Molecular Taxonomy and Phylogenetic Analysis of the Endangered Blunt-nosed Leopard Lizard, *Gambelia sila*

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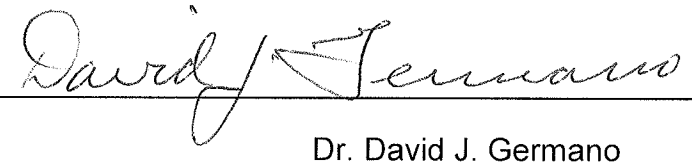

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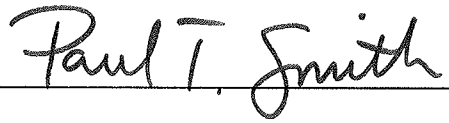

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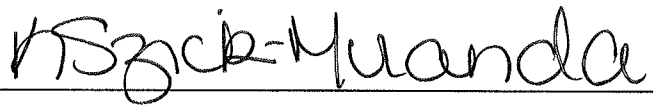

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Abstract

The blunt-nosed leopard lizard (*Gambelia sila*) is a federally and state-listed endangered species, endemic to the San Joaquin Valley, Carrizo and Elkhorn Plains, and Cuyama Valley of central California. Habitat degradation has had a profound impact on the historic distribution and population size of *G. sila*. Although recognition of *G. sila* as a distinct species has been questioned by some authors (e.g., Cope 1900, Smith 1946), it is currently recognized as a full species separate from the wide-ranging long-nosed leopard lizard (*G. wislizenii*); however, genetic support for the specific status of *G. sila* is lacking. Furthermore, the genetic identity of leopard lizards in the purported hybrid zone between these two species in the Cuyama Valley in Santa Barbara and Ventura counties has not been evaluated using modern molecular techniques. Understanding the genetic identity of leopard lizards in the Cuyama Valley has practical as well as systematic implications. I investigated the sister taxon relationship of *G. sila* and *G. wislizenii* using 603 base pairs of sequence from the mitochondrial cytochrome oxidase III (CO3) gene from 37 individuals representing the two species sampled from various populations in western North America. Phylogenetic analysis revealed 17 haplotypes that are partitioned into two major clades that correspond to the range of *G. sila* and that of *G. wislizenii* haplotype groups, thus supporting the recognition of both lizards as distinct species. Additionally, I sequenced 682 base pairs of the mitochondrial cytochrome oxidase *b* (cyt *b*) gene from 34 individuals representing six populations of *G. sila*, including lizards from a remnant hybrid population. Phylogenetic analysis indicated that the cyt *b* sequences consisted of 18 haplotypes that are partitioned into three geographic clades: northern, central, and southern. All lizards from the Cuyama Valley exhibited the *G. sila* mitochondrial DNA signature and formed the "southern" clade that was joined as a sister group to the "central" clade. My morphological analysis placed some leopard lizards from the hybrid zone with true *G. sila*, whereas some aggregated with *G. wislizenii*, indicative of hybrid status. However, genetic signatures suggest that all lizards in the hybrid zone are true *G. sila*, and not hybrids.

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CHAPTER 1

Purpose of the Study

The lizards that belong to the family Crotaphytidae (collard and leopard lizards) are desert dwelling reptiles that are native to the southwestern United States and northern Mexico. Currently there are 12 species in two genera that are recognized; four species in *Gambelia* (leopard lizards) and eight species in *Crotaphytus* (collard lizards). Leopard lizards occur in all four major North American deserts (Sonoran, Mojave, Great Basin, and Chihuahuan) from central Idaho in the north to northern Mexico in the south.

The taxonomic status of crotaphytid lizards has been debated since the first formal description of the leopard lizard, *Crotaphytus wislizenii* (Baird and Girard 1852). Stejneger (1890) was the first to describe and name the blunt-nosed leopard lizard (*Crotaphytus silus*) in the San Joaquin Valley of California (Fig. 1) as a species distinct from leopard lizards farther to the east. Cope (1900) disagreed with Stejneger and concluded that the leopard lizards in the San Joaquin Valley should be listed as a subspecies of the wide-ranging leopard lizard, *C. wislizenii silus.* In all cases, leopard lizards and collard lizards were considered to be in the same genus until Smith (1946) separated the two by placing leopard lizards in the genus *Gambelia*. Smith (1946) also placed the blunt-nosed leopard lizard as a subspecies of *G. wislizenii*. This generic and specific split has not been accepted by all lizard systematists even though the bases for the separation were differences in morphological, biological, ecological, and behavioral characteristics.

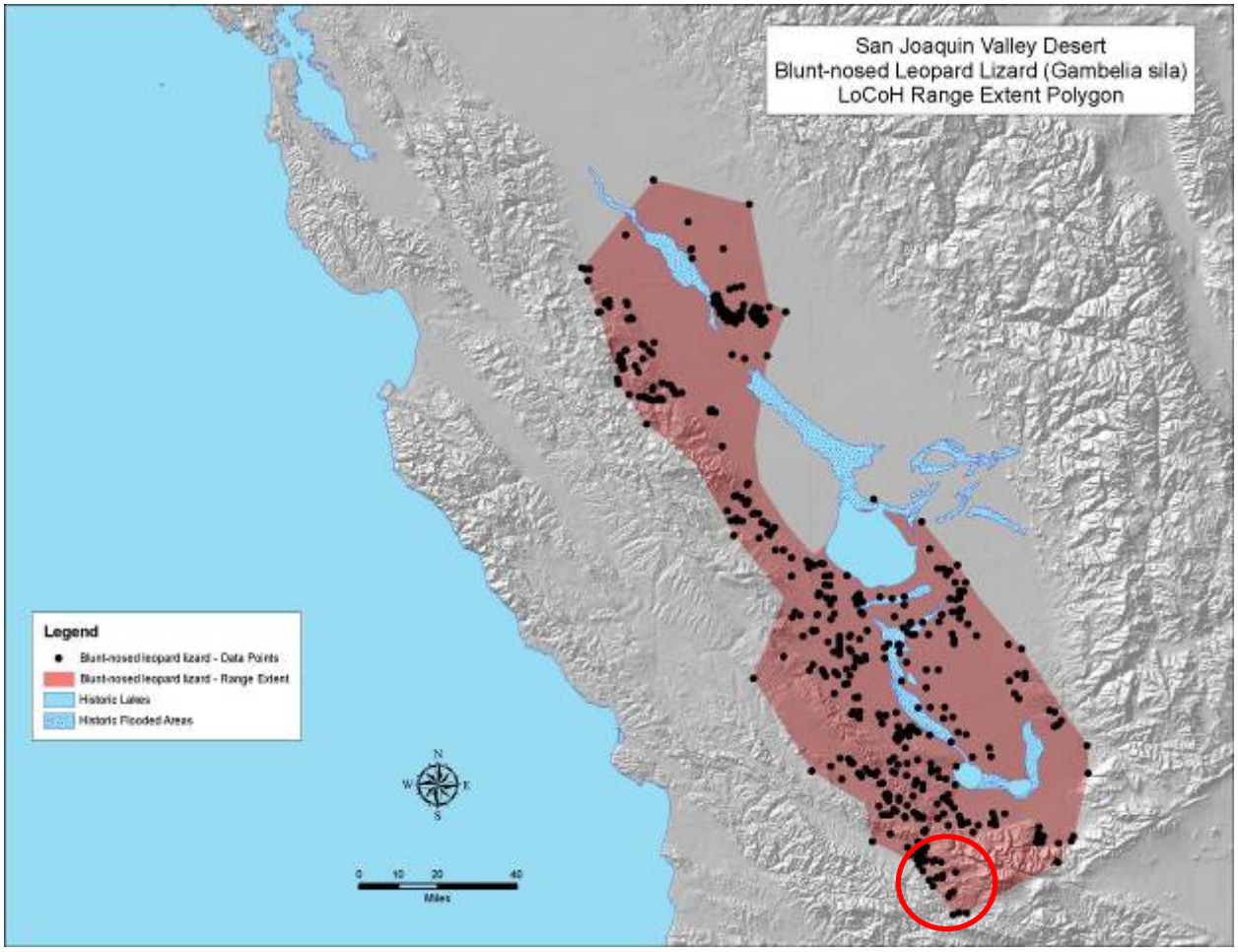

Figure 1. The historic distribution (red shading) of the blunt-nosed leopard lizard (*Gambelia sila*) in the San Joaquin Valley, Carrizo and Elkhorn Plains, and Cuyama Valley of California. The blue areas within the range of the lizard represent past wetlands that are absent now. The black dots are specific locality records. The red circle is over the purported hybrid zone of *G. sila* with *G. wislizenii*.

Both Sanders (1950) and Montanucci (1965) described a contact zone between long-nosed leopard lizards and blunt-nosed leopard lizards along the Cuyama River drainage system in Ventura and Santa Barbara counties, California, which theoretically allowed genetic interchange between these groups. Montanucci (1970) described hybrid leopard lizards based mainly on morphological traits and hypothesized that

interbreeding had been occurring between *G. sila* and a remnant hybrid population inhabiting the area. Tanner and Banta (1977) did not support the recognition of a separate genus for leopard lizards (*Gambelia*) distinct from collared lizards, nor Montanucci's (1969) recognition of *G. silus* as a distinct species from long-nosed leopard lizards because of the contact zone. Despite this challenge to the generic split and the specific status of blunt-nosed leopard lizards, *Gambelia* is currently recognized as a valid genus and *G. silus* as a full species. Jennings (1987; 1995) changed the specific epithet to *sila* to properly agree in gender with the genus. The present taxonomy of the blunt-nosed leopard lizard is, therefore, *Gambelia sila*, although there is only weak genetic support for this binomial.

Gambelia sila (Fig. 2) has been listed federally as endangered since 1967 and by the state since 1971, and protection of natural lands supporting *G. sila* has been necessary for their recovery (U.S. Fish and Wildlife Service, 1985; 1998). Upwards of 85% of the original natural communities in their range has been destroyed because of urbanization, oil development, and agriculture (U.S. Fish and Wildlife Service, 1985; Germano and Williams, 1992; Montanucci, 1965). Unlike the non-protected long-nosed leopard lizard (*G. wislizenii*; Fig. 2), the immense loss of habitat and fragmentation has caused isolated populations of *G. sila,* and its current endangered status. A priority for the recovery of *G. sila* has been to determine the genetic variation within and among isolated populations on fragmented land to identify significant conservation sites and enhance population movements between them (U.S. Fish and Wildlife Service, 1998).

Figure 2. Female blunt-nosed leopard lizard (*Gambelia sila*) from the San Joaquin Valley (top) and a female long-nosed leopard lizard (*Gambelia wislizenii*) from the Mojave Desert (bottom).

Determining the populations with unique genetic variation can lead to understanding how best to preserve and enhance the surrounding habitat to ensure recovery (U.S. Fish and Wildlife Service, 1998).

Of particular interest is the purported hybrid zone between *G. sila* and *G. wislizenii* in the Cuyama Valley. A reassessment of the hybrid zone using a comparison of the DNA barcodes of known *G. wislizenii* and *G. sila* to the barcodes of lizards inhabiting the hybrid zone should help determine the genetic status of these canyon lizards and help guide environmental assessments in the area. Also, some recent molecular-based studies that dealt with *Gambelia* (Orange et al. 1999; Corrigan 2002; McGuire et al. 2007) were unsuccessful at effectively addressing the recognition of *G. sila* as distinct from *G. wislizenii* due to insufficient taxon sampling. For this reason the present study was designed to build upon the work by Orange et al. (1999) by sequencing the same mitochondrial CO3 fragment from 10 additional *G. wislizenii* from California and 20 *G. sila* from various localities throughout California, including nine from the contact zone. By including approximately equal representation of both *G. sila* and *G. wislizenii* in my phylogenetic analyses, I can effectively assess the monophyly of each species and provide the first extensive molecular evidence concerning the specific status of the endangered blunt-nosed leopard lizard. Furthermore, to provide additional phylogenetic support for the relationships among *G. sila* populations, I designed my study to build upon the work of Corrigan (2002) by sequencing the same mitochondrial cytochrome oxidase *b* fragment from 23 additional *G. sila* from various localities throughout California, including 18 individuals from the contact zone (Fig. 3). I also used the mitochondrial cytochrome oxidase III (CO3) gene fragment that was analyzed

previously by Orange et al. (1999) as additional support. By analyzing the combined mitochondrial CO3 and cyt *b* gene sequences of *G.sila* individuals, I was able to provide more resolution for the relationships among *G.sila* populations.

