FINAL REPORT

CHARACTERIZATION OF GENETIC STRUCTURE AND PHYLOGENETIC RELATIONSHIPS OF RIPARIAN BRUSH RABBIT POPULATIONS

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INTRODUCTION

The brush rabbit (*Sylvilagus bachmani*) is found along the Pacific Coast from the Columbia River in Oregon, south through California to the Baja California peninsula. There are 13 known subspecies (Hall 1981). One subspecies of importance, the riparian brush rabbit (*Sylvilagus bachmani riparius*), is a listed Federal and State endangered species (Williams and Basey 1986; US Fish and Wildlife Service 1998, 2000). It occurs in the northern San Joaquin Valley, occupying riparian habitat predominantly composed of willow thickets, wild roses, and other successional shrubs and trees, as well as old-growth riparian forests dominated by valley oaks (*Quercus lobata*). The only known natural populations are confined to Caswell Memorial State Park (CMSP population) on the Stanislaus River (approximately 105 hectares), and among small patches of habitat on private lands (about 125 hectares) along the San Joaquin River (South Delta population), about 16 km northwest of Caswell MSP. A small, new population of riparian brush rabbits was discovered in 2003 in the town of Lathrop (specimens labeled Mossdale in Appendix, Table B-1), across the main channel of the San Joaquin River from the rest of the South Delta population. In addition, a population consisting of animals translocated from the controlled propagation program (South Delta stock), and their progeny, is located on the San Joaquin River National Wildlife Refuge (NWR) and on adjacent lands, southeast of Caswell MSP (Williams, et al. 2008). The natural populations are in San Joaquin County (Williams and Basey 1986; Williams et al. 2000), but the re-established population is in Stanislaus County(Figure 1).

The South Delta population is distributed in patches along Paradise Cut, Tom Paine Slough, the main channel of the San Joaquin River where it enters the Delta, and two railroad right-of-ways near crossing points at the channel (Williams and Hamilton 2002). All of the land except the Interstate Highway right-of-way is privately owned and is either managed for cultivated agriculture, transportation, or flood control. All other historical habitat along the San Joaquin River and its tributaries has been lost or degraded beyond use by irrigated agriculture, clearing of natural vegetation, and impoundment and channelization of streams and rivers.

The South Delta riparian community differs markedly from Caswell MSP. Caswell MSP consists mostly of old-growth Valley oak forest, with an abundance of grape vines cloaking trees and shrubs, and a large quantity of downed woody material littering the ground. The South Delta habitat represents various riparian successional stages from thickets of sand bar willows to large patches of an invasive weed, the white-topped peppergrass, *Lepidium latifolium* (Williams and Hamilton 2002).

The size of the Caswell MSP population was last estimated in 1993, when 43 individuals were captured in a 3-week census in 3 sections of the Park, with an estimated population size of 241. Numbers have declined significantly since 1993. Between 1997 and 2001, no more than 6 individuals were captured in an annual census (Williams 1993, Williams et al. 2000, unpubl. data). In 2001, only 2 rabbits were captured. An average of 16 rabbits per year were captured from 2002 to 2004. In 2005-2006, 6 and 9 rabbits were captured, respectively, both as a result of the annual census, and during trapping for a woodrat project.

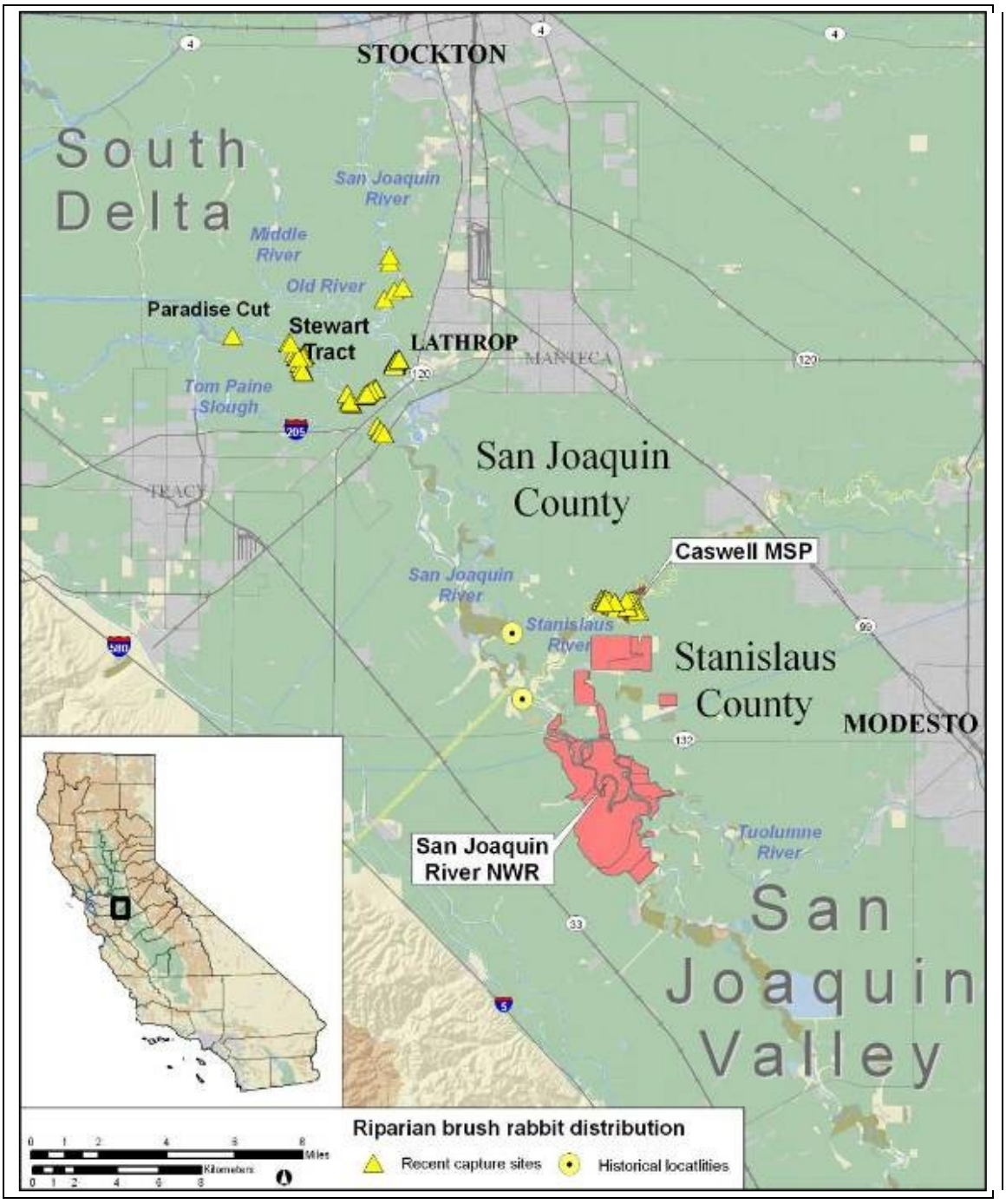

Figure 1. Historical and recent (current) records of natural distribution for the riparian brush rabbit, *Sylvilagus bachmani riparius*.

There has not been a population census of the South Delta population because of private property restrictions. Spot trapping over a 6-month period on the parcels of private property in 1998-1999 captured 18 riparian brush rabbits. In 2001 permission was granted to trap more extensively along Paradise Cut on Calilfia LLC property. During trapping over 4 nights and 5 days in August 2001, 21 riparian brush rabbits were captured at 3 sites. Brush rabbits were captured at 3 of 4 sites sampled in South Delta at a rate 3.3 times higher than the highest capture rate in

Caswell MSP (Williams and Hamilton 2002). The South Delta population is larger than the Caswell MSP population, most likely due to more available successional vegetation, a result of farming disturbance and flood control.

Additionally, in February 2003, a new population of riparian brush rabbits in the South Delta was discovered on the east side of the San Joaquin River within the city limits of Lathrop (Figure 1). The site occupies 27 acres with natural vegetation located south of the proposed Mossdale Landing development (Lloyd and Williams 2003). This is the only population that has been located in the South Delta on the east side of the main channel of the San Joaquin River. It is small and highly vulnerable.

Populations of riparian brush rabbits are under significant, proximate threats of extinction. Less than 1% of their historical habitat exists today. Principal causes of endangerment can be linked directly to construction of dams on the Stanislaus and San Joaquin rivers, and to canalization of the valley floor portions of these streams (U.S. Fish and Wildlife Service 1998). The population in Caswell MSP faces threats from inbreeding and loss of genetic diversity, random demographic events associated with small populations, wildfire, flooding, disease, predation exacerbated by high numbers of feral cats, and possibly from competition with desert cottontails (*S. audubonii*) (Williams and Basey 1986; Williams 1988, 1993; U.S. Fish and Wildlife Service 1998). The South Delta populations face threats from stochastic demographic and genetic events, flooding, disease, predation, competition, and habitat conversion on private and state lands.

The Recovery Plan for *S. b.riparius* calls for the establishment of three wild, self-sustaining populations in addition to the population at Caswell Memorial State Park (U.S. Fish and Wildlife Service 1998). With suitable, uninhabited sites available for reintroduction, ESRP began the process of controlled propagation of *S. b.riparius* in 2001, with animals originating from the South Delta. Six individuals were trapped and bred in large pens located west of Lodi in San Joaquin County in the South Delta. Over one year, 49 rabbits were captive-born, and then returned to the wild at a new location, in the San Joaquin River National Wildlife Refuge. Survival rate in the following year was approximately 60% (Williams et al. 2002).

Given the success of the captive breeding and translocation project, one potential strategy to augment the declining number of endangered riparian brush rabbits at Caswell MSP, and reduce the negative genetic impact of small population size, is to translocate rabbits from areas in the South Delta, or their progeny (from the controlled propagation program), into Caswell MSP. It is critical to understand the genetic relationship between these two populations before such augmentation of the Caswell MSP is attempted (Storfer 1999), and this requires comparing these two populations genetically with other geographically contiguous subspecies of *S. bachmani*.

Genetics is a useful tool to help us understand the structure of natural populations and to help us make informed decisions regarding wildlife conservation and management. As many species face an accelerated threat of extinction, biologists often are faced with making rapid decisions with regard to management. This sort of pressure has influenced the application of tools such as GPS technology, genomics, genetics and population biology, developed outside of this discipline, to be adapted for use in conservation and wildlife management (DeSalle and Amato 2004). Microsatellite analysis is one of four main genetic methods (including AFLPS- amplified fragment length polymorphisms, DNA sequencing and SNP-single nucleotide polymorphism analysis) that have been used increasingly for population level studies of animals by conservation geneticists (Bruford and Wayne 1993, DeSalle and Amato 2004, DeWoody 2005).

