Final Project Report

Desert Pocket Mouse
Landscape Genomics Project

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Project No. *2021-BEC-2095D*
Date *June 5, 2023*

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Table of Contents

List of Tables

List of Appendices

Appendix A. Figures

Abbreviations and Acronyms

AMOVA	Analysis of Molecular Variance
bp	base pairs
C. p.	*Chaetodipus penicillatus*
DCP	Desert Conservation Program
exp_het	Expected heterozygosity
FIS	inbreeding coefficients
HE	expected heterozygosity
HO	observed heterozygosity
K	number of populations
k	most likely number of genetic clusters
max_het	maximum heterozygosity
max_maf	maximum allele frequency of markers to keep
min_maf	minimum allele frequency of the markers to keep
MSHCP	Multiple Species Habitat Conservation Plan
mtDNA	mitochondrial DNA
NA	number of alleles
Ne	historical effective population size
NE	effective number of alleles
SNP	single nucleotide polymorphism

EXECUTIVE SUMMARY

As of the date of this report, the Clark County Desert Conservation Program (DCP) is pursuing a major amendment to the Clark County Multiple Species Habitat Conservation Plan (MSHCP) and the associated Endangered Species Act Section 10(a)(1)(B) incidental take permit. Objectives for the amendment include revising the list of species covered by the MSHCP and incidental take permit to focus mitigation efforts on species most impacted by private-land development activities, and to include conservation needs of newly Covered Species.

The desert pocket mouse (*Chaetodipus penicillatus*) has been identified as a species of conservation concern that warrants Covered Species status. However, unanswered questions about the taxonomic status, genetic diversity and dynamics, distribution and isolation, and overall health of the species hamper development of an effective conservation strategy and management actions for the species.

The DCP developed a habitat suitability model for *C. penicillatus* and inventoried small mammals in the Riparian Reserve System Units along the Muddy and Virgin rivers. The DCP then conducted surveys in potentially suitable habitat throughout Clark County to assess the presence/absence of *C. penicillatus* in areas identified for potential development, proposed for conservation, and in areas modeled as having a high habitat suitability rating. Three populations of *C. penicillatus* were identified: the Virgin and Muddy Rivers, Las Vegas Wash Wetland Park, and in riparian areas around Laughlin, Nevada.

The goal of this project was to provide information on population genomic structure and evaluate potential landscape connectivity of *C. penicillatus* populations and subspecies to aid in developing an effective conservation strategy for the species in Clark County. Specific objectives included documenting genetic diversity of *C. penicillatus* populations in Clark County; delineating the range of the subspecies in the area, particularly *C. p. sobrinus*; estimating current and past effective population sizes; determining extent and direction of gene flow; and begin identifying threats to long-term viability of the subspecies.

The project evaluated tissue samples from 114 specimens collected during the previous DCP projects and museum specimen tissues from throughout the southwest region to evaluate subspecies relationships. Tissue samples were processed, DNA was prepared, and genotypes were developed. The resulting genotypes were then evaluated to generate summary statistics used to address the above objectives of the study.

Evaluation of Results

Subspecies Distributions and Genetic Diversity. The current nature and distribution of Mojave Desert biota can be explained by glacial retreat and the gradual aridification of the region during the Late Pliocene through the Pleistocene and continuing today. The widespread rivers and pluvial lakes provided riparian habitat conducive to species such as *C. penicillatus.* As the water receded suitable habitat converted to upland vegetation and such species became increasingly isolated resulting in various degrees of divergent lineages recognized today as subspecies. This study identified both support for and discrepancies with the current classifications within *C. penicillatus*, as well as varying degrees of genetic interchange among them. However, the intent of this report is not to propose a reclassification of those subspecies. Regardless, the data indicates three populations of the species were documented as present in Clark County and they are *C. p. sobrinus*.

Distinct Populations and Effective Population Size of *C. p. sobrinus*. The three populations of *C. p. sobrinus* each possess significant genetic structuring which indicates the presence of three genetically distinct populations, likely the result of geographic isolation of the populations over thousands of years

due to the distances between them and the lack of suitable habitat along any potential movement corridors.

Additionally, effective population size for each distinct population was at or possibly below recommended population minimums for effective species conservation objectives and long-term population viability. Populations appear to have undergone recent demographic expansion which may indicate healthy population growth following a relatively recent bottle neck.

Evaluation of Risks and Potential Threats. A preliminary evaluation of risks and potential threats to the species resulted in a number of possible concerns. Habitat conversion to other uses or through encroachment of non-native species would cause the loss of individuals and may result in additional isolation. Increased anthropogenic extraction of water may affect the preferred shrub species within the remaining habitat. The continued aridification of the region due to climate change would continue to reduce the amount and quality of available habitat for the species. Other threats likely exist and an evaluation of these is discussed.

Recommendations

Confirm/Refine Habitat Preferences/Requirements. The preferred habitat for this species has been identified as including relatively dense shrubs with open interspace and gravelly to sandy soil. Studies to further quantify these habitat requirements could inform management plans to maintain or restore habitat.

Population Dynamics Studies and Long-term Monitoring. An evaluation of the population dynamics of the Clark County populations would provide valuable information on the health and condition of the populations in comparison to the genomics assessment from this report. This information also would allow establishment of baseline information for evaluating effectiveness of management actions.

Habitat Corridors Within/Among Currently Protected Areas. Connectivity within and possibly among these populations may be critical for their long-term viability. Identifying potential habitat corridors among the occupied protected areas should be considered.

Survey Intervening Colorado River for Additional Populations. Any additional populations in the County likely would be present in habitat patches along the Colorado River, such as the Overton Wildlife Management Area or small washes fed by springs. Locating such populations around Lake Mead and south to Laughlin would provide additional information on relationships among the populations and provide insight into potential connectivity corridors.

Determine Physiological Tolerances and Resilience to Climate/Anthropogenic Change. Climate change predictions and anthropogenic water use are significant threats likely to add physiological stress to these populations. Evaluating if they could tolerate or adapt to changing conditions and habitat given their isolation would provide additional insight into the vulnerability of the species.

Assess Potential Threats to the Populations. Conservation of the species in Clark County will require an evaluation of the threats to the species in general, and to the three populations individually, to develop sound management actions. Obvious threats include climate change, continued and increased anthropogenic use of water affecting vegetation in the washes, encroachment of non-native plant species, and actions on adjacent private land that may further alter habitat in the protected parcels.

Assess the Taxonomic Status of *C. penicillatus*. A re-evaluation of the subspecies taxonomy is warranted but would not alter the fact that Clark County contains three distinct populations of *C. penicillatus* and that those populations warrant conservation and management as distinct units.

1 INTRODUCTION

1.1 Project Need and Management Actions Addressed

The Clark County Desert Conservation Program (DCP) is currently pursuing a major amendment to the Clark County Multiple Species Habitat Conservation Plan (MSHCP) and associated Section 10(a)(1)(B) incidental take permit. The DCP has several objectives for the MSHCP amendment, but the two that are most pertinent for this project are:

- To revise the list of species covered by the MSHCP and associated incidental take permit to focus mitigation efforts on those species most impacted by private-land development activities.
- To revise the conservation strategy to address the conservation needs of new Covered Species and to improve mitigation effectiveness and program accountability.

The desert pocket mouse (*Chaetodipus penicillatus*) has been identified as a species of conservation concern that warrants status as a Covered Species under the MSHCP amendment. However, unanswered questions about the population dynamics of the species and range of various subspecies make developing an effective conservation strategy for the species difficult.