Figure 3. Male blunt-nosed leopard lizards (*Gambelia sila*) from the contact zone in the Cuyama Valley demonstrating morphological characteristics of hybrid leopard lizards.

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CHAPTER 2

Molecular Evidence Supports the Specific Status of the Endangered Blunt-nosed Leopard Lizard, *Gambelia sila* (Squamata: Iguanidae)

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Abstract

The systematic status of the federally and state-listed blunt-nosed leopard lizard (*Gambelia sila*, Stejneger 1890) has been questioned by some authors (e.g., Cope 1900, Smith 1946). Although current taxonomy separates *Gambelia sila* from *Gambelia wislizenii*, little genetic evidence supports this separation. We investigated the sister taxon relationship of *G. sila* and *G. wislizenii* (Baird & Girard 1852) using 603 base pairs of sequence from the mitochondrial cytochrome oxidase III (CO3) gene from 37 individuals representing the two species. Samples came from ten populations in western North America. Phylogenetic analysis revealed 17 haplotypes that are partitioned into two major clades, separated by approximately 4.1% sequence divergence. These major clades correspond to the *G. sila* and *G. wislizenii* haplotype groups, and, therefore, supports the specific status of *G. sila*.

Introduction

Currently, 12 species of collard lizards (*Crotaphytus* sp.) and leopard lizards (*Gambelia* sp.) are recognized in the family Crotaphytidae (McGuire 1996). Three

extant species in the genus *Gambelia* are found throughout arid regions of western North America; *G. sila* (Stejneger), a restricted endemic of the Central Valley of California, *G. copei* (Banta & Tanner 1977), endemic to Baja California, and *G. wislizenii* (Baird & Girard 1852), the most widely distributed member of this genus found in northern Baja California and elsewhere in Mexico, as far north as Idaho and Oregon, and eastward to west Texas (McGuirre 1996, Stebbins 2003). Leopard lizards were originally placed in the genus *Crotaphytus*. Baird and Girard (1852) published the first description of a leopard lizard, *Crotaphytus wislizenii*. Later, Baird (1858) proposed the subgenus *Gambelia* for *C. wislizenii*, but provided no morphological or geographical basis for the designation. Stejenger (1890) was the first to recognize *Crotaphytus silus*, found in the San Joaquin Valley of California, as a separate species. However, Cope (1900) later synonymized *C. silus* and *C. copeii* with *C. wislizenii*.

Over the next 75 years the taxonomy of crotaphytid lizards remained in a constant state of flux. Smith (1946) was the first to elevate *Gambelia* to generic level and reduced *G. silus* to a subspecies of *G. wislizenii*. This decision was not universally accepted and Cochran (1961) re-established G. *silus* as a distinct species, but as a member of *Crotaphytus (= C. silus*). Osteological work by Montanucci (1969) supported Cochran's (1961) recognition of *C. silus* as a separate species.

Montanucci *et al.* (1975) were the first to apply molecular methodology to resolving the *Crotaphytus-Gambelia* debate. The results of their protein electrophoretic studies supported the recognition of *Gambelia* as a valid genus; however, work by Tanner and Banta (1977) did not support the designation, nor Montanucci's (1969) recognition of *G. silus* as a distinct species. Currently, *Gambelia* is recognized as a

valid genus and *G. silus* as a full species. However, the specific epithet was changed to *sila* (Jennings 1987, 1995) to reflect correct gender agreement with the genus. The current taxonomy of the Blunt-nosed Leopard Lizard is, therefore, *Gambelia sila*, although there is only weak genetic support for specific status.

The debate surrounding the recognition of *G. sila* as a distinct species is due, in part, to the observation that some lizards exhibit morphological characteristics intermediate in form relative to the morphological criteria that has traditionally been used to distinguish between *G. sila* and G. *wislizenii*. Additionally, some have stated that lizards found in the canyons of Santa Barbara and Ventura counties of California at the extreme southern end of the range of *G. sila* are intermediate forms between *G. wislizenii* and *G. sila* and that this contact zone has allowed free genetic interchange between these taxa (Montanucci 1965; Sanders 1950). Montanucci (1965) reported that there were also possible intermediate forms found near Frazier Peak in Ventura County, California. Montanucci (1970) concluded that a remnant population of a hybrid origin existed in the contact zone and primarily interbred with *G*. *sila*.

McGuire *et al.* (2007) examined the phylogenetic relationships of crotaphytid lizards using partial sequences from the NADH dehydrogenase 2 (ND2), five tRNA, and cytochrome oxidase *b* genes. This study, which included two individual *G. sila* from northern California (Madera Co.) and > 50 *G. wislizenii* individuals, indicated that *G. sila* and *G. wislizenii* are sister taxa, but with relatively weak branch support. In another study that dealt exclusively with *G. wislizenii,* Orange *et al.* (1999) sequenced a portion of the mitochondrial cytochrome oxidase III (CO3) gene from 23 individuals and found that the lizards were partitioned into two major clades: Western (Mojave + Great Basin +

Colarado Plateau deserts) and Eastern (Chihuahuan Desert) clades. Only one of the *G. wislizenii* individuals included in the Orange *et al.* (1999) study was from California (San Bernardino Co.), however. Therefore, we designed the present study to increase the taxon sampling and population representation of both *G. sila* and *G. wislizenii* and build upon the work by Orange *et al.* (1999) by sequencing the same mitochondrial CO3 fragment from 10 additional *G. wislizenii* from California and 20 *G. sila* from various localities throughout California, including nine from the contact zone. By including approximately equal representation of both *G. sila* and *G. wislizenii* in our phylogenetic analyses, we can effectively assess the monophyly of each species and provide strong molecular evidence on the specific status of the endangered blunt-nosed leopard lizard.

Methods and Materials

We analyzed 37 mitochondrial cytochrome oxidase III (CO3) gene sequences representing 18 *G. sila*, 17 *G. wislizenii* and two *Crotaphytus* species that were used as outgroup taxa (Table 1). We extracted DNA from toe clippings using the DNAeasy tissue kit (QIAGEN, Valencia, California) according to the manufacture's instructions. Voucher genomic extracts are stored at the Department of Biology, California State University, Bakersfield. We used primer sequences from Orange *et al.* (1999) to amplify and sequence a ~600 bp portion of the mitochondrial cytochrome oxidase III (CO3) gene from all *G. wislizenii*. This primer set, however, inconsistently amplified extracts of *G. sila*, so an internal primer set was designed from a single *G. sila* sequence (see Table 2) and was used to amplify the cytochrome oxidase III (CO3) gene from all remaining *G. sila* samples.

We carried out polymerase chain reaction (PCR) amplifications in 20-50µl volume and annealing temperatures ranging between 48-55°C. We purified successfully amplified PCR products by either using QiaQuick PCR columns or using shrimp phosphatase and exonuclease (ExoSAPit). We submitted purified PCR products to the University of Florida's DNA Sequencing Core Facility for sequencing both forward and reverse strands on an ABI 377 DNA sequencer. DNA sequence electropherograms were read, edited, and aligned using Geneious v5.0 (Drummond *et al.* 2010). DNA sequence alignment was straight-forward and did not necessitate the insertion of any gaps.

We calculated summary statistics for the DNA sequence data using PAUP* (Swofford 2003) and Geneious v5.0 (Drummond *et al.* 2010). We estimated phylogenetic relationships using maximum parsimony (MP) and neighbor-joining (NJ) analysis in PAUP* and Bayesian methods using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) as implemented in Geneious v5.0. Parsimony analysis was carried out using the multiple equally parsimonious heuristic search option with tree bisection reconnection and 100 random addition sequence replicates. We conducted neighbor-joining analysis (Saitou and Nei 1987), as implemented in PAUP*4.0, based on the Tajima-Nei distance (Tajima and Nei 1984). We estimated support for specific nodes on the MP and NJ trees by bootstrap analysis (Felsenstein 1985; 1000 replications with 10 random addition sequence replicates).

The evolutionary model utilized for Bayesian analysis was selected by MODELTEST 3.7 (Posada and Crandall 1998, 2001), using the Akaike information criterion (AIC) (Posada and Buckley 2004), and corresponded to the GTR + I + G

model. We analyzed our dataset under the recommended model using a Markov chain Monte Carlo (MCMC) search strategy in MrBayes. We conducted two independent runs on the data and for each run the MCMC process was set so that four chains (three heated and one cold) ran simultaneously for a total of 1,340,000 generations (sampling every 100 generations). A majority rule consensus was created from the remaining trees (n = 72239).

Results and Discussion

We analyzed 603 aligned bases of DNA sequence of the CO3 gene from 39 taxa. Of the 603 characters, 112 (18%) were variable, and 69 (11%) were parsimony informative. The percentage divergence values for all pairwise comparisons among the various consensus groups varied from 4.3 – 23.9% (Table 3). Using percentage divergence values as benchmarks for the recognition of genera and/or species is not only controversial, but difficult to interpret as the same gene may evolve at different rates in different lineages (Hickerson *et al.* 2006; Rubinoff and Holland 2005).

Overall, there were 3.3% base pair differences between the *G. sila* and *G. wislizenii* consensus groups (16-25bp, 2.6-4.1% for individual sequences). In contrast, *C. collaris* and *C. bicinctores*, the two outgroup taxa used in this study, differed by 11%. Perhaps the only strong conclusions that can be made about this consensus exercise is that *Gambelia* and *Crotaphytus* are genetically closely related, and that the two *Gambelia* species diverged more recently than the two *Crotaphytus* species included in this study. Collectively, there were 17 unique *Gambelia* haplotypes, 9 for *G. sila* (A-I)

and 8 for *G. wislizenii* (J-Q; see Table 1). We found 17 haplotypes that could be distinguished based on diagnostic nucleotide sites (Table 4).

Parsimony analysis recovered 104 equally parsimonious trees (length= 141, CI= 0.88, RI= 0.95). The topologies of the trees resulting from the neighbor-joining and Bayesian analyses were largely congruent and differed only in the placement of the *G. sila* individuals from Panoche area in the northern part of the range of *G. sila*. The most significant result of the phylogenetic analyses was the partitioning of the *Gambelia* haplotypes into two distinct clades that correspond to the two species analyzed in this study. Haplotypes A-I, represent individuals from the range of *G. sila*, grouped together to form a monophyletic lineage that was joined as a sister group to all the samples from the range of *G. wislizenii* (Fig. 1). The tree estimated using Bayesian methods was largely congruent with the parsimony tree. As such, Bayesian posterior probabilities are included on the parsimony tree (Fig. 1). In general, clades strongly supported by bootstrap analysis were also supported by high Bayesian posterior probabilities.

One other significant result of our study concerned the phylogenetic placement of the *G. sila* individuals from the Panoche population, the most northern population analyzed in this study. Relative to all other *G. sila* samples, those from Panoche exhibited 10-12 (1.6-1.9%) base pair differences compared to all other *G. sila* samples. This percentage divergence value is significant when examined in the light of the 16-25 (2.6-4.1%) base pair differences that separate *G. sila* from *G. wislizenii* proper. These percentage divergence values clearly suggest that the Panoche population has been geographically and reproductively isolated from other *G. sila* populations. At a minimum, our data point to the need for further study of the population genetics of *G.*

sila throughout their range. Such a study would be highly informative with respect to conservation and/or translocation efforts.

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Table 1. List of samples of *Gambelia* and *Crotaphytus* analyzed, and their locality and GenBank accession numbers.