USE OF GENETIC ANALYSIS IN OTHER SPECIES

A state-listed endangered woodrat population, the eastern woodrat (*Neotoma floridana*) of Illinois, was, like the riparian brush rabbit, believed to be reduced to a single population until scientists found a few small, extant populations located within 2-14 km of each other. Microsatellite analysis at 6 loci revealed significant genetic variation between the small, isolated populations, with the most isolated population showing the lowest level of heterozygosity. Dispersal for this species was limited by anthropogenic and natural barriers, and fragmented habitat, a condition similar to what we find in the riparian brush rabbit. As with riparian brush rabbit, translocation has been considered as a method to boost woodrat numbers and to populate suitable uninhabited areas (Monty *et al*. 2003).

Microsatellite analysis has been conducted on the Allegheny Woodrat (*Neotoma magister*) in the Appalachian region of the eastern United States. This species is considered endangered or threatened by state governments in the states in which it occurs. Geographically distinct populations, and subpopulations within larger populations were examined. Statistical tests on eleven polymorphic microsatellite DNA loci revealed low gene flow among subpopulations (most likely due to limited dispersal) even among subpopulations as close as 3 km apart. This led to recommendations for management of individual subgroups, as designated by groups of geographically proximate rock outcrops or even individual rock outcrops in which the subpopulations are isolated (Castleberry *et al*. 2002). The potentially isolated subgroups of riparian brush rabbit need to be genetically examined in order to make decisions about management, similarly to the woodrats in this study.

Another type of genetic analysis, mitochondrial DNA analysis, was used to discriminate among small, isolated populations of Lower Keys marsh rabbits (*Sylvilagus palustris hefneri*) in the Lower Florida Keys. This subspecies is listed as federally endangered (USFWS 1990). Genetic analysis determined that the population was subdivided into two distinct groups, and the authors concluded that they should be managed as such. Translocations were implemented to offset declining populations, but it was suggested that the mixing of individuals from these two distinct groups should be avoided (Crouse *et al*. 2009). This is an issue that is paramount to our genetic study of the riparian brush rabbits.

In contrast, genetic studies on 27 small, isolated populations of white-footed mice (*Peromyscus leucopus*) at 8 loci found that habitat fragmentation had little effect on the genetic structure of *P. leucopus*. Although there was significant genetic variation between even closely spaced (500-2000 m apart), but isolated populations, the overall heterozygosity and number of alleles within populations did not differ significantly between such populations. Microsatellite studies allowed researchers to detect some level of gene-flow between populations, even though there were apparent barriers to movement (Mossman and Waser 2001). Molecular studies will allow us to find similar patterns, if they exist, in riparian brush rabbit.

Fragmentation of populations can have negative effects on genetic structure, by increasing reproductive isolation, which in turn reduces genetic variation as a result of genetic drift (Scribner, *et al*. 2005). Populations of riparian brush rabbits are currently fragmented, and may be experiencing these very processes. When genetic variation is low, the raw material for natural selection to act on is limited, and populations may find themselves unable to adapt to changing environments, changing food supplies, new diseases and parasites, and competitors (Lacy 1987). Inbreeding is also a consequence of reduced population size and reproductive isolation. Inbreeding can reduce the fecundity and survival of individuals in a population (inbreeding

depression), possibly due to expression of deleterious alleles in homozygotes, or from a reduction in heterozygotes that may have survival advantages over homozygotes for other reasons (Lacy 1997). Genetic mixing of such populations with captive breeding and translocations can reverse these trends, by increasing genetic variation and augmenting the pool of alleles. This may seem to be a straightforward solution, but without a thorough genetic analysis of the populations concerned, there is the danger of disrupting locally co-adapted genotypes, creating offspring that have reduced fitness compared to previous generations (Lynch 1991).

Genetic data should augment ecological and demographic studies of organisms when making management decisions. Population genetics is helpful for examining reproductive isolation, demographic independence of populations and spatial structure in the environment, especially when direct observations are difficult or trapping is not productive (Scribner *et al*. 2005). Knowledge of population locations, population size and demographics, and local barriers to movement make interpretation of genetic data more realistic and applicable to management decisions. Riparian brush rabbit populations have been studied for a number of years, and population size and habitat characteristics have been documented. (Williams and Basey 1986, Basey 1990, Williams 1993). Some previous genetic work has been accomplished (Williams et al. 2000, Williams et al. 2003), and this study was conducted to augment those genetic data on a larger scale, to better define regional populations of *S. bachmani*. These data will allow greater insight into interpopulation relationships and aid in management decisions regarding mixing populations of riparian brush rabbits in the wild or in captive breeding scenarios.

OBJECTIVES

This investigation had four main objectives:

1. Quantify within-population genetic variation of *S. b. riparius* at the South Delta (135 rabbits analyzed previously) and Caswell MSP (45 rabbits analyzed previously).
2. Quantify among-population genetic variation between South Delta and Caswell MSP.
3. Quantify among-population and within-population genetic variation in other subspecies of brush rabbits, including *S. b. mariposae* (23 completed), and *S. b. macrorhinus* (14 completed).
4. Compare the genetic variation of *S. b. riparius* populations to that found in other subspecies (*S. b. mariposae* and *S. b. macrorhinus*) to determine the significance of differentiation between *S. b. riparius* populations with respect to potential translocations between populations.

METHODS

SAMPLE COLLECTION

Rabbits were captured using Tomahawk™ traps of the double-door design that were set directly in runways or paths in dense vegetation. Traps were baited with a combination of walnut meats, rolled oats, molasses, and sliced apple. Traps were set in the afternoon or early evening, checked about 2 hours after dark and again in the early morning. Traps were left open around the clock

unless weather conditions threatened the health of the rabbits. Captured brush rabbits were permanently marked with metal ear tags and PIT tags, weighed, and measured. A 1-2 mm diameter plug of ear tissue was taken with a biopsy punch from brush rabbits and preserved in 95% ethanol (reagent grade). Ear punches were stored in a -20 °C freezer prior to extraction.

Individuals of *Sylvilagus bachmani riparius* were trapped at various areas within Caswell MSP, along the Stanislaus River (Caswell, Figure 2, Figure 3, Figure 4). A total of 96 rabbits at Caswell MSP were sampled for this study between 1998 and 2006. Individuals of *S. b. riparius* were also trapped in the South Delta area along the San Joaquin River in San Joaquin County at Paradise Cut, Tom Paine Slough, Mossdale and the main channel of the San Joaquin River (S. Delta, Figure 2, Figure 3, Figure 5). A total of 109 rabbits were sampled from this area for this study between 1999 and 2003.

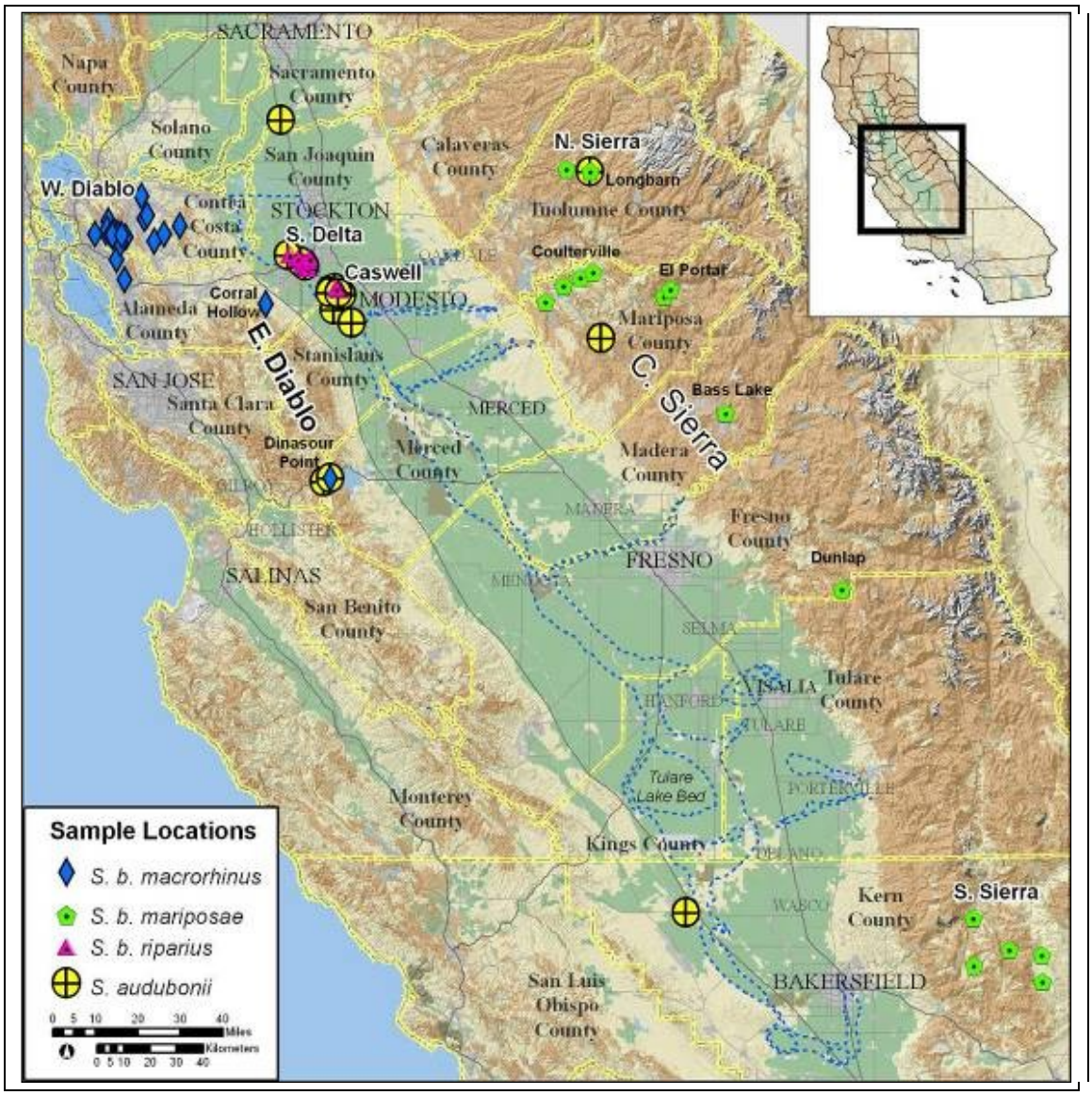

Figure 2. Locations of *Sylvilagus bachmani* and *Sylvilagus audubonii* samples used in the study and regions for grouped samples.

