies). Double-digest Restriction-site Associated DNA (ddRADseq) libraries were prepared following the protocol described in Peterson et al. (2012) with the following modifications: 500 ng of DNA from each individual was digested using 0.5 μl of EcoRI (0.1 U/μl) and 0.5 μl of SphI-HF (0.1 U/μl) at 37°C for 3 h. P1 and P2 barcoded adapters were ligated onto the ends of digested fragments and pooled into sets of 24 samples. Each pool was PCR-amplified and uniquely indexed with Illumina adapters (P5 and P7) prior to size selection. Fragments between 500 and 800 bp were selected using a Pippen Prep electrophoresis cassette (Sage Science). The pooled library was then tested for quality and quantity of DNA using quantitative PCR and sequenced on 78% of one lane of 150-bp single-end reads on an Illumina HiSeq 4000 platform at the Vincent J. Coates Genomics Sequencing Laboratory of the California Institute for Quantitative Biosciences.

We used the process_radtags function from the Stacks v2.53 pipeline (Catchen et al., 2013; Rochette et al., 2019) to demultiplex our reads. Each raw read fastq file was aligned to the *Eremophila alpestris* genome CLO_EAlp_1.0 (Mason et al., 2020; GenBank assembly accession: GCA_009792885.1) using the mem function in BWA v0.7.17-r1188 (Li & Durbin, 2009) with default settings. Files were converted from SAM to BAM format and reads were sorted using Samtools v1.10 (Li et al., 2009). The genome-aligned reads were assembled into RAD loci using ref_map.pl code implemented through Stacks v2.53 (Catchen et al., 2011, 2013; Rochette et al., 2019). The populations pipeline implemented through Stacks was used to export read data into various formats for subsequent analyses. We included only loci with a minimum of 80% completeness of individuals per population (-r flag) and with a minimum allele frequency (-min-maf flag) of 0.05. For analyses that required unlinked SNPs, we included the --write-random-snp flag implemented through populations to obtain only a single random SNP from each locus. Raw reads are available via the NCBI Short Read Archive (PRJNA769069).

2.1.4 | Environmental data

We used Bioclim environmental variables and ISRIC soil data to associate the genetic and morphological data with the environmental characteristics of specimen collecting localities. For each locality, we obtained 19 Bioclim variables (Fick & Hijmans, 2017) at a spatial resolution of 30 arc seconds through CHELSA (Karger et al., 2017), along with five measurements of soil data—bulk density, clay content, coarse fragment volume, sand volume, and silt volume—at a resolution of 250 m through the ISRIC SoilGrids database (https://www.isric.org/explore/soilgrids).

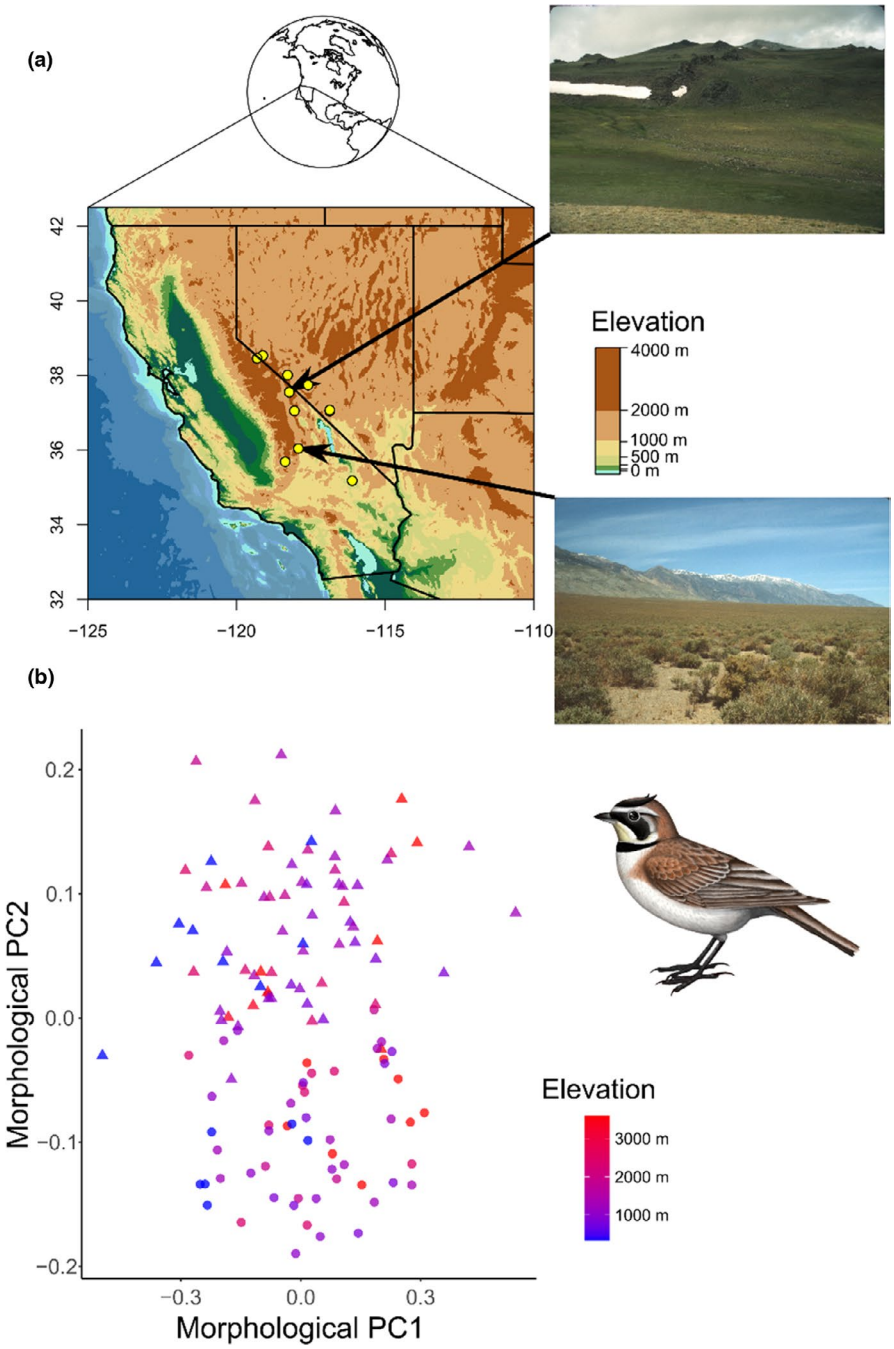

FIGURE 1 (a) Map depicting collecting localities across the southwestern USA along with photographs of a representative high-elevation and low-elevation habitat (image credit: Carla Cicero). (b) PCA summarizing the variance among individuals in morphology, incorporating variation among eight individual traits. Triangles represent females, circles represent males, and the colour of each point corresponds to elevation. Illustraton provided by Subir B. Shakya [Colour figure can be viewed at wileyonlinelibrary.com]