Species	Haplotype	Code/Locality	GenBank accession #
G. wislizenii	-	Clark, NV	AF095619*
G. wislizenii	-	Clark, NV	AF095621*
G. wislizenii	-	Mohave, AZ	AF095620*
G. wislizenii	-	Boxelder, UT	AF095613*
G. wislizenii	-	Boxelder, UT	AF095614*
G. wislizenii	-	Boxelder, UT	AF095615*
G. wislizenii	-	Navajo, AZ	AF095616*
G. wislizenii	M	1L Mojave, CA	GU550093
G. wislizenii	O	2L Mojave, CA	GU550095
G. wislizenii	J	3L Mojave, CA	GU550090
G. wislizenii	Q	4L Mojave, CA	GU550097
G. wislizenii	J	5L Mojave, CA	GU550090
G. wislizenii	P	7L Mojave, CA	GU550096
G. wislizenii	J	8L Mojave, CA	GU550090
G. wislizenii	N	9L Mojave, CA	GU550094
G. wislizenii	K	Wf Mojave, CA	GU550091
G. wislizenii	L	Lf Mojave, CA	GU550092
G. sila	I	22 Panoche, CA	GU550089
G. sila	I	27 Panoche, CA	GU550089
G. sila	D	30 Panoche, CA	GU550082
G. sila	E	F1 Lokern, CA	GU550083
G. sila	G	M2 Lokern, CA	GU550086
G. sila	G	F3 Lokern, CA	GU550086
G. sila	G	F4 Lokern, CA	GU550086
G. sila	G	F5 Lokern, CA	GU550086
G. sila	G	F6 Lokern, CA	GU550086
G. sila	G	2 Buena Vista, CA	GU550085
G. sila	H	03 Elkhorn, CA	GU550087
G. sila	B	12 Cuyama River, CA	GU550079
G. sila	C	15 Cuyama River, CA	GU550081
G. sila	H	18 Cuyama River, CA	GU550087
G. sila	F	1 Ballinger Canyon, CA	GU550084
G. sila	A	3 Ballinger Canyon, CA	GU550077
G. sila	A	19 Quatal Canyon, CA	GU550077
G. sila	A	6 Apache Canyon, CA	GU550078
G. sila	A	8 Apache Canyon, CA	GU550078
G. sila	B	10 Apache Canyon, CA	GU550080
C. bicinctores	-	Oregon	AF095611*
C. collaris	-	New Mexico	AF095612*

* previously published sequence obtained from GenBank

Table 2. Oligonucleotide primers used in the study of *Gambelia sila* and *G. wislizenii*, with GenBank accession numbers for sequences.

Primer	Sequence (5''-3')	GenBank accession nos.
CO3-F(L8618)*	CATGATAACACATAATGACCC	AF095613-AF095616 AF095619-AF095621
CO3-R(H9323)*	ACTACGTCTACGAAATGTCAG	
CO3-F	CCTTCTAATGACCTCCG	AF095613-AF095616 AF095619-AF095621
CO3-R	AAATGTCAGTATCATGCGG	

* from Orange et al. (1999)

Table 3. DNA sequence divergence (%) among different consensus groups. *Crotaphytus* (n=2), *Gambelia* (n=37), *G. sila* (n=20), *G. wislizenii* (n=17).

Consensus Sequence Comparison	% divergence
Crotaphytus vs *Gambelia*	23.9%
G. wislizenii vs. *G. sila*	4.3%
C. collaris vs. *C. bicinctores*	11.0%

Table 4. List of diagnostic nucleotide sites/columns that distinguish between the 17 mitochondrial cytochrome oxidase III haplotpes (A-Q) of *Gambelia sila* and *G. wislizenii*.

Haplotype	111112223333333333344444445555552334663344407911345677789113344834678980827825347464256873258461805572131139
A	ttatacaccaatggtaagcgtatcaactaattgccaat
B	ttatacaccaatggtaagcgtatcaattaattgccaat
C	ttatacaccaatggtaagcgtatcaattaatagccaat
D	ccgttcaccaatggtaagcacaccgaccaattatcaat
E	ttatacatcaatggtaagcgtatcaactaattgccagt
F	ttatacaccaatggtaagcgtatcaactacttgccaat
G	ttatacatcaatggtaagcgtatcaactaattgccaat
H	ttatacatcaatggtaaacgtatcaactaattgccaat
I	tcgttcaccaatggtaagcacaccgaccaattatcaat
J	tcgcatgccagcaacaagtacgtcgcccgattacagat
K	tcgcatgccggcaataggtacgtcgcccaattacagac
L	tcgcatgccggcaataggtacgtcgcccaattacagat
M	tcgcatgccagcaacgagtacgtcgcccgactacagat
N	tcgcatgccggcaataagtacgtcgcccaattacagat
O	tcgcatgcaagcaacaagtacgtcgcccgattacagat
P	tcgcatgccagcaacaagtacgttgcccgattacagat
Q	tcgcataccagcaacaagtacgtcgcccgattacagat

Table 5. Absolute pairwise distance matrix for 17 *Gambelia* haplotype taxa for the mitochondrial CO3 gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Gw8L California	-																
2 Gs19 CA Quatal Canyon	21	-															
3 Gs12 CA Cuyama River	22	1	-														
4 Gs15 CA Cuyama River	23	2	1	-													
5 GwWfLnM2 California	5	22	23	24	-												
6 GwLfLnF1 California	4	21	22	23	1	-											
7 Gs30 CA Panoche	18	11	12	13	19	18	-										
8 Gs CA LokernF1	23	2	3	4	24	23	13	-									
9 Gw1L California	2	23	24	25	7	6	20	25	-								
10 Gw9L California	3	20	21	22	2	1	17	22	5	-							
11 Gs1 CA Ballinger	22	1	2	3	23	22	12	3	24	21	-						
12 Gw2L California	1	22	23	24	6	5	19	24	3	4	23	-					
13 Gs CA LokernF5	22	1	2	3	23	22	12	1	24	21	2	23	-				
14 Gw7L California	1	22	23	24	6	5	19	24	3	4	23	2	23	-			
15 Gs18 CA Cuyama River	23	2	3	4	24	23	13	2	25	22	3	24	1	24	-		
16 Gw4L California	1	20	21	22	6	5	17	22	3	4	21	2	21	2	22	-	
17 Gs27 CA Panoche	17	10	11	12	18	17	1	12	19	16	11	18	11	18	12	16	-

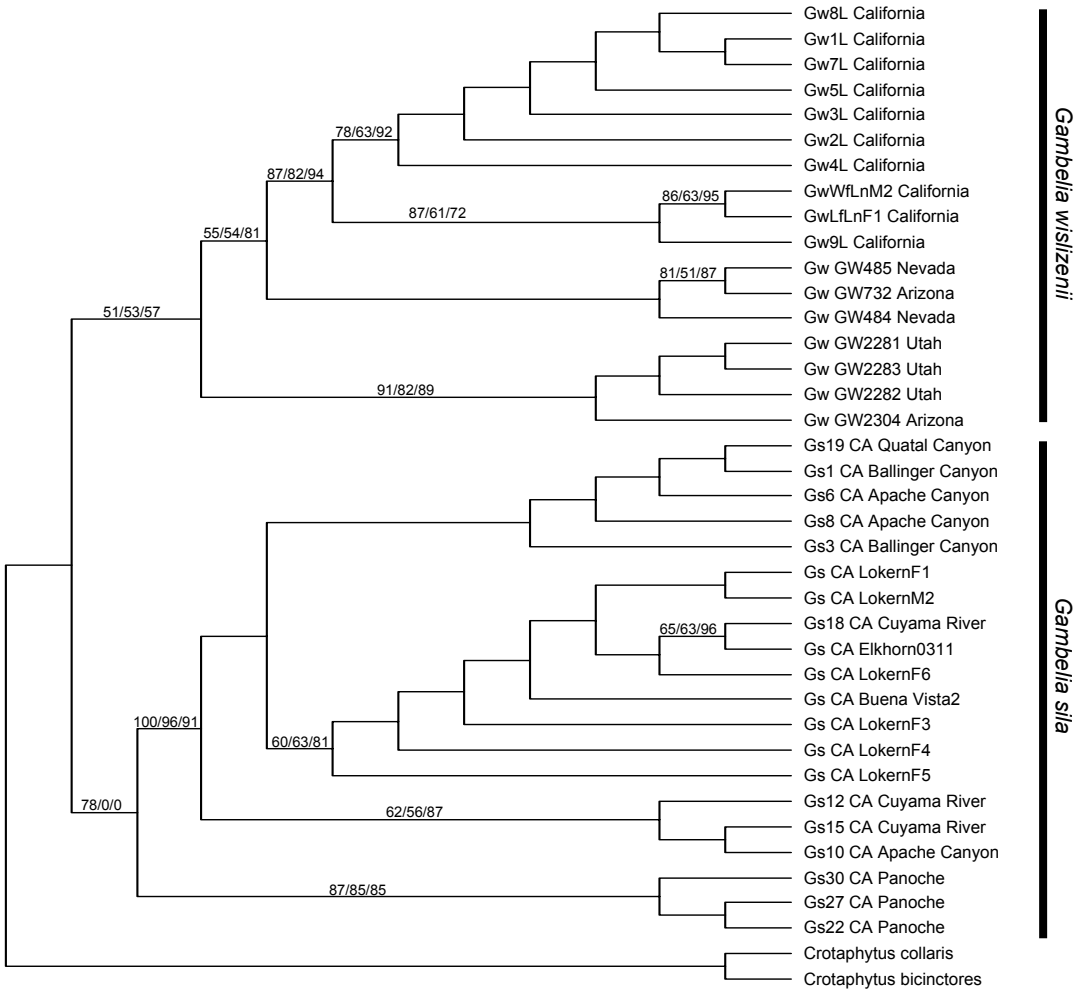

Figure 1. Phylogenetic analysis for 39 taxa in the family Crotaphytidae using the neighbor-joining tree method based on Tajima-Nei distance and combined sequence data from the mitochondrial cytochrome oxidase III gene. Tree length, 141; consistency index, 0.88; retention index, 0.95. Numbers above branches are neighbor-joining bootstrap values (%), parsimony bootstrap values (%), and Bayesian posterior probability values (%).

CHAPTER 3

Genetic Variation among Populations of *Gambelia sila* with Emphasis on a Purported Hybrid Zone

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Abstract

The blunt-nosed leopard lizard (*Gambelia sila*) is a federally and state-listed endangered species, endemic to the San Joaquin Valley, Carrizo and Elkhorn Plains, and Cuyama Valley of central California. Habitat degradation has had a profound impact on the historic distribution and population size of *G. sila*. Decreased population size coupled with decreased gene flow among adjacent populations, could result in the undesirable biological effects often associated with inbreeding. To assess the genetic diversity of *G. sila*, we sequenced 682 base pairs of the mitochondrial cytochrome oxidase *b* (cyt *b*) gene from 34 individuals representing six populations. We also sequenced 603 base pairs of the mitochondrial cytochrome oxidase III (CO3) gene from 32 individuals representing six natural populations. Lastly, we combined and analyzed 1,282 base pairs of the cyt *b* and CO3 gene from 17 individuals representing four natural populations. We found 18 mitochondrial DNA haplotypes among the 34 cyt *b* sequences across all populations. Phylogenetic analysis indicated that the 18 haplotypes are partitioned into three major clades, which correspond geographically as

northern, central, and southern haplotype groups. Of particular focus were individuals collected from the canyons leading into the Cuyama Valley in Ventura and Santa Barbara Counties where possible introgression occurs with hybrids of *G. wislizenii* to the east. All lizards from the Cuyama Valley exhibited the *G. sila* mitochondrial DNA signature and formed the "southern" clade that was joined as a sister group to the "central" clade. A morphological analysis placed some leopard lizards from the hybrid zone with true *G. sila*, whereas some aggregated with *G. wislizenii*, indicative of hybrid status. However, genetic signatures suggest that all lizards in the hybrid zone are true *G. sila*, and not hybrids.

Introduction

The blunt-nosed leopard lizard (*Gambelia sila*) has been listed federally endangered since 1967 and state endangered since 1971 with approximately 85% of its original habitat lost to agriculture, oil development, and urbanization (U.S Fish and Wildlife Service, 1985; Germano and Williams, 1992; Jennings, 1995). The range of *G. sila* was once spread throughout most of the San Joaquin Valley and its surrounding foothills, portions of the Cuyama Valley, and the Carrizo Plain (Montanucci, 1965; McGuire, 1996). Because it is an endangered species, it could be important to determine the genetic variation within and among isolated populations on fragmented land to identify significant conservation sites and enhance population movements between them (U.S Fish and Wildlife Service, 1998).

Small isolated populations of *G. sila* on fragmented land caused by the destruction of habitat in the San Joaquin Valley likely has resulted in decreased gene

flow among adjacent populations. Fragmentation of habitat may significantly reduce or even prevent gene flow through migration or gamete exchange, which could result in the adverse biological effects often associated with inbreeding depression (Klug et al., 2005). In small populations, random genetic drift may cause the attrition of genetic diversity by overwhelming the force of natural selection and resulting in the loss of evolutionary potential (Hartl, 2000; Keyghobadi *et al.*, 2005; Klug *et al.* 2005). Empirical studies on low genetic variation support the hypothesis that inbreeding caused by random genetic drift lowers individual and mean population fitness and the likelihood of population perseverance (O'Brien *et al.* 1987; Ralls *et al.* 1988). Low genetic diversity can have deleterious genetic effects upon phenotypic traits, which leads to decreased fecundity, growth rates, fertility, and offspring viability (Charlesworth and Charlesworth, 1987). Loss of genetic variation by random genetic drift in small populations may influence dynamics and ultimately increase the possibility of inbreeding depression, outbreeding depression, and the risk for population extinction (Charlesworth and Charlesworth, 1987; Lynch, 1991; Newman and Pilson, 1997).

