Genetic Analysis of Harbison's Dun Skipper to Inform Population Management and Restoration on Conserved Lands in San Diego County

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Data Availability Statement

Single Nucleotide Polymorphism genotype data and sample data are available in Vandergast and others (2025; https://doi.org/10.5066/P13VRALN).

Artificial Intelligence Use Disclosure Statement

No artificial intelligence tools were used in the design or implementation of this study, nor in the preparation of this report.

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Conversion Factors

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
meter (m)	3.281	foot (ft)
kilometer (km)	0.6214	mile (mi)
kilometer (km)	0.5400	mile, nautical (nmi)
meter (m)	1.094	yard (yd)
	Mass	
gram (g)	0.03527	ounce, avoirdupois (oz)
kilogram (kg)	2.205	pound avoirdupois (lb)
metric ton (t)	1.102	ton, short [2,000 lb]
metric ton (t)	0.9842	ton, long [2,240 lb]

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

°F = (1.8 × °C) + 32.

Abbreviations

DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
RAD	Restriction-Associated DNA
SDMMP	San Diego Management and Monitoring Program
SNP	single nucleotide polymorphism
USGS	U.S. Geological Survey

Abstract

This report details the development and analysis of single nucleotide polymorphic loci to understand population genetic structure and diversity among local populations of the Harbison's dun skipper, *Euphyes vestris harbisoni,* primarily in San Diego County, California, USA. We developed a set of 2984 SNPs. Local populations were clustered into two to three regional genetic clusters throughout the San Diego County study area: a southeast cluster, an admixed northeast cluster, and a cluster comprised of the two local populations sampled west of Interstate 15 in Lake Hodges and Elfin Forest. Increasing genetic isolation with geographic distance was significant among local populations. While effective population size estimates calculated for the 2016 cohorts in the Southeast and Lake Hodges clusters were both high (point estimates above 500), individual heterozygosity appeared to decline in both clusters over time, and notably so in the Lake Hodges cluster after 2016, suggesting that this cluster may have lost genetic diversity over time. The Southeast cluster appears to have the highest observed heterozygosity across all surveyed areas, but sample sizes in the Northeast cluster were low which may affect these estimates. Future collection efforts may benefit from additional sampling in the Northeast cluster to improve representation. The management plan for Harbison's dun skipper encourages restoration and re-establishment efforts in unoccupied or recently extirpated sites, particularly in the central portion of the range. Re-establishment efforts could target individuals from large and annually stable local populations in the Southeast portion of the range for transplant, as these populations were also the most genetically diverse.

Introduction

Genomic data are increasingly being used to help understand the health and connectedness of populations of rare species to inform conservation and management. Genetic diversity patterns can reflect breeding population sizes, gene flow and selection, and genetic diversity is a necessary component of future fitness, persistence and adaptive potential (Hoffmann and others, 2017; Kardos and others, 2021). Populations with low genetic diversity and small sizes, and populations that are isolated because natural dispersal and migration patterns are inhibited, can be identified for management or enhancement. These populations may be more vulnerable to local extinction without management action (Spielman and others, 2004; Bozzuto and others, 2019). Monitoring genetic diversity over several generations can also reveal trends over time, particularly in rapidly changing environments (Hoban and others, 2014).

The Harbison's dun skipper, *Euphyes vestris harbisoni* (Brown and McGuire 1983; Lepidoptera: Hesperiidae), is a rare subspecies with a limited distribution centered across the foothills in southern San Diego County, with a northern extent reaching western Riverside County and southern Orange County and southern extent into northern Baja California (Marschalek and Deutschman 2015). Over the past several decades, the Harbison's dun skipper (hereafter skipper) has measurably declined in population sizes and number of occurrences within its range (Marschalek and others, 2019). Given these declines, the skipper is a target species of San Diego County conservation plans with specific conservation and restoration objectives recommended by the San Diego Management and Monitoring Program (SDMMP).

Within its limited range, the skipper is a habitat specialist found in riparian oak woodlands, or chaparral with narrow canyons or drainages that contain the only documented

larval host plant, San Diego sedge (*Carex spissa*; Brown and McGuire, 1983; Marschalek and Deutschman, 2015). Adult skippers also rely on a variety of native and introduced nectar sources including morning glory (*Calystegia macrostegia* subsp. *tenuifolia*), red thistle (*Cirsium occidentale*), loosestrife (*Lythrum californicum*), and less frequently golden yarrow (*Eriophyllum confertiflorum*) and black mustard (*Brassica nigra*). There are 20 documented nectar plant species, nearly all of which have white, purple, or pink flowers (Marschalek and Deutschman, 2015).

