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Genetic Approaches to Population Ecology and Conservation of the Sacramento Mountain Salamander

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GENETIC APPROACHES TO POPULATION ECOLOGY AND CONSERVATION OF THE SACRAMENTO MOUNTAIN SALAMANDER

by

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ABSTRACT

Aneides hardii (Sacramento Mountain salamander) is restricted to high-elevation habitat that is fragmented by low-elevation piñon-juniper woodland. *Aneides hardii* is a species of conservation concern in New Mexico, in part, because disease and climate change endanger its long-term persistence. In this study, we use sequence data from the mitochondrial cytochrome b gene and a microsatellite locus of *A. hardii* to addresses the timing and scale of population subdivision. We also calculated genetic diversity measures and compared them among mountain ranges to explore demographic history of *A. hardii*. Twenty-six haplotypes and three genetically distinct lineages were identified, and each haplotype was restricted to a single mountain range (Capitan, White, or Sacramento Mountains). Mitochondrial data indicated divergence between disjunct *A. hardii* populations consistent with early Pleistocene climatic changes, characterized by glacial and interglacial cycles, that isolated salamanders to 'sky island' habitats and distinct lineages. Cooler, wetter periods of the Pleistocene would have been a time of possible

range expansion for *A. hardii* because of increased available habitat in lower elevations. Fine scale structure was detected in *A. hardii* lineages and could be indicative of a small home range. Mitochondrial and microsatellite data showed differences in the magnitude of fine scale patterns that could be an indication of sex based dispersal. Our results genetic diversity statistics varied among mountain-tops, and therefore, we propose Environmentally Significant Units (ESUs) to manage this species. ESUs prioritize species management of historic lineages because they are evolutionarily significant, e.g. White and east Capitan Mountains populations. Furthermore, we developed environmental DNA (*eDNA*) methodology to indicate the presence/absence of *A. hardii*. Our true positive detection rate was 25%, and we found an eDNA persistence time of at least one week. Our eDNA methodology could be utilized in other terrestrial Plethodontidae, especially endangered species (e.g. *Plethodon neomexicanus*). Our genetic approaches aim to assist in the conservation management of the *A. hardii* based on genetic methodology and quantitative data analysis.

TABLE OF CONTENTS

Chapter 1

Introduction to *Aneides hardii*

Background

Members of family Plethodontidae are lungless salamanders that rely on moisture for cutaneous respiration and have minimal dispersal capabilities. Plethodontidae are characterized by costal grooves and a slit from the nostril to the mouth called the nasolabial groove (Degenhardt et al. 2005, Petranka 1998). Unlike most amphibians, plethodontid salamanders do not metamorphose or have an aquatic larval stage. Plethodontid eggs hatch as smaller forms of adults in a process called direct development (Degenhardt et al. 2005). Plethodontid salamanders are unique in their physiological constraints, and therefore, they are also uniquely adapted to moist terrestrial habitats that are distinct from stream or pond dwelling amphibians.

Plethodontidae have an expansive geographic distribution and evolutionary history that began in North America approximately 66 million years ago (Mya) (Shen et al. 2015). Plethodontid salamanders are paraphyletic and diversified into distinct lineages between 65-33 Mya in the Pacific Northwest, which is corroborated by fossils, morphology, mitochondrial DNA, and nuclear DNA evidence (Shen et al. 2015). Eastern and western members of the genus of Plethodon diverged around 40 Mya, and the divergence of the genus Aneides followed about 28 Mya (Shen et al. 2015). *Aneides hardii* (Sacramento Mountain salamander) is of interest because of their geographic isolation from the nearest coastal species of the genus Aneides. New Mexico has high elevation mixed conifer habitats similar to coastal plethodontid habitat (Lowe 1950).

Taxonomy

Initially named *Plethodon hardii*, Charles Lowe renamed this species *Aneides hardii*, and it is the sister taxon to *Aneides lugubis* (Lowe 1950). *Aneides hardii* is characterized by a nasolabial groove, 14-15 costal grooves, five toes on the hind feet, and sexual dimorphism (Lowe 1950, Ramotnik 1997).