2.2 | Population structure analyses and data filtering for neo-sex chromosomes

Using the vcf file generated through the pipeline populations, we assessed population structure with STRUCTURE v2.3.4 (Pritchard et al., 2000) implemented through ParallelStructure (Besnier & Glover, 2013) on the CIPRES Science Gateway (Miller et al., 2010). STRUCTURE was initiated for 50,000 generations with a burnin of 5,000 for k ranging from 1 to 6. For each value of k, we ran five independent runs. We used the program StructureHarvester (Earl & vonHoldt, 2012), which makes use of the Evanno et al. (2005) method, to identify the best value of k. We visualized the data with principal component analysis (PCA) using the glPCA function from the R-package adegenet v2.0.1 (Jombart, 2008).

Both the STRUCTURE and PCA analyses resulted in clusters by sex when all loci were considered together. Because at least three autosomes have fused with sex chromosomes to form neo-sex chromosomes in larks (Dierickx et al., 2020; Sigeman et al., 2019), and such fusion is likely to affect the STRUCTURE and PCA plots as well as any downstream analyses, we analysed autosomal and neo-sex scaffolds separately. Hence, we created two separate data sets: (1) Z chromosome and associated putative neo-sex chromosome loci (subsequently referred to as the neo-sex data set) and, (2) autosomal loci. Dierickx et al. (2020) and Sigeman

et al. (2019) showed chromosomes corresponding to Zebra Finch (*Taeniopygia guttata*) chromosomes Z and portions of chromosomes 3, 4A, and 5 are associated with the neo-sex chromosomes in Alaudidae. Because the Horned Lark genome has not been annotated, we identified Z chromosomes and associated neo-sex chromosomes by aligning protein coding sequences from the chromosome-level genome assembly of Zebra Finch (Warren et al., 2010) to the Horned Lark. To achieve this, we created a BLAST database using the *makeblastdb* function of the NCBI blast+ package (Camacho et al., 2009), and then used coding sequences from the Zebra Finch assembly bTaeGut1_v1 (GenBank assembly accession: GCA_003957565.2) to align Zebra Finch coding sequences to the Horned Lark assembly. We kept only the best hit for each coding sequence, with e-values less than $1e^{-10}$, and tabulated the number of coding sequences for each of the Zebra Finch chromosomes present in a scaffold of Horned Lark. Where five or more Zebra Finch coding sequences mapped to a Horned Lark scaffold, we assigned the corresponding Zebra Finch chromosome number to that scaffold. Because of the strong synteny present among bird genomes (Zhang et al., 2014), most Horned Lark scaffolds were assigned to only one Zebra Finch chromosome. Two scaffolds (WMCF01000023.1 and WMCF01000024.1) aligned to two different Zebra Finch chromosomes (Z and 5, and Z and 4A, respectively). Of the 2714 scaffolds, 2106 scaffolds (77.6%) did not match any Zebra Finch chromosome. However, the 2106 scaffolds correspond to only 0.0168 Gb of the 1.0410 Gb Horned Lark genome (1.6%). Using data from Dierickx et al. (2020) and Sigeman et al. (2019) as well as our mapping data, we separated the scaffolds corresponding to Zebra Finch chromosomes Z, 3, 4A, and 5 from the vcf files and combined them to make the neo-sex data set. We decided to use all the scaffolds corresponding to choromosomes 3, 4A, and 5 because we could not confidently place individual scaffolds from these chromosomes as autosomal or neo-sex.

We verified the assignments to Horned Lark scaffolds as autosomal or sex-linked by calculating the average coverage for each scaffold for all males and all females, and then calculating the ratio of coverage of scaffold to average coverage of autosomal scaffold. Average coverage of autosomal scaffold was estimated by calculating mean coverage of all scaffolds not assigned to Zebra Finch chromosome 3, 4A, 5 and Z (Figure S1). Among the first 100 scaffolds, four of the eight scaffolds assigned to the Z chromosomes had half the coverage in females with very little variability. The two neo-sex scaffolds (WMCF01000023.1 and WMCF01000024.1) had intermediate coverage, 0.76 (s.d. 0.03) and 0.62 (0.04) respectively, between those for autosomes and Z chromosome in females (Figure S1). This variability in coverage supports our decision to include all scaffolds mapping to 3, 4A, 5, and Z as part of the neo-sex data set, because we cannot confidently assess the chromosomal locality of all scaffolds without a chromosome-level reference assembly.

We also attempted to categorize and phase out neo-Z and neo-W SNPs from neo-sex scaffolds. To ensure that we could phase the data, we calculated the frequency of homozygous and heterozygous alleles for each highly differentiated SNP (i.e., SNPs with greater than 3 standard deviation values of loadings on PC1 axis for that particular scaffold) in four scaffolds with the strongest neo-Z and neo-W associations. We then checked to see if the SNPs segregated into homozygous for males (0/0 or 1/1) and heterozygous for females (0/1). We expected this pattern as males have two copies of the Z chromosome and females have one each of the Z and W chromosome. Hence, if the SNP is part of both neo-Z and neo-W, females will be heterozygous and males will be homozygous. We checked this association for 66 SNPs from the four scaffolds. Because only 34 of the 66 SNPs showed this pattern, we did not move forward with phasing the data into neo-Z and neo-W (Figure S2).

2.3 | Association of morphological data with environment and geographic distance

We ran a PCA for the morphological, environmental, and soil data sets using the function prcomp in R v3.6.1 (R Core Team, 2017). To account for spatial autocorrelation, we calculated Moran's eigenvector maps (MEMs) using genetic data, repeated independently for the autosomal and neo-sex data sets, and cartesian coordinates of each site using the mgQuick function in the package memgene (Galpern et al., 2014). Since both the autosome and neo-sex data sets produced similar values for MEMs, we only used the values obtained from the autosomal data for further analyses. To look for associations between the morphological and environmental data sets, we compared PC1 and PC2 of the morphological data, along with each individual morphological character, with PC1 of the environmental data and PC1 of the soil data using a linear mixed model implemented with the lme function of the R package nlme (Pinheiro et al., 2021). We also included sex and MEMs for MEMGENE1 as a fixed effect. We performed a redundancy analysis (RDA) using the function rda from the package vegan (Dixon, 2003) for the morphological PC axes to check for linear dependence with environmental and soil data.