Landscape connectivity, the extent to which the various land uses within an area affect the movement and behavior of an organism, is an important practical consideration in restoration and conservation planning. As land use intensity increases the landscape becomes ever more fragmented into a mosaic of small patches of suitable land cover types. How a species utilizes the mosaic will influence important processes such as dispersal, emigration, and immigration which are important components to metapopulation dynamics in a fragmented landscape. The size and connectivity of habitat patches affect local inbreeding and effective population size, as well as adaptation over longer periods of time, affecting the long-term viability of the species in a region.

Therefore, consideration of the degree of isolation and how populations are connected across the current landscape is important, as is the potential effects of future development and climate change on those connections and the populations. It is also prudent to understand the current distribution of genetic diversity to better understand how connections (natural and artificial) might maintain or enhance that diversity within and among the small populations and conservation areas. Given the desert pocket mouse is found in few widely distributed small patches of habitat throughout Clark County, Nevada (Clark County), the species may be vulnerable to a wide variety of these threats.

Further, additional information on the potential distribution and validity of the two subspecies potentially present within the County may provide further insight into the management and conservation of the species.

1.2 Project Background

The DCP commissioned development of habitat suitability models for multiple species to provide resource managers a tool for developing conservation goals and management strategies for species of concern, including *C. penicillatus* (Nussear 2020). These models are extremely useful tools, but their effectiveness is tied directly to the number of confirmed observations of the particular species, and relatively few observations were available for developing the model.

In 2020, DCP contracted BEC Environmental, Inc. (BEC) to conduct an inventory of the small mammal community within the Riparian Reserve System Units established along the Muddy River and the Virgin River in eastern Clark County (BEC 2020). The project included establishing transects within parcels of

the Riparian Reserve Units, ensuring the parcels and habitats within each of the units were represented in the effort. BEC biologists captured *C. penicillatus*, a MSHCP Evaluation Species at the time, in all but one of the surveyed sites. It was primarily found in the sandy habitats of the floodplains of the Muddy and Virgin Rivers where vegetation density was low enough to allow exposure of bare soils.

DCP contracted BEC to assess the distribution of *C. penicillatus* throughout Clark County in 2021. Determining the presence or absence of the species in areas identified for potential development or for proposed for conservation was of particular interest for the project (BEC 2021). An additional objective was to investigate areas modeled as having a high habitat suitability rating to identify potential occupied habitat patches and further inform the model. Despite the wide distribution of trapping effort, the species was only located in the riparian habitat along the Muddy and Virgin Rivers, in the Las Vegas Wash Wetland Park, and in riparian areas around Laughlin. See Appendix A - Figure 1 for a distribution of the surveyed areas and locations of successful ("positive") captures (from BEC 2021).

1.3   Project Goals and Objectives

The goal of this project is to provide information on population genomic structure and potential landscape connectivity of *C. penicillatus* populations and subspecies that will aid in developing an effective conservation strategy for the species in Clark County.

Specific objectives of this project included:

1. Document genetic diversity of *C. penicillatus* populations throughout the range of the species in Clark County.
2. Delineate the range of the subspecies, with particular focus on determining the extent of *C. p. sobrinus* range.
3. Estimate current and past effective population sizes.
4. Determine direction and extent of gene flow between populations.
5. Begin identification of potential threats to long-term viability of the *C. p. sobrinus* subspecies warranting further evaluation or management action.

1.4   Project Description

The project entailed evaluating the genomics of individuals within populations of *C. penicillatus* throughout the County and the region to address the varied objectives. Representative specimens of the species captured during the previous DCP projects were euthanized for morphological evaluation and tissue samples were collected for future use. These tissues were the primary basis for the analyses in this study. Additionally, tissue samples from specimens of *C. penicillatus* collected by other mammalogists throughout the region were obtained from scientific collections for use in this study.

These tissues were then processed and analyzed to identify the unique genotype of each individual specimen. The resulting information was then used to evaluate genetic diversity among the subspecies of *C. penicillatus* in the region, to identify distinct populations of *C. p. sobrinus,* to evaluate the degree of introgression of *C. p penicillatus*, and to assess the effective population size of the populations and the degree and direction of gene flow.

2 METHODS AND MATERIALS

2.1 Specimen Collection and Processing

Tissue samples were a combination of tissues collected in previous DCP projects supplemented with museum tissues. The majority of the samples included are from Clark County, which is the focus of the current study.

Tissue samples, typically liver or kidney, from specimens were collected in the field from euthanized animals during standard museum specimen preparation, stored in 70% ethanol or temporarily flash frozen in liquid nitrogen and moved to permanent storage in a -80 °C freezer.

Tissue samples and nondestructive ear-clips from additional museum specimens were included to ensure most subspecies were represented and the entire geographic distribution of the species was covered. The subspecies *C. p. seri*¸ known only from Isla Tiburón in the Gulf of California, was not included in this study.

DNA was extracted from tissues using either DNeasy extraction kits (Qiagen Inc, Valencia, CA) or a lysis buffer protocol (Longmire et al. 1997). A number of DNA samples, especially those representing subspecies outside of Clark County, were extracted prior to the start of this study and utilized a lysis buffer protocol. More recently collected samples, including those processed from Clark County, were extracted using the DNeasy kits following manufacturer's protocol. Following extraction, DNA was visualized using electrophoresis on a 0.8% agarose gel for confirmation of large molecular weight and estimation of total concentration. Approximately 500 nanograms of DNA per specimen was provided to CD Genomics (Shirley, NY) for double digest restriction-site associated DNA (ddDNA) via Illumina sequencing. The sequenced DNA were utilized for genotyping and analysis as described below.

2.2 Genotyping

The qualified genomic DNA of each sample was double digested by restriction enzymes PstI_MspI and incubated at 37°C for 16–20 hours. Fragmented DNA was then purified with Agencourt AMPureXP beads (Beckman Coulter, Inc., Brea, CA) and ligated to barcoded adapters. Samples with different barcodes were pooled on multiplexing batches and bead purified. The samples were loaded onto a BluePippin instrument (Sage Science Inc., Beverly, MA) for size selection. The eluted fraction was amplified with oligo primers that introduced TruSeq indexes and subsequently bead purified. The resulting libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Inc., Carlsbad, CA) and Bioanalyzer DNA assay (Agilent Technologies, Inc., Santa Clara, CA). The constructed libraries were then sequenced with Illumina NovaSeq6000 paired end 150 base pairs (bp).

Raw Illumina reads were processed using the process_radtags module of STACKS 2.5 (Rochette et al. 2019). After filtering out low-quality reads, the remaining data from each individual were aligned to a *Perognathus* reference genome assembly (*Perognathus longimembris pacificus*; Genbank assembly ASM2315922v1). Ref_map and populations pipelines were run within STACKS. Loci found in at least 65% of samples (r = 0.65) with a minor allele frequency of at least 5% (min_maf = 0.05) and heterozygosity upper bound of 0.8 (max_het = 0.8) were retained. One single nucleotide polymorphism (SNP) per locus (--write_single_snp) was retained to meet the assumptions of linkage equilibrium. The VCF file output was converted to appropriate input files using PGDSpider 2.1.1.5 (Lischer and Excoffier 2012)

2.3 Analyses

2.3.1 Genetic Diversity of Subspecies (Objectives 1 and 2)

The geographic distribution of genetic variation was explored in a number of ways. First, we estimated the number of populations (K) using STRUCTURE ver. 2.3.4 (Pritchard et al. 2000). STRUCTURE uses a Bayesian algorithm to assign individuals to a value of K clusters. We utilized the structure input file from STACKS 2.5 to execute runs with a burn-in of 100,000 iterations followed by 100,000 iterations and performed 3 replicates for K=2 through K=10. We examined the mean alpha and mean likelihood scores for evidence of plateau and implemented Structure Harvester (Earl and VonHoldt 2012) which uses the method of Evanno et al. (2005) to determine the most likely number of genetic clusters (k). This exploratory approach allowed us to examine genetic structure broadly across the entire species and determined how many genetic groups are most likely given our data, without any *a priori* information such as geographic distribution.