Major threats to the persistence of the skipper are habitat loss, fragmentation, and habitat degradation. In particular, the loss of San Diego sedge can occur after wildfire and drought, growth of invasive plants, destruction of overstory shade plants by invasive insects, grazing, bank alterations, stream pollution and scouring after extreme rain events (Marschalek and Deutschman, 2015). In addition to reducing population size, habitat loss and fragmentation can lead to reduced dispersal and gene flow among occurrences, which is especially concerning when isolated occurrences are small, as documented with the skipper (Marschalek and Deutschman, 2015).

Further, local populations may be contracting. Recent comprehensive field surveys conducted between 2014 – 2017 uncovered many locations on conserved lands that could support skippers based on the presence of San Diego sedge. Approximately 66% of locations were occupied but all population sizes were small (Marschalek and others, 2019). This updates prior work by Brown (1982), who reported that the skipper was found in nearly all sedge patches in the early 1980s. Continued monitoring in 2021 – 2025 determined that local populations remained relatively small but that both adult and larval skippers were consistently observed at

sites where they were previously known to be present (Lyons and others, 2024; Marschalek and Faulkner, 2025).

Management actions to restore skippers in San Diego County may include habitat restoration and reestablishing occupancy in locally extirpated sites (Marschalek and Deutschman, 2018). This genetic study was undertaken to provide information on genetic connectivity across the skipper range and levels of genetic diversity within persistent populations. Together with larval and adult survey counts, genetic information can be used by managers to assess patterns of genetic connectivity across the range, and to help select among possible source populations for re-establishment efforts in unoccupied suitable habitat.

Purpose and Scope

Here we developed a set of Single Nucleotide Polymorphic loci (SNPs) and genotyped individuals collected throughout the subspecies range between 2013 and 2023. Using these data, we examined population genetic structure and patterns of genomic diversity across the landscape and across sampling years to examine population trends and inform future management of this rare subspecies.

Methods

Field collections

Samples were collected during field surveys conducted between 2013 and 2023 from monitored local populations throughout the subspecies range in San Diego, Riverside and Orange counties, California, USA (Figure 1). Adult skippers were captured by hand net, and one leg was removed and placed into a tube with 95% ethanol. Samples were were kept frozen at -80

°C until further processing. Skippers were marked with permanent marker to avoid recapturing and released. Due to the small local population sizes, samples were obtained from as many individuals as possible.

Figure 1. Harbison's dun skipper local populations sampled between 2013 and 2023 across San Diego Riverside and Orange counties, California, USA. Sites included Hot Springs Canyon, Fox Spring, Daley Ranch, Hellhole Canyon County Preserve, Pamo Valley, Boden Canyon, Lake Hodges, Elfin Forest, Crestridge Ecological Reserve, Loveland Reservoir, Sycuan Peak, Skye Valley Road, North and South Barret Lake, and Hollenbeck Canyon Wildlife Area.

DNA extractions and library preparations

DNA was extracted from ethanol preserved leg tissue. First, legs were rinsed and air dried to remove ethanol. Samples were then soaked overnight in proteinase K and lysis buffer at 55 ℃, and then genomic DNA was extracted using the Qiagen puregene kit, and following manufacturer's protocols, with genomic DNA eluted in 25-30 ul of 1X Tris-EDTA (TE). All extractions were quantified on a Qubit fluorometer using the double stranded DNA, high sensitivity assay kit (Thermofisher Scientific).

We followed the 3RAD (restriction-associated DNA) sequencing protocol (Bayona-Vásquez and others, 2019) to generate genome-wide single nucleotide polymorphism (SNP) data. We prepared 3RAD libraries with 100 ng of sample DNA and used the restriction enzymes EcoRI, MseI, and CviQI. We conducted individual Polymerase Chain Reactions (PCRs), pooled PCR product by volume and selected fragments using a Pippin Prep size fractionator (Sage Science). Final libraries were bead-cleaned and quantified using the Qubit fluorometer (Life Technologies) and sequenced (150 bases, paired-end reads) on the Illumina NovaSeq platform at MedGenome, Inc. (Foster City, CA).

Raw sequence demultiplexing, quality filtering, and genotyping were performed on the USGS Hovenweep High Performance Computing platform (Falgout and others, 2024). We used BWA 0.7.17 to align demultiplexed forward reads to the *Ephyes dion* reference genome using the 'mem' command with default settings. These reads were then exported and sorted as .bam files using SAMtools 1.10, and used as input for the gstacks program within Stacks v2.66 to build a catalog of individual RAD loci. These were further filtered for quality. We used plink 1.9

(https://www.cog-genomics.org/plink/1.9/) to assess the attribute quality of data and removed samples that had greater than 30 percent missing data. For population structure analyses, one SNP was randomly chosen from each locus and a minimum allele frequency (MAF) filter of 0.05 was applied. Genotypes were exported in a variant call format for further analysis.