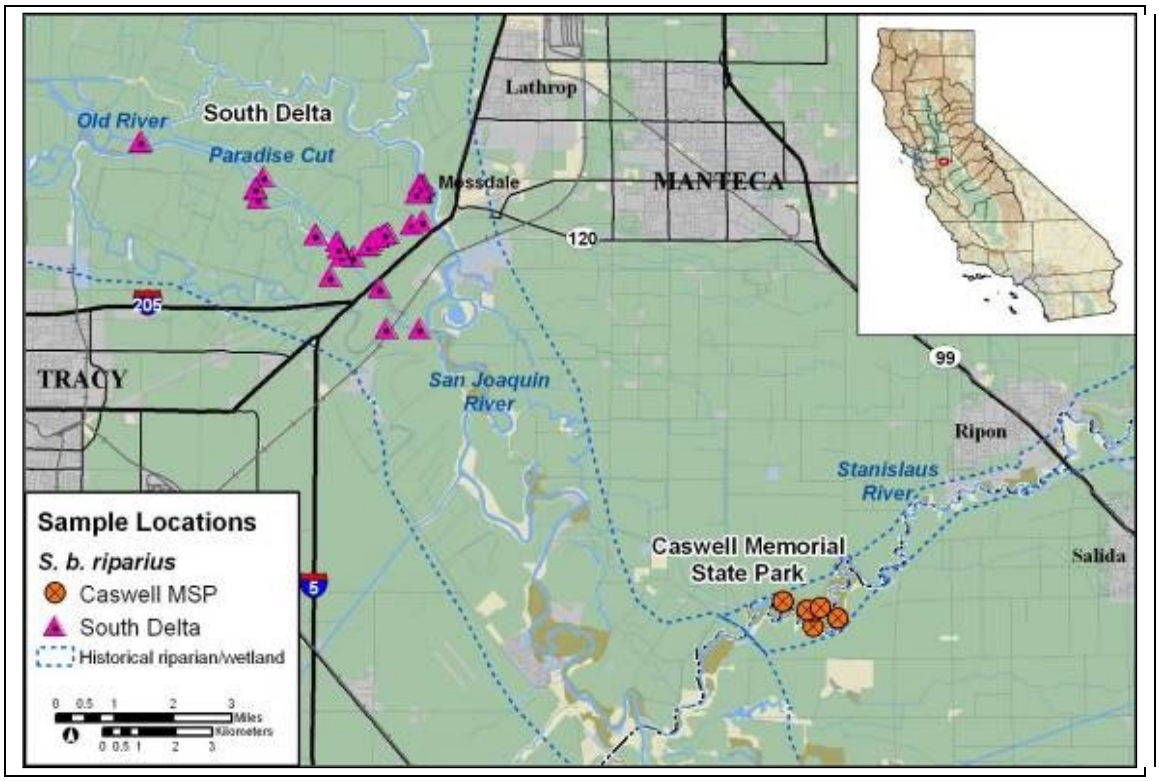

Figure 3. General geographic locations of *S. b. riparius* samples in the South Delta and Caswell Memorial State park.

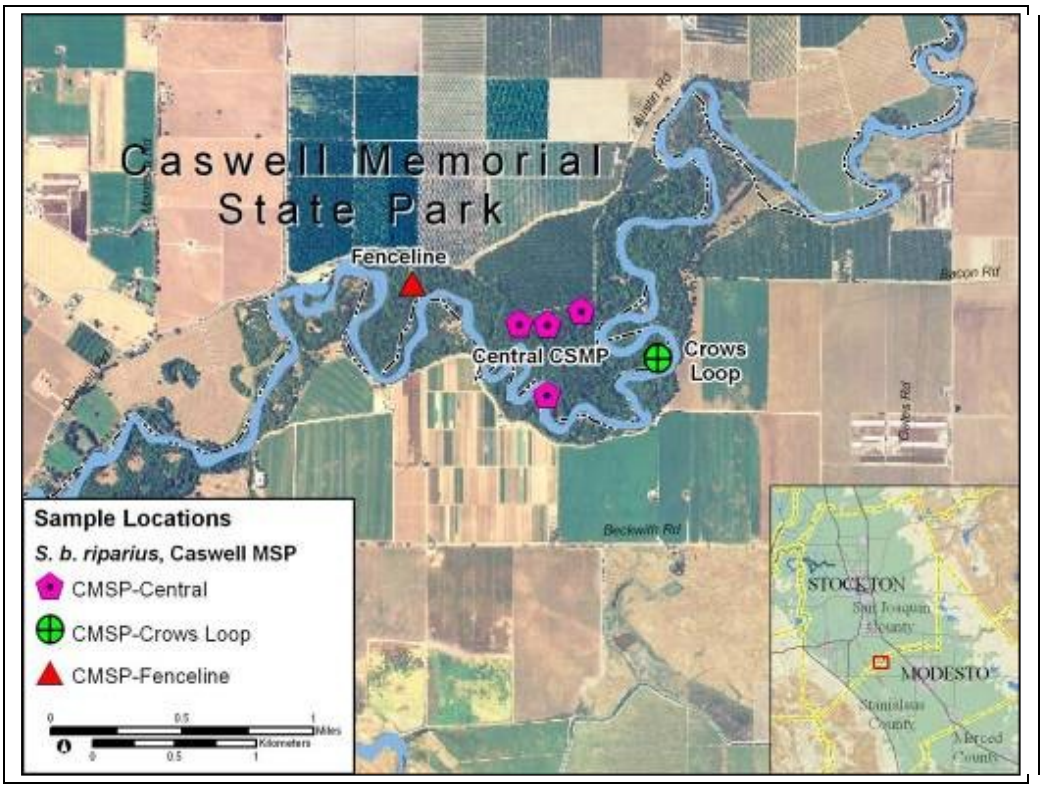

Figure 4. Geographic sub-locations of S. b. riparius samples, Caswell Memorial State park.

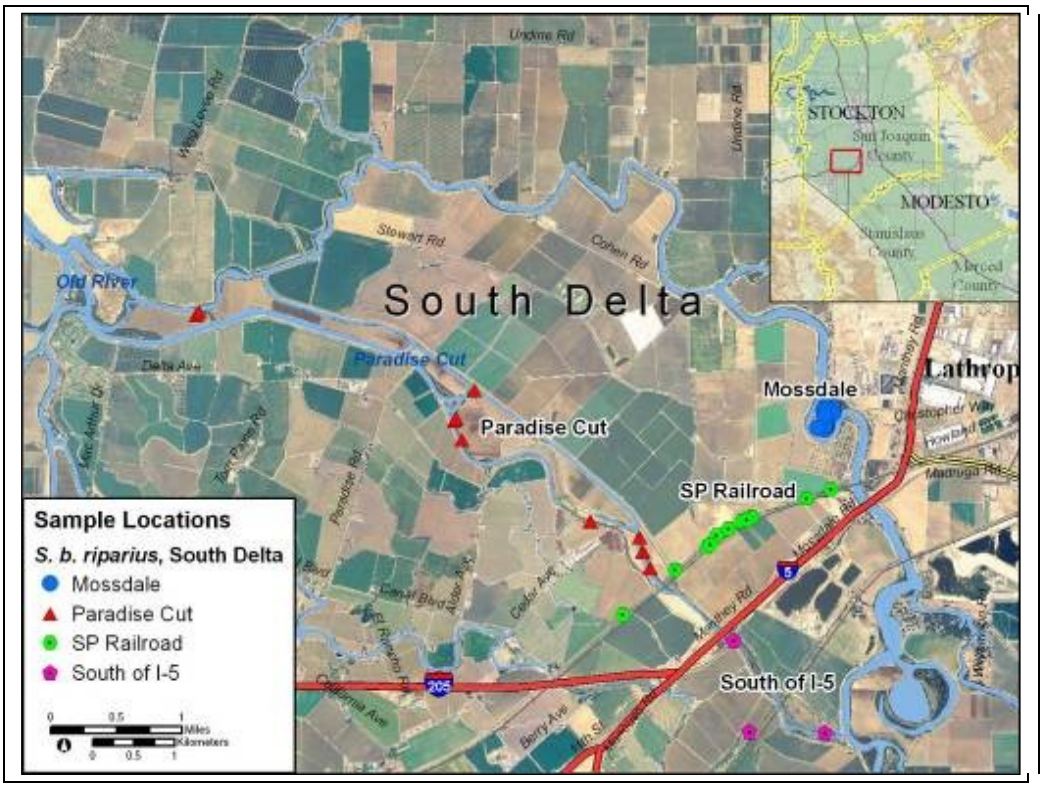

Figure 5. Geographic sub-locations of S. b. riparius samples, South Delta area.

Twenty-one rabbits of subspecies *S. b. mariposae* were trapped at Longbarn and Twain Harte (N. Sierra region, Figure 2, Appendix A) from 1999 to 2000. Nineteen other samples of *S. b. mariposae* were obtained from museum skin and tissue samples from the University of California-Berkeley Museum of Vertebrate Zoology (MVZ) collected in the central and southern Sierra Nevada (C. Sierra and S. Sierra, Figure 2, Appendix A). Some of these specimens were over 80 years old. Tissue (3mm x 3mm) was cut from the seam area on the belly created during museum skin preparation. Two of the samples consisted of preserved muscle tissue.

Fourteen rabbits of subspecies *S. b. macrorhinus* were trapped at Corral Hollow and Pacheco State Park/Dinosaur Point (E. Diablo, Figure 2). Thirty-two additional samples (ear tissue up to 6 mm x 6 mm) were donated by the Lindsay Wildlife Hospital/Museum in Walnut Creek, CA. from the area of western Contra Costa County, California (W. Diablo, Figure 2). These samples were obtained from rabbits that were brought to the facility either injured or dead.

We collected or obtained an additional 47 samples of a more common rabbit species (desert cottontail, *S. audubonii*) to allow comparisons of the within-species variation of *S. bachmani* with interspecific variation of *S. bachmani* and *S. audubonii*. Samples were collected from various locations in central California (Figure 2).

DNA EXTRACTION

DNA was extracted from ear punches and ear tissue using the Qiagen DNeasy Blood and Tissue Kit (ref), with a few modifications. Samples were weighed prior to extraction, and 6.0 - 25.0 mg of tissue was used in each extraction. Extra tissue was returned to the vial with 95% ethanol and placed in the -20 °C freezer. Scalpel and tweezers were flame sterilized over a Bunsen burner

prior to each sample processing. Excess hair was scraped off of the tissue prior to maceration, as hair pigments have been found to inhibit downstream PCR reactions in some cases (Müller, et al 2007). Ear tissue was macerated with the scalpel prior to extraction.

Tissue was placed in a 1.5 ml microfuge tube with 180 µl ATL buffer and 20 µl proteinase K. Tissue and buffers were incubated in a 56 °C water bath for 18-48 hours with intermittent vortexing. (This is a deviation from the Qiagen protocol that calls for 1-8 hours to overnight incubation.)

Museum skin samples were incubated for the longest (36-48 hours) and some were incubated with double the volume (40 µl) of proteinase K, to ensure complete breakdown of the proteins in this tougher, dried tissue. Samples incubated for longer than 24 hours had the incubator temperature reduced to 37 °C for the remainder of the incubation, to lengthen the activity of the Proteinase K.

Following incubation, samples were vortexed for 15 seconds and centrifuged in the microcentrifuge for 10 seconds. AL buffer and 96-100% ethanol (200 µl each) were added to each tube, which was then vortexed and centrifuged briefly. Samples were pipeted into a DNeasy Mini spin column, and centrifuged at 8000 rpm for 1 minute. (DNA becomes bound to the filter membrane at this stage until chemical release.) Flow- through was discarded. Washing buffer AW1 (500 µl) was added to the tube, which was then centrifuged at 8000 rpm for 1 minute. Flow- through was discarded. Washing buffer AW2 (500 µl) was then added to the tube, which was centrifuged at 12,700 rpm for 3.5 minutes (Qiagen protocol calls for 14,000 rpm for 3 minutes, but the top speed on our microcentrifuge was 12,700 rpm). Flow- through was discarded.