2.4 | Association of genetic data with environment and geographic distance

We analysed the autosomal data set using STRUCTURE and PCA as described above. For each value of k (1–5), we initiated five independent STRUCTURE runs for 50,000 generations with a burnin of 5000. Using the Evanno method (Evanno et al., 2005), we estimated the value of k with the best likelihood score, and we then initiated 10 independent STRUCTURE runs for that value of k for 500,000 generations with a burnin of 50,000.

We also ran PCA analyses for each individual scaffold in the neo-sex data set using glPCA to assess variation among the scaffolds. We were only able to generate PCAs for 25 of the 53 scaffolds of the neo-sex data set because the remaining scaffolds did not have enough variants for glPCA to generate PC axes. For three neo-sex scaffolds (WMCF01000011.1, WMCF01000023.1, and WMCF01000024.1), we visualized synteny with the Zebra Finch

using the coding sequence matches generated from BLAST. Since these scaffolds cluster by sex, we calculated Weir and Cockerham F_{ST} (Weir & Cockerham, 1984) implemented through vcftools v0.1.13 (Danecek et al., 2011) between males and females in order to identify regions within the scaffold that are specific to either sex.

To assess geographic patterns of genetic structure and admixture, we used the program SpaceMix v0.13 (Bradburd et al., 2016) as implemented through R v3.6.1 (R Core Team, 2017). This program requires input from a data set of unlinked biallelic SNPs, which we generated with one SNP per RAD locus for the autosomal and neo-sex data sets separately. We omitted the more distantly related Colombian birds from this and subsequent analyses, because their substantial genetic differentiation from the focal group obscured resolution among our focal Californian individuals. We transformed the data to the input required by SpaceMix using R, and then used the sample function in base R to randomly select which allele would be counted for each locus. We collated SNP calls by population and used population values as input for SpaceMix. We implemented 10 fast runs iterated for 10,000 generations to inform the program about an appropriate starting state and used the best run to conduct a long run iterated for 5,000,000 generations. Convergence and run statistics were assessed in R using functions that are described in the documentation of SpaceMix.

To examine statistical associations between individual SNPs and elevation, we performed an association analysis with PLINK v1.90b6.18 (Purcell et al., 2007) under default settings. We also used BEDASSLE v 1.5 (Bradburd et al., 2013), implemented through R v3.6.1 (R Core Team, 2017) on both the autosomal and neo-sex data sets, to better understand the effects of geographic and environmental variation on genetic variation among our sampled populations. Geographic distance between the locality coordinates of each site was calculated using the spDist function in the sp package in R (Bivand et al., 2013; Pebesma & Bivand, 2005). We analysed three different environmental variables associated with each sampling locality using BEDASSLE: (1) elevation, (2) mean temperature of wettest quarter (Bioclim variable 8), and (3) annual precipitation (Bioclim variable 12). The two individual Bioclim variables were selected because of their high loadings in the bioclimatic PCA (Table S3), suggesting these two variables are major drivers of environmental difference among our sampled sites. Geographic and environmental distances were scaled by dividing each value by the maximum value for each data set. Two independent beta-binomial BEDASSLE MCMC runs were computed for 5 million generations for each of the three environmental variables, sampling every 1,000 generations. Convergence among chains was assessed using R in accordance with the BEDASSLE documentation. A burnin of 2.5 million generations (50%) was used based on the stabilization of the MCMC chain as inspected by trace plots of the posterior distribution in R.

3 | RESULTS

3.1 | ddRAD-loci processing

We recovered a total of 483,801 RAD loci using the Stacks ref_map pipeline with a mean effective per-individual coverage of 33.4x (st. dev. = 14.6×). The mean number of sites per locus was 145.

3.2 | Morphological variation

The PCA of morphological data resulted in a first PC axis with loadings corresponding to variation in body size that explained 56.72% of the total variation (Table S2). The second morphological PC axis loaded positively with spur length and wing length but negatively with tail length, and accounted for 12.53% of the total variation (Table S2). Individuals were separated by sex but not by elevation along the two PC axes (Figure 1b; Table S2). Specifically, males had smaller PC1 values on average, indicating that they were larger bodied than females (Figure S3; Table S2).

Analysis of spatial autocorrelation using memgene suggested the first MEM axis, MEMGENE1, accounted for almost all of the variation for both autosomal and neo-sex data sets. The MEMs form two groups with the southern-most populations forming one group and the northern-most populations forming another, with the geographically intermediate populations falling between them (Figure S4).

When we constructed linear mixed models, we found that the PC1 scores of morphological measurements were positively associated with bioclimatic PC1 (Table 3, Table S3), such that larger bodied birds are associated with less seasonal sites and higher annual precipitation. In contrast, morphology PC1 was unassociated with elevation, MEM, and soil PC1 (Table 3, Table S4). Morphology PC2 was positively associated with elevation and negatively associated with soil PC1 (Table 3), such that birds with longer spurs, larger wings, and shorter tails occurred at higher elevations with soils that had less sand and fewer coarse fragments. There was no association between morphology PC2 and bioclimatic variation. When morphological characters were considered individually, we found that wing length, tarsus length, and bill depth were negatively associated with bioclimatic PC1, revealing a bioclimatic association similar to the one seen with morphology PC1. Finally, spur length was negatively associated with soil PC1, such that longer spurs occurred in soils that had less sand and coarse fragments and more clay and silt content (Table 3).

RDA analysis using bioclimatic variables suggested only 13.5% of the variation was accounted for by the constrained RD axes. RDA using soil variables suggested only 4.2% of the variation was accounted for by the constrained RD axes. In both cases, most of the variation was accounted for by the PC1 axis, 78.4% for bioclimatic variables and 86.7% for soil variables.

3.3 | Population structure

A data set comprising 28,474 variant sites was used for the STRUCTURE and PCA analyses. For STRUCTURE, the highest support was obtained for a value of $k = 3$, which separated the Colombian individuals (subspecies *peregrina*) from the remaining individuals, and the non-Colombian individuals into two groups that generally corresponded to males and females and not to geography or elevation (Figure 2a). The PCA plot for the first two principal component (PC) axes showed similar patterns, first separating Colombian individuals and then segregating the remaining birds by sex (Figure 2b).

