Lower Colorado River Multi-Species Conservation Program

Balancing Resource Use and Conservation

Genetic Characterization of the California Leaf-nosed Bat (*Macrotus californicus*) Along the Lower Colorado River

U.S. DEPARTMENT OF THE INTERIOR
BUREAU OF RECLAMATION

February 2016

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Lower Colorado River
Multi-Species Conservation Program

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ACRONYMS AND ABBREVIATIONS

CLNB	California leaf-nosed bat (*Macrotus californicus*)
DNA	deoxyribonucleic acid
LCR	lower Colorado River
LCR MSCP	Lower Colorado River Multi-Species Conservation Program
mtDNA	mitochondrial deoxyribonucleic acid
NGS	Next-generation sequencing (whole genome)
PCR	polymerase chain reaction

Symbols

#	number
%	percent

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ABSTRACT

This project aims to describe the current genetic diversity and demographic history of the California leaf-nosed bat (*Macrotus californicus)* (CLNB) through deoxyribonucleic acid (DNA) sequencing and analysis. The main questions guiding this study are: What is the phylogeographic history of this unique bat; how does the lower Colorado River and historic and current human impacts within the species' range affect population connectivity; and how do the populations in the lower Colorado River region relate to the genetic diversity across the entire range? By answering these questions, this study will contribute to the understanding of the poorly understood demographics of the CLNB and may be used to inform future conservation measures for the species. To date, 916 base pairs of the cytochrome-b mitochondrial DNA (mtDNA) gene have been sequenced for 99 bats from 20 locations along the lower Colorado River, the Southwestern United States, Baja California, and northern Mexico. There were 16 haplotypes identified across the range, with 5 haplotypes present in the samples taken from the lower Colorado River and 3 haplotypes unique to the river. A forthcoming microsatellite DNA analysis will attempt to reveal any fine-scale genetic diversity within and among sampled populations, demographic change across time, and population connectivity among collection sites.

INTRODUCTION

The California leaf-nosed bat (*Macrotus californicus*) (CLNB) is one of two bat species under evaluation by the Lower Colorado River Multi-Species Conservation Program (LCR MSCP). The CLNB is a non-migratory, colonial, cave-roosting bat. This species is not readily detected using acoustic surveys because of its low decibel calls and the similarity of its calls to other species. It also has a relatively low capture rate in mist netting surveys along the lower Colorado River (LCR) (Calvert 2015a, 2015b); however, the species appears to be fairly common at several roost sites in the vicinity of the LCR (Brown 2010). The difficulty in remotely detecting this species using acoustic surveys and mist netting precludes using classic mark-recapture or telemetry techniques to understand even the basic demographics of the population along the LCR – in particular, those subsets of the population using LCR MSCP conservation areas.

New molecular techniques for estimating population demographics, such as movements, current and historic effective population size, and site fidelity, are opening the doors for studying the natural history of rare and elusive species (for a review see Waits and Paetkau 2005). Historically, the mitochondrial cytochrome-b gene has proven effective for evaluating deep population level divergence in mammals and other taxa (Bradley and Baker 2001) and has been used as a preliminary marker to identify any older divergences in CLNB populations in this study.

In addition to mitochondrial deoxyribonucleic acid (mtDNA) analysis, nuclear microsatellites are proving to be a relatively inexpensive yet effective method for identifying the population structure and gene flow in both a historic and contemporary context (Hoshino et al. 2012; Paetkau et al. 1995). Microsatellites have already been developed for Waterhouse's leaf-nosed bat (*Macrotus waterhousii*) (Murray et al. 2008), and they could be applicable to the closely related CLNB. Because most of the cost of microsatellites is in identifying and developing informative sets, the availability of known microsatellites can significantly reduce the cost associated with producing these data. In recent years, the development of Next-generation sequencing (NGS) techniques has made sequencing whole genomes fiscally viable for researchers (Ekblom and Galindo 2011). NGS technology is being used in this study to identify informative microsatellites for use in population analyses.

GOALS

The goals of this study were originally stated in Reclamation (2012) as the following:

1. Document genetic structuring of roost sites along the LCR during the winter and breeding seasons.

2. Provide estimates for the current and historic effective population size of the LCR population, including the timing of changes in population size (increase and/or decrease).