The last step in the extraction called for DNA to be eluted or released off of the filter with a proprietary Qiagen AE buffer. AE (100 µl) was added prior to centrifuging at 8000 rpm for 1 minute. DNA was collected at the bottom of a reservoir tube. This process was repeated with a second reservoir tube, to obtain any remaining DNA. To facilitate this process, the AE buffer was heated in the 56 °C incubator prior to elution, to increase yield. (This is a deviation from the Qiagen protocol, which does not require the heating of the AE buffer prior to elution).

DNA QUANTIFICATION

DNA samples were quantified using a spectrophotometer (Eppendorf BioPhotometer) in units of ng/µl. Each sample was measured 2 or 3 times to ensure accurate quantification. If 1st and 2nd elutions for a sample were similar in concentration, then they were combined into a single tube. Otherwise, they were maintained in separate tubes. (Appendix)

If a sample registered fewer than 5 ng/µl after quantification, remaining tissue was re-extracted. This led to successful DNA samples in all but 2 cases. Concentrations ranged from 5 ng/µl to 120 ng/µl. There was no apparent correlation between weight of extracted tissue and final ng/µl yield after extraction.

After quantification, dilutions were made of each sample, for a working DNA concentration of 6 ng/µl. Samples were diluted with either purified water or AE buffer. Dilutions made with AE buffer had a longer shelf life for successful amplification. Dilutions were stored in the refrigerator at 4 °C, while original concentrated DNA was stored at –20 °C.

PCR TECHNIQUES

A total of 9 loci were examined in this study, but one was discarded because of very low variability. A 10th locus was screened, but found to have poor amplification, which was difficult to interpret, so it was discarded from the analysis. Of the remaining loci, 5 amplified regions containing di-nucleotide repeats, the number of repeats representing different alleles in an individual. Three loci contained tetra-nucleotide repeats, or more complex repeats (tetra- plus di-nucleotide repeats within the same segment). Primers were chosen from the literature as successfully amplifying rabbit DNA, but were not specifically designed for use in *S. bachmani*. Data from 3 additional loci were examined, and 2 of these were used to augment data in the analysis. Table 1 shows all primer sequences used and their references.) Primers were manufactured by Integrated DNA Technologies, or were provided from a previous study.

DNA fragments (loci) were amplified using the Qiagen Multiplex PCR Kit, which consists of HotStarTaq DNA polymerase, nucleotides (dNTP's), MgCl and Multiplex PCR buffer, combined in their proprietary 2X Qiagen Multiplex PCR Master Mix. To this mix, a pair of forward and reverse primers was added (one of which was labeled with a fluorescent tag), at concentrations of 0.2µm per primer. PCR reactions were processed in volumes of 10 µl to 50 µl, depending on the samples being amplified. For most samples, a concentration of 6 ng of DNA in a 10µl reaction gave good amplification while conserving the Qiagen Master Mix stock. Lower quantity or quality DNA samples were run at larger reaction volumes, which improved PCR results. The most difficult samples to amplify, the MVZ skins, had better success in some cases with proportionally less DNA in a larger PCR reaction volume. This technique was verbally confirmed by Dr. Eileen Lacey of the MVZ, as a strategy for working with museum skin DNA. Conversely, older DNA extractions (DNA previously extracted and stored over the last 3 to 7 years) sometimes had improved amplification by addition of larger volumes (higher concentration) of DNA extract in the PCR mixture.

PCR reactions were amplified in Applied Biosystem's 9600 Thermocycler with 96 wells. Protocol for amplification consisted of:

1. 10 minutes at 96 °C (initial denature),
2. Approximately 40 cycles of
   a. 30 seconds at 95 °C (denature),
   b. 1 minute 30 seconds at the locus-dependent annealing temperature, between 67-54 °C (primer anneal), and
   c. 1 minute at 72 °C (chain building).
3. The annealing temperature in the first 10 to 15 cycles was programmed to gradually "touch down" every cycle from a higher to lower annealing temperature. The melting point of the primers sets the upper limit of annealing temperature. Using the touchdown procedure, the majority of the amplification is based on a higher first possible annealing temperature, where primer binding is most specific. This provides later amplification cycles with the most specific target material.
4. Following these 40 cycles, the samples were subjected to 30 minutes at 60 °C, to finish building PCR fragments.
5. Lastly, samples are cooled to 4 °C in the thermocycler indefinitely, until the reactions can be placed in the refrigerator.

Table 1. Primer sequences used.

SAT12	Forward 5' CTT GAG TTT TAA ATT CGG GC 3'	Mougel *et al.* 1997
GenBank # X99891	Reverse 5' GTT TGG ATG CTA TCT CAG TCC 3'	
A10	Forward 5' TCC CAC TAG AAA CTT TCA AAA C 3'	Estes-Zumpf *et al.* 2008
GenBank # EF672485	Reverse 5' CAC GTT AGC ACA GAG TTG TAT C 3'	
D106	Forward 5' GCA CAA ATA ATA CAT ACG TCT G 3'	Estes-Zumpf *et al.* 2008
GenBank # EF672484	Reverse 5' ATC CAT CCA TCT ATC CAC TC 3'	
SOL44	Forward 5' GGC CCT AGT CTG ACT CTG ATT G 3'	Surridge *et al.* 1997
Genbank # X94684	Reverse 5' GGT GGG GCG GCG GGT CTG AAA C 3'	
SAT7	Forward 5' GTA ACC ACC CAT GCA CAC TC 3'	Mougel *et al.* 1997
Genbank # X99888	Reverse 5' GCA CAA TAC CTG GGA TGT AG 3'	
SAT16	Forward 5' ATT CAG CCT CTA TGA ATT CCC 3'	Mougel *et al.* 1997
GenBank # X99890	Reverse 5' AAT GCT ACA TGG TAA CCA GGC 3'	
SOL8	Forward 5' GGA TTG GGC CCT TTG CTC ACA CTT G 3'	Rico *et al.* 1994
GenBank # X79217	Reverse 5' ATC GCA GCC ATA TCT GAG AGA ACT C 3'	
SOL30	Forward 5' CCC GAG CCC GAG ATA TTG TTA CCA 3'	Rico *et al.* 1994
GenBank # X79215	Reverse 5' TGC AGC ACT TCA TAG TCT CAG GTC 3'	
OCLS1B	Forward 5' ACT GCT ATA TCA AAG GCA TGA CCC 3'	van Haeringen *et al.* 1996/97
	Reverse 5' TCA GGT ATT TGG AAA GTG AAT CCC 3'	
OCBGLX	Forward 5' TCT AGG AAG AAG CTT TAT CCC TC 3'	van Haeringen *et al.* 1996/97
	Reverse 5' GTT TTC TCA TCA GAA ATC CAC C 3'	

Samples that were difficult to amplify with low quantity or degraded DNA, especially the MVZ skin samples, were processed at slightly lower annealing temperatures, to assist the primer in more easily attaching to degraded DNA. The touch-down protocol was still applied to these samples, but the starting and ending annealing temperatures were up to 5° C lower than that used for ear tissue.

PCR products were visualized on 2% agarose minigels stained with ethidium bromide, which binds to DNA and fluoresces under UV light for DNA fragment detection. Minigels were viewed on a UV lightbox with camera attached, and computer assisted images were produced using the program Kodak ImageReady 2.0. Photographs were stored as JPEG files and printed. The brightness of a sample band on the minigel was an indication of the concentration of the PCR product.

PCR products were diluted with water, with the general ratio of 1 µl of PCR product to 100 µl of water. Using the minigel image as a guide, fainter PCR products were either diluted by only 50 µl of water, or were used undiluted. PCR products or dilutions, at a volume of 1.5 µl, were pipetted into a 96-well half-skirted plate, the individual sample reservoirs were capped, and the sealed plate genotyped at the Iowa State University DNA Facility.

GENOTYPING

Genotyping took place on an ABI PRISM® 3100 Genetic Analyzer, with a 500 base-pair ladder run simultaneously with each PCR product for sizing. Peak graphs generated by the 3100 were interpreted using Applied Biosystem's GeneMapper software, available remotely on the Iowa

State University server. PCR products were sized between 95 and 300 base pairs, with each base pair size representing a unique allele.

Allele sizes were recorded for each sample (Appendix C). Most individuals were amplified at a particular locus more than once, to confirm sizes and definitively label homozygotes (Gagneux et al. 1997). Genotypes from the previous study were scaled to match current sizes, and generally had global shifts from 2 to 7 base-pairs.

DATA ANALYSIS

The program Microchecker (Oosterhout, *et. al*. 2004) was used to screen the microsatellite allele data for genotyping errors, including the presence of null alleles. Null alleles are those alleles that are actually present, but fail to amplify during the PCR process, because of a mutation at the annealing site (site of attachment) of the primer (Holme *et al*., 2001). The presence of a null allele appears just like that of a homozygote after PCR, because only one allele is visible at the locus for individuals carrying the null allele. Microchecker uses a Monte Carlo simulation to determine expected homozygote and heterozygote frequencies of the allele sizes present in the population. Hardy-Weinberg equilibrium theory is used to calculate the frequency of null alleles by comparing expected and actual allele frequencies. An excess of apparent homozygotes often flags the presence of a null allele at the locus. The program adjusts the observed allele and genotype frequencies to account for null alleles, which permits the data to be used in subsequent genetic analysis programs. This results in the second allele of a subset of genotyped homozygotes being categorized as a missing data point. Both original and null-adjusted data were used in later analyses (*STRUCTURE*, *adegenet* and *Arlequin*), and results were compared. Microchecker also scans the data for out of range and zero allele sizes, as well as values that are not positive integers.

Individual populations were visually screened for the presence of private alleles, alleles that are only found in a particular population and no others. If two populations have a different set of private alleles, they have not been interbreeding for some time, enough to allow the accumulation of these allelic variants. The ratio of the number of private alleles to the total number of alleles found in the population is a useful statistic to describe the level of separation of one population from another over time.