We further visualized the data using phylogenetic methods. A pairwise distance matrix generated using VCF2Phylip2.0 (Ortiz 2019) was analyzed using the NeighborNet method in SplitsTree4 (Huson and Bryant 2006) to produce an unrooted phylogenetic network.

To generate summary statistics for subspecies we assigned individuals to the appropriate subspecies based on geographic location with a few exceptions. Based on previous mtDNA results (e.g., Jezkova et al. 2009) and morphological data (Hoffmeister and Lee 1967), the northern portion of *C. p. angustirostris* was separated from locations in the south (Appendix A – Figure 2). One of the objectives of the current study was to evaluate the southern boundary of *C. p. sobrinus* in Clark County due to previous 15-mile discrepancy among authorities on the location of the boundary between *C. p. penicillatus* and *C. p. sobrinus.* Hoffmeister (1986) placed the boundary at Davis Dam while Hall (1995) defined it "at least as far north as Searchlight". Recognizing the Colorado River does not appear to be a significant corridor for *C. penicillatus* (Jezkova et al. 2009, Wood et al. 2013, Hoffmeister and Lee 1967) and the lack of any obvious hard biogeographic barrier between *C. p. sobrinus* and the northern distribution of *C. p. penicillatus*, the majority of the evidence supports some level of introgression between the two currently described subspecies occurs in southern Nevada. This introgression is to be expected between subspecies at contact zones. Given recognition of subspecies (especially nonallopatric subspecies) can be contentious (Burbrink et al. 2022) methodology has been developed and proposed for delineating subspecies in rodents (Patton and Conroy 2017).

To generate summary statistics for this study we included all Clark County samples in *C. p. sobrinus* and evaluated the level and extent of introgression using the above STRUCTURE analysis for the entire species and applied population level analyses on smaller subsets of the Clark County populations as outlined below.

2.3.2 Identification of Distinct Populations of *C. p. sobrinus* in Clark County (Part of Objective 2)

We defined three distinct populations within Clark County to further analyze population demographics within the focal study area. The populations were largely based on geographic features and the known natural distribution of the species following several years of fieldwork aimed at this endeavor (BEC 2020, BEC 2021) and further supported by some of the preliminary results of the exploratory data analysis (see Appendix A – Figure 2 as adapted from BEC 2021). These three populations are:

1. <u>Muddy/Virgin Population</u>: Muddy River, Virgin River and Colorado River north of Lake Mead
2. <u>Las Vegas Wetlands Population</u>: Springs Preserve and the Wetlands in Las Vegas, Nevada
3. <u>Laughlin Population</u>: Areas around Laughlin, Nevada

The following analyses treated each population separately. We used the populations module in STACKS software we calculated numbers of alleles (NA), effective numbers of alleles (NE), expected (HE) and observed (HO) heterozygosity, and inbreeding coefficients (FIS) for each sampled population. We estimated Fu's F and Tajima's D in Arlequin 3.5.2.2 (Excoffier et al. 2005) on the subsets of individuals to test for departures from a Wright-Fisher model of neutral evolution (Tajima1989, Fu 1997). We also tested for significant population structure of the three *C. p. sobrinus* populations using an AMOVA in Arlequin 3.5.2.2 (Excoffier et al. 2005).

2.3.3   Historical Effective Population Size and Gene Flow (Objective 3 & 4)

We estimated historical effective population size (Ne) using SNeP v1.11 (Barbato et al. 2015). We set the number of bins to 8, minimum number of items to 2, and minimum distance between SNPs to 1000 and analyzed all chromosomes with sufficient data following thinning. We used coalescent simulations to estimate the direction and magnitude of gene flow among populations in fastsimcoal2 (Excoffier et al. 2021). We estimated all possible combinations of gene flow among populations. We restricted both analyses to the dataset of the three populations of *C. p. sobrinus* in the County.

3   RESULTS AND EVIDENCE OF THE RESULTS

3.1   *Sample Processing, DNA Sequencing, and Genotyping*

The final dataset totaled 114 samples. Initially 130 samples were provided to CD Genomics. The genomic library preparation failed for three samples, and the lab was unable to produce results. An additional sample required further troubleshooting in the lab and was successfully sequenced; however, it was not finished in time to be included in the results presented here. Seven samples were discovered to have been misidentified in the field by the collectors. These other species were not included in the analyses presented here but will be used for other phylogenetic analyses in the future. An additional five samples were excluded due to a significant amount of missing genetic data. A total of 45,641 single nucleotide polymorphisms were identified from STACKS analyses following filtering. The sequenced DNA data were submitted to the DCP separately in March 2023.

The sequenced DNA were utilized for genotyping and then the analyses described below.

3.2   *Subspecies Genetic Diversity and Distribution (Objectives 1 and 2)*

We found support for both the Jezkova et al. (2009) hypothesis and further subspecific structuring highlighting the complex phylogeographic history of the species. The Structure Harvester results supported three genetic clusters (Appendix A - Figure 4), two of which support a Mojave and Sonoran group with admixture in western Arizona through Laughlin into Las Vegas.

Higher levels of K provided additional resolution in geographically defined genetic structure (not shown) but additional details of phylogenetic structure are evident in the phylogenetic network (Appendix A - Figure 5). Distinct clusters that coincide with geographically isolated populations in the County provide further support for the conclusion of isolated populations.

Significant population structuring was found among the populations we identified in Table 1. Approximately 37% of the genetic variation was among the three groups. Most of the variation (54%) was within individuals reflecting the high level of rare alleles.

Table 1. Analysis of Molecular Variance (AMOVA) conducted in Arlequin only for *C. p. sobrinus* populations.

Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation
Among groups	2	2082.156	25.37679 Va	36.85
Among populations within groups	3	266.744	2.44692 Vb	3.55
Among individuals within populations	59	2612.331	3.24224 Vc	4.71
Within individuals	65	2456.5	37.79231 Vd	54.88
Total	**129**	**7417.731**	**68.85826**	**99.99**

Summary statistics for each of the currently defined subgroups are presented in Table 2, Table 3, and Table 4. As seen in Table 4, Tajima's D and Fu's F were statistically negative for the Muddy/Virgin Population of *C. p. sobrinus*. The other populations of *C. p. sobrinus* had nonsignificant negative values.

Table 2. Number of private alleles for different subspecies and specific populations of *C. p. sobrinus*.

ID	Private Alleles	Obs_Het	Var	Obs_Hom	Var	Exp_Het	StdErr	Exp_Hom	Var
penicillatus	10503	0.06059	0.0119	0.93941	0.0119	0.07997	0.00067	0.92003	0.0161
pricei	7428	0.08781	0.0181	0.91219	0.0181	0.1149	0.00085	0.8851	0.02229
Muddy/Virgin	4879	0.01753	0.0055	0.98247	0.0055	0.01959	0.00038	0.98041	0.00536
Las Vegas Wetlands	1137	0.02018	0.0071	0.97982	0.0071	0.02397	0.00049	0.97603	0.00762
Laughlin	2998	0.04592	0.0134	0.95408	0.0134	0.05164	0.00063	0.94836	0.01423
angustirostris	1395	0.03215	0.0119	0.96785	0.0119	0.03843	0.00057	0.96157	0.01252
stephensi	588	0.01648	0.0089	0.98352	0.0089	0.01427	0.00037	0.98573	0.00502

Table 3. Calculated Pi and Fis for different subspecies and specific populations of *C. p. sobrinus*.