3. Provide information about the space use and foraging habitat requirements of CLNB along the LCR.

METHODS

Bats were mist netted both at their roosts and in foraging areas situated close to their roosts throughout the range (figure 1). Where sampling was not feasible, museum specimens were obtained (attachment 1). Once a bat was captured, a 3-millimeter wing biopsy punch was taken and stored in a 2-milliliter tube filled with ethanol. In the laboratory, tissues were extracted using a DNeasy kit (Qiagen Inc., Germantown, Maryland). Extracted deoxyribonucleic acid (DNA) was prepared with two previously described mtDNA cytochrome-b primers developed for the Waterhouse's leaf-nosed bat (Hoffman and Baker 2001) and amplified through a polymerase chain reaction (PCR) at 95 degrees for 45 seconds, 53 degrees for 45 seconds, and 60 degrees for 45 seconds at 34 cycles. The PCR product was cleaned and run through an ABI 3130 automated sequencer (Applied Biosystems, Foster City, California), checked for ambiguous base calls in Sequencher 4.8 (Gene Codes Corporation, Ann Arbor Michigan), and aligned in MEGA (Tamura et al. 2007). Haplotype maps were created in ArcGIS. In addition to the mtDNA analysis, a nuclear microsatellite analysis is being performed. Whole genome sequencing was carried out on six individuals from across the species' range using Illumina sequencing at the Research and Testing Laboratory (Lubbock, Texas) using methods outlined in Malausa et al. 2011.

STUDY AREA

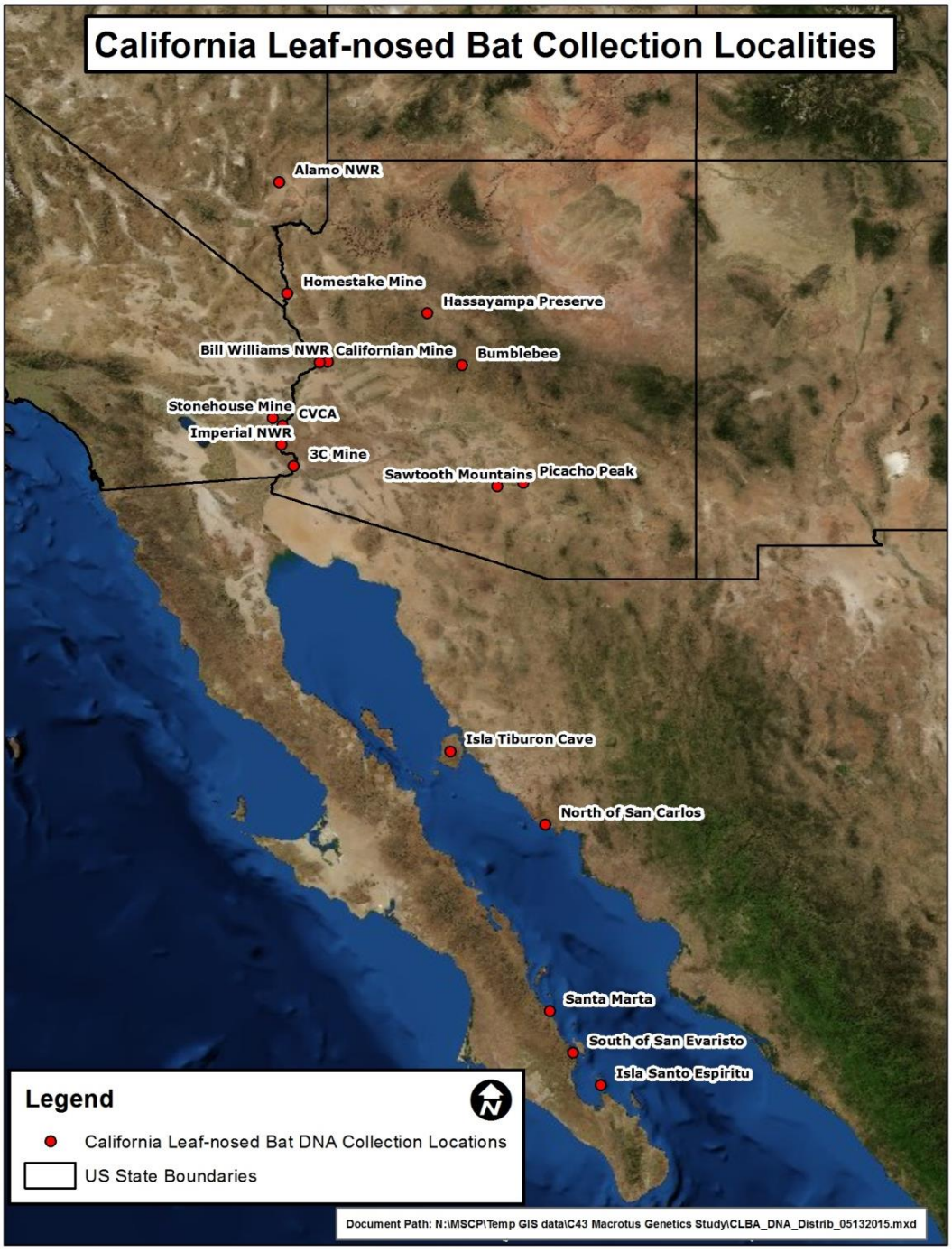

Figure 1.—CLNB sample localities.