Data were analyzed using *STRUCTURE* (Pritchard, *et al*. 2000, Falush *et al*. 2003), a genetic analysis program designed to search for the number and identity of genetically homogeneous groups of individuals using a Bayesian algorithm. This model searches for the presence of Hardy Weinberg or linkage disequilibrium, and attempts to group the data into clusters (*K*) in a way that minimizes linkage disequilibrium, effectively assigning individuals to populations which are characterized by their allele frequencies (Appendix B) at each locus studied. This program has been shown to be effective using smaller numbers of microsatellite loci (as low as 7 loci still demonstrate accurate results) (Pritchard, *et al*. 2000), and the samples for this project were analyzed at 8 or 11 loci, depending on the sample. Population data were compared between *S. b. riparius* at two locations separated by landscape barriers (Caswell MSP and South Delta, Figure 3), between the 2 subspecies *S .b. mariposae* and *S. b. macrorhinus* separated by the Central Valley of California, and with all subspecies included (*S. b. riparius*, *S .b. mariposae* and *S. b. macrorhinus*). Desert cottontails (*S. audubonii*) were also added as an outgroup representing a different species. *STRUCTURE* is designed to be sensitive enough to discriminate between closely related populations, and therefore is important for discriminating

between the 2 closely related *S. b. riparius* subpopulations. We ran 10 replicates[1] assuming clusters (*K*) of 1 through 8[2] using 30,000 burn-in steps and 30,000 Markov Chain Monte Carlo (MCMC) steps. We assumed admixture (potential mating) and allowed the program to infer alpha (the degree of admixture), and also ran *STRUCTURE* assuming no admixture, which would be expected for some of the geographically separated populations. Output of independent runs of *STRUCTURE* (10 runs per cluster) were combined using program *CLUMPP* (Jakobsson and Rosenberg 2007) and displayed graphically using the program *DISTRUCT* (Rosenberg 2004).

Evanno calculations (Evanno *et al*. 2005) were applied to the results of *STRUCTURE*. This calculation measures the rate of change of the log probability of data to belong to perceived clusters, delta K, and is a better predictor of the real number of groups among the data than *STRUCTURE* alone, when migration among populations is uneven. Both *STRUCTURE* and the subsequent Evanno calculations provide only an estimate of K, however, since the population model used in *STRUCTURE* is an idealization. Prior knowledge of the biological system must also be taken into consideration with either method. *STRUCTURE* and the Evanno method also are limited to detecting the highest level of hierarchy in a given data set, and internal clusters in the data must be tested for substructure, which we have done. Analysis of *S. b. riparius* subpopulations alone, *S. bachmani* as a group, and all *Sylvilagus* samples were tested independently, as noted previously.

Data were also examined using the *adegenet* package for the *R* software, designed for multivariate analysis of molecular marker data (Jombart 2008). This has advantages over the program *STRUCTURE*, which uses Bayesian clustering. The program *adegenet*, which uses multivariate methods, does not require assumptions (e.g., Hardy Weinberg equilibrium or the absence of linkage disequilibrium) concerning the underlying genetics model. *Adegenet* summarizes genetic variability to reveal genetic structuring, without prior supposition of population structure. This program also runs much faster than *STRUCTURE*, which often requires more than 24 hours of program run time for a particular data set.

We used *adegenet* to summarize data by transforming genetic variables into principle components and examined scatterplots of components for comparison to results produced by *STRUCTURE*. This allowed us to conduct additional exploratory analysis by visualizing scatterplots of samples grouped by both taxonomic groups and geographic locations. Data were copied from an Excel spreadsheet into an R dataframe (Appendix G) and null alleles were replaced by the mean of that allele from all available observations[3]. Principle component analysis (PCA) was conducted using the *dudi.pca* command from the *ade4* package in *R* (Chessel et al. 2004) and plotted with the *ade4* package *s.class*. *R* language code used for this procedure is included in Appendix G.

Finally, data were examined with the program *Arlequin* (Excoffier, et al. 2005) to examine general population level statistics for each group of animals. Data were examined for private alleles, null alleles, heterozygosity levels, Hardy-Weinberg equilibrium, linkage disequilibrium, and the fixation index, F_{ST}, and R_{ST}. Alleles were divided into 2 major subgroups, to minimize missing data in the analysis, since *Arlequin* will throw out entire loci if more than 5% of values

[1] Ten is a typical number of replicates allowing users to check for consistency between runs.

[2] During exploratory analysis we found that using more than eight clusters (K) did not add usefull results.

[3] This is necessary for PCA analysis. Replacing null values with 0 (zero), rather than mean values, produced consistent results.

are missing. One subgroup (Subgroup I) contained 8 loci with the most recent amplifications of microsatellites, including 3 tetranucleotide repeat loci (with some gaps filled in by previous data). A second subgroup (Subgroup II) contained 7 loci (5 loci in common with the recent subgroup), using primarily previous microsatellite amplifications derived from 7 dinucleotide repeat loci, (with some gaps filled in by current amplifications). For each subgroup, we examined both original data, and data with loci adjusted for possible null alleles, according to the procedure of Microchecker. For the Hardy Weinberg equilibrium test, we set the program to use 1,000,000 Markov chain steps, and 100,000 dememorization steps ("burn in" steps). For the linkage disequilibrium test, we set the program to use 50,000 permutations, and 5 initial conditions, as recommended by the program authors.

RESULTS

NULL ALLELES

Individuals of *S. b. mariposae* from the Central and Southern Sierra regions (Figure 2) had 4 loci with possible null alleles (Table 2). Individuals of *S. b. macrorhinus* from the Western Diablo region displayed 3 loci with possible null alleles. Individuals of *S. b. riparius* from Caswell MSP displayed 3 loci with possible null alleles. Across subspecies of *S. bachmani*, the locus D106 had the most instances of possible null alleles (in 4 subpopulations). The locus A10 showed 3 subpopulations with possible null alleles. Many of these possible null alleles occurred in only a few individuals within the population.

Table 2. Null alleles present at different loci for three subspecies of *S. bachmani*.

	Null Alleles Present					
	S. b. riparius		***S. b. macrorhinus***		***S. b. mariposae***	
Locus	CMSP	South Delta	E. Diablo	W. Diablo	N. Sierra	C./S. Sierra
SAT7	no	no	no	no	no	no
SAT16	no	no	no	no	no	no
SOL8	no	no	no	yes	no	no
SOL30	yes	no	no	yes	no	yes
SOL44	no	no	no	yes	no	yes
A10	no	no	yes	no	yes	yes
SAT12	no	no	no	no	no	no
D106	yes	yes	yes	no	no	yes
OCR4	yes	no	no	x	no	x
OCLS	no	no	yes	x	no	x
OcBGLX	no	no	no	x	no	x
Hardy-W Equilibrium	possibly	possibly	possibly	possibly	possibly	possibly

Null alleles corrected where indicated, given the recommendations of Microchecker. Both data sets (with and without null alleles corrected) were used in further analyses.

Private alleles, or those unique to one grouping of samples exclusively, were tallied for S. bachmani subspecies and for the Caswell MSP and South Delta populations of *S. b. riparius* (Table 3). We found that two of the South Delta samples had allele frequencies that, while unique to South Delta - *S. b. riparious*, were common with *S.audubonii* samples. We suspect

that these samples were mislabeled at some point in the data collection process. Considering potentially mislabeled samples, the South Delta had between 4-6 private alleles, 0-2 less than the number of private alleles for Caswell MSP (6).

Table 3. Frequency of alleles private to *S. bachmani* subspecies and *S. b. riparius* in the Caswell State Memorial Park and South Delta populations.

		Frequency of individuals					
				S. b. riparius			Grand
Locus	Allele	*S. b. macrorhinus*	*S. b. mariposae*	Caswell MSP	South Delta	Total	Total
SAT7	184				2	2	2
	186			1		1	1
	202			21		21	21
SAT16	112		1				1
	138	1					1
SOL8	103	1					1
SOL30	162		7				7
	172		11				11
	174		8				8
	176		9				9
	178				1*	1	1
	180		1				1
	184		2				2
SOL44	191	1					1
	193		1				1
	211		1				1
	233				2*	2	2
A10	210			1		1	1
	223		1				1
	225				2	2	2
SAT12	126	1					1
	130	1					1
D106	173	3					3
	197		1				1
OCLS	151			1		1	1
	161			1		1	1
	165				22	22	22
	173				62	62	62
	181	1					1
OCBGLX	237			4		4	4
TOTAL		**9**	**43**	**29**	**89-91***	**120**	**172**
Private Alleles		**7**	**11**	**6**	**4-6***	**10-12***	

* SOL30-178 and SOL44-233 are from two individual samples that may be misidentified *S. audubonii* samples. Both alleles are common to other *S. audubonii* samples.

STRUCTURE RESULTS

There were no differences in results between data sets with original alleles and corrected null alleles. The population of *S. b. riparius* from the South Delta appeared to be distinct from the population at Caswell MSP. Figure 6, Figure 7, Appendix D). This result held whether or not the program used information concerning the geographical origin of the data (pre-assigned Population structure).

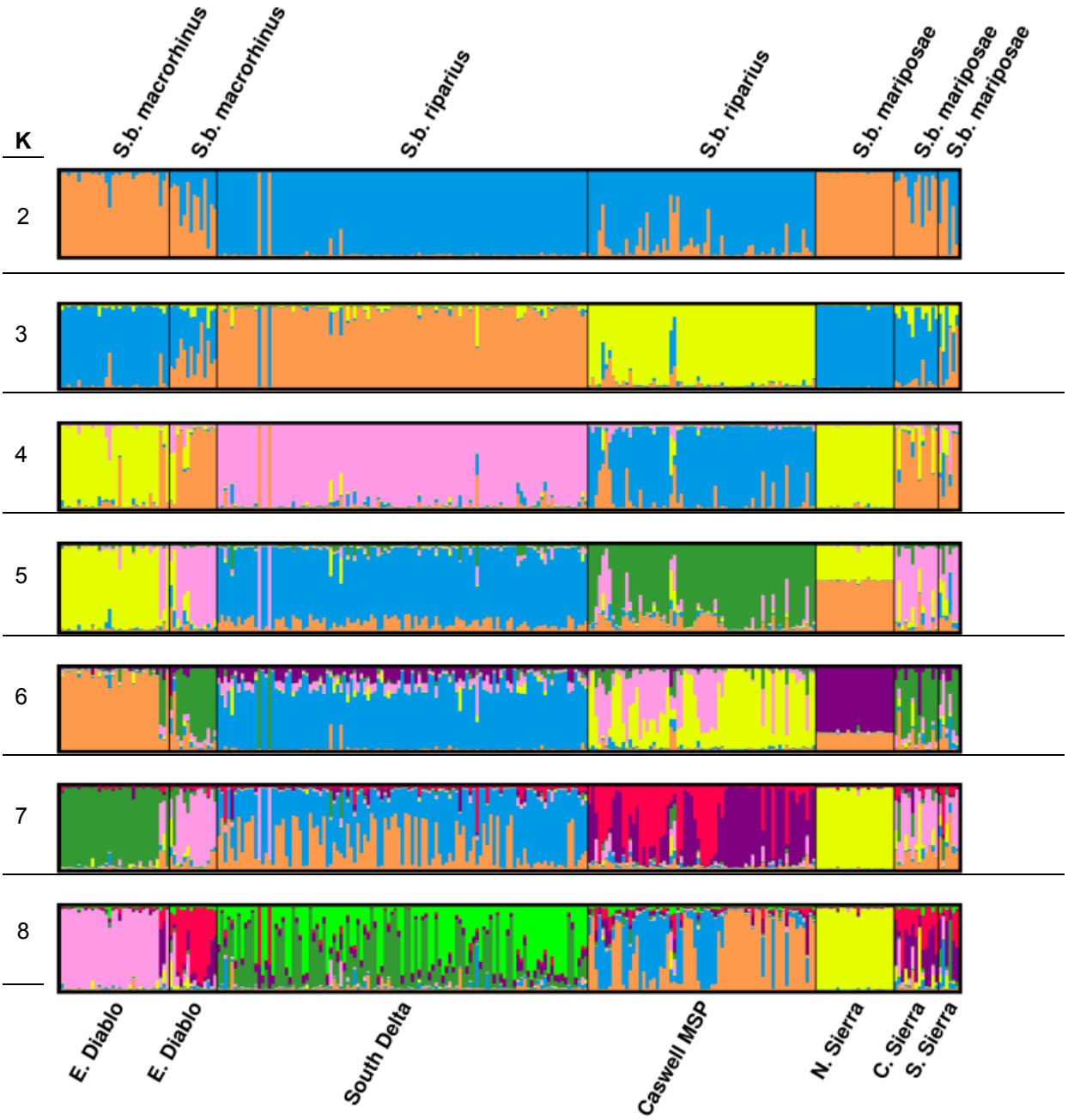

Figure 6. Association of *Sylvilagus bachmani* ssp samples with clusters (*K*). Each chart shows the association of each sample with a cluster (1-*K*) where there is assumed K number of clusters using the *STRUCTURE* admixture model.