Group ID	Pi	Var	StdErr	Fis	Var	StdErr
penicillatus	0.08147	0.01671	0.0007	0.11153	0.077	0.00846
pricei	0.12199	0.02514	0.0009	0.10281	0.0863	0.00286
Muddy/Virgin	0.01986	0.00551	0.0004	0.01243	0.0117	0.01032
Las Vegas Wetlands	0.0252	0.00843	0.0005	0.01435	0.0151	0.00429
Laughlin	0.05344	0.01524	0.0007	0.026	0.0247	0.00547
angustirostris	0.04153	0.01462	0.0006	0.02235	0.0254	0.00182
stephensi	0.01631	0.00656	0.0004	0.00023	0.0073	0.00014

Table 4. Calculations of Tajima's D and Fu's F statistics for different subspecies and specific populations of *C. p. sobrinus*.

ID	Tajima's D	p-value	Fu's Fs	p-value
penicillatus	-1.70315	0.014	-5.977	0.02
pricei	-0.90213	0.184	0.7555	0.423
Muddy/Virgin	-2.06059	0.004	-24.401	<0.001
Las Vegas Wetlands	-0.84044	0.234	-3.1175	0.069
Laughlin	-0.49059	0.38	-3.2358	0.056
angustirostris	-0.12803	0.496	-0.3806	0.265
stephensi	-0.46097	0.352	-0.0441	0.324

3.3   Effective Population Size (Objectives 3)

Historical effective population size has dramatically dropped for the Clark County populations (Figure 6). Oldest estimates are for 5,000-6,000 generations ago which can be roughly translated to similar number of years if it is assumed one generation/year for this mouse although the oldest estimates from SNeP should be viewed with caution, so they are not reported here. Historical effective population sizes for each of the populations are estimated to have been as high as approximately 4000 in the Muddy/Virgin population in the distant past and declined to approximately 200 recently. Similar declines are seen in the other two Clark County populations to levels below 100.

3.4   Extent and Direction of Gene Flow (Objective 4)

We included this objective to evaluate the historical direction and extent of gene flow among the *C. p. sobrinus* populations in Clark County. However, despite multiple attempts to conduct the analysis, we could not achieve a convergence of results from independent runs of the fastsimcoal2 analysis. A reliable reliable estimate of the amount or direction of flow of genetic material could not be obtained. The problem appears to be due to the programming in the analytical tools. We were not able to produce results we felt confident in presenting in this report. We are investigating alternative analyses, including treemix (Pickrell and Pritchard 2012) and moments (Jouganous et al. 2017), that may allow us to generate more robust and reliable results in the future.

3.5   Begin Assessing Threats to Populations (Objective 5)

The potential threats to the populations are reviewed and discussed in Section 4.

4   EVALUATION/DISCUSSION OF RESULTS

4.1   Subspecies Distributions and Phylogeography of the Mojave Desert (Objectives 1 and 2)

The Late Pliocene to Pleistocene development of the Mojave Desert biota described in Bell et al. (2010) proposes a number of distinct geophysical and climatic phenomena responsible for the current distribution of species lineages that occupy the desert southwest. This includes the mtDNA hypothesis for *C. penicillatus* of Jezkova et al. (2009). Of particular interest is the early Pleistocene regional isolation that divided *C. penicillatus* into north and south Mojave and Sonoran clades followed by secondary contact post-Pleistocene glacial retreat (6,000 years ago to present). The widespread pluvial lakes and largely continuous riverine systems during the Pleistocene (Lancaster and Tchekarian 2003, Enzel et al. 2003) would have likely favored expanding populations of *C. penicillatus*, given our understanding of their

current habitat requirements of denser brush vegetation and proximity to water. Near the end of the Pleistocene and, more recently, the aridification of the Mojave Desert has resulted in the desiccation of the widespread rivers and Pluvial lakes severing connections among the northern populations (Jezkova et al. 2009).

Although the intention of this report is not to recommend taxonomic changes, we feel it appropriate to comment on the support and possible discrepancies our data provide with the current subspecific classification of *C. penicillatus*. While the STRUCTURE results appear to support the hypothesis of introgression between a northern and southern lineage of *C. penicillatus*, we also find clearly distinct geographically isolated populations within *C. p. sobrinus*. The large number of SNP nuclear markers certainly allows for greater resolution than morphology or mtDNA could provide at the population level. Continuing with the above phylogeographic model, we find support for the hypothesis that numerous remnant populations of the various subspecies within the Mojave Desert have been isolated, scattered across the region. This includes *C. p. stephensi*, portions of what is currently *C. p. angustirostris*, and at least two populations within *C. p. sobrinus* along the remaining tributaries of the Colorado River (Muddy/Virgin Rivers and Las Vegas Wash) in Clark County. These populations are apparently isolated, and over the last 5000+ years (using a one-year generation time estimate), inbreeding has led to dramatic declines in effective population size as expected (addressed in section 4.2). Additionally, the population of *C. p. sobrinus* near Laughlin appears to be an introgression zone for *C. p. sobrinus* and *C. p. penicillatus* which may extend south into Arizona.

We identified other notable findings. Southern portions of current *C. p. angustirostris* populations are clearly genetically related to *C. p. penicillatus*. The Gila River appears to be a soft border between *C. p. penicillatus* and *C. p. pricei*, consistent with previous investigators' findings (e.g., Patton et al. 1981, Jezkova et al. 2009). Further evaluate of these results in the future and reevaluation of the taxonomy of the subspecies is recommended based on these data.

4.2   Distinct Populations and Effective Population Size of C. p. sobrinus (Objective 3)

The distinct populations of *C. p. sobrinus* are of particular interest for conservation. Each population should be treated as distinct units and managed separately. The small effective population size and significant population structuring suggest the populations have been geographically isolated for some time. It is important to emphasize that effective population size is not census size and these estimates should not be interpreted as the number of individuals in each population, which would be larger than these estimates of effective population size. Effective population size is the size of an idealized population that would give the same genetic parameter as the population under study and in SNeP v1.11 it is calculated using linkage disequilibrium among multiple unlinked loci (Barbato et al. 2015). Numerous factors can influence the value including selection, sex ratios, and statistical choices so the specific values should be considered with caution while the relative values and changes overtime can provide useful insight into population structuring.

Currently, small effective population sizes were calculated in each of the three Clark County populations. The results of the study suggest a gradual decline in effective population size over time, recently to values between 50 and 250 for the different Clark County populations. The general consensus in the literature has been that effective population sizes above 50 are sufficient for conservation purposes and long-term viability (Franklin 1980); however, recently, that value has been called into question following 40 years of research, and values of 100 or more might be more appropriate depending on objectives (Frankham et al. 2016).

The results from the neutrality tests (Fu's Fs and Tajima's D) indicate significant departures from neutral evolution, particularly in the Muddy/Virgin population (Table 4). Values not different from zero (for example Laughlin and Las Vegas Wetlands) indicate populations at equilibrium between mutation and drift. Negative values for these two statistics indicate an excess of rare alleles which can be interpreted as recent population expansion following a bottleneck (Tajima 1989, Fu 1997). This phenomenon can also be seen in the long individual branches of the phylogenetic network and the high proportion of genetic variation within individuals in the AMOVA (Appendix A - Figure 5 and Table 1). An alternative hypothesis is that a strong selection sweep for a new beneficial characteristic or mutation has occurred in this population. A new beneficial characteristic or mutation cannot be ruled out with the analyses presented here, but we believe it is less likely the entire genome (represented by genome wide SNPs) is involved in the sweep. More likely, some of the loci could be under selection or near regions that are being selected for.

The genetically unique northernmost Muddy/Virgin population is of particular interest. Peripheral populations are ideal for studying adaptation because they tend to be at a physiological extreme providing a strong selective pressure and those populations tend to be small which together allow for very rapid evolution which may provide important variation when conditions change (MacDonald et al. 2017). Future analyses should focus on determining what genes (if any) in these peripheral populations are under selection and the adaptive potential of the populations under different climate change and disturbance scenarios.