Habitat

In general, plethodontid salamanders have a small home range (Merchant 1972, Kleeberger and Werner 1982). *Aneides hardii* is fossorial and spends most of its life below ground. It emerges during the short and sporadic monsoon season when moisture levels permit respiration through their skin and activity above ground. During the monsoon, *A. hardii* are nocturnal and sleep during the day under cover objects, e.g. rocks, decaying logs, leaf litter, and bark. The habitat is composed of Rocky Mountain Douglas fir (*Pseudotsuga menziesii*), Rocky Mountain maple (*Acer glabrum*), ponderosa pine (*Pinus ponde*rosa), white fir (*Abies concolor*), blue spruce (*Picea pungens*), and/or quaking aspen (*Populus tremuloides*) (Lowe 1950, Ramotnik 1997). Canopy cover regulates temperature and moisture and is an essential component of *A. hardii* habitat.

Throughout New Mexico, *A. hardii* inhabit high-elevation sky islands with intervening natural barriers to movement like deserts, low-elevation habitats, piñon-juniper, and grass valley habitats. High-elevation forests have a suite of suitable *A. hardii* microhabitat characteristics that include: moisture, soil, cover objects, and canopy cover. Mixed conifer forest in New Mexico highlight an essential habitat type to native species assemblages, e.g. Sacramento Mountain salamander, Jemez Mountains salamander, Mexican spotted owl, Southwestern Willow flycatcher and New Mexico meadow

jumping mouse (New Mexico Department of Game and Fish 2016; Federal Register 2016).

The U.S. Forest Service and New Mexico Department of Game and Fish survey for *A. hardii* in habitat prior to ground disturbing activities (e.g. road construction, logging, fire, and thinning) to collect presence/absence data as needed (S. Cordova, personal observation). Plethodontids emerge when conditions allow for lungless respiration, but presence/absence surveys are not a true indication of plethodontid absence if conditions are too dry or hot for emergence. Additionally, presence/absence data are qualitative, so resource managers lack quantitative data to address the effectiveness of management practices and stochastic events on *A. hardii*. *Aneides hardii* spends a minimum of nine months of the year below ground, but it is unknown how long these species can survive below ground without emerging. Since *A. hardii* is a cryptic species, this study developed genetic methods and quantitative data analysis to address the evolutionary and demographic history of this species and the utility of DNA-based approaches for species detection.

New Mexico forests vary from wet to dry depending on climatic oscillations of El Niño/La Niña weather patterns and decadal oscillation (D'Arrigo and Jacoby 1991). Historically in the Southwest, low-grade fires were typical in ponderosa pine, and mixed-conifer forests had less frequent, patchy crown fires (Touchan et al. 1996). Despite plethodontid physiological constraints, *A. hardii* is relatively drought and fire adapted (Ramotnik 1997). Low-grade fires are not a significant threat to *A. hardii*, but high-intensity fires are a significant threat to plethodontid persistence and habitat.

Threats to persistence

Aneides hardii is at risk of fungal and viral diseases that are attributed to global amphibian decline, e.g. *Batrachochytrium dendrobatidis* (Bd), *Batrachochytrium salamandrivorans* (Bsal), and ranavirus (Longcore et al. 1999, Richgels et al. 2016). In 2007, a *Plethodon neomexicanus* individual tested positive for Bd in northern New Mexico, and Bd has not yet been found in *A. hardii* (Cummer et al. 2005). In the United States, Bsal has not been detected, but this fungus has decimated salamander species in Europe (Grant et al. 2016). In this study, biosecurity protocols, e.g. equipment sanitation and salamander handling, were tailored to plethodontid salamanders and preventing the spread of infectious disease. Climate change and disease are dynamic processes that ultimately influence the persistence, abundance, and distribution of plethodontid salamanders, like *A. hardii*. Climate change and disease can elicit a negative feedback loop to amphibians because climate-stressed amphibians are more susceptible to disease (Rollins-Smith 2017). We suggest *A. hardii* conservation and management goals should promote species and habitat resilience. Undoubtedly, resilient salamanders and habitat will have the best opportunity for long-term persistence.

Conservation status

Aneides hardii is locally abundant in the southern range in the Sacramento Mountains, and northern *A. hardii* populations in the White and Capitan Mountains are patchily distributed and locally variable in abundance across high-elevation habitats (S. Cordova, personal observation). *Aneides hardii* is protected by the State of New Mexico and designated a threatened species (New Mexico Game and Fish 2000). In this study, historical sites in the southern Lincoln National Forest were consistently occupied (S.

Cordova, personal observation), but some important questions arose regarding rarity, persistence, and population resilience of *A. hardii*. For example, how fragmented are *A. hardii* populations? Specifically, is there gene flow within and between the Sacramento, White, and Capitan Mountains *A. hardii* populations? Do patterns of genetic diversity differ between them? Finally, what are the management consequences of distinct lineages if they exist?