The use of molecular phylogenies for conservation can be important when developing effective conservation and management strategies (Mace, 2004). Molecular characters provide a key source of information allowing the use of molecular systematics to expose the phylogenies of alleles within species (Moritz, 1995). Determining populations with unique genetic variation can lead to understanding how best to preserve and enhance surrounding habitat to ensure recovery of the species (U.S Fish and Wildlife Service, 1998).

Few phylogenetic studies concerning the relationships of *Gambelia sila* have

been conducted since the federal and state listing of this species as endangered in the early 1970s. Corrigan (2002) was the first to examine mitochondrial cytochrome oxidase *b* gene sequences of eight populations of *G. sila* including individuals collected from the Cuyama Valley hybrid zone. Corrigan (2002) found 17 unique haplotypes that partitioned into three major clades that correspond geographically as northern, central, and southern haplotype groups. To provide additional phylogenetic support for the relationships of *G. sila* populations, the present study was designed to build upon the work by Corrigan (2002) by sequencing the same mitochondrial cytochrome oxidase *b* fragment from 23 additional *G. sila* individuals from various localities throughout California. The mitochondrial cytochrome oxidase III (CO3) gene fragment that was analyzed previously by Orange *et al.* (1999) was used as additional phylogenetic support for the findings of Corrigan (2002). By analyzing the combined mitochondrial CO3 and cyt *b* gene sequences of *G. sila* individuals, we can effectively provide more resolution for the relationships of *G. sila* populations.

One area of *G. sila* of highest priority for protection is the natural lands in upper Cuyama Valley (U.S Fish and Wildlife Service, 1998). This area is of particular interest because it is a contact zone between *G. sila* and *G. wislizenii* (Sanders 1950) Studies by Montanucci (1970, 1978) described the electropheretic and morphological characters of hybrid lizards. Because hybrid lizards may not be protected under the federal Endangered Species Act (ESA) nor by the State of California's Fully Protected Species designation, projects that occur in the hybrid zone potentially may not have to take these purported hybrids into account when studying environmental effects. Lizards that can be classified as *G. sila*, which must be protected, also occur in the area and the

actual genetic identity of hybrid individuals entering into the range of *G. sila* is not currently known. Also, the distribution of lizards that could be classified as hybrids may have changed over the past four decades since the mapping of the hybrid zone by Montanucci (1970). DNA barcoding is a relatively new technique that uses a short DNA sequence from a standardized position in the genome as a molecular diagnostic for species-level identification (Hebert *et al.* 2003). A comparison of the DNA barcodes of known *G. wislizenii* and known *G. sila* to the barcodes of lizards inhabiting the hybrid zone should help elucidate the genetic identity of leopard lizards in the Cuyama River and canyons east of the river.

The goal of this study is to assess the genetic diversity and structure of *G. sila* populations throughout their current range in California to assist conservation efforts. We determined the level of genetic variation of *G. sila* populations using phylogenetic and population genetic methods. Besides a range-wide analysis, we also focused on the purported hybrid population of leopard lizards in the Cuyama Valley. We measured both morphological and genetic characters of lizards in the hybrid area and compared this information to population of *G. sila* and *G. wislizenii* outside the hybrid zone.

Methods and Materials

Phylogenetic analysis–We analyzed 34 cytochrome oxidase *b* (cyt *b*) gene sequences representing 31 *Gambelia sila* representing six populations. We used three *G. wislizenii* as the outgroup taxa (Table 1). We also analyzed 32 mitochondrial cytochrome oxidase III (CO3) gene sequences representing 30 *G. sila*, with two *G. wislizenii* as the outgroup taxa (Table 1). We also combined and analyzed both the cyt

b and CO3 gene sequences representing 17 *G.sila*, with two *G. wislizenii* as the outgroup taxa (Table 1). We extracted DNA from toe clippings using the DNAeasy tissue kit (QIAGEN, Valencia, California) according to the manufacture's instructions. Voucher genomic extracts are stored at the Department of Biology, California State University, Bakersfield. We used primer sequences from Pearse and Pogson (2000) to amplify and sequence an ~682 bp portion of the mitochondrial cytochrome oxidase *b* gene (cyt *b*) from all *Gambelia* individuals. We also used primer sequences from Orange *et al.* (1999) to amplify and sequence an ~603 bp portion of the mitochondrial cytochrome oxidase III (CO3) gene from all *Gambelia* individuals. This primer set, however, inconsistently amplified extracts of *G. sila*, so an internal primer set was designed from a single *G. sila* sequence (see Table 2) and used to amplify the cytochrome oxidase III (CO3) gene from all remaining *G. sila* samples.

We carried out polymerase chain reaction (PCR) amplifications in 20-50µl volume and annealing temperatures ranging between 47.8°C and 52.8°C for the cyt *b* gene. We also carried out polymerase chain reaction (PCR) amplifications in 20-50µl volume and annealing temperatures ranging between 48-55° C for the CO3 gene. Successfully amplified PCR products were purified by either using QiaQuick PCR columns or using shrimp phosphatase and exonuclease (ExoSAPit). We submitted purified PCR products to the University of Florida's DNA Sequencing Core Facility for sequencing both forward and reverse strands on an ABI 377 DNA sequencer. DNA sequence electropherograms were read, edited, and aligned using Geneious v5.0 (Drummond *et al.* 2010). DNA sequence alignment was straight-forward and did not necessitate the insertion of any gaps.

We calculated summary statistics for the DNA sequence data using PAUP* 4.0b10 (Swofford 2003). We estimated phylogenetic relationships using maximum parsimony (MP) analysis in PAUP* and Bayesian methods using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Parsimony analysis was carried out using the multiple equally parsimonious heuristic search option with tree bisection reconnection and 100 random addition sequence replicates. We estimated support for specific nodes on the MP trees by bootstrap analysis (Felsenstein 1985; 1000 replications with 10 random addition sequence replicates).

The evolutionary model used for Bayesian analysis was selected by MODELTEST 3.7 (Posada and Crandall 1998, 2001) using the Akaike information criterion (AIC; Posada and Buckley 2004), which corresponded to the GTR + I + G model. We analyzed our dataset under the recommended model using a Markov chain Monte Carlo (MCMC) search strategy in MrBayes. We conducted two independent runs on the data and for each run the MCMC process was set so that four chains (three heated and one cold) ran simultaneously for 2,500,000 generations (sampling every 2500 generations) for the cyt *b* gene and 1,500,000 generations (sampling every 1500 generations) for the CO3 gene. A majority rule consensus tree was calculated from the remaining trees for each data matrix.

Morphometric Analysis of Hybrid Lizards– We recorded eight morphometric measurements from nine *G. sila* and nine *G. wislizenii* collected from areas in their range, and 30 *Gambelia* collected from the purported hybrid zone in the Cuyama Valley. On each individual collected, we measured snout-eye length (tip of the snout to the anterior of the eye), snout-ear length (tip of snout to the anterior of the ear), head width

(widest part of the head), and snout-vent length (tip of snout to posterior of vent) to the nearest 0.1 mm using digital calipers or a ruler. We also analyzed and scored (in parentheses) five color-pattern characters: 1) dorsal spots - few as in *sila* (0), numerous as in *wislizenii* (1); 2) dorsal band width - broad as in *sila* (0), narrow as in *wislizenii* (1); 3) cross band termination - lateral termination as in *sila* (0), dorsal termination as in *wislizenii* (1); 4) dorsal spot orientation - linear to band as in *sila* (0), sporadic as in *wislizenii* (1); 5) dorsal spot ornamentation – none as in *sila* (0), dots or fragmented lines as in *wislizenii* (1). Character state scores were added to the morphometric analysis. Details of diagnostic features along with the scope of variation are discussed by Montanucci (1970).

We compared mean snout-vent lengths (SVL) among individuals of each group using analysis of variance ($\alpha = 0.05$). To asses morphometric differences independent of size, we transformed data by using the residuals from regression analyses of each morphometric trait to SVL to remove as much of the effect of size as possible (Reist, 1985). Pooled within-group slopes for each variable on SVL (Thorpe, 1976; Reist, 1985) were used along with the grand mean of SVL to remove size effects. After morphometric measurements were adjusted for size, we used principal components analysis (Minitab 15 Statistical Software, 2007) using morphometric and character state variables to determine if *G. sila* and *G. wislizenii* differed in morphometric space. If groups were apparent after PCA, we then used multivariate analysis of variance (Minitab 15 Statistical Software, 2007) to determine if there were significant differences in morphometric and character state variables among *G. sila*, *G. wislizenii*, and leopard lizards from the hybrid area. Significance of MANOVAs was determined using the

greatest characteristic root (gcr; Harris, 1985). If the overall MANOVA was significant, lower order discriminant functions were similarly tested. We used ANOVAs to determine which characters varied significantly across samples when treating characters independently, with overall significance tested using post hoc critical values (CV; Harris, 1985). These post hoc critical values are more stringent than F values derived for independent ANOVAs. For those ANOVAs that were significant, we compared means among groups using Student-Newman-Keuls (SNK) test.

Results

Phylogenetic analysis– We analyzed 682 aligned bases of DNA sequence of the cyt *b* gene from 34 individuals. Of the 682 characters, 49 (7%) were variable, and 34 (5%) were parsimony informative. We also analyzed 603 aligned bases of DNA sequence of the CO3 gene from 32 individuals and 31 (5%) were variable, and 26 (4%) were parsimony informative. Of the 1285 aligned bases of DNA sequence of the cyt *b* and CO3 gene combined from 17 individuals, 328 (25%) were variable, and 200 (15%) were parsimony informative. We found 18 unique *Gambelia* haplotypes that partitioned into three major clades that correspond geographically as northern, central, and southern haplotype groups, five for the northern clade (L-P), nine for the central clade (C-K), and four for the southern clade (A-B, F, Q; see Table 3). We found 18 haplotypes that could be distinguished based on diagnostic nucleotide sites (Table 4).

We used MEGA v5.0 to analyze within and among haplotype group sequence divergence (Table 5). In this regard, we conducted pairwise comparisons among the northern, central, and southern haplotype groups. The most differentiated group was the

northern group (Table 5). As expected, mean sequence diversity was higher for among group comparisons than for within group comparisons (Table 5).

Parsimony analysis recovered three equally parsimonious trees (length = 54, CI = 0.91, RI = 0.95) for the cyt *b* gene (Fig. 1), two equally parsimonious trees (length = 31, CI = 1.0, RI = 1.0) for the CO3 gene (Fig. 2), and one parsimonious tree (length = 392, CI = 0.95, RI = 0.91) for the combined cyt *b* and CO3 gene (Fig. 3). The topologies of the trees resulting from the parsimony and Bayesian analysis for the cyt *b* gene were largely congruent and differed only in the placement of the *G. sila* individuals from Panoche area in the northern part of the range of *G. sila*. The parsimony analysis showed Panoche being unresolved with relation to *G. sila* individuals from Pixley and Semitrophic area. The Bayesian analysis provided more resolution for the relationship of the *G. sila* individuals from the Panoche area, which formed a sister group relationship with the *G. sila* individuals from Pixley and Semitrophic area (Fig. 4). The Bayesian analysis showed that the *G. sila* individuals from the Pixley and Semitrophic area are more closely related to the remaining *G. sila* individuals than the Panoche individuals.

The topologies of the trees resulting from the parsimony and Bayesian analysis for the CO3 gene were exactly congruent and placed the *G. sila* individuals from Panoche area as a sister group to all the other *G. sila* individuals (Fig. 5). The parsimony and Bayesian analysis showed individuals from the southern clade being unresolved with relation to *G. sila* individuals that formed the central clade. Lastly, the topologies of the trees resulting from the parsimony and Bayesian analysis for the combined cyt *b* and CO3 gene were mostly congruent and differed only with the

relationship of *G. sila* individuals from the central clade. The Bayesian analysis provided more resolution for the relationship of the *G. sila* individuals from the central clade (Fig. 6).