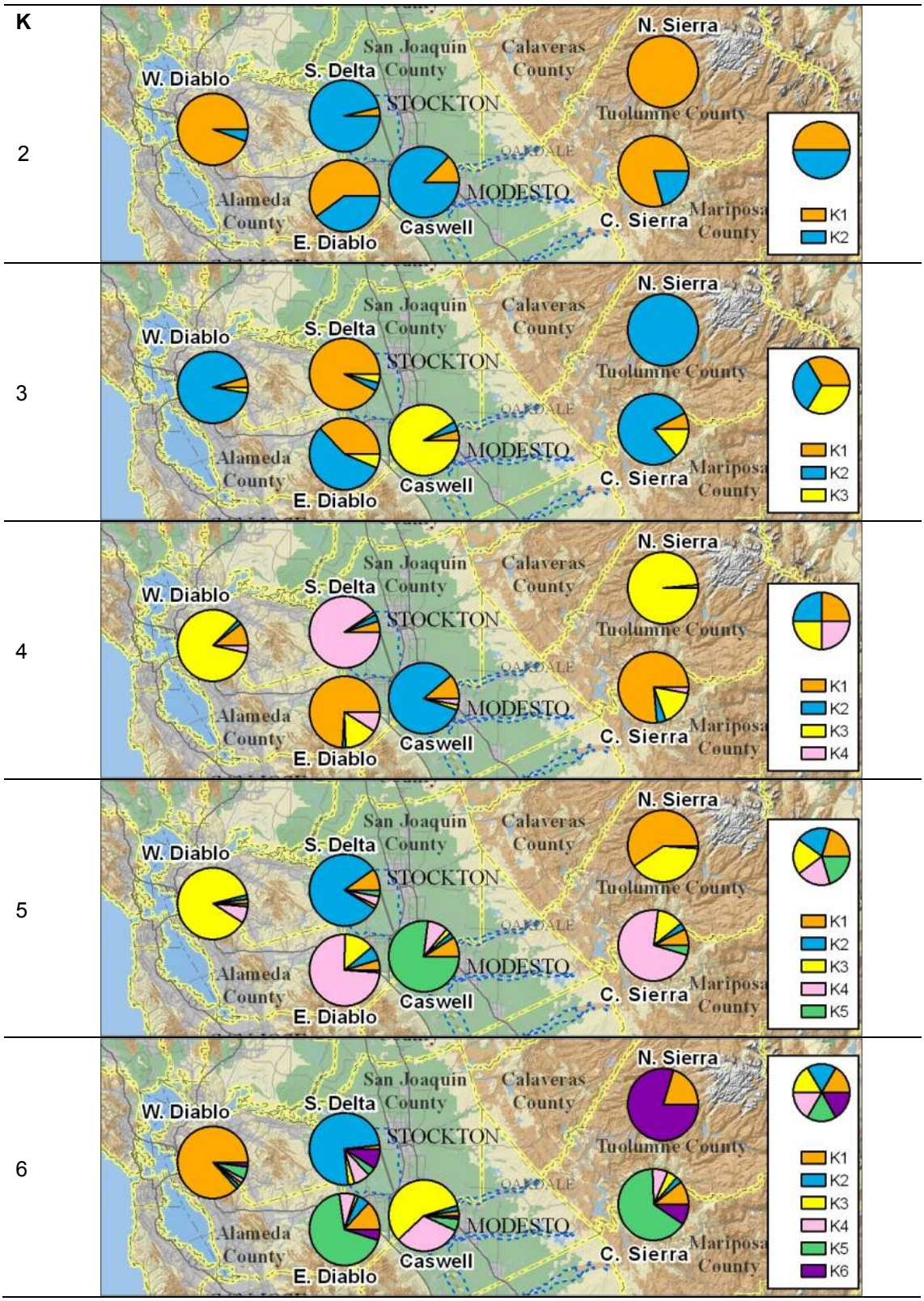

Figure 7. Association of *Sylvilagus bachmani* ssp samples with clusters (*K*) grouped by general location. Each map charts the average association of individual samples for each general location with one of 1-*K* clusters.

When all three brush rabbit subspecies were analyzed together, each subspecies was an independent group, with the two locations of *S. b. riparius* (South Delta and Caswell MSP) remaining as two distinct entities. When forced to fit into K=2 populations, the groups were *S. b. riparius* and *S. b. mariposae-S. b. macrorhinus*. When forced to fit into K=3 populations, the individuals were clustered as: *S. b. riparius* (Caswell MSP); *S. b. riparius* (South Delta); and the combination of *S. b. mariposae* and *S. b. macrorhinus*. With a designation of K=4 populations, the individuals were separated into *S. b. riparius* (Caswell MSP); *S. b. riparius* (South Delta); a combination of *S. b. mariposae* (N. Sierra) and *S. b. macrorhinus* (E. Diablo); and a combination o f *S. b. mariposae* (C. Sierra and S. Sierra) and *S. b. macrorhinus* (W. Diablo).

EVANNO CALCULATIONS

Whether or not the population locations for *S. b. riparius* were designated prior to running the program, and whether or not null alleles were adjusted in the data set, Evanno calculations clearly separated individuals into 2 populations (South Delta and Caswell MSP). Within *S. b. riparius*, the geographically separated Mossdale rabbits did not sort out as a subpopulation distinct from other South Delta rabbits. With data from all subspecies of brush rabbits included, regardless of using geographical information as another factor, and with or without null alleles adjusted, the Evanno method calculated K=3 populations as the best fit. Samples with null alleles adjusted, and no population locations designated were exceptions; results of both scenarios tied for 3 and 4 populations as possible best fits. Rather than the data separating into 3 groups representing different subspecies, the data separated into Caswell MSP; South Delta; and the samples of *S. b. mariposae* and *S. b. macrorhinus*. With K=4 populations, *S. b. mariposae* and *S. b. macrorhinus* were separated, as were Caswell MSP and South Delta. With only *S. b. riparius* specimens, Caswell MSP and South Delta rabbits were grouped as two distinct populations. When *S. b. mariposae* and *S. b. macrorhinus* specimens were analyzed together, they were separated into two 2 subspecies by the Evanno calculations However, there is further sub-structuring in both of these subspecies by collection locality. There were differences between the northern Sierra population of *S. b. mariposae* and the central and southern populations, samples of which were widely separated (Figure 2), though brush rabbits are distributed throughout the intervening areas in appropriate habitat. Differentiation was apparent between the Corral Hollow sample of *S. b. macrorhinus* and those from East Diablo and West Diablo (Figure 2).

PRINCIPAL COMPONENT ANALYSIS.

Clustering of samples by principal component coordinates was consistent with the basic patterns of the *STRUCTURE* analyses, namely the separation of riparian brush rabbit into two distinctive subpopulations (South Delta and Caswell MSP. Figure 8 and Figure 9 show clusting between samples grouped by subspecies and general location (e.g., South Delta and Caswell MSP) using the first and second principal components that respectively represent approximately 11% and 7% of variance (Appendix F). We also examined more specific locations (sublocations) of riparian brush rabbit samples (Figure 10, Figure 11) and found a consistent pattern of differentiaton between South Delta and Caswell MSP sublocations.

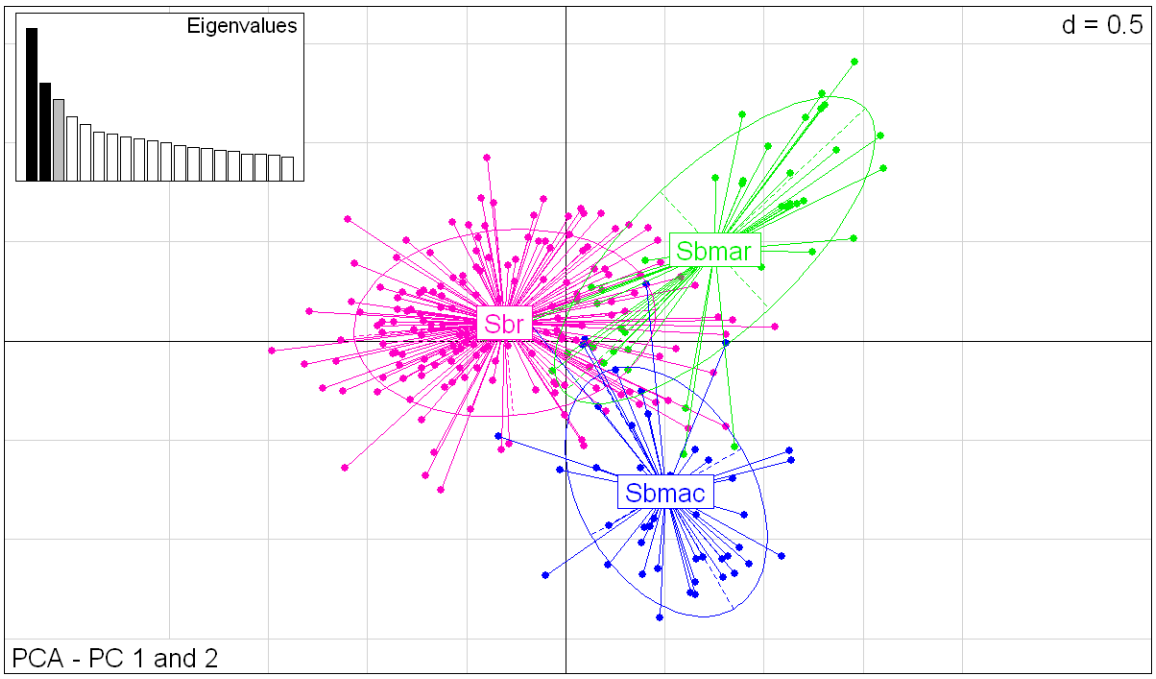

Figure 8. Principal components 1 and 2 using three subspecies of *S. bachmani* grouped by subspecies. Eigenvalues are listed in Appendix F (*S. bachmani* ssp. samples).