RESULTS

Sequencing resulted in 916 base pairs of usable sequence of the mitochondrial cytochrome-b gene. An analysis revealed 16 total haplotypes in 91 samples from 18 localities across the range, with 5 haplotypes present within 8 localities from the LCR region, 3 of which were found nowhere else in the range (figure 2, table 1). Haplotype 1 (red) is common and broadly distributed across the LCR and adjacent survey sites in the Southwestern United States. Haplotype 2 (orange) was detected in bats collected from the Californian, Stonehouse, and 3C Mines as well as the Imperial National Wildlife Refuge. Haplotype 3 (magenta) was only detected in the Californian Mine. Haplotype 6 (turquoise) was detected in the 3C and Stonehouse Mines as well as the Cibola Valley Conservation Area. Two haplotypes present in the Sonoran portion of the range occurred in the Southwestern United States (light blue and yellow). Whole genome sequencing produced hundreds to thousands of microsatellite loci for each sample (table 2).

DISCUSSION

The first goal of this project was to document genetic structuring of roosts along the LCR. The mtDNA sequencing revealed higher genetic structuring than would be expected in a completely panmictic population. It is difficult to infer much about the LCR populations without a more complete sampling of the range, as this structuring may be due to incomplete sampling. The mtDNA results are also an incomplete picture of the genetic structuring in the range, as mtDNA is maternally inherited and represents only the female lineage. While difficult to say with certainty, given the uneven sampling, there seems to exist within the LCR samples a gradient of high diversity to low diversity from the center of the species' range to the northern periphery. This is in line with what is seen in other species (Garner et al. 2003; Hutchinson 2003; Schwartz et al. 2003), as the edge of the species' range is presumably less suitable, exacerbating the effects of genetic drift due to a reduced subset of the breeding population.

The high diversity of haplotypes in the LCR region relative to the southern portion of the range may be an artifact of sampling biased toward the northern portion of the range. Alternatively, it may reflect the Colorado River Valley's role as a center of genetic diversity for the species. The Lower Colorado River Valley retained relatively warmer conditions during the last glacial maximum (Thompson and Anderson 2000) and may have played the role of a refugium for warm desert species, leading to a high degree of genetic diversity relative to the rest of the home range, as has been documented in studies of warm-desert adapted heteromyid rodents (Jezkova et al. 2009).