Genetic Analyses

The final SNP dataset was analyzed for population genetic structure and diversity. Genetic clustering analyses were used to explore structure across the study area. We applied the maximum-likelihood approach of ADMIXTURE (Alexander and others, 2009) to define the best supported number of genetic clusters (K) in the data when fit to a population genetic model that assumes Hardy-Weinberg equilibrium. We performed 10 replicate analyses to evaluate up to 10 clusters. To assess the best value of K, we performed 5-fold cross-validation and determined the K-values with the lowest cross validation error and examined the individual assignment plots. In addition, a principal component analysis (PCA) was implemented in adegenet 2.1.3 (Jombart and Ahmed, 2011), using R 4.4.1 (R Core Team, 2026), to visualize the genotypes in multidimensional space. Plots were visually assessed for geographic structure.

Genetic differentiation among local populations was examined with pairwise estimates of Nei's D. We tested for isolation by distance among local populations using a mantel test for matrix correlation in the program IBD (Bohonak, 2002).

Genetic diversity indices which included observed heterozygosity (Ho), unbiased expected heterozygosity (uHe) and inbreeding coefficients (FIS), were estimated with dartR v.2.0.4 (Mijangos and others, 2022). Estimates are reported for each sampling location with greater than five samples and grouped by region. We estimated contemporary effective

population size (Ne) for each genetically defined cluster using the linkage disequilibrium method (Waples and Do, 2010) within the program NeEstimator 2.1 (Do and others, 2014). We assumed random mating for each group, applied a minimum allele frequency of 2% and calculated 95% confidence intervals for point estimates by jackknifing across samples (Jones and others, 2016). Finally individual heterozygosity estimates were calculated using GENHET (Coulon, 2010) and plotted by regional cluster across collection years to assess trends in heterozygosity over generations.

Results and Discussion

We prepared and sequenced 182 Harbison's dun skipper individuals. After removing low quality samples and loci with high missing data, the final genomic dataset consisted of 148 individuals genotyped at 2984 SNPs with 10.62% missing data overall. Applying a minimum allele count of 3 reduced the number of SNPs to 1335. Clustering results were very similar between the two datasets, so here we present results with the full 2984 SNPs. The SNP dataset is available in Vandergast and others (2025). Diversity statistics, including percent polymorphic loci and observed and expected heterozygosity were generally highest in the southeast region and lowest in the Lake Hodges cluster (Table 1). Inbreeding coefficients were low in all three regional clusters (Table 1).

Table 1. Genetic diversity statistics for genetic clusters and by local populations within clusters for Harbison's dun skippers. We report the number of individuals analyzed (N), the number of loci (N Loci), the percentage of polymorphic loci, the observed heterozygosity (Ho), the unbiased expected heterozygosity (uHe) and the inbreeding coefficient (FIS). NA, not applicable, UHe and FIS are not reported for sample sizes < 3.

Cluster/Local Population	N	N Loci	% polymorphic loci	Ho	uHe	FIS
Southeast	**108**	**2984**	**81.57**	**0.0847**	**0.0813**	**0.0031**
Crestridge Ecological Reserve	5	2980	19.56	0.0727	0.0737	0.0183
Loveland Reservoir	21	2984	42.06	0.0873	0.0832	-0.0075
Sycuan Peak	13	2984	33.24	0.0859	0.0808	-0.0296
Skye Valley Road	26	2984	40.11	0.0837	0.0805	-0.0057
North Barrett Lake	9	2984	29.22	0.0855	0.0812	-0.0261
South Barrett Lake	15	2984	35.52	0.0869	0.0805	-0.0328
Hollenbeck Canyon Wildlife Area	19	2984	34.18	0.0834	0.0791	-0.0178
Lake Hodges	**28**	**2984**	**32.14**	**0.0698**	**0.0654**	**-0.0200**
Lake Hodges	26	2984	31.74	0.0701	0.0651	-0.0280
Elfin Forest	2	2981	10.80	0.0669	NA	NA
Northeast	**12**	**2984**	**33.48**	**0.0831**	**0.0835**	**0.0208**
Hot Spring Canyon	1	2490	34.42	0.0775	NA	NA
Fox Spring	1	2721	19.08	0.0757	NA	NA
Daley Ranch	4	2984	20.44	0.0841	0.0813	-0.0231
Hellhole Canyon County Preserve	1	2966	8.40	0.0779	NA	NA
Pamo Valley	3	2983	18.17	0.0871	0.0818	-0.0480
Boden Canyon	2	2982	13.88	0.0832	NA	NA

Admixture analysis best supported two genetic clusters across the study area (Figures 2 & 3). Individuals sampled at Lake Hodges and Elfin Forest (both west of Interstate 15; hereafter Lake Hodges cluster) comprised one cluster (Figure 2). Sites in the southeastern portion of San Diego County (hereafter Southeast cluster) were mainly composed of a second cluster, with a small number of individuals of admixed assignment scattered among the local populations (Figure. 2). Sites sampled in the northeastern portion of San Diego County, east of Interstate highway 15, along with Fox Spring (Riverside County) and Hot Spring Canyon (Orange County)

were all admixed. The PCA analysis further distinguished this northeast group (hereafter Northeast cluster), separating three groups along the first two axes which accounted for 6.96% and 2% of the variation, respectively (Figure 4).