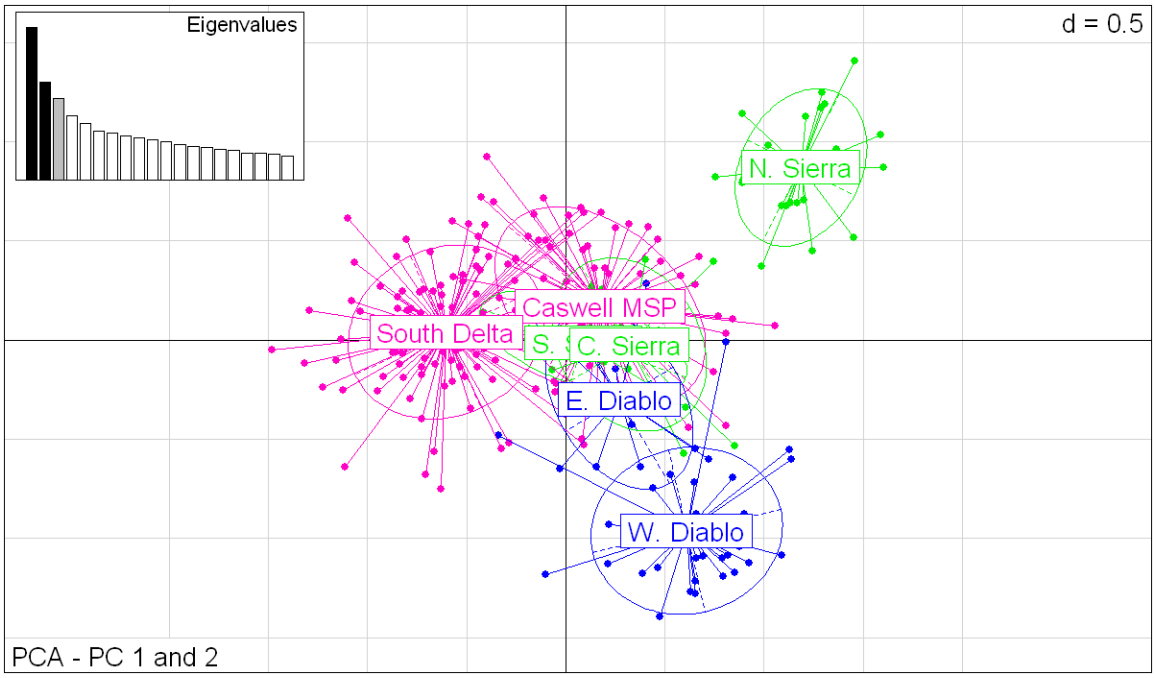

Figure 9. Principal components 1 and 2 using three subspecies of *S. bachmani* grouped by general geographic location.. Eigenvalues are listed in Appendix F (*S. bachmani* ssp. samples).

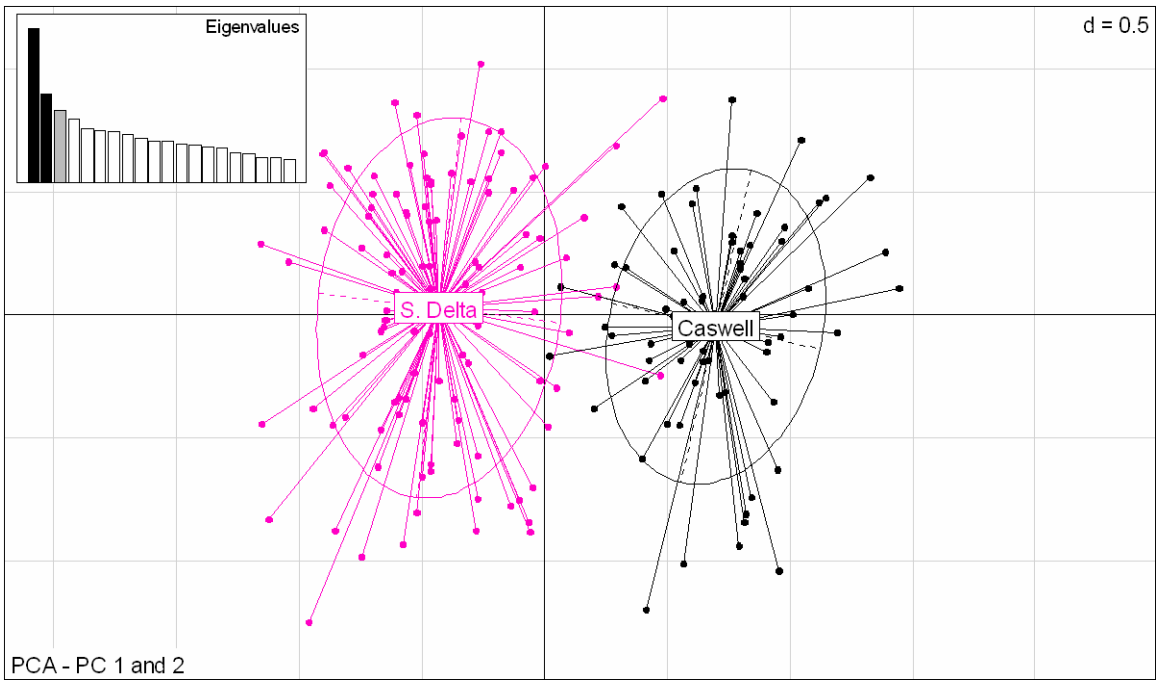

Figure 10. Principal components 1 and 2 using of *S. bachmani* grouped by general geographic location (Figure 3). Eigenvalues are listed in Appendix F (*S. b. riparius* samples).

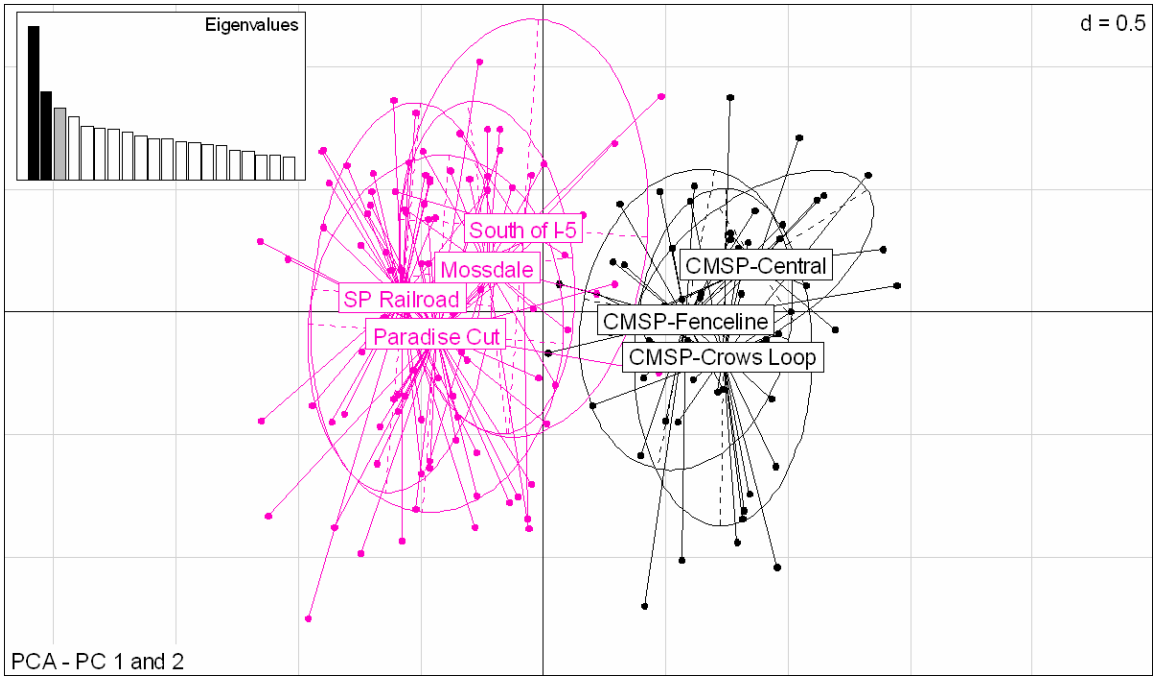

Figure 11. Principal components 1 and 2 using of *S. b. bachmani* grouped by geographic sub-locations (Figure 4,Figure 5). Eigenvalues are listed in Appendix F (*S.b. riparius* samples).

ARLEQUIN RESULTS

The groups *S. b. mariposae*, *S. b. macrorhinus*, *S .b. riparius*-South Delta and *S. b. riparius*-Caswell MSP were examined independently for Hardy Weinberg equilibrium and linkage disequilibrium between loci. Each group had some loci that were not in Hardy Weinberg equilibrium some loci within each group appeared to have linkage to other loci. (Table 4, Table 5)

Table 4. Hardy-Weinberg equilibrium for *S. b. riparius* at Caswell Memorial State Park and the South Delta.

Location and sample size	Locus	#Genot	Obs.Het.	Exp.Het.	*P*-value	Std.Dev.
Caswell MSP	1	37	0.70270	0.76083	0.28315	0.00049
(*n*=67)	2	37	0.70270	0.71159	0.12651	0.00035
	3	38	0.63158	0.79158	0.00399	0.00007
	4	39	0.74359	0.79421	0.01525	0.00010
	5	39	0.82051	0.79021	0.23626	0.00038
	6	38	0.71053	0.77965	0.06167	0.00017
	7	37	0.37838	0.48834	0.13166	0.00033
	8	36	0.72222	0.64124	0.00001	0.00000
South Delta	1	54	0.66667	0.65178	0.02239	0.00015
(*n*=109)	2	54	0.66667	0.72066	0.15717	0.00029
	3	54	0.81481	0.68882	0.31031	0.00043
	4	54	0.72222	0.73884	0.00832	0.00008
	5	54	0.75926	0.82953	0.01427	0.00012
	6	54	0.79630	0.83887	0.04305	0.00020
	7	54	0.64815	0.71599	0.81747	0.00038
	8	54	0.62963	0.69938	0.00000	0.00000

Table 5. Significant linkage disequilibrium for *S. b. riparius* at Caswell Memorial State Park and the South Delta.

Caswell MSP									South Delta								
Locus	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Locus**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0		+	-	-	+	-	-	-	**0**		-	-	-	-	-	-	-
1	+		+	-	+	+	-	-	**1**	-		-	-	+	-	-	-
2	-	+		-	-	+	-	-	**2**	-	-		-	+	-	+	-
3	-	-	-		-	-	-	-	**3**	-	-	-		+	+	+	+
4	+	+	-	-		-	-	+	**4**	-	+	+	+		+	+	+
5	-	+	+	-	-		+	-	**5**	-	-	-	+	+		-	-
6	-	-	-	-	-	+		-	**6**	-	-	+	+	+	-		+
7	-	-	-	-	+	-	-		**7**	-	-	-	+	+	-	+	

+ = Significant, - = not significant (*a* = 0.05)

For F_{ST} and R_{ST} values, runs comparing populations of "riparian brush rabbits only", "all brush rabbits", and "all Sylvilagus" were examined. Original alleles and null-adjusted loci were used independently. F_{ST} and R_{ST} values were significantly different between subpopulations of riparian brush rabbits (South Delta and Caswell MSP). . Significant differentces between

subspecies and those between cottontails and brush rabbits were apparent when using both original data, and when using null-adjusted data (Table 6, Table 7).