To determine whether the clustering by sex was due to only a few scaffolds, we mapped each scaffold to the respective Zebra Finch chromosome using coding sequences. Each Horned Lark scaffold corresponded to one or two Zebra Finch chromosomal scaffolds. The scaffolds that mapped to two Zebra Finch chromosomal scaffolds included large portions of both the Zebra Finch autosome and the Z chromosome. The potential neo-sex scaffolds WMCF01000011.1, WMCF01000023.1, and WMCF01000024.1 corresponded to segments of Zebra Finch chromosomes 3, 5, and 4A, respectively. For WMCF01000023.1 and WMCF01000024.1, a large portion of the scaffold also contained regions that corresponded to the Zebra Finch Z chromosome (Figure 2c). Elevated F_{ST} values between males and females correspond exactly to the regions on scaffolds WMCF01000023.1, and WMCF01000024.1 that map to Zebra Finch chromosomes 5 and 4A, respectively, but not to the Zebra Finch Z chromosome (Figure 2c).

As mentioned in the methods, we split the RAD loci data set into neo-sex (6320 SNPs) and autosomal subsets (22,154 SNPs); the neo-sex data set was based on scaffolds corresponding to the Zebra Finch Z chromosome as well chromosomes 3, 4A, and 5 that contain putative neo-sex chromosomal regions. The PCA plot for the autosomal data set showed limited clustering with elevation (Figure 3a). For the neo-sex data set, the PCA plot still showed segregation by sex but also exhibited strong associations with elevation along the PC1 and PC2 axes in addition to three distinct clusters along PC2 axis (Figure 3b). Subsequent STRUCTURE analyses for the autosomal data again supported $k = 3$. However, the population assignments using autosomal data related more to geography and elevation and not to sex (Figure 3c).

Individual PCA plots for 25 putative neo-sex scaffolds revealed three clear patterns (Figure S5). First, 18 scaffolds corresponding to some portion of Zebra Finch chromosomes Z, 3, 4A, and 5 did not show any apparent association with elevation, PC1, or PC2. Three scaffolds which mapped to the Zebra Finch Z-chromosome (WMCF01000034.1, WMCF01000084.1, and WMCF01000089.1) showed PCA patterns in which three distinct clusters were associated with elevation in some individuals. Both males and females were present in two of the clusters, whereas the intermediate cluster contained only males. The remaining four scaffolds (WMCF01000003.1, WMCF01000011.1, WMCF01000023.1, and WMCF01000024.1) segregated by sex and elevation. This pattern was less pronounced on scaffolds WMCF01000003.1 and WMCF01000023.1, with the former corresponding to Zebra Finch chromosome 5 and the latter containing portions of chromosome 5 and the Z-chromosome. In scaffolds WMCF01000024.1 (corresponding to parts of Zebra Finch chromosome 4A and Z) and WMCF01000011.1 (corresponding to Zebra Finch chromosome 3), males and females were clearly separated. Within each male and female cluster, high elevation and low elevation clusters also could be observed easily.

SpaceMix results from both the autosomal and the neo-sex data sets suggest three geogenetic clusters (Figure 3c; Figure S6). Two major clusters featured samples from the three southernmost arid regions and from the northern alpine regions, respectively. In the neo-sex data set, one polygon encompassing individuals from the White Mountains was placed distantly from the northern cluster.

3.4 | Associations of genetic data and environmental variables

SNPs that were strongly associated with elevation were all located on scaffolds that corresponded to the Zebra Finch Z chromosome (Figure 4). Results from our BEDASSLE analysis showed that geographic distance plays a larger role in explaining genetic variation than environmental variables (Table 3). Furthermore, the neo-sex data set (average $\alpha E/\alpha D = 1.43 \times 10^{-1}$) is affected to a greater degree by changes in elevation than the autosomal data set (average $\alpha E/\alpha D = 6.65 \times 10^{-3}$).

4 | DISCUSSION

We found that geographic distance was more significantly associated with genomic variation in Horned Lark populations along our elevational gradient compared to environmental variation (Figure 3, Table 3). However, we also found associations between elevation and sex-linked chromosomal loci (Figure 4, Table 3), suggesting that patterns of IBE vary among regions of the genome. Horned Larks have unusually large sex chromosomes (Bulatova, 1973; Dierickx et al., 2020; Sigeman et al., 2019), and differentiation at sex-linked loci may be particularly prevalent in early stages of differentiation among birds more generally (Hooper et al., 2019; Irwin, 2018; Sigeman et al., 2019). The Horned Larks in our study system occupy varying elevations with different habitats (Table 1). These localities differ in soil conditions such as coarse fragment size and are characterized by variable bioclimatic conditions—namely annual precipitation and temperature seasonality. Living in such variable environments, Horned Larks are likely to be adapted to local environmental conditions. The genetic basis of environmental adaptation in larks may lie in these chromosomal rearrangements and with other structural changes throughout the genome. Therefore, differences among populations may be facilitated by structural changes in the genome that could alter gene expression or change epistasis among loci, rather than by other neutral genomic processes.