Significant genetic isolation among the three populations of *C. p. sobrinus* is further supported by the apparent absence of this species in the intervening areas (BEC 2020, 2021) and the habitat loss associated with climate shifts over the last 10,000 years. The most likely routes of connectivity would either be along the Colorado River where there is no obvious habitat (although additional field efforts should focus in these areas) or in the intervening dry valleys throughout Clark County. The Colorado River water system seems most likely for route connectivity given the historical connections among the populations associated with the Pluvial Lake system that occupied the area now consists of unsuitable dry lake beds. With this in mind, the County's current and future system of protected areas appears to cover each of these populations. The prudent way forward would be to work toward understanding the physiological and ecological needs of these populations and focus on the three distinct areas separately. Future research needs for these populations should include understanding how climate change and anthropogenic water use will impact these populations and their adaptive potential given the declining genetic diversity.

4.3 Direction and Extent of Gene Flow (Objective 4)

One objective of the study was to evaluate the historical direction and extent of gene flow among the *C. p. sobrinus* populations in Clark County. The analyses appear to support the hypothesis that there is very little to no genetic connectivity among the Clark County populations, and we could not attain reliable estimates of the amount or direction of flow of genetic material from the fastsimcoal2 analysis. The problem appears to be due to the programming being used in the analytical tools. Alternative analyses, including treemix (Pickrell and Pritchard 2012) and moments (Jouganous et al. 2017), that may allow for more robust and reliable results in the future are being evaluated.

4.4 Evaluation of Risks (Objective 5)

Based on the results of this and previous studies of the species in Clark County, *C. penicillatus* is represented in the area as three known genetically distinct populations with the northern two being populations of the subspecies *C. p. sobrinus* and the third being an introgression of *C. p. penicillatus* and *C. p. sobrinus*. Additionally, results indicate these populations have been isolated for an extended period of time resulting in steadily declining effective population size within each to the point that the viability

of the populations is at risk. Finally, the cause of this isolation is the loss of suitable habitat in the intervening areas which has resulted from the long-term desertification of the Mojave Desert and further exacerbated by the development and management of the Colorado River system, land development, and now climate change.

Given this assessment of the status of the species in Clark County, we identify a series of threats to the long-term viability of the species for further evaluation and consideration for conservation and management of the species.

Habitat Conversion to Other Uses
The amount of habitat available for this species is limited to the riparian corridors and habitats along the Virgin and Muddy Rivers, the Las Vegas Wash, and around Laughlin. While parcels of land in these areas have been afforded various degrees of protection through existing ownership or recent acquisitions by the County or other entities for conservation, large areas remain in private ownership. Development of those lands would further reduce potential habitat for the species.

Loss of Habitat Quality on Protected Parcels
The quality of the habitat on many of the protected parcels may not be optimal for the species despite the fact the species is present. Many of these parcels have varying degrees of infestation of invasive plant species which displace the native shrubs and become monocultures with no interspace as required by the species. Continued encroachment of invasive plants places the species at further risk. Additionally, activities or conditions on adjacent private parcels may impact the protected parcels.

Connectivity
Connectivity among groups of the species has been identified as an important factor in the viability of populations and subspecies of throughout southern Nevada. Connectivity was evaluated in this study, and while the degree and direction of geneflow could not be calculated, the results indicate minimal connectivity among the populations. As stated above, the three populations of the species have been isolated for an extended period and by significant distance. Restoring connection between the populations may be unfeasible. However, maintaining or improving connectivity within the three populations may provide conservation benefits to the populations where such actions are possible.

Disease
Given the individuals within the three populations possess low genetic diversity, these individuals may be at higher risk from new diseases to which they have not been previously exposed.

Climate Change
The continued effects of climate change and the increased desertification of the region will result in changes to the vegetation communities, including the riparian shrub habitats required by this species. The increased demand for water will further reduce the groundwater and soil moisture required to maintain these riparian habitats. As these habitats are further depleted, the threats to this species and others increase.

5   SUMMARY AND CONCLUSIONS

Subspecies Distributions and Genetic Diversity
- Evidence indicates a degree of admixture has occurred among some subspecies in southern Nevada, particularly *C. p. penicillatus* and *C. p. sobrinus* in the southern portions of Clark County.

Distinct Populations of *C. p. sobrinus*
- Significant genetic structuring indicates the presence of three genetically distinct, geographically isolated populations.
- Current system of protected lands contains portions of each of these populations.
- Each population should be managed as a distinct population. Moving individuals between populations to increase diversity is not currently advised.

Effective Population Size of *C. p. sobrinus*
- Effective population size for each distinct population is at or possibly below recommended population minimums for effective species conservation objectives and long-term viability.
- Populations appear to have undergone recent demographic expansion which may indicate healthy population growth following a bottle neck. Future efforts should evaluate the adaptive potential of these peripheral populations.

Evaluation of Potential Threats
- Multiple potential threats have been identified for further evaluation and consideration during development of conservation and management plans:
    o Habitat conversion to other uses
    o Loss of habitat quality on protected parcels
    o Connectivity
    o Disease
    o Climate change

6   RECOMMENDATIONS

With the potential classification of *C. penicillatus* as a Covered Species under the MSHCP, robust management plans goals must be established to support the conservation success of the species. Based on the results of the previous surveys, the species has limited distribution in the County. Based on the results of this study, the three known populations are relatively isolated from each other, and the two northern populations are isolated from any other populations of the species.

While the subspecies status of the population in Laughlin is unclear, the two northern populations of the *C. p. sobrinus* subspecies are distinct populations. Additionally, the results of this study clearly indicate the effective population size of the populations are at or below the thresholds typically indicative of at-risk species. Therefore it is critical to develop and implement management and conservation actions soon and ensure they are based in evidence. As such, the following recommendations have been developed for consideration for future efforts to develop the information needed to make sound management decisions.

Confirm/Refine Habitat Preferences/Requirements
The preferred habitat for this species has been identified as requiring relatively dense shrubs with open interspace, and with gravelly to sandy soil. Studies to further define these habitat requirements would be valuable for informing development of simple cost-effective steps to maintain or restore habitat conditions in currently protected areas. This should include measures of population density in different substrate and vegetation conditions for each population to determine what conditions are ideal for long term persistence of the population.

Population Dynamics Studies and Long-term Monitoring
In coordination with the habitat preferences study, an evaluation of the population dynamics of each population of the species would support the establishment of a long-term monitoring program. One objective would be to assess the genomic and population stability of the species

throughout these areas for comparison with the genomics assessment from this report. The second objective would be to establish a baseline for comparison to similar data collected in the future to assess the effectiveness of management plans.

Identify Habitat Corridors Within/Among Currently Protected Areas

Identification of potential habitat corridors among currently protected areas would allow a continuous connection with each population to ensure the largest network possible. This could include remote sensing and geospatial mapping at a landscape level combined with the genetic data presented here.

Survey Intervening Colorado River for Additional Occupied Areas in Clark County

Surveys conducted thus far have exclusively sampled lands managed by the County and the Bureau of Land Management. If additional populations are present in the County, they likely are present along the Colorado River. While much of this habitat is in private ownership, the Overton Wildlife Management Area may possess suitable habitat. It is likely that little habitat for this species occurs in the Lake Mead National Recreation Area north of the Hoover Dam. Much of the habitat was lost due to anthropogenic changes along the river, including damming and flooding of the river to create Lake Mead and Lake Powell. Inconsistent water levels in Lake Mead make it unlikely that native vegetation will recolonize to create the dense shrub habitat preferred by *C. penicillatus*. However, a few springs located in the Stewarts Point area may retain sufficient vegetation to support small populations. If populations exist in new locations, some individuals could be submitted for ddRAD to assign to populations and further refine our understanding of the distribution and connectivity.