Morphometric Analysis–PCA yielded seven principal components (PC) with eiganvalues ≥ 1. The first two PCs separated *Gambelia* into two groups, which were identifiable as *G. sila* and *G. wislizenii* (Fig. 7). Because of collinearity among variables, we could only use snout-eye length, dorsal spots, and dorsal band width in MANOVA. The overall MANOVA was significant ($F_{[2,46]} = 54.72$, $P < 0.001$), as was the second discriminant functions (DF; $F_{[2,46]} = 5.34$, $P < 0.001$). The first two DFs separated *G. sila* from *G. wislizenii*. The second DF only accounted for 8.9% of the variance. Leopard lizards from the hybrid zone congregated either with one of the other species (Fig. 8).

Discussion

Estimates of evolutionary divergence over sequence pairs between groups (Table 5) indicated that the northern haplotype group has diverged significantly from the central (7.9 bp) + southern (6.9 bp) groups where the mean divergence was only 1.9 base pairs between the central and southern groups, respectively. This finding suggests that the northern haplotype group has been reproductively isolated for a much longer period of time than the central and southern groups have been isolated. A possible explanation for this result was likely an initial range expansion of *G. sila* to the northern regions of the San Joaquin Valley followed by population retreat that resulted in isolated pockets of *G. sila* populations in favorable habitats. The phylogenetic

analysis provides support for the partitioning of the 18 *G. sila* haplotypes into three distinct clades that are geographically northern, central, and southern haplotype groups. The phylogenetic analysis also showed that all the lizards from the Cuyama Valley exhibited the *G. sila* DNA signature and formed the southern clade.

Montanucci (1970) described and designated hybrid leopard lizards in the Cuyama Valley based mainly on intermediate morphological traits and electrophoretic characters. Montanucci (1970) found these morphological "hybrids" in Ballinger, Quatal, and Burges canyons and in the Cuyama River next to these canyons. The hybrid zone extended too much of the area along Highway 33. He also found what he called G. wislizenii in Apache Canyon, Dry Canyon, and throughout the Lockwood Valley area to the east of the hybrid area. He determined that *G. sila* were north of Ballinger Canyon. Montanucci (1970) also hypothesized that interbreeding has been occurring between *G. sila* and a remnant hybrid population inhabiting the area. If this hypothesis is true, then we should have been able to detect unique DNA signatures for *G. wislizenii*, *G. sila* and the remnant hybrid population. On the other hand, enough time (~40 years) has elapsed since the hybrid zone was first delineated that *G. sila* and the remnant hybrid population may have become genetically homogeneous due to continuous interbreeding.

We found individuals during our surveys that morphologically appeared to be hybrids, as well as leopard lizards that we would classify as *G. sila* or *G. wislizenii* based on morphology. Yet, the modern genetic techniques we employed did not support these morphological designations. Indeed, all lizards from the purported hybrid zone exhibited the *G. sila* mitochondrial DNA signature. If *G. wislizenii* were indeed

present in the canyons and Cuyama Valley, this may have suggested one-way compatibility between male *G. wislizenii* and female *G. sila*; however, because DNA barcoding failed to recover a single lizard from the canyons that exhibited the *known G. wislizenii* mitochondrial DNA signature (even from those lizards that were morphologically classified as *G. wislizenii*), we can exclude this as being a possibility. Therefore, we conclude that true *G. wislizenii* and true *G. sila* are allopatric and are not actively producing hybrids in the canyons and Cuyama Valley. Furthermore, our results indicate that all leopard lizards in the hybrid zone should be classified as *G. sila* and not as hybrids. Thus our findings suggest that the significant difference in morphometric characteristics of the three *G.sila* populations disagree with the phylogenetics analysis. The morphological similarity of some *G. sila* that inhabit the canyons and Cuyama Valley to *G. wislizenii* is likely due to as yet undetermined environmental factors.

One significant result of this analysis is that although the Cuyama Valley leopard lizards grouped either with blunt-nosed leopard lizards or long-nosed leopard lizards, all Cuyama Valley leopard lizards exhibited the mitochondrial DNA signature of G.sila and formed the unique southern clade.

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Table 1. List of samples analyzed along with their locality and GenBank accession numbers.

Species	Haplotype	Locality	GenBank accession #
G. wislizenii	-	Mojave, CA	--
G. wislizenii	-	Mojave, CA	--
G. wislizenii	-	Mojave, CA	--
G. sila	A	3 Cuyama River, CA	--
G. sila	B	Cuyama River, CA	--
G. sila	C	1 Buena Vista, CA	--
G. sila	D	2 Buena Vista, CA	--
G. sila	E	3 Buena Vista, CA	--
G. sila	F	4 Buena Vista, CA	--
G. sila	G	Elkhorn Plains, CA	--
G. sila	H	1 Lokern, CA	--
G. sila	I	2 Lokern, CA	--
G. sila	J	3 Lokern, CA	--
G. sila	K	4 Lokern, CA	--
G. sila	L	1 Panoche, CA	--
G. sila	M	1 Pixley, CA	--
G. sila	N	2 Pixley, CA	--
G. sila	O	1 Semitropic, CA	--
G. sila	P	2 Semitropic, CA	--
G. sila	Q	1 Cuyama River, CA	--
G. sila	R	2b Buena Vista, CA	--
G. sila	L	2 Panoche, CA	--
G. sila	B	10 Apache Canyon, CA	--
G. sila	B	11 Apache Canyon, CA	--
G. sila	B	13 Apache Canyon, CA	--
G. sila	B	14 Apache Canyon, CA	--
G. sila	A	Cuyama River, CA	--
G. sila	F	2 Cuyama River, CA	--
G. sila	A	4 Apache Canyon, CA	--
G. sila	B	5 Apache Canyon, CA	--
G. sila	A	6 Apache Canyon, CA	--
G. sila	A	8 Apache Canyon, CA	--
G. sila	B	9 Apache Canyon, CA	--
G. sila	F	1a Buena Vista, CA	--

Table 2. Oligonucleotide primers used in this study with GenBank accession numbers for sequences.

Gene and primer code*	Sequence (5'-3')	Reference	GenBank accession nos.
CO3-F(L8618)	CATGATAACACATAATGACCC	Orange et al. (1999)	AF095613-AF095616 AF095619-AF095621
CO3-R(H9323)	ACTACGTCTACGAAATGTCAG	Orange et al. (1999)	
CO3-F	CCTTCTAATGACCTCCG		AF095613-AF095616 AF095619-AF095621
CO3-R	AAATGTCAGTATCATGCGG		

Table 3. List of diagnostic nucleotide sites that distinguish between the (18) mitochondrial cytochrome oxidase *b* haplotpes (A-R).

Haplotype	111111222223333333333344444445555666666666 233679167788355680022234468901256671678001223456 7 309415773956714342803692489297853862391567698201 7
A	CTTCTCTTTCCACTACTAAGCCTCGCAGATTTTAACCTGCGCTGTCTTC
B	CTTCTCTTTCCACTACTAAGCCTCGCAGGTTTTAACCTGCGCTGTCTTC
C	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGCGCTGTCTTC
D	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGCGCTGTCTCT
E	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGCGCTGTATTT
F	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGCGCTGTCTTT
G	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGTGCTGTCTTT
H	CTTCTCTTTCCACTACTAAGCCCCGTAGATTTTAACCTGCGCTGTCTTC
I	CTTCTCTTTCCACTACTAAGCCTCGTAGATTTTGACCTGCGCTGTCTTT
J	CTTGTCTTTCCACTACTAAGCCTCGTAGATTTTAACCTGCGCTGTCTTT
K	CTTCTCTTTCCACTACTAAGCCTTGTAGATTTTAACCTGCGCTGTCTTT
L	CCTCCCTTTCCATTATTGAATCTCGCAAATCTTGATCTACACTGTCCTC
M	CTTCCCTTTCCATTATTGAATCTCGCAAATCTTGATCTACACTGTCCTC
N	CTCCCTTCTTCACTACTGAGCCTCGCAGATTTTGACCTGCGCTGCCTTC
O	CTCCCTTCTTCACTACTGAGCTTCGCAGATTTTGACCTGCGCTGCCTTC
P	TTCCCTTCTTCACTACTGAGCCTCGCAGATTTTGACCTGCACTGCCCTC
Q	TTCCCTCCTTCACTACTGAGCCTCGCAGATTTTGACCTGCACTGCCCTC
R	CTTCTCTTTCTACTACTAAGCCTCGCAGATTTTAACCTGCGCTGTCTTC

Table 4. Absolute pairwise distance matrix for 34 *Gambelia* taxa for the mitochondrial cytochrome oxidase *b* gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Ballinger Canyon	-																
2 Quatal Canyon	1	-															
3 Buena Vista 1	1	2	-														
4 Buena Vista 2	3	4	2	-													
5 Buena Vista 3	3	4	2	2	-												
6 Buena Vista 4	2	3	1	1	1	-											
7 Elkhorn Plains	3	4	2	2	2	1	-										
8 Lokern 1	2	3	1	3	3	2	3	-									
9 Lokern 2	3	4	2	2	2	1	2	3	-								
10 Lokern 3	3	4	2	2	2	1	2	3	2	-							
11 Lokern 4	3	4	2	2	2	1	2	3	2	2	-						
12 Panoche 1	14	15	15	17	17	16	17	16	15	17	17	-					
13 Panoche 2	13	14	14	16	16	15	16	15	14	16	16	1	-				
14 Pixley 1	8	9	9	11	11	10	11	10	9	11	11	16	15	-			
15 Pixley 2	9	10	10	12	12	11	12	11	10	12	12	17	16	1	-		
16 Semitropic 1	11	12	12	14	14	13	14	13	12	14	14	15	14	3	4	-	
17 Semitropic 2	12	13	13	15	15	14	15	14	13	15	15	16	15	4	5	1	-
18 Apache Canyon 10	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
19 Apache Canyon 11	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
20 Apache Canyon 13	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
21 Apache Canyon 14	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
22 Ballinger Canyon	0	1	1	3	3	2	3	2	3	3	3	14	13	8	9	11	12
23 Ballinger Canyon	2	3	1	1	1	0	1	2	1	1	1	16	15	10	11	13	14
24 Ballinger Canyon	1	2	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
25 Apache Canyon 4	1	2	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
26 Apache Canyon 5	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
27 Apache Canyon 6	0	1	1	3	3	2	3	2	3	3	3	14	13	8	9	11	12
28 Apache Canyon 8	0	1	1	3	3	2	3	2	3	3	3	14	13	8	9	11	12
29 Apache Canyon 9	1	0	2	4	4	3	4	3	4	4	4	15	14	9	10	12	13
30 Buena Vista 1	2	3	1	1	1	0	1	2	1	1	1	16	15	10	11	13	14
31 Buena Vista 2	4	5	3	3	3	2	1	4	3	3	3	18	17	12	13	15	16
32 Long nosed 1	15	16	16	18	18	17	18	17	16	18	18	18	18	18	19	18	19
33 Long nosed 2	15	16	16	18	18	17	18	17	16	18	18	20	20	18	19	18	19
34 Long nosed 3	14	15	15	17	17	16	17	16	15	17	17	19	19	17	18	17	18

	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
18 Apache Canyon 10	-																
19 Apache Canyon 11	0	-															
20 Apache Canyon 13	0	0	-														
21 Apache Canyon 14	0	0	0	-													
22 Ballinger Canyon	1	1	1	1	-												
23 Ballinger Canyon	3	3	3	3	2	-											
24 Ballinger Canyon	2	2	2	2	1	3	-										
25 Apache Canyon 4	2	2	2	2	1	3	0	-									
26 Apache Canyon 5	0	0	0	0	1	3	2	2	-								
27 Apache Canyon 6	1	1	1	1	0	2	1	1	1	-							
28 Apache Canyon 8	1	1	1	1	0	2	1	1	1	0	-						
29 Apache Canyon 9	0	0	0	0	1	3	2	2	0	1	1	-					
30 Buena Vista 1	3	3	3	3	2	0	3	3	3	2	2	3	-				
31 Buena Vista 2	5	5	5	5	4	2	5	5	5	4	4	5	2	-			
32 Long nosed 1	16	16	16	16	15	17	16	16	16	15	15	16	17	19	-		
33 Long nosed 2	16	16	16	16	15	17	16	16	16	15	15	16	17	19	12	-	
34 Long nosed 3	15	15	15	15	14	16	15	15	15	14	14	15	16	18	1	11	-

Table 5. Evolutionary distance and diversity estimates for *G. sila* (n=29) cyt *b* sequences. Standard error estimates were obtained by using a bootstrap procedure (500 replicates). All calculations were conducted in MEGA v5.0.