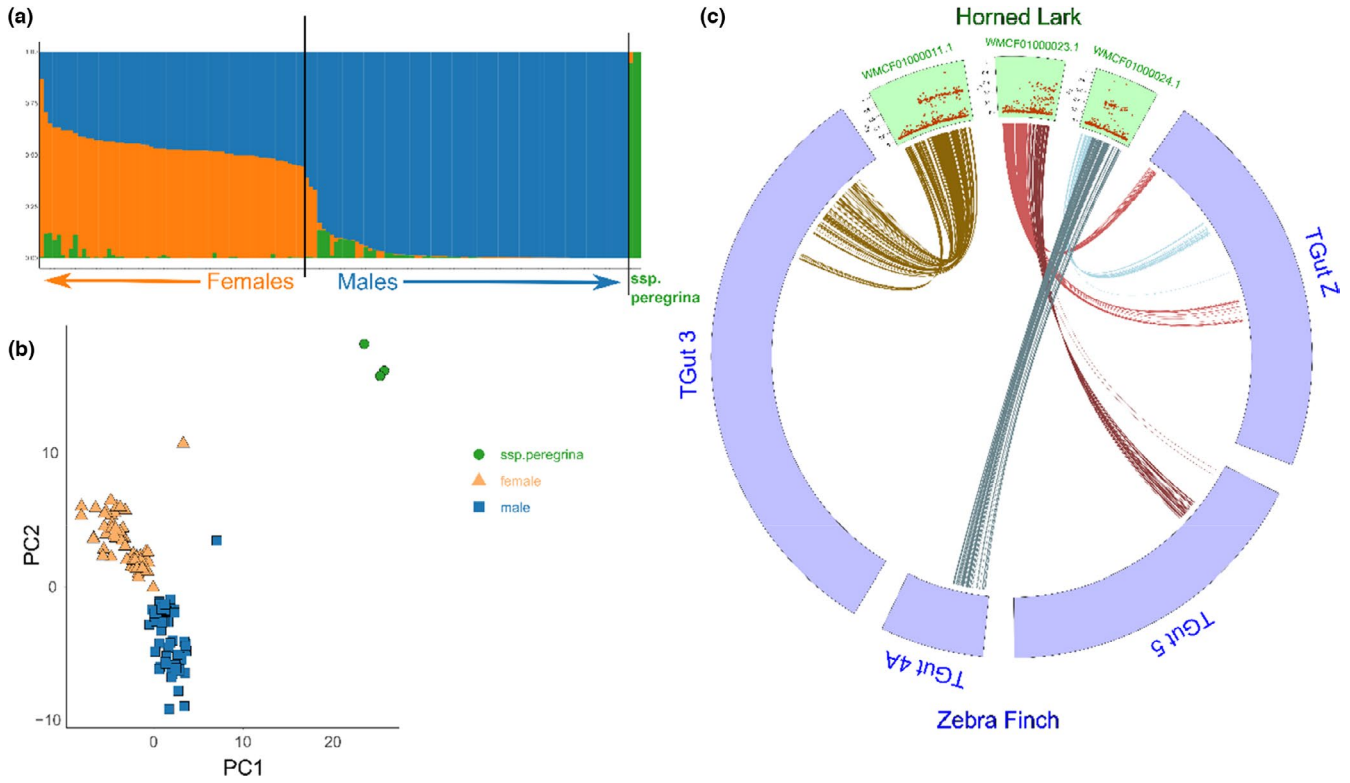

FIGURE 2 (a) STRUCTURE plot for all individuals using the complete SNP data set (28,474 SNPs). All stacks are arranged by sex except for the Colombian population (subspecies *peregrina*). (b) PCA plot for all individuals using the complete SNP data set. Males are represented by blue squares; females are represented by orange triangles; the Colombian population *peregrina* is represented by green circles. (c) Circos plot showing synteny of three putative neo-sex scaffolds and associated regions on the Zebra Finch chromosomes 3, 4A, 5, and Z. Green boxes show F_{ST} values between males and females highlighting the elevated levels of F_{ST} divergence between males and females that correspond to regions of the Horned Lark genome mapping to chromosomes 3, 4A, and 5 but not the Z chromosome of the Zebra Finch [Colour figure can be viewed at wileyonlinelibrary.com]

We found that Horned Lark populations at similar elevations are more similar to each other than expected by IBE alone. This finding implies that ecological differences across elevational bands may not prevent genetic connectivity among populations at different elevations (Figure 3c). In corroboration of this idea, we found that IBD had a larger effect on population structuring of Horned Larks compared to IBE (Table 2). The BEDASSLE results further suggest that neo-sex loci might be more affected by environment than autosomal loci. Taken together, these two findings suggest that geographic distance outweighs elevational distance in shaping genomic differentiation among Horned Larks, and that elevational associations are most pronounced among neo-sex chromosomes.

4.1 | Effects of environment on morphology

We found that Horned Larks tended to be larger in wetter, less seasonal environments, and that larks at higher elevation sites tended to have larger tarsi. Overall body size (i.e., morphology PC1) was not associated with elevation in our linear mixed models (Table 3); although there is extensive variation in the body size among Horned Larks, this variation did not correspond directly with temperature or elevation in our data set.

We also uncovered associations between hallux length and different soil types, such that larks in habitat with soil rich in clay and silt had longer halluces compared to larks in soil with lots of sand and coarse fragments. Larks are terrestrial birds that frequently run and walk across the substrate. Thus, this variation in claw length may represent a cursorial adaptation for mobility and traction in different habitats across the elevational gradient. A comparative analysis across the lark family found that larks in grassy habitats tended to have longer toes and claws compared to those occupying habitat with bare ground (Green et al., 2009). The associations we observed between hallux length and soil conditions among populations of Horned Larks may reflect general ecomorphological trends in foot morphology among passerines. However, as the hallux is used for digging and locomotion across substrates, differences across elevations in hallux length may be the result of wear through consistent friction in different soil types. Associations between soil conditions and foot morphology have been observed in other bird species (Kaboli et al., 2007), and similar associations among lark populations and species could be further explored in a phylogenetic comparative framework.