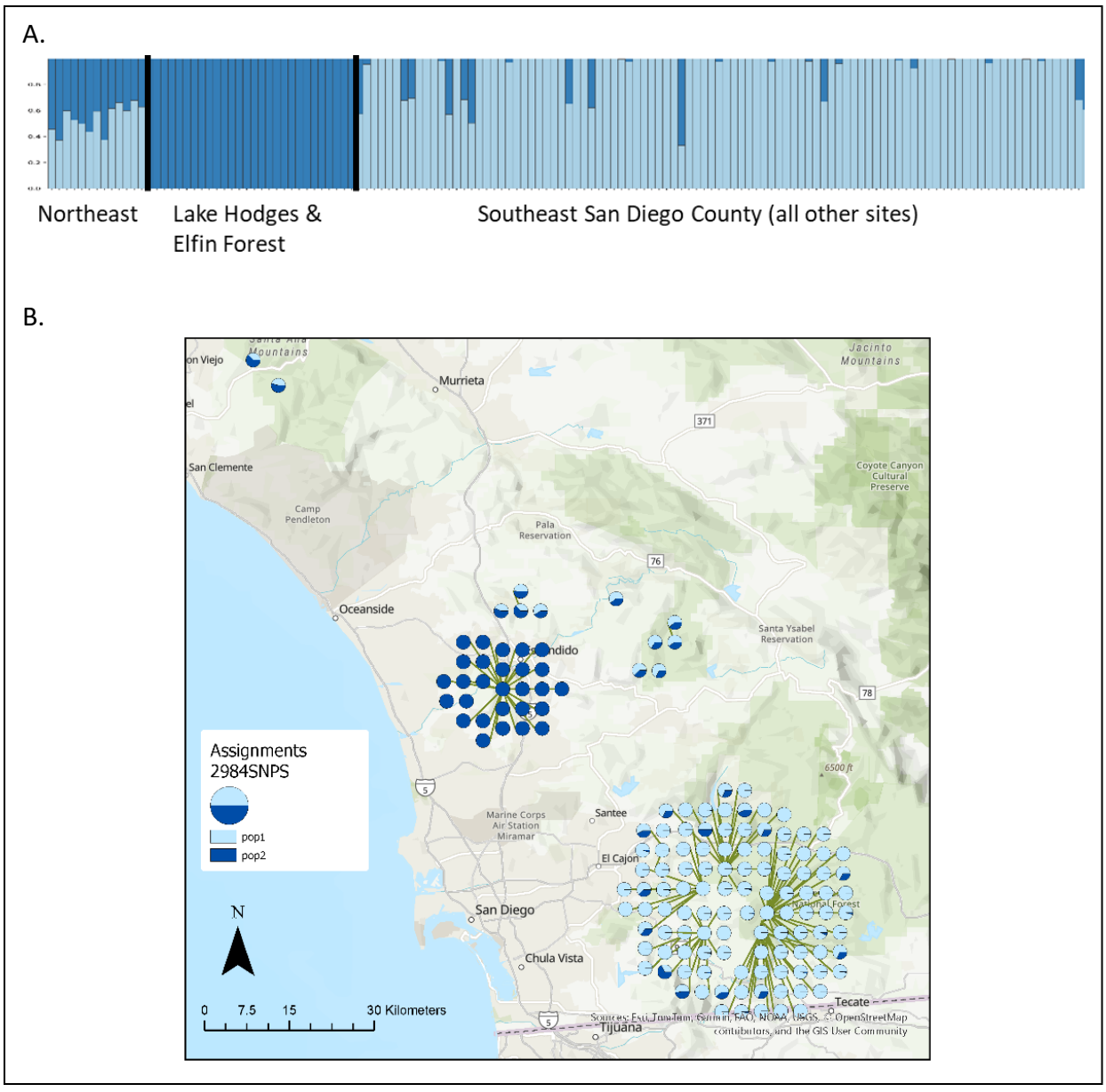

Figure 2. (A) Plot of individual Harbison's dun skipper assignments from admixture analysis of 2984 SNPS showing the optimal two clusters or genetic populations, indicated by light blue (pop/cluster 1) and dark blue (pop/cluster 2). Individuals are arranged from north to south. (B) Individuals clustered geographically, with Lake Hodges and Elfin Forest (west of I-15) clustering separately from Southeast San Diego and Northeast individuals showing mainly admixed assignments. The Southeast cluster also contains some individuals with mixed assignment across several local populations.