Parameter/Comparison	Divergence (# of bp)	SE
Distance		
Mean Distance (Total)	6.3	1.2
Between Group Evolutionary Distance		
Northern vs Central	7.9	-
Northern vs Southern	6.7	-
Central vs Southern	1.9	-
Within Group Evolutionary Distance		
Northern	7.5	2.0
Central	1.9	0.6
Southern	0.9	0.6
Diversity		
Mean Diversity in Entire Population	6.3	1.2
Mean Diversity Within Subpopulations	4.1	0.7

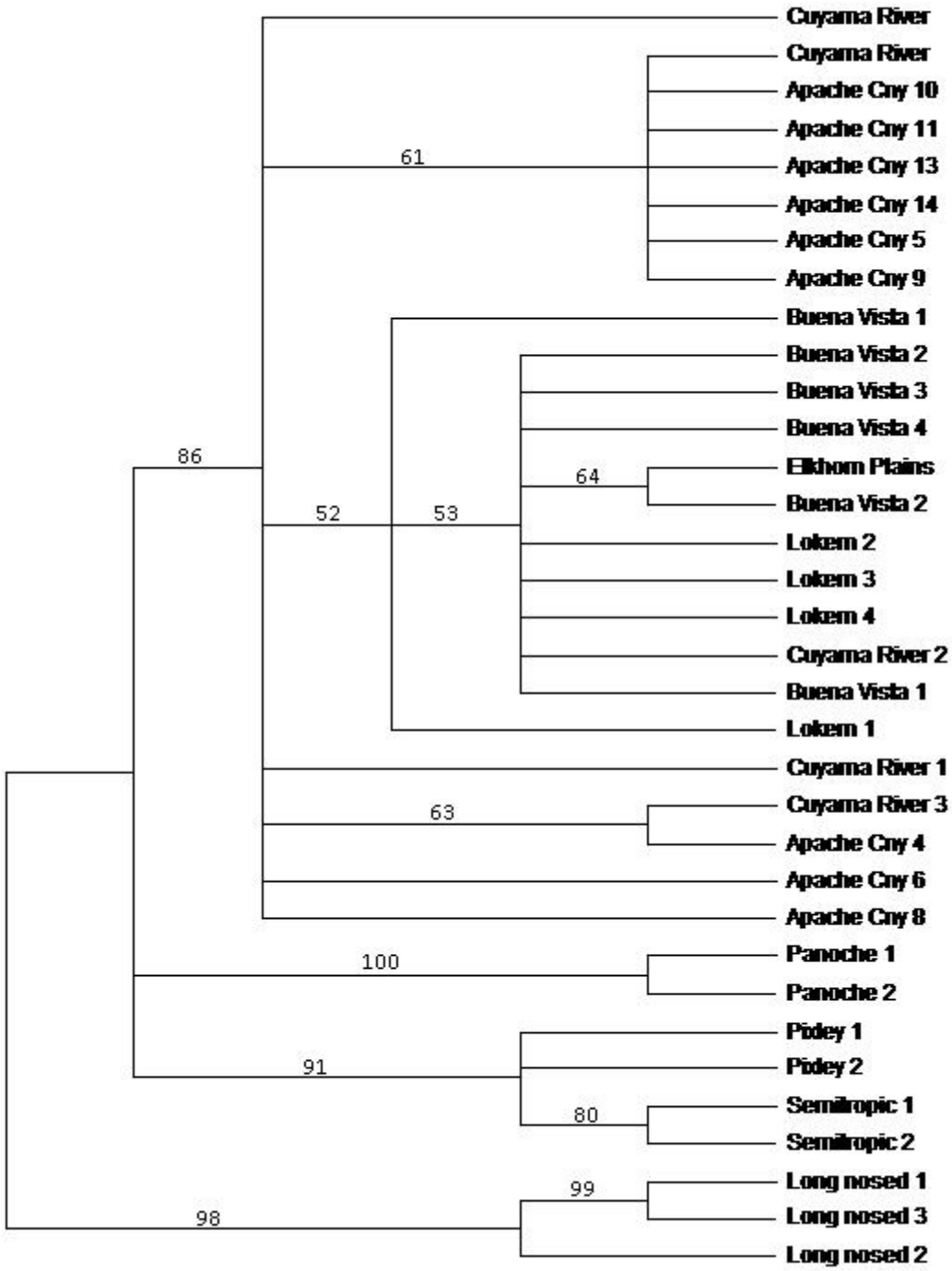

Figure 1. Strict consensus of three equally parsimonious trees based on unweighted parsimony analysis of a 682-bp portion of the mitochondrial cytochrome oxidase *b* gene for 34 *Gambelia* taxa. Tree length: 54; consistency index, 0.91; retention index, 0.95. Numbers above branches are bootstrap values (%).

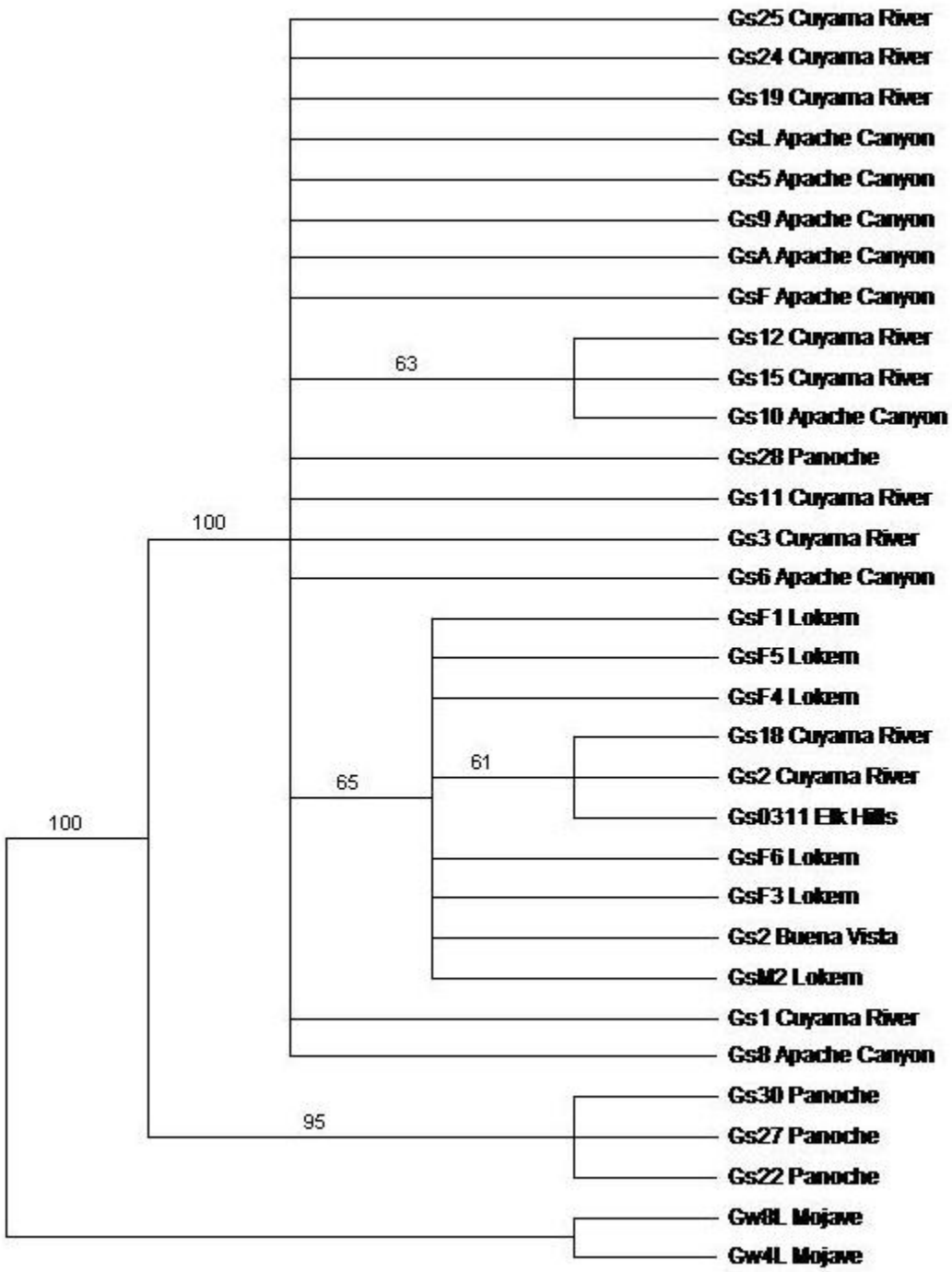

Figure 2. Strict consensus of two equally parsimonious trees based on unweighted parsimony analysis of a 603-bp portion of the mitochondrial cytochrome oxidase III gene for 32 *Gambelia* taxa. Tree length: 31; consistency index, 1.0; retention index, 1.0. Numbers above branches are bootstrap values (%).

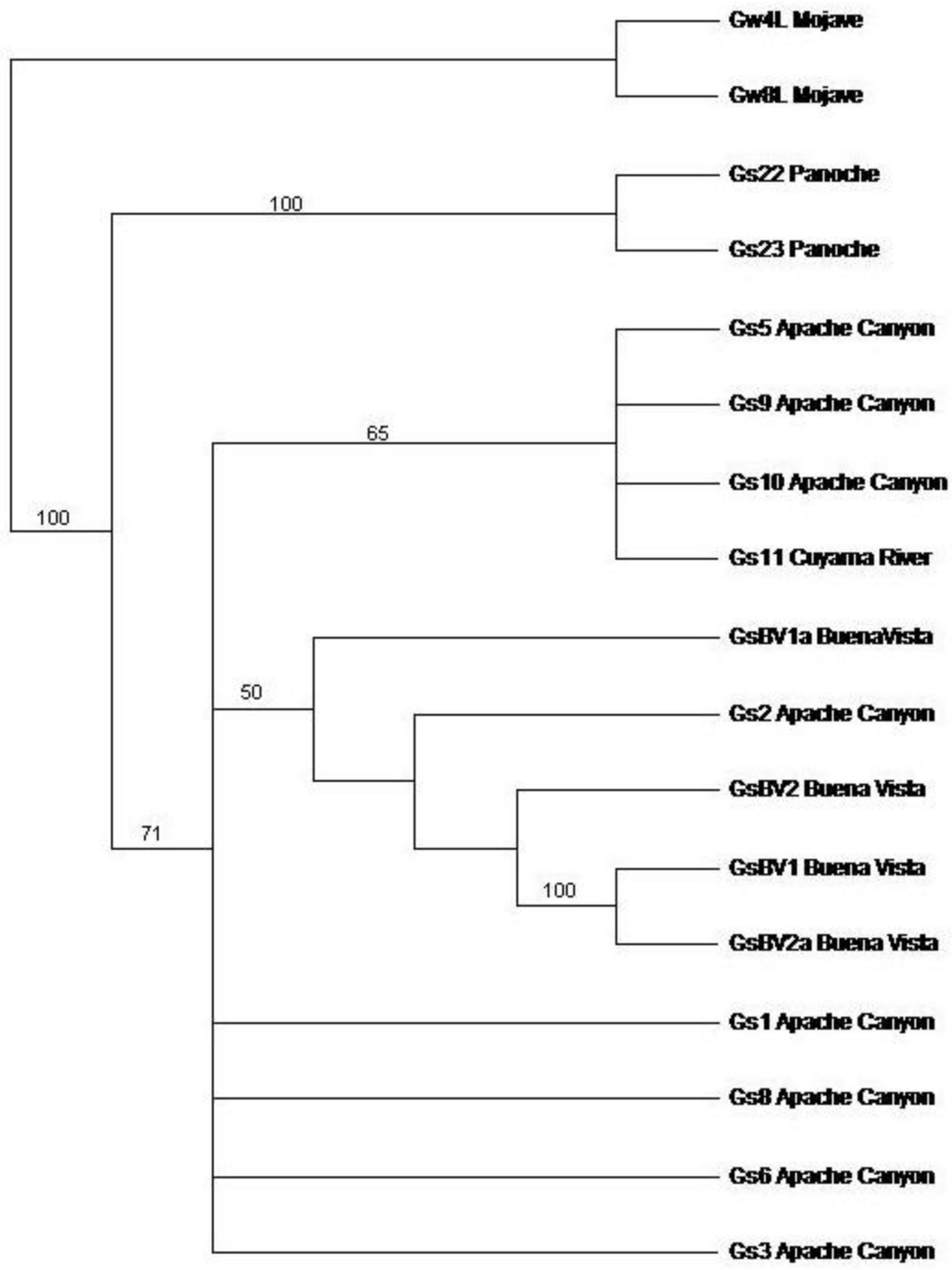

Figure 3. Single most parsimonious tree based on unweighted parsimony analysis of a 1285-bp portion of the mitochondrial cytochrome oxidase *b* gene and mitochondrial cytochrome oxidase III gene for 17 *Gambelia*. Tree length: 392; consistency index, 0.95; retention index, 0.91. Numbers above branches are bootstrap values (%).