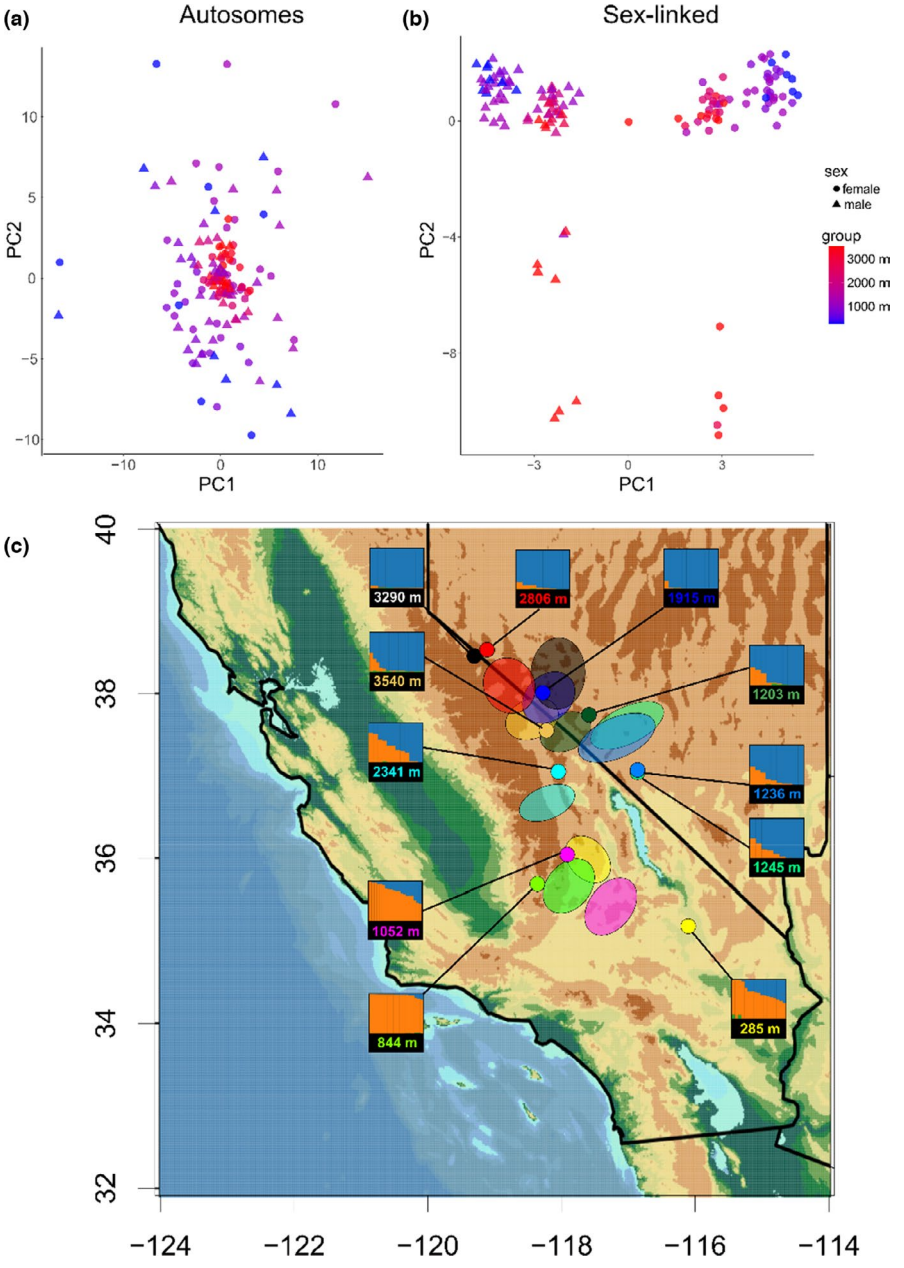

FIGURE 3 (a) PCA for autosomes (i.e., excluding the combined chromosomes Z, 3, 4A, and 5 as mapped to Zebra Finch; 22,154 SNPs); colours correspond with sampling elevation. (b) PCA for Z-linked and chromosomes 3, 4A, and 5 as mapped to Zebra Finch (6,320 SNPs). (c) STRUCTURE plots for autosomes mapped to corresponding sampling localities. Elevation of each site represented below STRUCTURE stacks; Colombian individuals were omitted for this study and are not shown on map. Ellipses correspond to the geogenetic location of each population with geographical locations shown as corresponding coloured points [Colour figure can be viewed at wileyonlinelibrary.com]

4.2 | The role of neo-sex chromosomes in lark differentiation along an elevational gradient

Polymorphisms in loci on sex chromosomes, with their higher mutation rates, greater probability of fixation, and potential role in the evolution of sexual antagonism, can play an outsized role in phenotypic evolution, speciation, and diversification in birds (Irwin, 2018). Various traits—from feather coloration (Bourgeois et al., 2020; Kim et al., 2019; Toomey et al., 2018) to sperm motility (Kim et al., 2017; Knief et al., 2017)—have been attributed to genic regions on the sex chromosomes of birds. Although avian sex chromosomes were thought to be highly stable in structure (Ellegren, 2010), this paradigm is being called into question with new empirical data reporting inversions, transversions, and translocations in the sex chromosomes of many diverse lineages of birds (da Silva et al., 2019; Gunski et al., 2019; Hooper et al., 2019; Hooper & Price, 2017; Kretschmer et al., 2020; Sigeman et al., 2019, 2020; Yazdi & Ellegren, 2018). Translocations of small to large fragments from autosomes to sex chromosomes, forming neo-sex chromosomes, further increase the potential role of sex chromosomes in speciation (Dierickx et al., 2020; Gan et al., 2019; Kretschmer et al., 2020; Sigeman et al., 2020). Characterizing sex-chromosome structure and understanding the interplay of loci within sex chromosomes are emerging as important aspects of studying the evolution of local adaptation and differentiation across landscapes (Connallon et al., 2018; Guerrero & Kirkpatrick, 2014; Lasne et al.,2017).

The presence of large neo-sex chromosomes in Horned Larks has previously been reported (De et al., 2010; Bulatova, 1973; Dierickx et al., 2020; Sigeman et al., 2019), but the exact nature and compositions of these fusions was heretofore unknown (but see

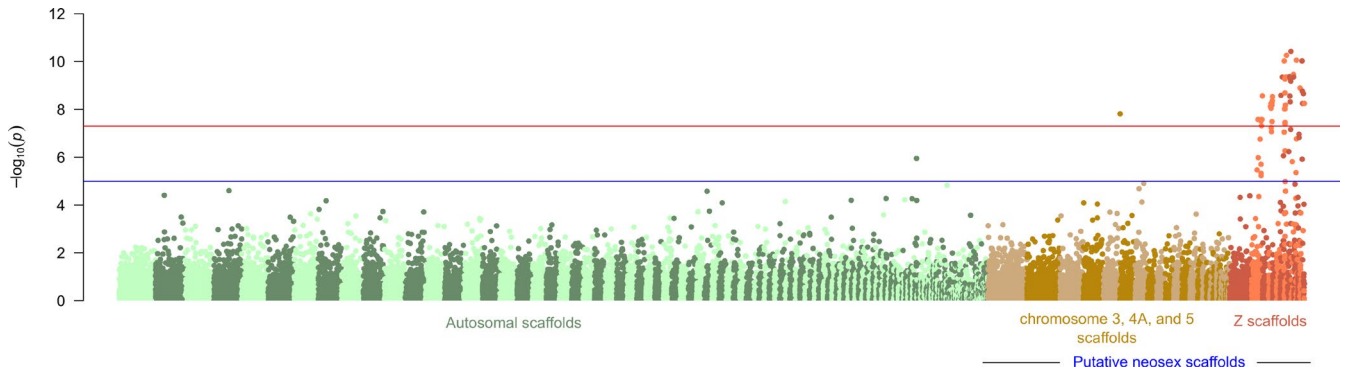

FIGURE 4 Negative logarithms of *p*-values of association of genetic data (complete SNP data set) with elevation arranged by scaffold. Green points are autosomal scaffolds, brown points are scaffolds corresponding to Zebra Finch chromosome 3, 4A, and 5, and red points are Z chromosome scaffolds. Brown and red points represent putative neo-sex scaffolds. The horizontal blue line corresponds to a *p*-value of $-\log_{10}(1 \times 10^{-5})$ while the horizontal red line corresponds to a *p*-value of $-\log10(5 \times 10^{-8})$ [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Results of two replicates of six BEDASSLE runs using autosomal and neo-sex data sets for comparison of relative effects of geographical distance with elevation, mean temperature of wettest quarter (Bioclim variable 8) and annual precipitation (Bioclim variable 12)