Table 6. Fixation indices (F_{ST} and R_{ST}) between S. bachmani subspecies.

Statistic	Taxonomic group	Allele values	*S. b. macrorhinus*	*S. b. riparius*	*S. b. mariposae*
F_{ST}	***S. b. macrorhinus***	Original	-	0.12752	0.10749
		Null-adjusted	-	0.11205	0.0972
	S. b. riparius	Original	0.12752	-	0.08402
		Null-adjusted	0.11205	-	0.07194
	S. b. mariposae	Original	0.10749	0.08402	-
		Null-adjusted	0.09720	0.07194	-
R_{ST}	***S. b. macrorhinus***	Original	-	0.09810	0.27458
		Null-adjusted	-	0.10942	0.02952
	S. b. riparius	Original	0.09810	-	0.18815
		Null-adjusted	0.10942	-	0.03233
	S. b. mariposae	Original	0.27458	0.18815	-
		Null-adjusted	0.02952	0.03233	-

Table 7. Fixation indices (F_{ST} and R_{ST}) between S. bachmani subspecies with S. b. riparius divided between South Delta and Caswell Memorial State Park samples.

Statistic	Taxonomic group	General location	Allele values	*S. b. macrorhinus*	*S. b. riparius*		*S. b. mariposae*
					South Delta	Caswell MSP	
F_{ST}	***S. b. macrorhinus***		Original	-	0.16004	0.14300	0.10749
			Null-adjusted	-	0.13822	0.11246	0.11148
	S. b. riparius	South Delta	Original	0.16004	-	0.11095	0.12087
			Null-adjusted	0.13822	-	0.12035	0.12165
		Caswell MSP	Original	0.14300	0.11095	-	0.08999
			Null-adjusted	0.11246	0.12035	-	0.07801
	S. b. mariposae		Original	0.10749	0.12087	0.08999	-
			Null-adjusted	0.11148	0.12165	0.07801	-
R_{ST}	***S. b. macrorhinus***		Original	-	0.09990	0.15016	0.27458
			Null-adjusted	-	0.10477	0.01312	0.02952
	S. b. riparius	South Delta	Original	0.09990	-	0.05408	0.20785
			Null-adjusted	0.10477	-	0.08374	0.06045
		Caswell MSP	Original	0.15016	0.05408	-	0.18223
			Null-adjusted	0.01312	0.08374	-	0.05380
	S. b. mariposae		Original	0.27458	0.20785	0.18223	-
			Null-adjusted	0.02952	0.06045	0.05380	-

DISCUSSION

Both *STRUCTURE* and *Principal Components Analysis* (*PCA*) revealed substructure in the brush rabbit populations of this study. Tissue samples of *S. b. mariposae* came from a large geographic area in the Sierra Nevada (approximately 360 km/220 miles). There are no known ecogeographic barriers and habitat is presumed to be relatively continuous along the chaparral communities of the western Sierran slope. Of our samples, only the northernmost population (N. Sierra) demonstrated some genetic differences from the remaining *S. b. mariposae*.

Likewise, *S. b. macrorhinus* had within-group structure between individuals from Corral Hollow and Dinosaur Point (E. Diablo) and the samples from Lindsay Wildlife Hospital (W. Diablo), (Figure 2). The *STRUCTURE* analysis, at K=4, showed clear division between these subgroups

within *S. b. mariposae* and *S. b. macrorhinus*. At K=2, there was less clarity, with some overlap (i.e., overlap in the clusters that individuals were assigned to) between *S. b. macrorhinus* (E. Diablo) and *S. b. mariposae*. Some of this overlap may be attributed to homoplasy (in this case the convergence of genotypes due to random mutations over time), rather than recent gene exchange.

There was some similar overlap in clustering between the central and southern samples *S. b. mariposae* and the rabbits from Caswell MSP, especially in the PCA results (Figure 9). This may indicate a more recent evolutionary ancestry between *S. b. mariposae* and *S. b. riparius* from Caswell MSP, but the northern samples of *mariposae* are closer and one would therefore expect greater similarity between them and those from Caswell MSP. Historically, the Sierra Nevada foothills and the confluence of the Stanislaus and San Joaquin rivers were connected by riparian habitat. This continuity in habitat provided by riparian plant communities also suggested in genetic analyses on riparian woodrats, *Neotoma macrotis* (Matocq, 2007).

However, the most striking finding of this study was the degree of differentiation between Caswell MSP and South Delta populations of *S. b. riparius*. To rigorously analyze this and other relationships, multiple approaches were used:

1. Adjustment of a proportion of apparent homozygotes due to null alleles—recommended by the program Microchecker—by replacing some homozygote alleles as "unknown," in addition to unadjusted data with greater than expected homozygosity.
2. Inclusion, and alternately not using, the geographical location of the samples from the program parameters.
3. Including, and alternately removing, entire samples with multiple missing alleles.
4. Assuming, and alternately removing, the admixture assumption of the model (admixture assumes individuals are close enough to come into contact for potential interbreeding).

Null alleles were present at some loci, in some groups of rabbits (Table 2). Primers designed for amplifying a locus of one species, but used for a species in a related genus (i.e., *Oryctolagus, Sylvilagus)*, may sometimes cause the appearance of null alleles due to the accumulation of mutations over time in the flanking region where the primers must anneal. The presence of null alleles may lead to overestimation of F_{ST} and genetic distance, and low levels of gene flow may increase this bias (Chapuis and Estoup, 2007). To correct this problem, the primers may be redesigned and all samples could be re-amplified at the locus in question, but this is a more costly, time-consuming option. The Microchecker program was designed to adjust the allele sizes in the population to reduce null allele issues in genetic analysis, and thus make the data usable for further analyses by programs such as *STRUCTURE* and *Arlequin*. We ran both null-adjusted and original data through both programs and found significant F_{ST} distances with either treatment. This gives us confidence that our original data with an excess of homozygotes accurately reflect the population structure represented by our brush rabbit samples.

The sample groups—*S. b. mariposae*, *S. b. macrorhinus*, *S. b. riparius* South Delta, and *S. b. riparius* Caswell MSP—had some loci that were not in Hardy Weinberg equilibrium (Table 2). In very small populations, allele frequencies may change dramatically from one generation to the next, due to nonrandom mating, sampling errors, or other factors.

Some loci within each group appeared to have linkage to other loci (Table 5). In a small population, the degree of disequilibrium may be substantial due to genetic drift. Gene flow may also produce significant levels of linkage disequilibrium in a population. High levels of homozygosity limit the effectiveness of recombination, and therefore, may promote linkage disequilibrium (or reduce its decay) in the population.

There is also a possibility that excess homozygosity could be attributed to inbreeding. If Caswell MSP rabbits are as effectively isolated from those of the South Delta as they appear to be, then closely related individuals likely would be breeding and this would result in offspring with more homozygous genotypes. The only positive way to distinguish the presence of null alleles from true homozygotes would be to redesign the primers at those loci with an excess of homozygotes. If the redesigned primers were able to amplify a new allele size for some of the homozygous individuals that were flagged as potentially having null alleles, then we could be certain that they are falsely homozygous rather than true homozygotes resulting from inbreeding. Although homozygosity and linkage disequilibrium reduces the effectiveness of F_{ST} and R_{ST} comparisons, *PCA* is not affected by these factors, and confirms the results of *Arlequin*.

In all cases, Caswell MSP and South Delta populations clearly separated from each other genetically. At all but one locus, individuals from the South Delta had one or more private alleles (for a total of 16 alleles at 9 loci). Caswell MSP additionally had 8 alleles at 5 loci that were unique to Caswell MSP rabbits. The genetic separation between the South Delta and Caswell MSP populations must have been in existence for some time to allow the accumulation of so many private alleles. Though the two populations are in much closer proximity to each other (9 miles/15 km) than *S. b. mariposae* and *S. b. macrorhinus* are to each other, the relatively large numbers of private alleles in the *S. b. riparius* populations also suggest genetic isolation.

Based on past surveys, the general lack of contiguous, suitable habitat between the Caswell MSP and South Delta populations (Williams and Basey, 1986; ESRP, unpubl. data; Greg Miller, pers. comm.), and examination of aerial photographs of unsurveyed private lands, it seems likely that the two populations are completely isolated from each other. The north bank of the Stanislaus River, where Caswell MSP is located, is on the eastern side of the San Joaquin River, whereas all the South Delta rabbit populations, except those from the Mossdale Oxbow Preserve, are on the western side of the San Joaquin River. Flowing rivers do not appear to be absolute barriers to rabbit movement (ESRP, unpubl. data), but they likely act as filter barriers, restricting gene flow.

Given the number of private alleles that have developed separately in the Caswell MSP and South Delta populations, it appears that they have been separated for a significant amount of time. The results of the *STRUCTURE* and *PCA* analyses indicate that the two populations are almost as different from each other as they are from *S. b. mariposae* and *S. b. macrorhinus*. It may be argued that, although sample sizes for Caswell MSP and South Delta were robust in this study, sample sizes for *S. b. mariposae* and *S. b. macrorhinus* were somewhat smaller, and if they had been larger, the F_{ST} and R_{ST} values between them may have been larger. Homoplasy may also cause these two geographically separated subspecies to appear more similar than they otherwise would be. Regardless, Caswell MSP and South Delta are still genetically differentiated to a degree that warrants careful consideration before either population is 'augmented' with breeders from the other.

There appear to be both positive and negative consequences to interbreeding the two populations (Crandall et al. 2000, Fraser and Bernatchez 2001, Storfer 1999). Potential negative consequences of mixing genes of two differentiated populations include constraining (slowing)

local adaptation and breaking down coadapted gene complexes (outbreeding depression). And, in populations with conflicting selection pressures, foreign alleles may overwhelm the local adaptations and may even prevent the evolutionary development of new species.

Potential benefits include maintaining genetic variation—especially when habitat fragmentation and degradation leads to the loss of variation—and preventing inbreeding depression. Augmenting a population also could increase the probability of introducing breeding stock with favorable adaptations and high fitness into the local population. Further, there may be less concern about genetic differentiation between two populations if that differentiation does not represent adaptation to local conditions (Fraser and Bernatchez 2001), and especially so in the context of landscape level changes resulting from climate change (*cf*. California LCC: http://californialcc.org/about.html).

We believe that this study of the genetics of riparian brush rabbit populations and their relationships to other brush rabbit subspecies has generated important information, but it has generated also many questions. Accordingly, we have a closing recommendation that the U.S. Fish and Wildlife Service and the California Department of Fish and Game convene a workshop in spring 2011 with a select group of mammalian population geneticists and key stakeholders, who have already reviewed the draft report, to discuss it and provide guidance to the agencies and to the authors.

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