Determine Physiological Tolerances and Resilience to Climate/Anthropogenic Change

Future climate change predictions and anthropogenic water use (in particular, declining levels of above ground water) are significant threats that are likely to add physiological stress to these peripherally isolated populations. The question of whether these populations could tolerate or adapt to changing conditions, given that dispersal to new favorable areas will be unlikely, should be addressed. This could be assessed through documenting the use of torpor for managing stress and temperature and humidity tolerances in each population. Additional analysis of the genomic data presented in this report would allow for cursory assessment of adaptive potential.

Assess Potential Threats to the Populations

The current protected areas in Clark County cover only a portion of each of the three populations. While the land management agencies can establish management plans to protect and even enhance the habitat in these areas for this species and many others that reside in these parcels, many factors are outside their control. Climate change has and will continue to affect vegetation communities in the region. Continued and increased anthropogenic use of water may further affect availability of shrub species and available habitat. Additionally, the agencies managing these protected areas have little control over what happens on neighboring private land. Activities on private land that may alter the protected areas, such as habitat removal and land development, expanded grazing, or other activities. A thorough threat assessment should be conducted to identify and address a range of "worst case" scenarios and develop measures to reduce the risks and provide potential responses to risk occurrences.

Assess the Taxonomic Status of *C. penicillatus*

A re-evaluation of the subspecies taxonomy is warranted. Recognizing that Clark County contains three distinct populations of *C. penicillatus* (regardless of which subspecies they are taxonomically assigned to) is an appropriate way to move forward in the management of this species. The two northern populations are likely at higher threat. The Muddy/Virgin area is

threatened by isolation and connection to the small tributaries which could see reduced flow in the near future, and the Las Vegas wetlands area is threatened due to its small size and urban development nearly entirely encompassing it.

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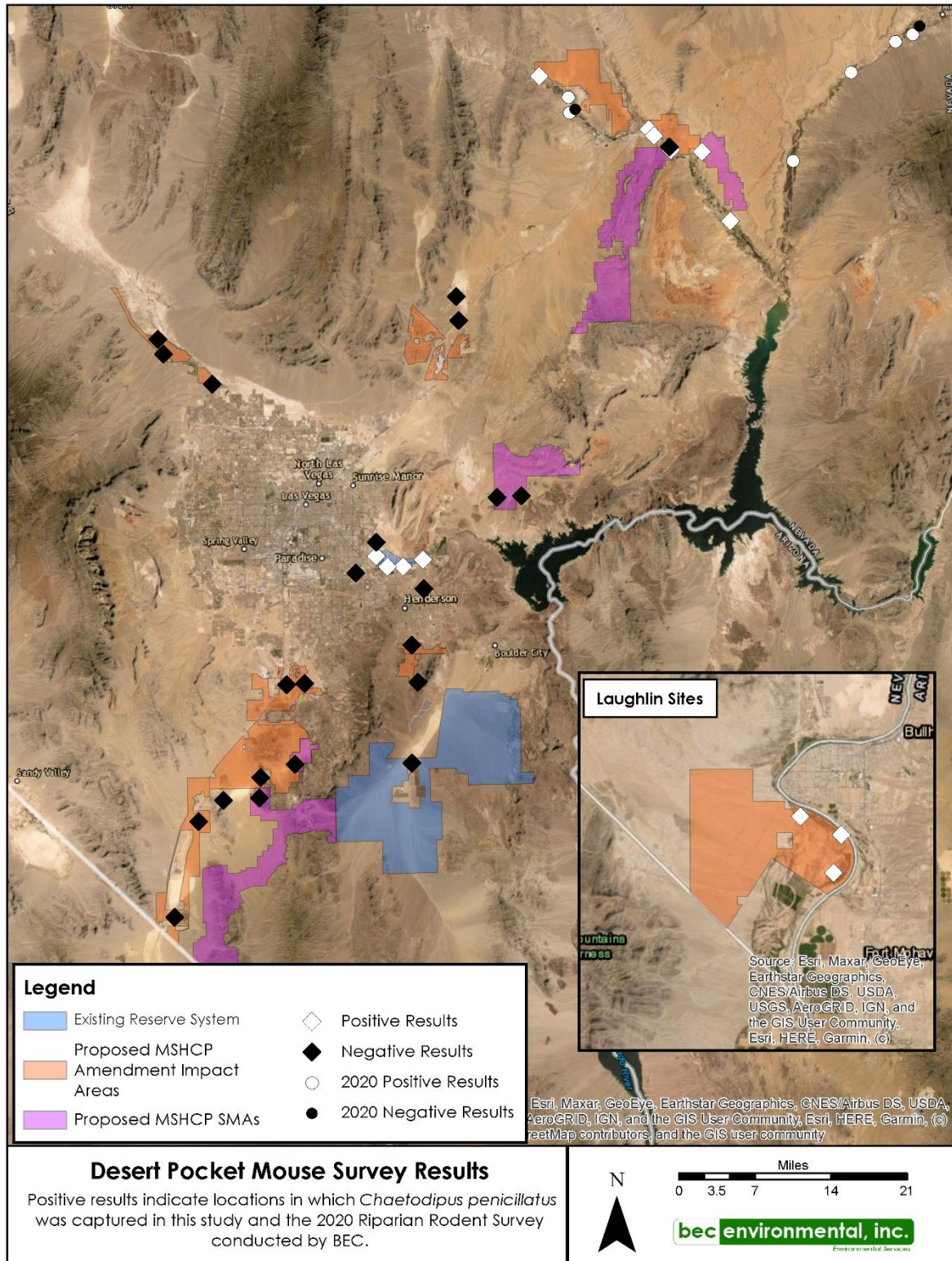
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APPENDIX A
Figures