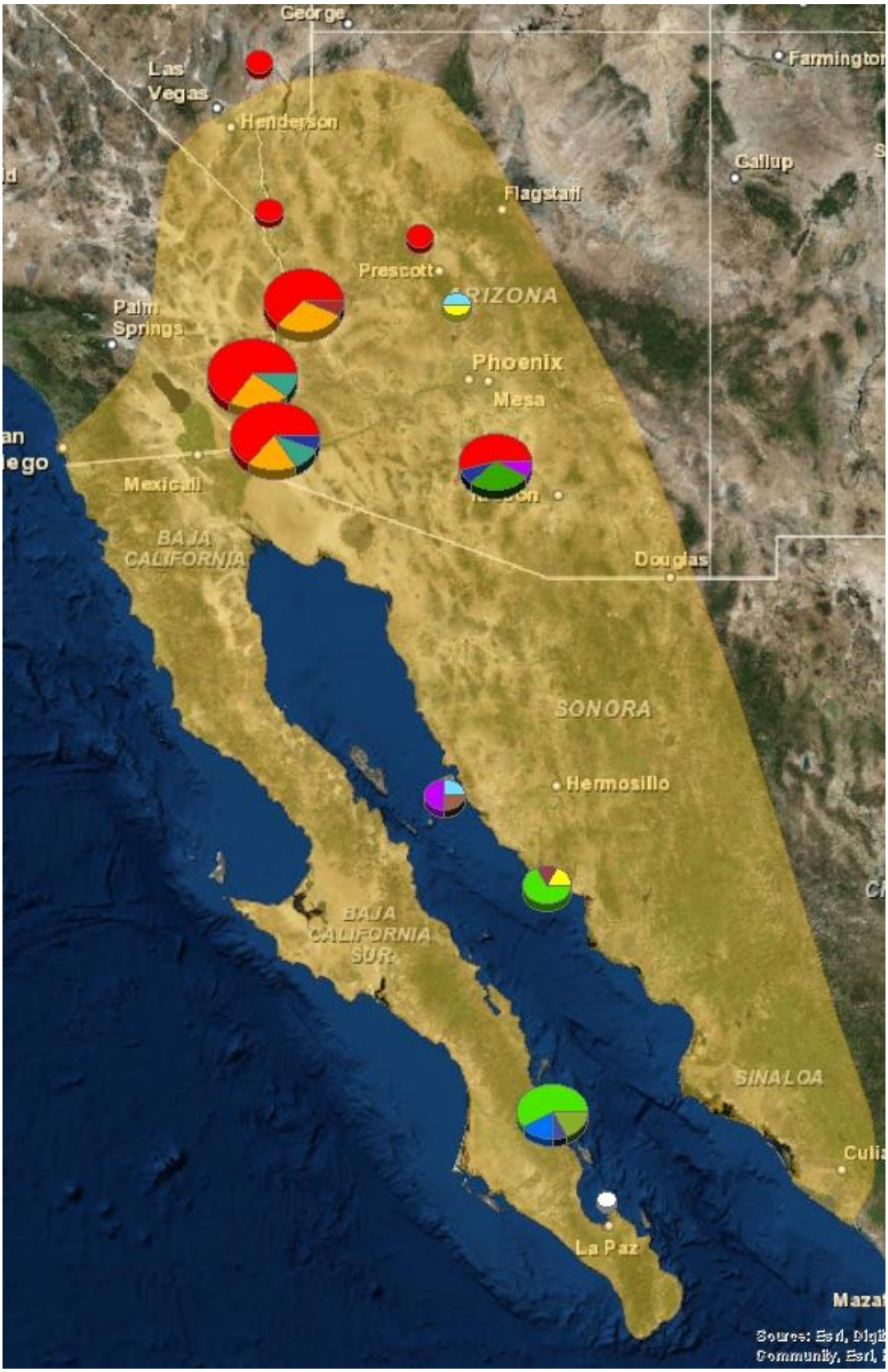

Figure 2.—Haplotype map (yellow overlay is species' range).

Table 1.—Complete list of collection localities, samples, and haplotypes present (H1 – H16)

Locality	# of samples	Haplotypes															
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16
Alamo National Wildlife Refuge	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Homestake Mine	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hassayampa Preserve	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bill Williams River National Wildlife Refuge	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Californian Mine	15	9	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Bumblebee	2	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Stonehouse Mine	16	10	5	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Cibola Valley Conservation Area	4	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Imperial National Wildlife Refuge	4	2	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
3C Mine	15	10	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Picacho Peak	4	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Sawtooth Mountains	9	4	0	0	0	0	0	1	3	1	0	0	0	0	0	0	0
Isla Tiburon Cave	4	0	0	0	1	0	0	0	0	2	1	0	0	0	0	0	0
North of San Carlos	2	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Santa Marta	6	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
South of San Evaristo	6	0	0	0	0	0	0	0	0	0	0	0	1	2	1	2	0
Isla Santo Espiritu	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Table 2.—Whole genome sequencing results for six CLNB
("Validated loci" are the total number of microsatellites isolated from each sample.)

Sample name	Validated loci	% loci identified from several sequences	Perfect microsatellites	Compound microsatellites
LVT2433	4,661	23	2,094	455
LVT4992	5,163	28	2,225	509
LVT10055	1,544	30	593	139
LVT10057	2,147	25	888	193
NK11120	3,274	27	1,403	336
NK80553	2,696	25	1,052	269

If the genetic structuring of roosts obtained from goal 1 is sufficiently distinct among roosts along the LCR, tissue sampling in future surveys could be used to estimate where individuals captured in restoration sites are roosting during both winter and summer. CLNB display high winter roost site fidelity, which may result in genetic structuring among the winter roosts.

When considering conservation efforts for a species, deciding how to prioritize efforts in a manner that best utilizes available funds can prove difficult without adequate information on the species. Should CLNB be considered for future conservation efforts by the LCR MSCP or other agencies, a detailed account of the genetic diversity of roosts along the LCR will provide information useful when determining how to best allocate funds and prioritize efforts. When faced with a limited budget for conservation, roosts that show a high degree of genetic diversity or roosts with a high number of unique haplotypes can be chosen for protection, thereby reducing the effects of genetic drift in future generations. Microsatellite sequencing will provide a better estimate of gene flow among roosts. This information can be used to identify chains of roosts important for preserving genetic diversity within the LCR region of the species' range.