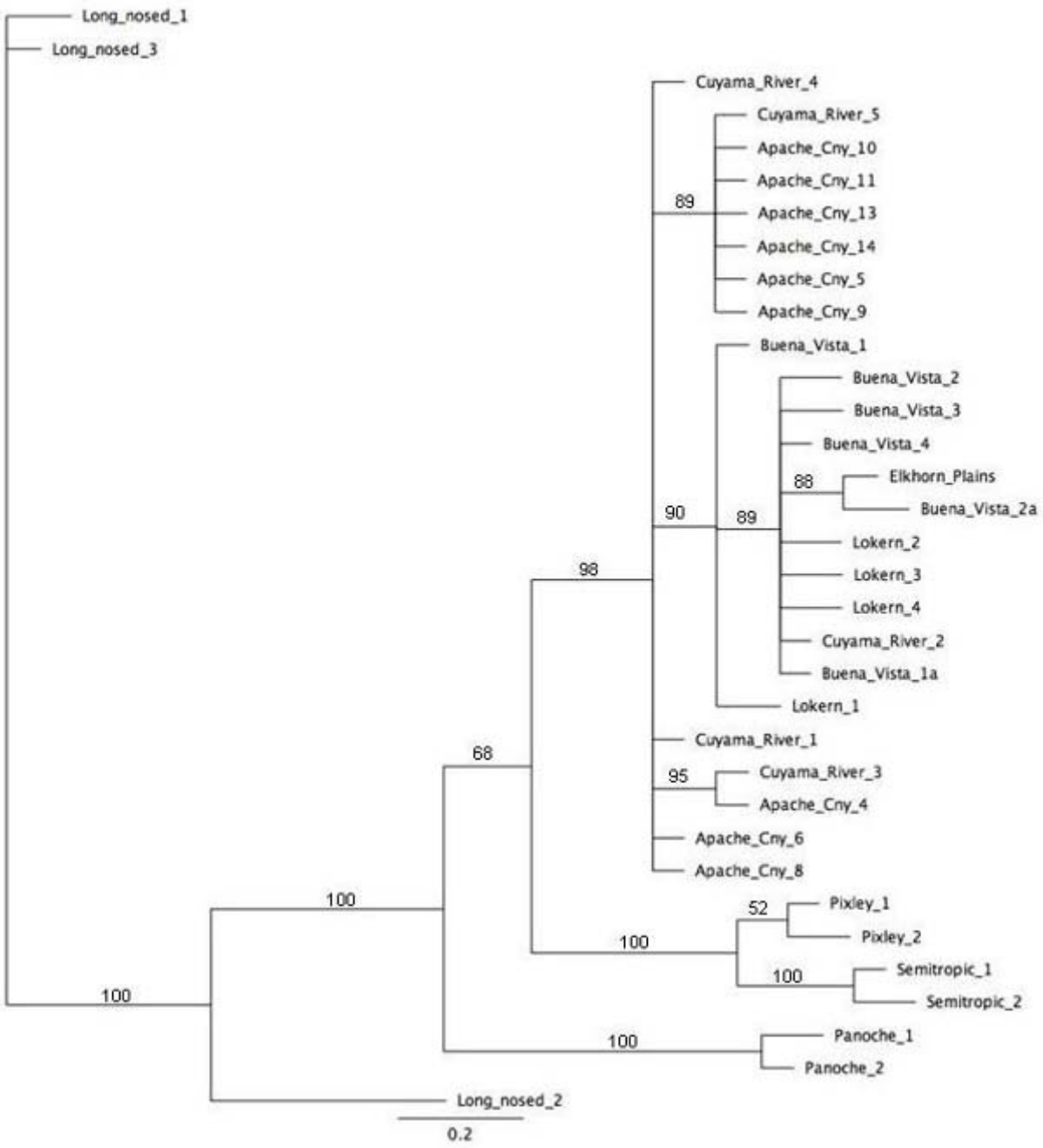

Figure 4. Bayesian phylogenetic tree constructed using the Cyt *b* gene of 34 taxa in the family Crotaphytidae. Average standard deviation split frequencies = 0.010. Posterior probabilities for Bayesian analysis located at nodes.

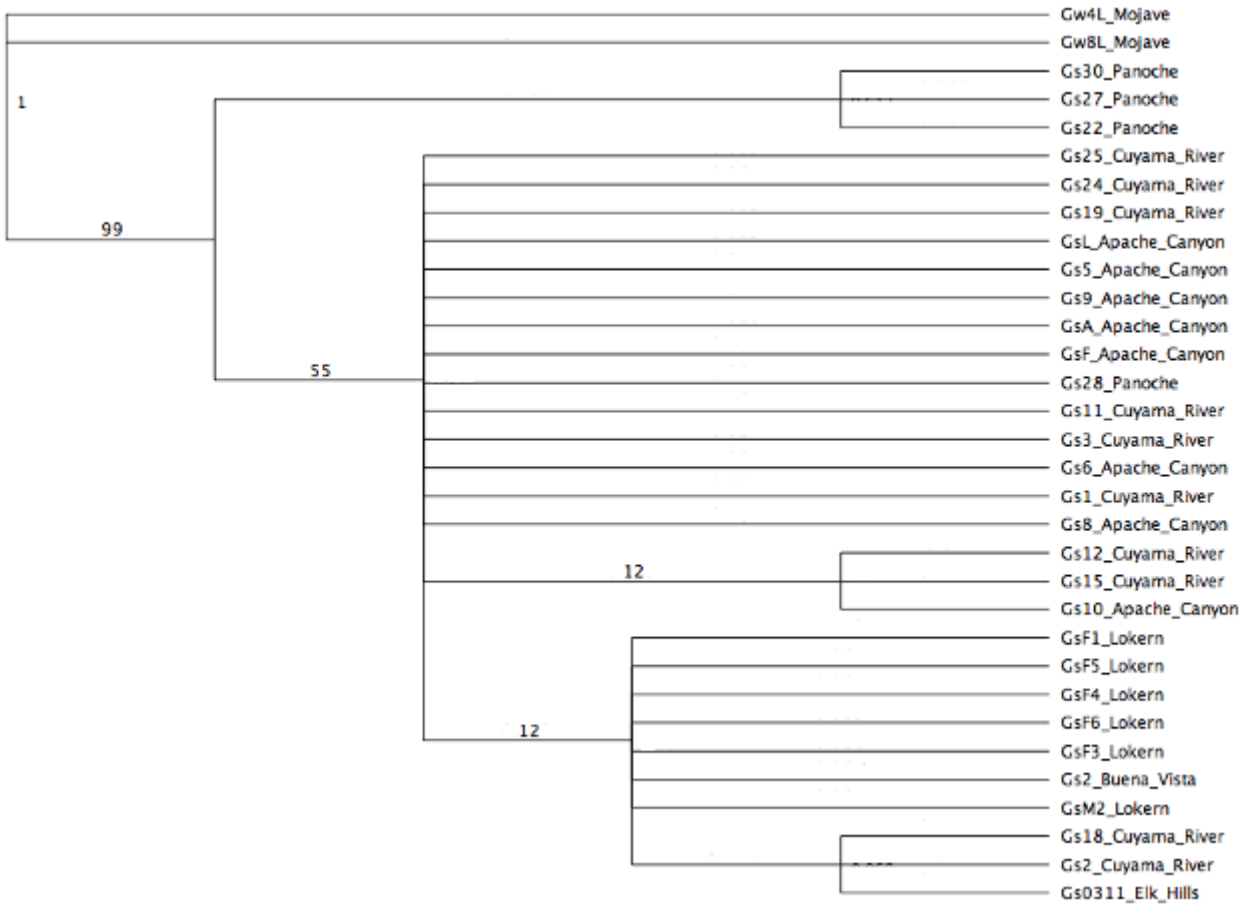

Figure 5. Bayesian phylogenetic tree constructed using the CO3 gene of 32 taxa in the family Crotaphytidae. Average standard deviation split frequencies = 0.010. Posterior probabilities for Bayesian analysis located at nodes.

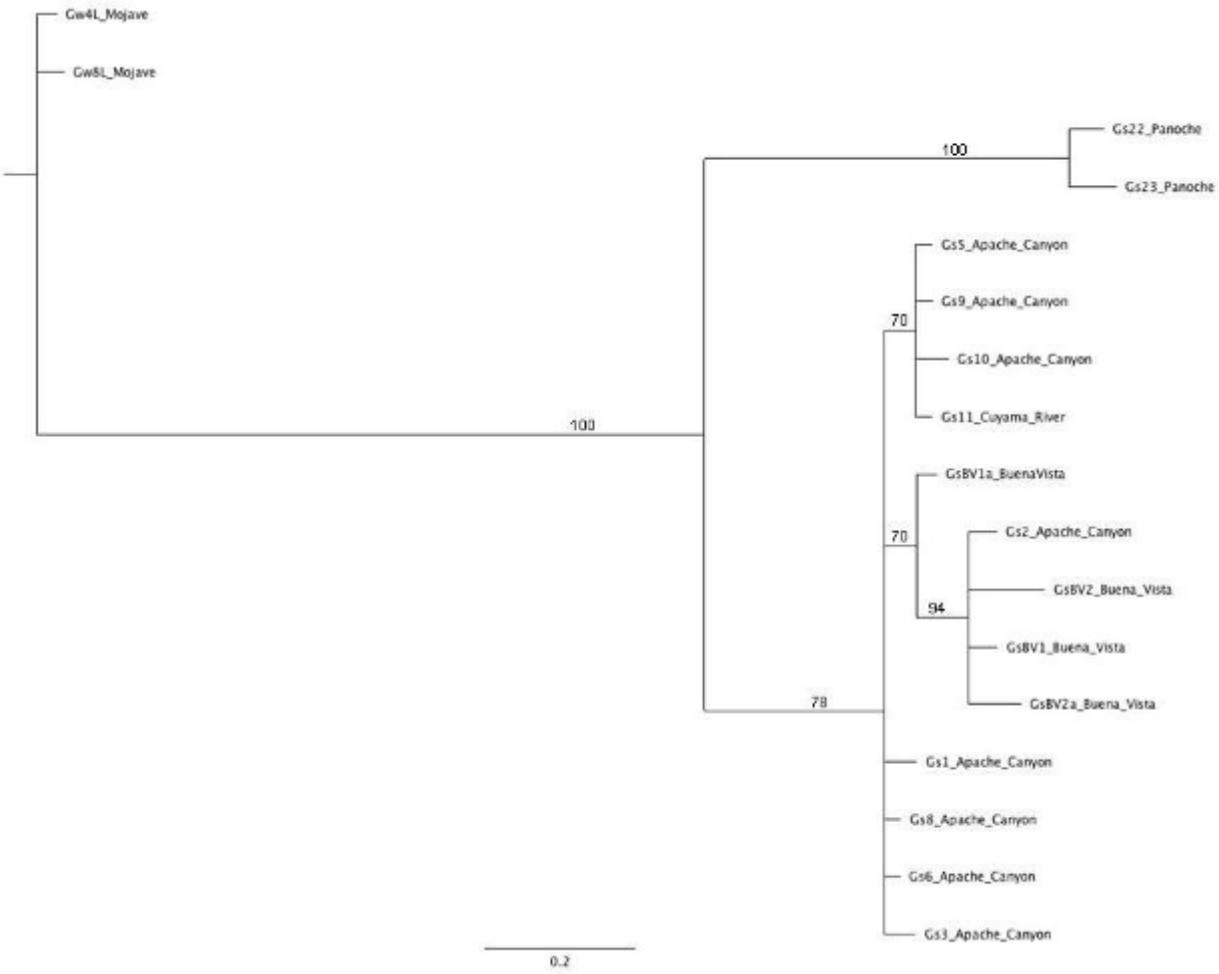

Figure 6. Bayesian phylogenetic tree constructed using the combined Cyt *b* and CO3 gene of 17 taxa in the family Crotaphytidae. Average standard deviation split frequencies = 0.010. Posterior probabilities for Bayesian analysis located at nodes.