Figure 1. Results of Previous *Chaetodipus penicillatus* Surveys in Clark County, NV (Figure from BEC 2021).
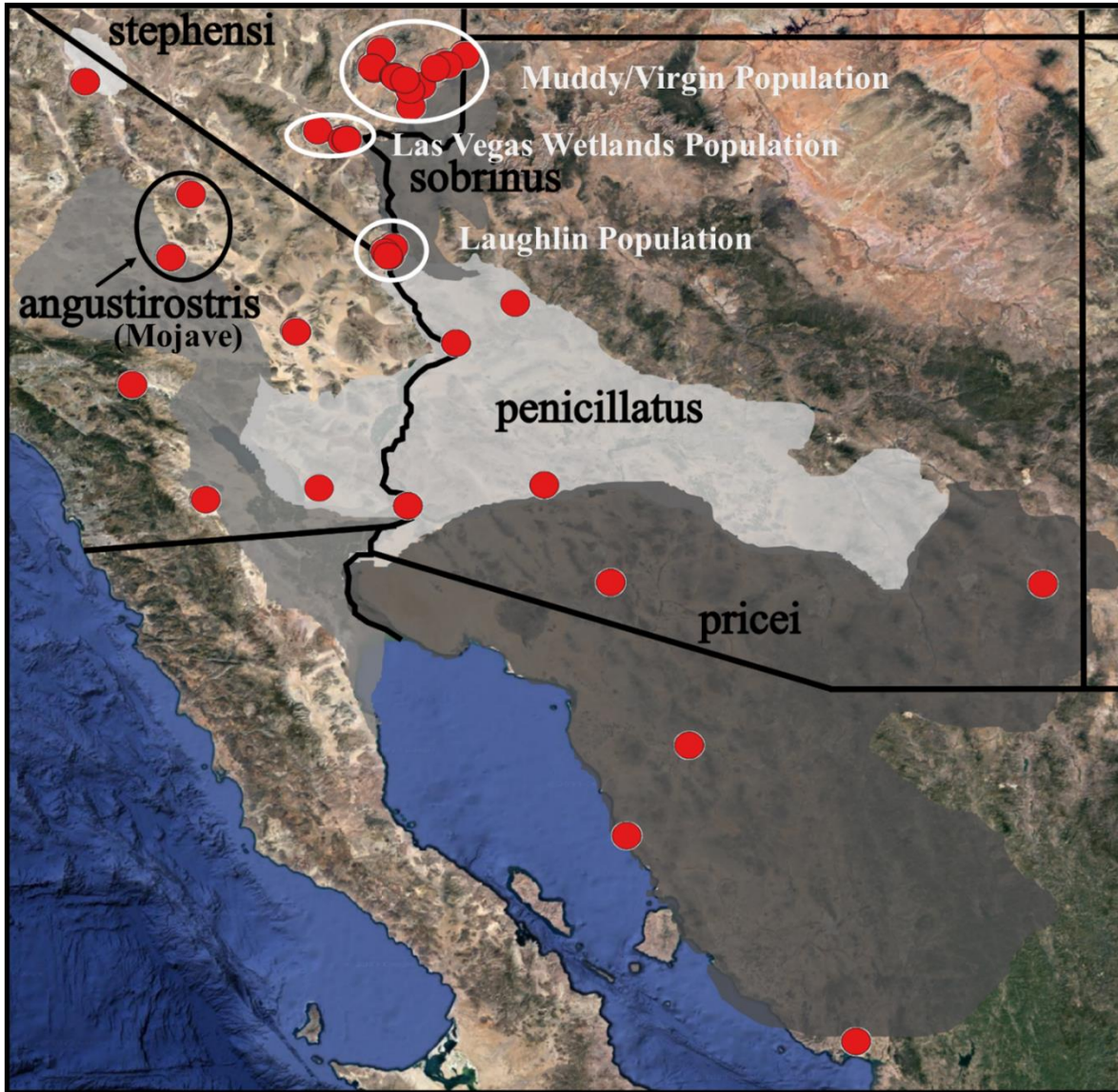
Figure 2. Locations of samples used in the analysis showing subspecies distribution (following Hoffmeister and Lee 1967), and population assignments used in this study.
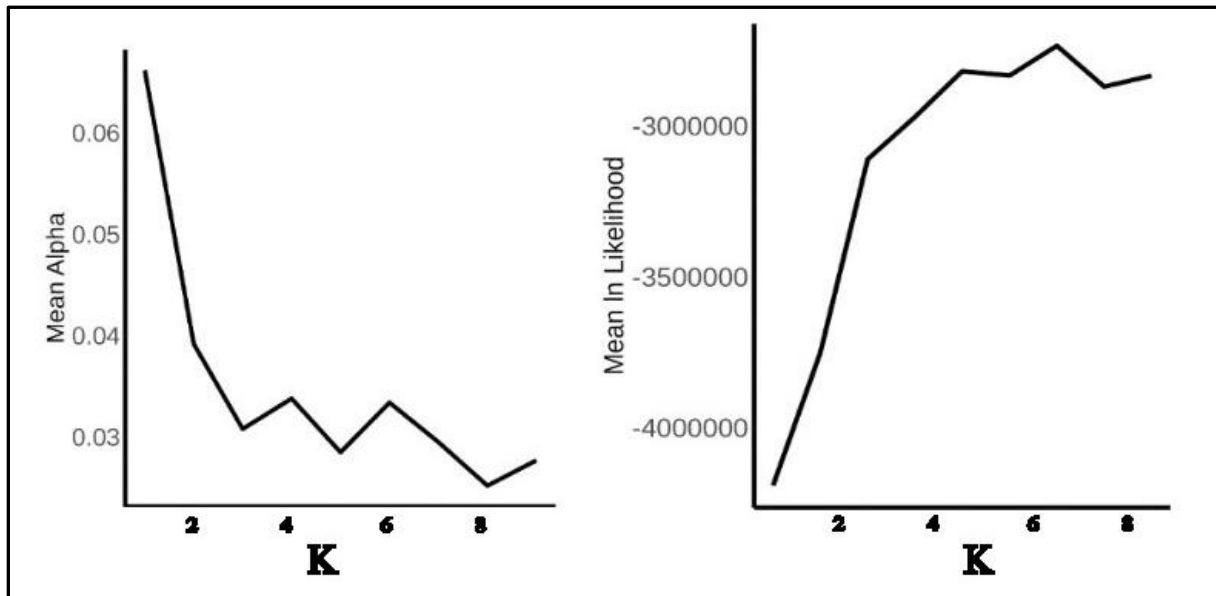
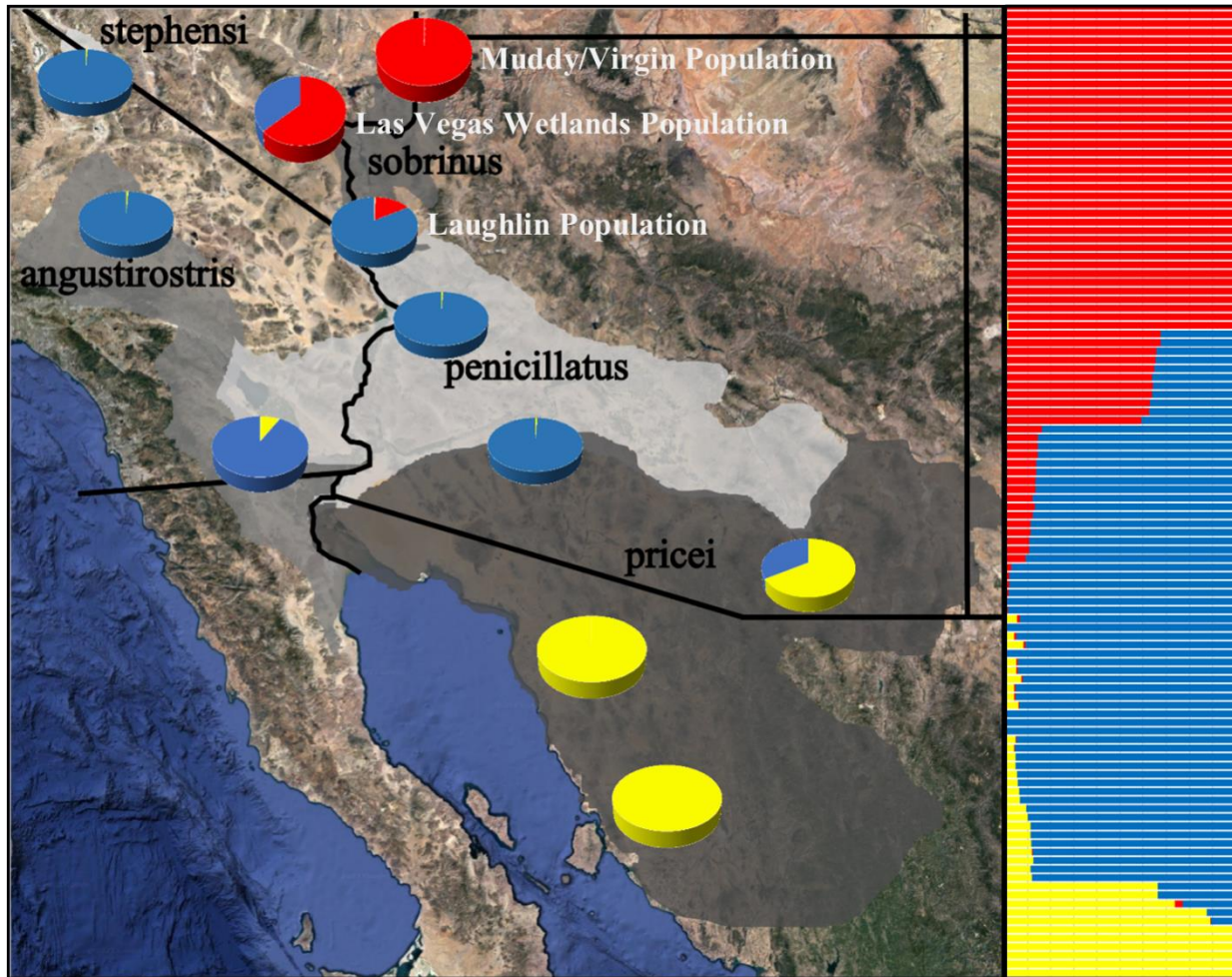**Figure 3. STRUCTURE Harvester results and choice of K. Method of Evanno et al. (2005) selected K=3 as best.**