Data set	Environmental variable	Replicate	Mean aE/aD
Autosomal	Elevation	1	7.83×10^{-03}
		2	5.46×10^{-03}
	Mean temperature of wettest quarter	1	2.67×10^{-03}
		2	3.23×10^{-04}
	Annual precipitation	1	3.55×10^{-03}
		2	3.23×10^{-03}
Neo-sex	Elevation	1	9.77×10^{-02}
		2	1.88×10^{-01}
	Mean temperature of wettest quarter	1	1.60×10^{-03}
		2	9.71×10^{-03}
	Annual precipitation	1	5.75×10^{-03}
		2	2.14×10^{-02}

Sigeman et al., 2021). At least three chromosomes have signals of translocation, corresponding to Zebra Finch chromosomes 3, 4A, and 5 (Figure S1; Dierickx et al., 2020; Sigeman et al., 2019). In our data set, two scaffolds showed clear evidence of fusions between autosomal and sex chromosomes: (1) WMCF01000023.1, containing regions homologous to part of Zebra Finch chromosome 5 and parts of chromosome Z; and (2) WMCF01000024.1, containing regions homologous to parts of Zebra Finch chromosome 4A and parts of chromosome Z. While we are unable to determine the entire scale of the fusions using our RAD data, the existence of these large translocations from multiple chromosomes to form a neo-sex chromosome could allow for the proliferation of novel mutations within these potentially recombination-suppressed regions (Bergero & Charlesworth, 2009). We found that polymorphisms in sex-linked loci were associated with different elevations among populations of Horned Larks, and that neo-sex chromosomes may contain genes that facilitate living in these different environments.

The formation of neo-sex chromosomes in Horned Larks may extend to the W chromosome in addition to the Z chromosome (Bourgeois et al., 2020; Sigeman et al., 2019). Our initial approach to characterizing population structure among individuals was hampered by differences in polymorphisms between males and females. In the two scaffolds containing regions of both an autosome and the Z chromosome, F_{ST} comparison between males and females recovered regions of elevated F_{ST} that corresponded to Zebra Finch autosomal regions but not to the Z chromosome (Figure 2c). This can be explained if the autosomal regions that correspond to elevated F_{ST} are present in both the Z and W sex chromosomes. Our reference genome is from a male Horned Lark (Mason et al., 2020) and therefore lacks the W chromosome present among females. Reads from resequenced females (which have both Z and W chromosomes) corresponding to the fused autosomal portions of chromosome 4A and 5 map only to the homologous region on the neo-Z chromosome of the male. The analysis of genomic coverage supports this idea as the coverage of the regions are between those of pure Z chromosome scaffolds and regular autosomal scaffolds (Figure S1). Independent translocations to both the Z and W chromosomes or integration of fused elements of Z chromosome pseudoautosomal regions (PARs) to the W chromosome (Sigeman et al., 2019, 2021) would allow these regions to subsequently diverge, since there is

TABLE 3 Results of linear mixed models (LMMs) comparing morphological data to MEMGENE (genetic variation shaped by spatial autocorrelation), environmental data, and sex

Morphological data	R^2_m	R^2_c	Environmental data + sex	*T*-value	Degrees of freedom	*p*-value
Morphological PC1	0.785	0.819	Elevation	−0.964	6	.373
			MEMGENE1	−0.278	6	.791
			Bioclimatic PC1	2.807	**6**	**.031**
			Soil PC1	−0.841	6	.433
			Sex	−21.988	**113**	**<.001**
Morphological PC2	0.179	0.191	Elevation	3.427	**6**	**.014**
			MEMGENE1	1.201	6	.275
			Bioclimatic PC1	−2.098	6	.081
			Soil PC1	−3.474	**6**	**.013**
			Sex	0.148	113	.883
Wing length	0.767	0.802	Elevation	1.544	6	.173
			MEMGENE1	0.830	6	.438
			Bioclimatic PC1	−3.839	**6**	**.009**
			Soil PC1	0.815	6	.446
			Sex	20.199	**113**	**<.001**
Tail length	0.750	0.780	Elevation	0.338	6	.747
			MEMGENE1	−0.291	6	.781
			Bioclimatic PC1	−1.593	6	.162
			Soil PC1	0.796	6	.456
			Sex	19.931	**113**	**<.001**
Tarsus length	0.258	0.284	Elevation	1.557	6	.171
			MEMGENE1	−0.178	6	.864
			Bioclimatic PC1	−2.928	**6**	**.026**
			Soil PC1	−0.699	6	.511
			Sex	4.208	**113**	**<.001**
Middle toe length	0.115	0.242	Elevation	0.475	6	.652
			MEMGENE1	0.221	6	.832
			Bioclimatic PC1	−1.779	6	.125
			Soil PC1	0.133	6	.898
			Sex	2.480	**113**	**.015**
Spur length	0.157	0.337	Elevation	1.640	6	.152
			MEMGENE1	0.394	6	.707
			Bioclimatic PC1	−0.025	6	.981
			Soil PC1	−2.434	6	.051
			Sex	−0.149	113	.882
Bill length	0.264	0.418	Elevation	−0.239	6	.819
			MEMGENE1	−0.255	6	.808
			Bioclimatic PC1	0.766	6	.473
			Soil PC1	−0.756	6	.478
			Sex	7.106	**113**	**<.001**

TABLE 3 (Continued)

Morphological data	R^2_m	R^2_c	Environmental data + sex	*T*-value	Degrees of freedom	*p*-value
Bill depth	0.265	0.265	Elevation	0.326	6	.756
			MEMGENE1	0.654	6	.538
			Bioclimatic PC1	−2.828	**6**	**.030**
			Soil PC1	1.699	6	.140
			Sex	5.660	**113**	**<.001**
Bill width	0.156	0.178	Elevation	−0.066	6	.950
			MEMGENE1	0.621	6	.558
			Bioclimatic PC1	−0.908	6	.399
			Soil PC1	0.545	6	.605
			Sex	4.378	**113**	**<.001**

Note: R^2_m indicates the marginal likelihood of the LMM, whereas R^2_c indicates the conditional likelihood. *p*-values that are <.05 are considered statistically significant and are shown in bold font. Loadings for the morphological PC1 corresponded to body size, whereas morphological PC2 corresponded to a trade-off between wing, spur, and tarsus length versus tail length.

little recombination between the Z and W chromosomes. This interpretation is further supported by karyotypic data from Horned Larks and other lark species that show enlargement of both the Z and W chromosomes (Bulatova, 1973).