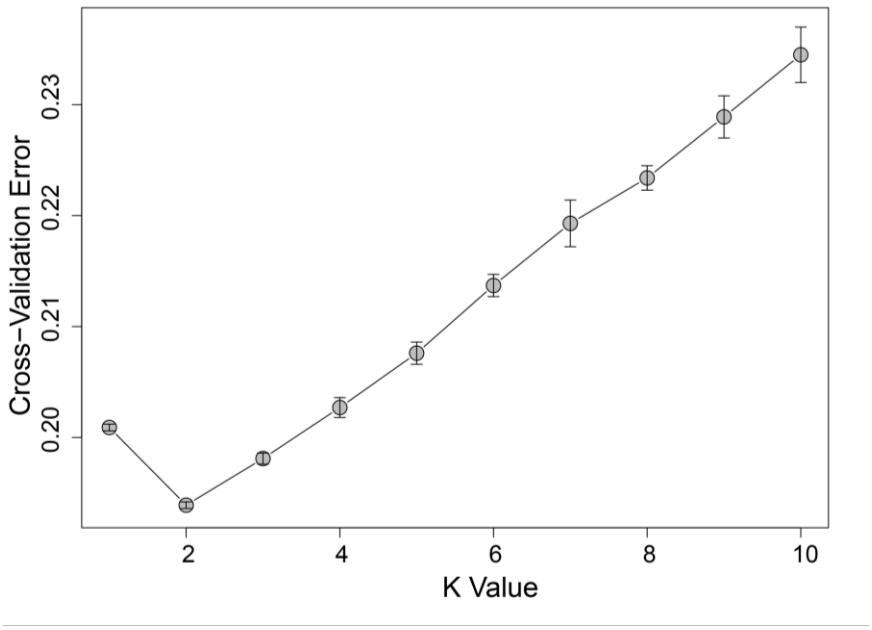

Figure 3. Cross validation error plotted by assessed K-values (number of clusters) showing lowest error and thus best support for two genetic clusters of Harbison's dun skippers in the Admixture analysis.