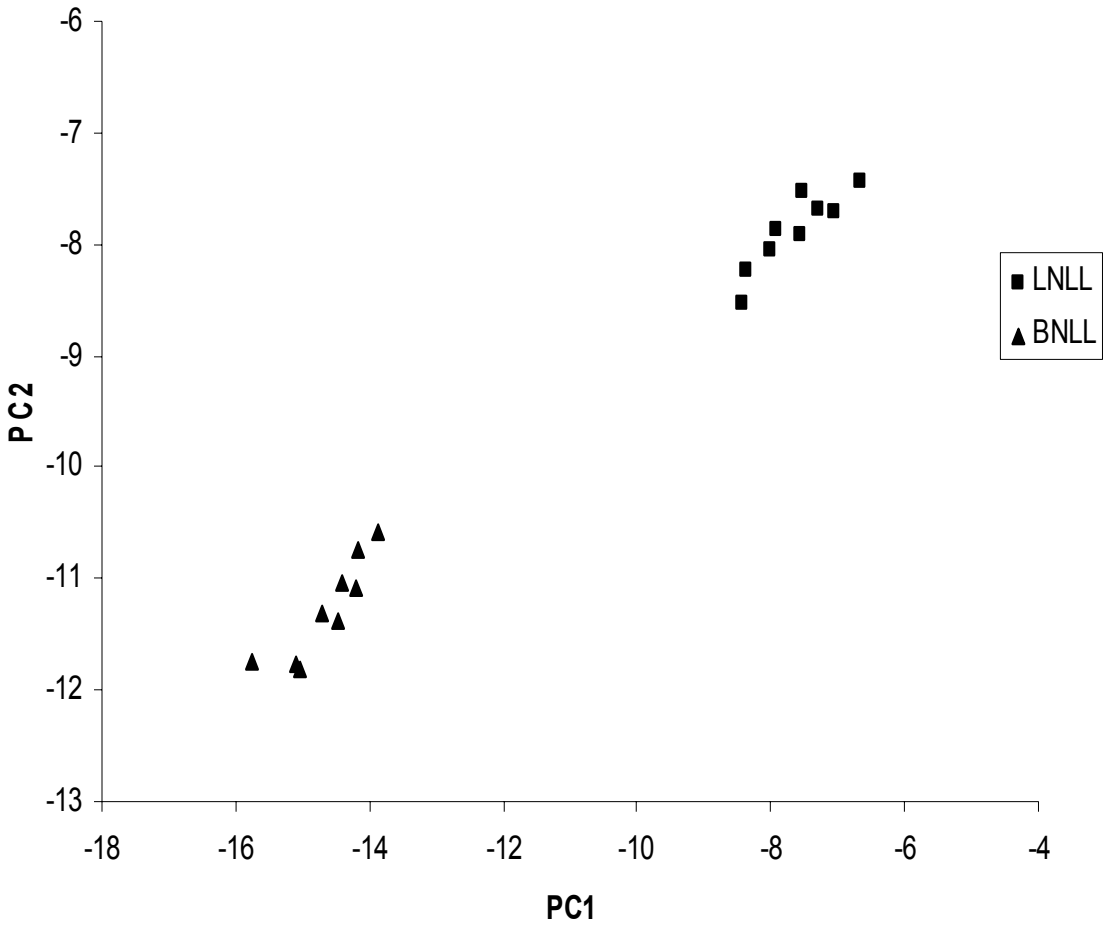

Figure 7. Morphometric space of two *Gambelia* species based on the first two Principle Components (PC) of size adjusted morphometric measurements and categorical characters. LNLL = long-nosed leopard lizard (*G.wislizenii*; n=9); BNLL = blunt-nosed leopard lizard (*G.sila*; n=9).

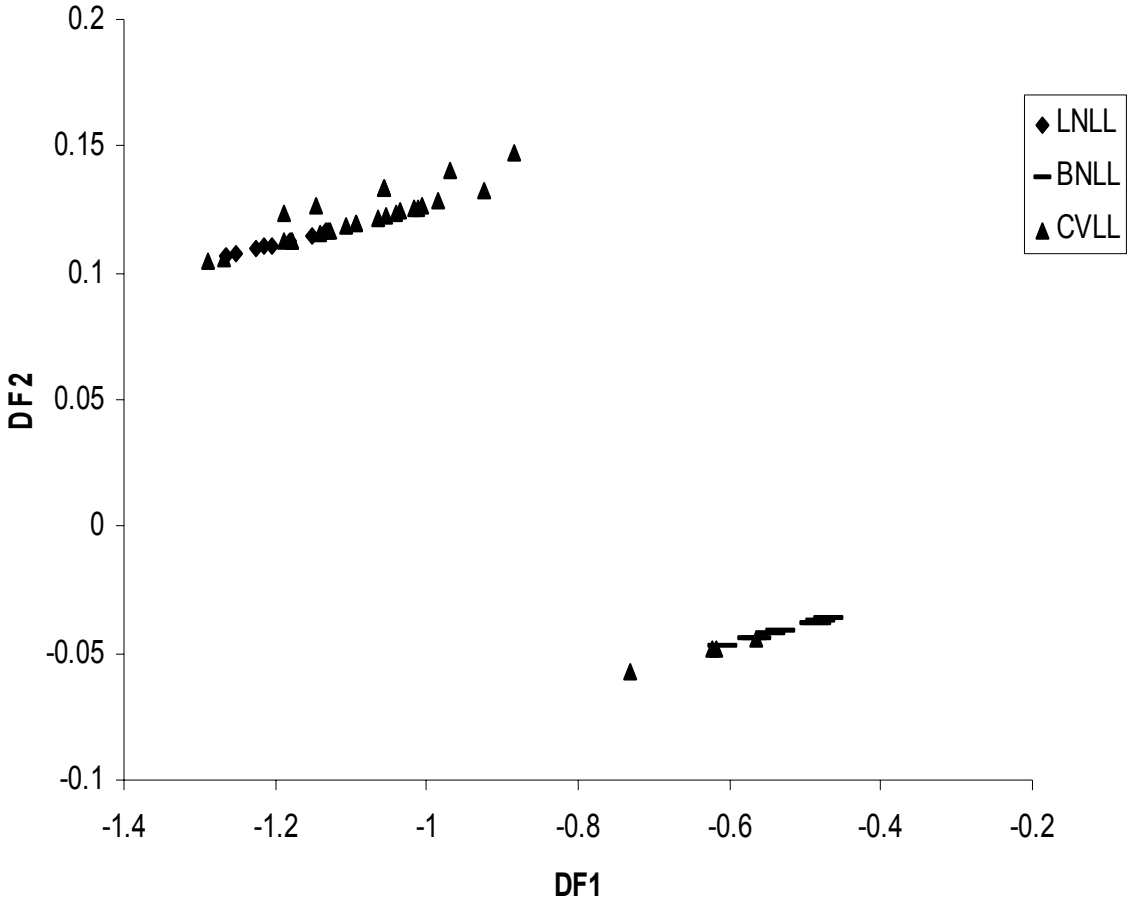

Figure 8. Morphometric space of three populations of *Gambelia* based on the first two Discriminate Functions (DF) from MANOVA of size-adjusted morphometric measurements and categorical characters. LNLL = long-nosed leopard lizard (*G.wislizenii*; n=9); BNLL = blunt-nosed leopard lizard (*G.sila*; n=9); CVLL = Cuyama Valley leopard lizard (individuals from the purported hybrid zone; n=31). DF1 accounts for 91.1% of the variance and DF2 accounts for 8.9%.

CHAPTER 4

Conclusions From Presented Studies

The systematic status of leopard lizards from the San Joaquin Valley in California has been debated ever since their first description. Having a resolved molecular phylogenetic estimate for the leopard lizards is essential due to the protection afforded *G.sila*. The molecular evidence in this study coupled with geographic distribution and morphology, supports the recognition of *G. sila* as separate, distinct sister-species to *G. wislizenii*. Within *G. sila*. phylogenetic analysis of mitochondrial gene sequences recovered 18 haplotypes that were distributed into three major clades that corresponds geographically as northern, central, and southern haplotype groups. The northern clade included individuals from Panoche, Semitropic, and Pixley populations and individuals from the Panoche population exhibited 13-18 (1.9-2.6%) base pair differences compared to all other *G. sila* samples. The individuals from the Semitropic and Pixley populations exhibited 6-16 (0.9-2.3%) base pair differences compared to all other *G. sila* samples. The analysis suggest that the northern clade and especially the Panoche population has been reproductively and geographically isolated from the other *G. sila* populations; however, the Semitropic and Pixley population exhibited 4-5 (0.5-0.7%) base pair differences suggesting that management efforts to improve movement and gene flow between the Semitropic and Pixley populations including translocations would be practical. The strong genetic structure of *G. sila* suggests that the impacts of oil, agriculture, and urban development over the past 131 years may have only augmented the genetic partitioning that we observe today.

The Central haplotype group included individuals from the Lokern, Buena Vista, Elk horn Plains and were nested within the *G. sila* clade. The *G. sila* from the Cuyama Valley and canyons formed the unique Southern clade and sister group relationship with the Central clade. The Central clade populations exhibited a greater number of haplotypes (10 haplotypes C-K, R) compared to the Northern (5 haplotypes L-P) and Southern (4 haplotypes A,B,F,Q) clade. The restoration of key habitat areas and the preservation of existing wildlife corridors among the Central calde populations would ensure connectivity and potentially enhance gene flow.

The individuals from the Cuyama Valley that we designated as the Southern clade all possessed the *G. sila* mitochondrial DNA signature despite the fact that we observed individuals that morphologically appeared to be *G. wislizenii* including hybrids as defined by Montinucci 1970. The greatest extent of suitable habitat remaining for members of the Southern Clade is along the Cuyama River, the canyons proper, and the habitat on terraces within the Cuyama River. Because of current agricultural and mining activity, little habitat for leopard lizards occurs along Highway 33, the base of hillsides to the east of Highway 33, or flat lands adjacent to the Cuyama River.

The discriminate functions (DF) analysis concluded that individuals sampled from the purported hybrid zone grouped with both *G. sila* and *G. wislizenii* which disagree with the molecular analysis; however, these results are similar to the findings of Montinucci 1970 where he observed intermediates that had morphological characteristics more similar to both *G. sila* and *G. wislizenii*.

The majority of the samples from the Cuyama Valley exhibited morphological characteristics that were similar to *G. wislizenii*; however, the sample size was relatively

small. The traditional morphological criterion used to distinguish *G. sila* and *G. wislizenii* can not be used to differentiate leopard lizard individuals in the Cuyama Valley and the canyons proper. The morphological similarity of some *G. sila* that inhabit the canyons and Cuyama Valley to *G. wislizenii* maybe the result of the narrow ecotonal area along the Cuyama River drainage system where numerous tributaries assemble. This area is unique because the Tehachapi Mountains enter from the northeast, the Transverse Ranges enter from the southeast and the Central and Southern Coast Ranges also merge into the area where Mount Pinos forms a node where all these ranges meet (Montanucci 1970).

The vegetation in the Cuyama Valley is composed of a transitional type of vegetation that forms unique plant assemblages composed of coastal species, chaparral species, montane species, valley-grassland species, and Mojave Desert species (Twisselman 1967; Montanucci 1970). Pinyon-junper woodlands, Chaparral, and California steppe are present and found throughout the Cuyama Valley badlands or in the vicinity of this area (Twisselman 1967; Montanucci 1970).

Wildlife in the Cuyama Valley is typical of species found in a variety of regions throughout California. Similar to the transitional state of vegetation types in the area, wildlife is composed of montane, desert, valley, and coast range species. The Cuyama Valley is also thought to be a wildlife corridor that connects the coastal areas to the San Joaquin Valley, as well as species moving from the Carrizo Plain area to the montane areas partially encompassed by the Los Padres National Park (Anderson *et al.* 2009).

Additional searches for leopard lizards should occur in Ballinger, Quatal, Burges, and Dry canyons, and in BLM land north of Highway 33 to determine if leopard lizards still occur there. There are several areas containing suitable habitat throughout the Lockwood Valley that need further investigation. A larger sample size and sample locations of *G. sila* individuals throughout California and an extensive study of the genetically isolated Panoche population would be extremely beneficial to help guide environmental assessments and better preserve the species.

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