Parts of the Horned Lark scaffolds that correspond to the Zebra Finch chromosome 3 also show elevated F_{ST} regions between males and females, but these do not have any regions associated with the Zebra Finch Z chromosome. In larks, chromosome 3 has been shown to exhibit different degrees of recombination suppression (Dierickx et al., 2020; Sigeman et al., 2019) and may be subject to a more complex pattern of translocation into sex-chromosomes. One interpretation for this different degree of recombination suppression is that translocated parts of chromosome 3 in Horned Larks are present on both the W chromosome and the Z chromosome, but the regions involved are probably different on each chromosome. Further, different lark species and populations may show variation in the extent to which loci typically found on the Zebra Finch chromosome 3 have been translocated and fused with different lark chromosomes, a question that begs further investigation (Dierickx et al., 2020). Our genome for the Horned Lark is from a Colombian bird, and differences in regions corresponding to chromosome 3 between Colombian and US birds might account for the pattern observed in scaffolds corresponding to chromosome 3.

Differences among sex chromosomes due to translocation present a nuanced scenario for landscape genomics. Translocated regions on the Z chromosome can recombine in male birds, which are the homogametic sex (ZZ). However, homologous translocated regions on the Z and W chromosomes in females (ZW) may suppress recombination and thereby allow the translocated regions on the W chromosomes to freely evolve, independent of homologous regions on the Z chromosome. Elevated F_{ST} between male and female Horned Larks in these regions suggest the two sex chromosomes have accrued different mutations in the translocated regions. The loci within these regions are evolving at least partially, if not completely, independently; hence potential co-option, neofunctionalization, or even pseudogenization of different genes may have occurred (Lynch & Walsh, 2007). Sex-chromosome diversity and evolution is remarkable across nature, enabling a wide suite of adaptations (Furman et al., 2020). For example, rapid evolution and diversification of genes on neo-sex chromosomes have been reported in facilitating diversity and tolerance to a large array of host plants in torticid moths (Nguyen et al., 2013). Results from our association mapping suggest strong associations between the Z chromosome and elevation, and such patterns may be driven by selective forces acting on regions of Zebra Finch autosomes that have been translocated into regions of the Z and W chromosomes of Horned Larks. Further studies using whole genomes are needed to better understand the distribution of regions in Z and W chromosomes and how they facilitate elevational adaptation in larks.

4.3 | Inversions in the Z chromosome

Chromosomal inversions are another potent mechanism that can facilitate divergence and speciation (Faria & Navarro, 2010; Hoffmann & Rieseberg, 2008; Hooper et al., 2019; Hooper & Price, 2017; Rieseberg, 2001; Wilson & Makova, 2009). Inversions in birds have been associated with phenotypic variation between species pairs and between populations (Hooper et al., 2019; Hooper & Price, 2017; Küpper et al., 2015; Lamichhaney et al., 2015; Poelstra et al., 2014). Hooper and Price (2015, 2017) found that inversions were more common among sympatric sister species of birds than in allopatric species, suggesting that inversions may play a larger role in speciation and in preventing populations from fusing back together upon secondary contact than previously thought. Inversions suppress recombination and allow for increased mutation persistence and subsequent phenotypic differentiation, hence, inverted regions can act as supergenes (Kirubakaran et al., 2016; Kunte et al., 2014; Küpper et al., 2015).

Three scaffolds in our Horned Lark study (WMCF01000034.1, WMCF01000084.1, and WMCF01000089.1) show clustering patterns consistent with inversions in PCA plots (Figure S5). These scaffolds correspond to regions in the Zebra Finch Z chromosome and are also most strongly associated with elevation in our association mapping analysis (Figure 4). They also lie between regions in the Z chromosome that potentially contain contiguous regions of autosomal translocations corresponding to Zebra Finch chromosome 3, 4A, and 5. We are unable to fully describe the extent and the haplotypes of these potential inversions using only RAD data, although individuals in the PCA are organized into three distinct clusters that appear to represent the two homozygous (with and without inversion) conditions and one heterozygous condition. Among the three groups, the middle group can be inferred to be intermediate between the two homozygous inversion conditions. This is further supported by the observation that this group contained only males, which can therefore be heterozygous for Z-linked loci. Females have either one of the two extreme conditions and therefore cannot be heterozygous for the inversion.

While most individuals in our study did not have the putative inversion, 11 birds had at least one copy of it. The nonintermediate smaller cluster (Figure S5), corresponding to the homozygous condition with the inversion, comprised seven individuals: six from the White Mountains in eastern California (3540 m) and one from Bald Mountain in western Nevada (2806 m). These sites are among the highest elevations we sampled. Of the four intermediate birds, three were also from the White Mountains, with the fourth from Rose Valley, California (1052 m). The White Mountains are central to the distribution of the putative inversion clusters. Among the 17 birds from that locality, seven share the same haplotypes as birds from across the full range we sampled. The association of inversions with high elevation sites suggests that genes within the inverted region may facilitate local adaptation to these habitats. Further studies using whole genomes are needed to better understand the nature of the inversion and the genes within it.

5 | CONCLUSION

Geographic distance (IBD) outweighs environmental distance (IBE) in shaping genomic differentiation among Horned Larks, although some genomic associations with elevation do exist. Specifically, elevational associations are more pronounced among neo-sex chromosomes than autosomes, suggesting that differences among populations of Horned Lark are facilitated primarily by structural changes to the genome. Further research with whole genomes is necessary if we are to fully understand these associations and to characterize particular genetic loci that may facilitate living under different environmental conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Nicholas A. Mason, Carla Cicero and Rauri C. K. Bowie designed the research project. Ned Johnson (NJK), to whom this study is dedicated, collected the morphological data, and NJK and Carla Cicero collected most of the samples. Cynthia Y. Wang-Claypool collected the genetic data. Subir B. Shakya and Nicholas A. Mason analysed the data. Subir B. Shakya and Nicholas A. Mason wrote the manuscript with feedback from remaining authors.

DATA AVAILABILITY STATEMENT

All raw data from genomic resequencing has been made available via the NCBI short-read archive: PRJNA769069. Morphological measurements along with raw values for morphological, soil, and environmental PCA are included in a supplementary table.

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