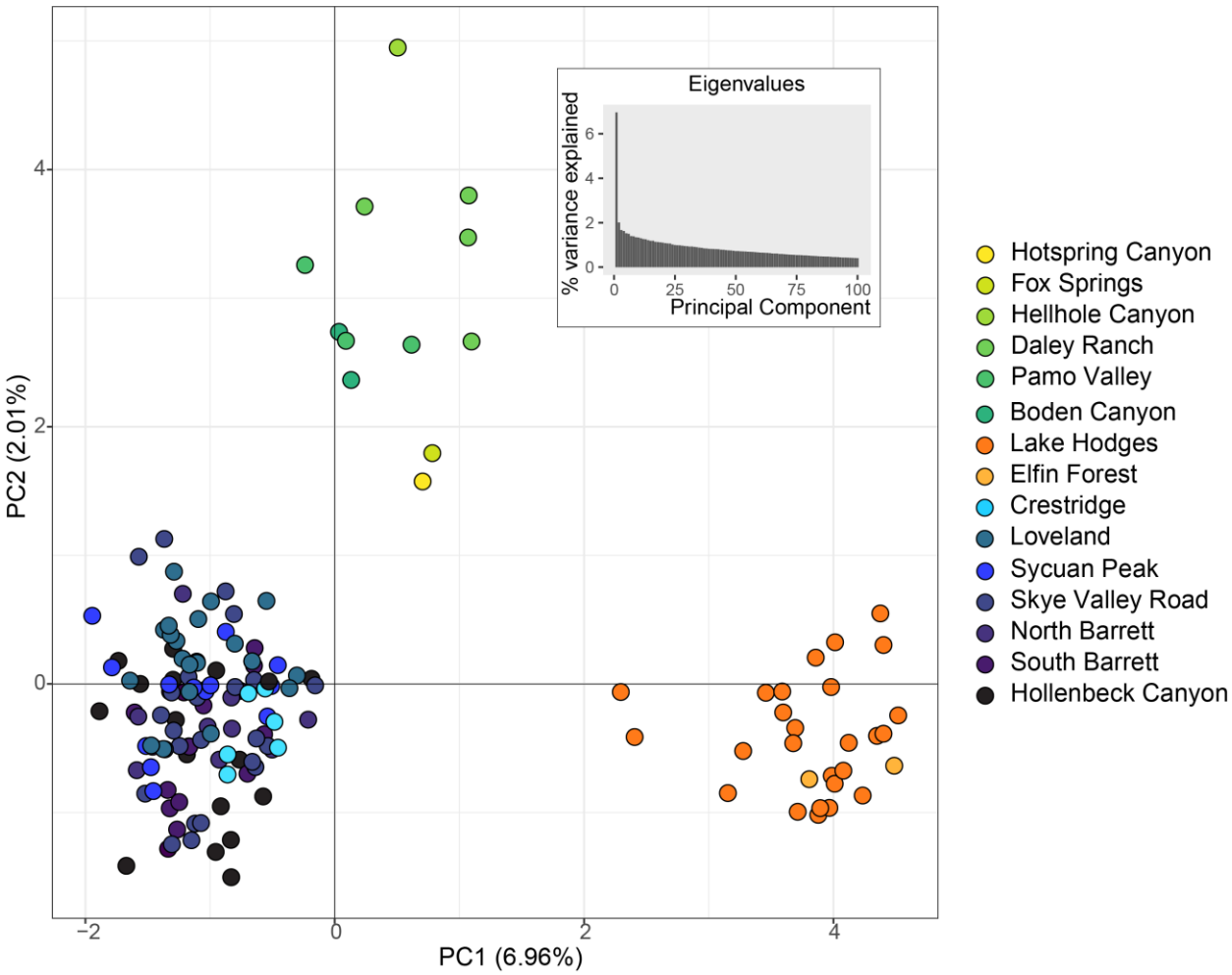

Figure 4. Principal Components Analysis (PCA) results separating geographic groupings of Harbison's dun skippers. Principal Component (PC) 1 separated the Lake Hodges cluster from other local populations (6.96% of the variance). PC 2 (2.0% of the variance) further distinguishes the Northeast cluster (Hot Springs, Fox Springs, Hellhole Canyon, Daley Ranch and Pamo Valley) and Southeast cluster (Crestridge, Loveland Reservoir, Sycuan Peak, Skye Valley Road, Barret Lake and Hollenbeck Canyon). Inset graph shows the percentage of variation explained by each principal component.

Similarly, we found significant genetic isolation by geographic distance across the range (Figure 5; Mantel $r = 0.5738$, $p \leq 0.001$), suggesting that there is likely a stepping-stone model of gene flow among local populations in this subspecies. Given that the Lake Hodges and Elfin Forest populations are more strongly genetically differentiated than other sites and form a unique cluster, it's possible that these western populations are more isolated from the other sampled populations because of habitat fragmentation.

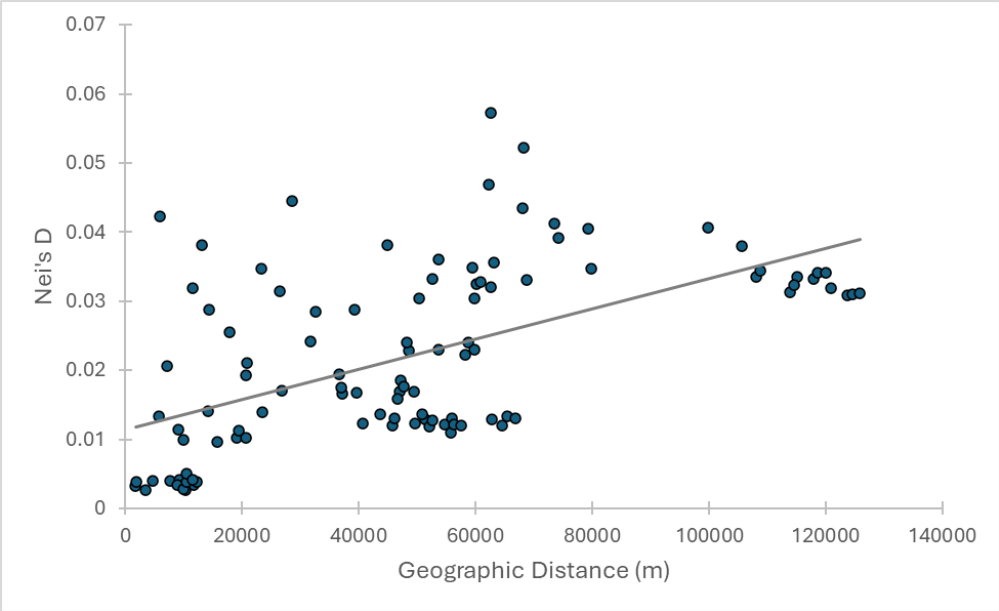

Figure 5. Nei's D, a measure of genetic distance, calculated among pairs of local populations of Harbison's dun skipper plotted by geographic distance among local populations.