Figure 4. Results of structure for K=3. Histogram shows proportion of assignment to each group. Pie charts show proportion of representative populations belonging to each group. Some populations are combined for visuals purposes.
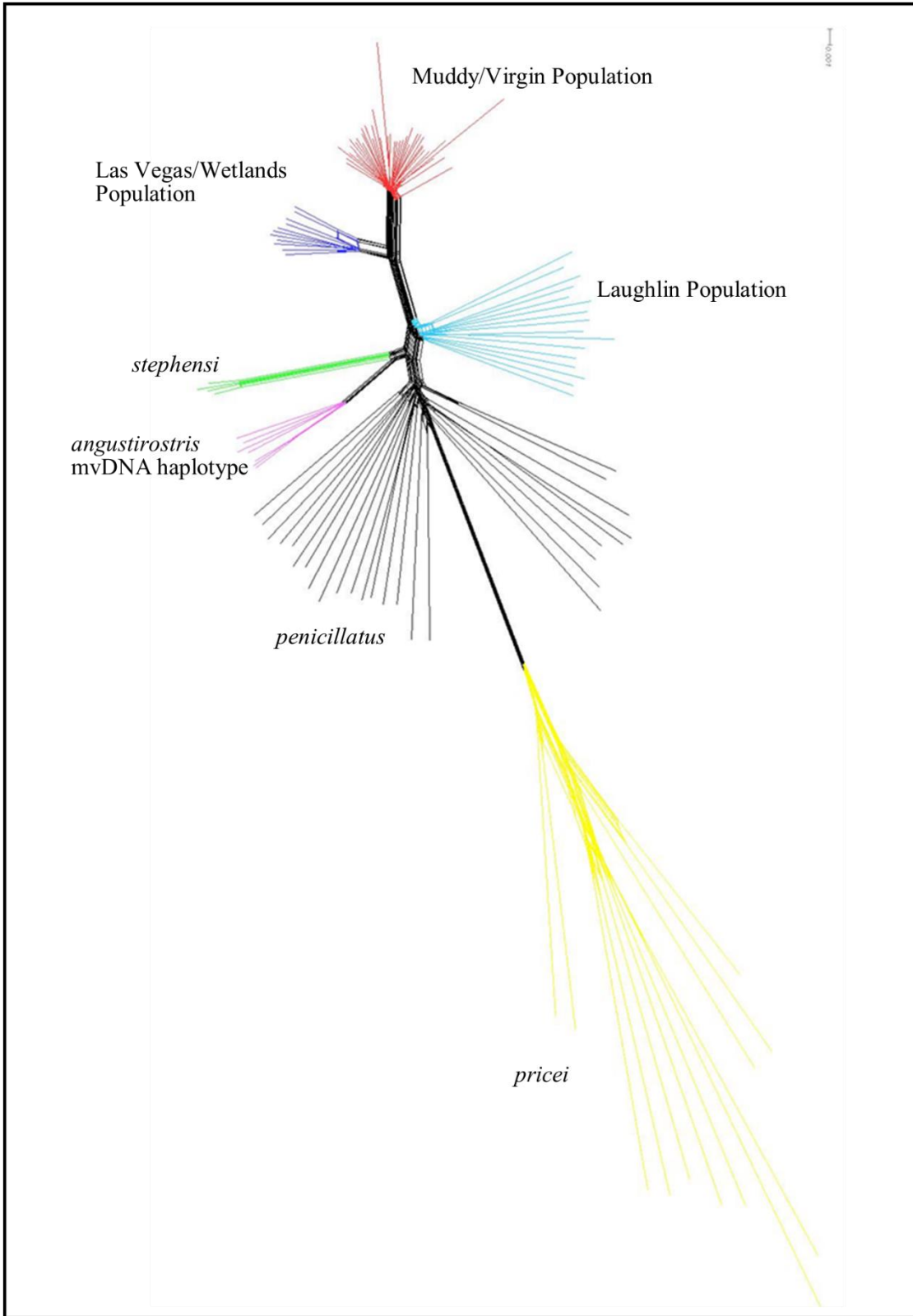
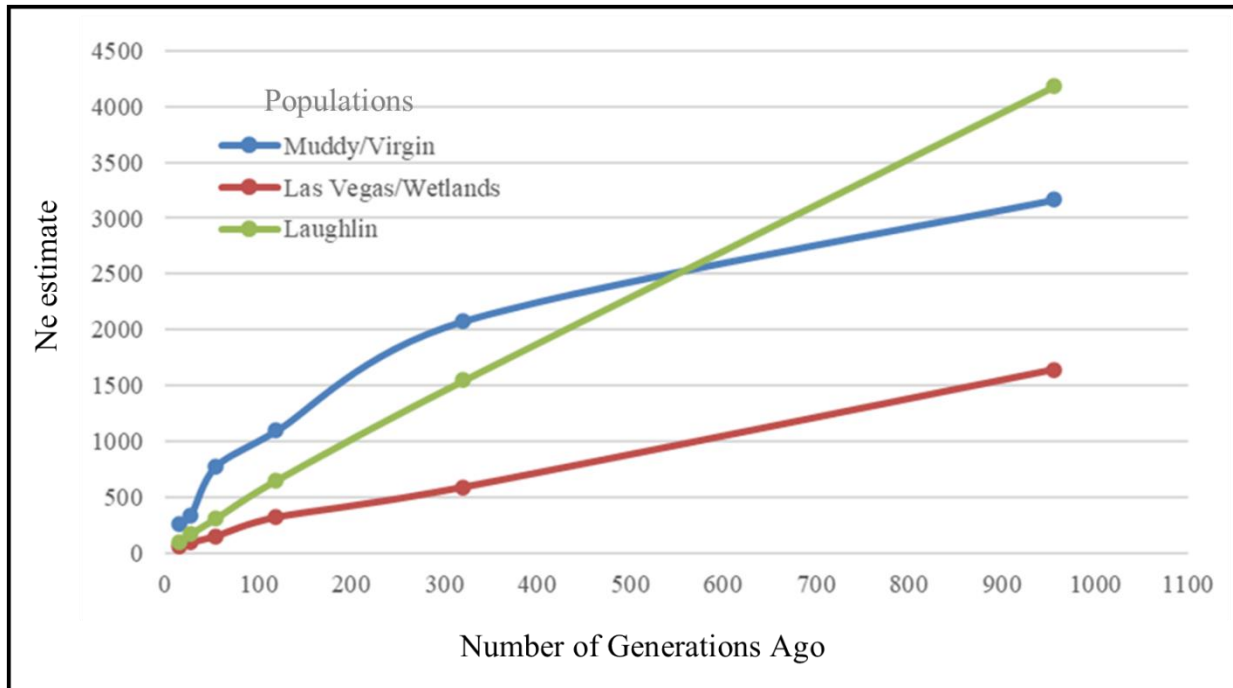**Figure 5. Phylogenetic network with populations and subspecies labeled.**

Figure 6. Estimate of historical effective population sizes (Ne) of *C. P. sobrinus* populations in Clark County, NV.