The presence of three unique haplotypes in the range has a number of possible reasons. One possibility is that the populations along the LCR may have been isolated for long enough to accrue unique polymorphisms. Another more likely possibility is that these unique haplotypes may be present throughout the range and missed due to the limited sample size from outside of the LCR. The presence of shared haplotypes between northern and southern reaches of the species' range may be due to shared ancestry, as of yet undocumented long-distance migration among populations, or incomplete lineage sorting during genetic analyses. Incomplete lineage sorting creates a discrepancy between gene trees (in this case, cytochrome b) and the overall species-level phylogenetic tree due to shared haplotypes being present in recently divergent populations. A solution to the

potential for incomplete lineage sorting is to evaluate concordance across a number of markers. The forthcoming microsatellite analysis will include dozens of markers and will help to alleviate this problem.

The second goal of the project was to provide estimates for the current and historic effective population size of the LCR population, including the timing of changes in population size (increase and/or decrease). Population genetic methods allow the estimation of both current effective population size (N_e, number of breeding individuals) and also historic N_e. The historic estimate can be used to causally associate events in the past (e.g., mine closures or habitat restoration) to changes in population size. How quickly a population loses genetic diversity is directly related to effective population size rather than the actual number of individuals present (Lande and Barrowclough 1987). CLNB exhibit a lek mating system whereby relatively few males are successful at mating. As few male individuals contribute to the gene pool of the next generation, the result is a more drastic reduction of the effective population size than would be seen in a species in which mating success is more evenly distributed. The reduced effective population size may amplify the loss of genetic diversity, making this species particularly sensitive to the effects of genetic drift and other stochastic genetic effects.

Whole genome sequencing produced thousands of microsatellite loci. The next step in the process is to run bioinformatics programs to narrow down the number of loci to microsatellites that are variable enough to be used in population-level analyses. Once the appropriate suite of microsatellites is chosen, sequencing can be run for the remaining samples and an analysis completed for the resulting dataset.

Based on the mtDNA results, the hypothesis that genetic structuring would differ enough among roosts that individual bats could be identified to their roost may not be true. As is evident from the haplotype map, there are many haplotypes per roost that occur along the range. The microsatellite dataset will offer finer resolution and will be assessed to see if it may provide a level of structuring that would make it possible to identify individuals to roosts.

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ATTACHMENT 1

Collection Localities, Tissue IDs, and Collection Methods

Locality	ID	# of samples	Collected or museum
Alamo National Wildlife Refuge	AWC-157 LVT4992	2	Collected
Homestake Mine	MACA1 MACA2	2	Collected
Hassayampa Preserve	JRH001 JRH002	2	Collected
Bill Williams River National Wildlife Refuge	JRH008	1	Collected
Californian Mine	MACA3 MACA4 MACA5 MACA6 MACA7 MACA8 MACA10 MACA11 MACA12 MACA13 MACA14 MACA15 MACA16 MACA17 MACA18	15	Collected
Bumblebee	LVT10052 LVT10053	2	Collected
Stonehouse Mine	MACA19 MACA20 MACA21 MACA22 MACA23 MACA24 MACA25 MACA26 MACA27 MACA28 MACA29 MACA30 MACA31 MACA33 MACA34 MACA35	16	Collected
Cibola Valley Conservation Area	MACA38 MACA39 MACA40 MACA41	4	Collected

Locality	ID	# of samples	Collected or museum
Imperial National Wildlife Refuge	JRH006 JRH007 JRH009 JRH010	4	Collected
3C Mine	MACA42 MACA43 MACA44 MACA45 MACA46 MACA47 MACA48 MACA49 MACA50 MACA51 MACA52 MACA53 MACA54 MACA55 MACA56	15	Collected
Picacho Peak	JRH012 JRH013 JRH014 JRH015	4	Collected
Sawtooth Mountains	JRH016 JRH017 JRH018 JRH019 JRH020 JRH021 JRH022 JRH023 JRH024	9	Collected
Isla Tiburon Cave	NK42845 NK42858 NK42875 NK42877 NK42850	5	Museum
North of San Carlos	NK6643 NK17599	2	Museum
Santa Marta	B02 B04 B06 B08 B09 B11	6	Museum

Locality	ID	# of samples	Collected or museum
South of San Evaristo	MACA001-11 MACA002-11 MACA003 MACA004-11 MACA513 MACA515 MACA805	6	Museum
Isla Santo Espiritu	LVT2433	1	Museum