Overall, our clustering results support that there is some movement and gene flow among local populations within clusters and support recent connectivity between the Northeast and Southeast clusters. Lyons and others (2024) evaluated marked individuals to identify movement among local populations in the Southeast cluster. Although they did not observe much movement, they suggested that the skippers are not sedentary based on two observations: low numbers of recaptures which suggest that skippers could be moving in and out of the sampled

areas, and their rapid recolonization following fire in two locations (Skye Valley Road and Barrett Lake burned in 2020 but skippers were observed in 2021; Lyons and others, 2024).

Effective population (Ne) sizes in the 2016 cohort were moderately high in both the Southeast and Lake Hodges genetic clusters. Ne point estimates were 968 and 639, respectively, with upper 95% confidence intervals exceeding 1000 in both clusters (Table 2). Although the lower 95% confidence interval for the Lake Hodges cluster was estimated to be below 500 at 338. Theoretically, effective population sizes above 50 to 100 individuals can avoid short term inbreeding effects, while above 500-1000 can preserve the evolutionary potential of populations in the longer term (Frankham and others, 2014). However, individual heterozygosity did decline in both clusters after 2016, particularly in the Lake Hodges cluster (Figure 6), suggesting that population sizes have decreased over this period. This loss of heterozygosity is consistent with the decline in occupancy and abundance between 2013-2017 reported in Lyons and others (2024). These declines could be linked to drought. Marschalek and Deutschman (2018) noted that during multiple drought years in the period between 2014-2018, San Diego sedge plants dried out; when all sedge plants exhibited heavy drought stress in a survey area, Harbison's dun skipper larvae or adults were undetectable.

Table 2. Effective population size estimates (Ne) with lower and upper 95% confidence intervals for the Southeast and Lake Hodges clusters calculated for the 2016 cohort of Harbison's dun skippers. Calculations were made using the linkage disequilibrium method. N is the number of samples included in the analysis. No estimate could be obtained from the Northeast cluster because of low sample size.

Cluster	Ne	Lower 95% CI	Upper 95% CI	N
Southeast	968	733	1418	63
Lake Hodges	639	338	4999	24

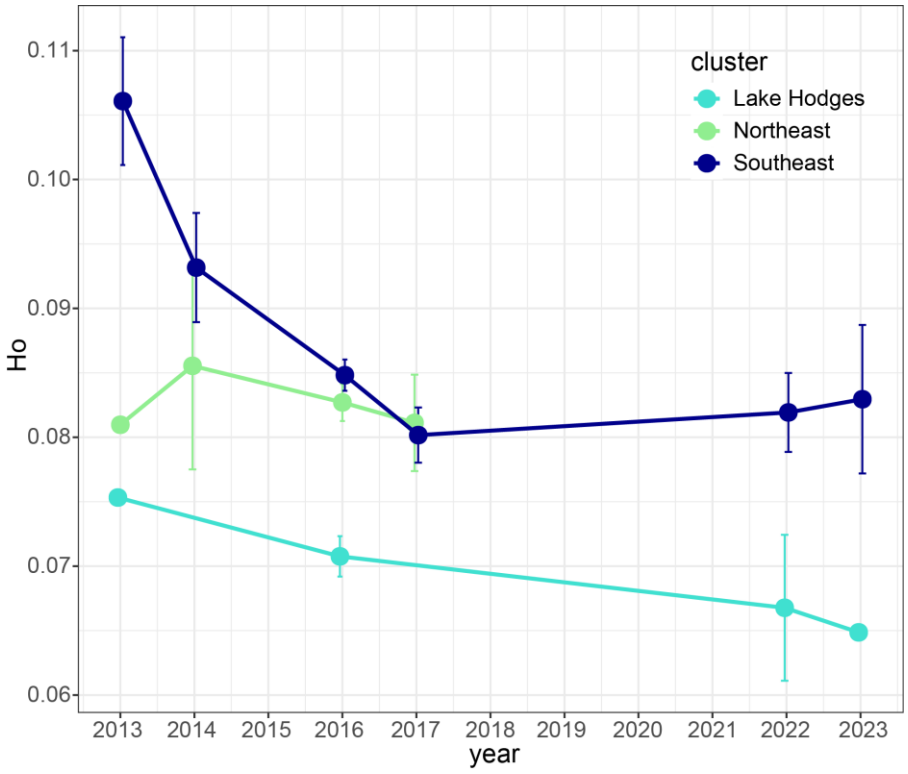

Figure 6. Individual observed heterozygosity (mean and standard error) over sampling year for Harbison's dun skipper individuals grouped by genetic cluster. Both the Southeast cluster and the Lake Hodges cluster showed declines in observed heterozygosity over time. The Lake Hodges cluster also had lower heterozygosity than the other two regional clusters. Loss of heterozygosity can be an indication of population size decline and increasing inbreeding.

Existing population information and genetic collections remain sparse in the northeastern portion of the range. Future efforts to survey this area and collect more samples could help better determine the levels of genetic diversity and genetic connectivity in this region and uncover additional local populations of skippers. Collection of leg tissue is likely the best source of DNA available from non-lethal sampling of adult skippers. Preliminary extraction results of wing clips of closely related skipper species yielded lower DNA volumes than leg tissues. However, we found that the amount of DNA recovered from these legs was variable, but generally low for

genomic level analysis. Current protocols collect legs in 95% ethanol, however, collection in cell lysis buffer may improve genomic DNA yields and should be tested in future efforts.

The Harbison's dun skipper habitat and conservation management plan (Marschalek and Deutschman, 2018) calls for enhancement of habitat in small, extant populations and efforts to restore the distribution, particularly in the central portion of San Diego County (Figure 7). Emerging genetic differences reflected in the two to three clusters and loss of genetic diversity support restoration in this central portion of San Diego County could be beneficial for restoring genetic connectivity. Restoration activities could include restoration of *Carex spissa* (San Diego sedge) and nectar plants in historically occupied areas and could include translocation efforts from currently occupied sites. The current genomic data collected and analyzed here suggests that local populations within the Southeast cluster are more genetically diverse than other areas which may make them appropriate candidate source sites for future translocations. The isolation by distance genetic pattern also suggests that utilizing source sites that are closest to the restoration site may help preserve existing genetic structure. While effective population size estimates in 2016 were moderately high, measured reductions in genetic diversity over time coupled with field survey results that found declines in local population abundance warrant consideration of restoration actions for Harbison's dun skipper.

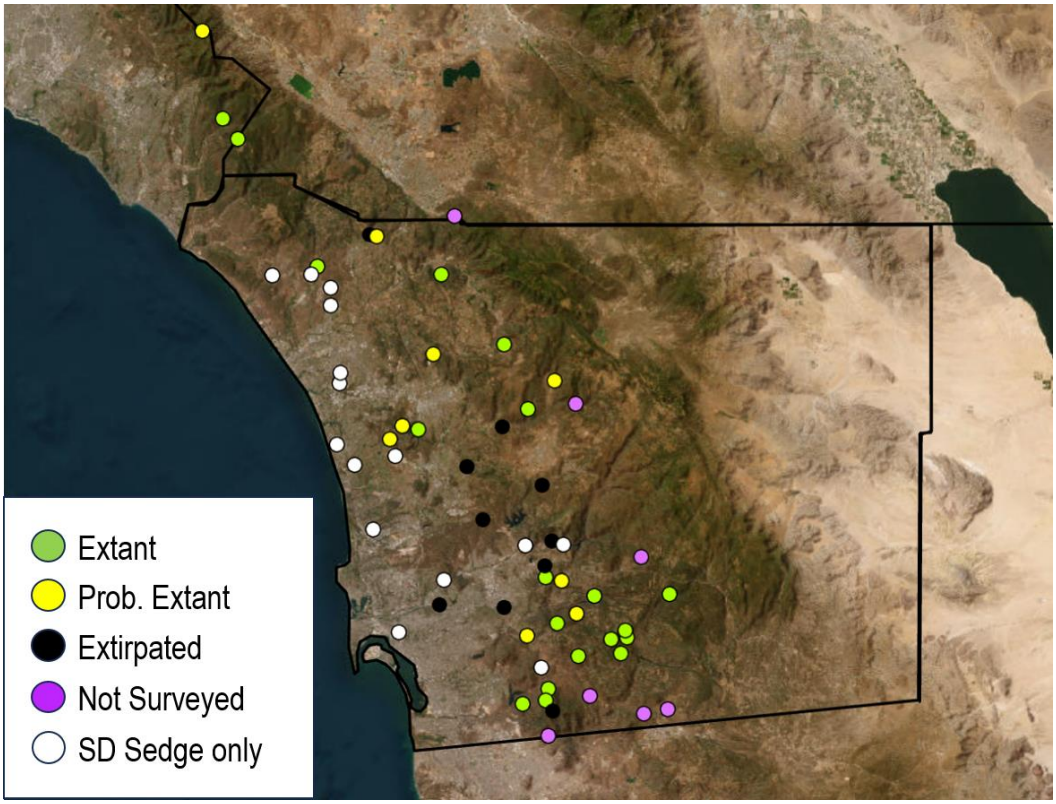

Figure 7. Distribution of Harbison's dun skipper extant local populations surveyed in 2024. The Southeast cluster appears separated from the Lake Hodges and Northeast clusters by surveyed but unoccupied (white) and extirpated sites (black). Reproduced from Marschalek and Faulkner (2025).

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