Besides presence/absence surveys for management purposes, *A. hardii* has been largely understudied since the early work of Cindy Ramotnik of the USGS in the 1990's (Ramotnik 1997). A baseline genetic sampling of *A. hardii* would assist resource managers in making informed management decisions. In this study, we surveyed wild *A. hardii* populations with presence/absence surveys coupled with non-lethal genetic sampling. In addition, we utilized the captive population of *A. hardii* population at the Albuquerque BioPark to develop environmental DNA (eDNA) methodology using environmental soil sampling.

Chapter 2

Genetically Distinct Mountain-Top Lineages of *Aneides hardii*

Introduction

Sky islands are terrain comprised of valleys and mountains that are barriers or bridges to colonization (Warshall 1995). Sky islands are vertically stacked biotic communities with clinal migration over short or long periods of time, and these complexes often have greater species diversity, endemism, and clinal variation than other inland areas (Warshall 1995). Previous work indicated sky islands can create genetically-isolated populations that in turn creates genetic structure. Restrictions to gene flow allow genetic differences to accumulate between populations called population structure, and the degree of structure, i.e. high or low structure, depends on how long local populations have been isolated and the size of local populations (Wright 1965). For example, highly structured populations were identified in the New Mexico ridge-nosed rattlesnake (*Crotalus willardi obscurus*, Holycross and Douglas 2007) and the Mexican jay (*Aphelocoma wsultramarina*, McCormack et al. 2008).

In the Pleistocene, inter-glacial periods lasted 10-20,000 years and reoccurred every 100,000 years or so (Fawcett et al. 2011). The climate was warmer and drier, and inter-glacial periods would have been a time where species, like *A. hardii*, could have utilized sky island habitat and clinal migration to escape the warming climate (Warshall 1950). During inter-glacial periods and even today, *A. hardii* populations were likely separated by uninhabitable low-elevation habitat allowing sequence divergence to accumulate between isolated populations in the absence of migration.

In plethodontid salamanders, population fragmentation can promote species diversity and rapid diversification as seen in eastern Plethodontidae (Kozak et al. 2002). On the contrary, isolation and small effective population size can result in loss of genetic diversity, which has been reported in fire salamanders (Álvarez et al. 2015) and long-toed salamander (*Ambystoma macrodactulym*) over ecological time (Giordano et al. 1999).

Aneides hardii is found on high-elevation sky islands in southern New Mexico to the east of the Madrean sky island archipelago, which lies between the southern Rocky Mountains and Sierra Madre Occidental (Warshall 1995). *Aneides hardii* depend on cool, moist islands of montane forests, talus rock, and soil. *Aneides hardii's* range is distributed across approximately 160 kilometers encompassing three mountains ranges, Capitan, White, and Sacramento Mountains (Degenhardt et al. 2005, Pope and Highton 1980). *Aneides hardii*, like most Plethodontidae, live at least 10 years and have a small home range (Ramotnik 1997, Merchant 1972, Kleeberger and Werner 1982).

Based on allozyme data, Pope and Highton (1980) found that Sacramento, White, and Capitan Mountains populations of *A. hardii* were genetically divergent. There were 21 loci examined with five polymorphic loci and only three statistically significant polymorphisms. However, sampling was limited to single sites representing each mountain range.

Aneides hardii populations are also fragmented by anthropogenic and natural barriers, e.g. roads, intervening desert habitats, low-elevation piñon-juniper woodland and grass valleys. We hypothesize that the main source of population fragmentation in *A. hardii* is long-term, low-elevation barriers to dispersal that created population structure

from tens of thousands to millions of years before present. For example, the northern Capitan Mountains population is separated from the White Mountains population by approximately 15 miles of low elevation piñon-juniper woodland.

It is also possible that anthropogenic fragmentation from roads and urban development can be a barrier to plethodontid salamander movement, e.g. fragmentation of the eastern red-backed salamander due to impacts of land use (Noël et al. 2006). Fragmentation of plethodontid populations separates large and demographically connected into small and secluded subpopulations. Smaller populations are prone to adverse demographic and genetic effects such as local extirpation, inbreeding, and loss of diversity (Frankham 2005). Hence, understanding patterns of population subdivision and the scale of movement both within and between populations can identify evolutionarily distinct lineages with a highly polymorphic mitochondrial sequence. Here, we use mitochondrial DNA data and a microsatellite locus to examine the scale and timing of population fragmentation. We predict the divergence of lineages corresponds to the Pleistocene. We also examined genetic diversity within each mountain population to make inferences about demographic history of the populations.

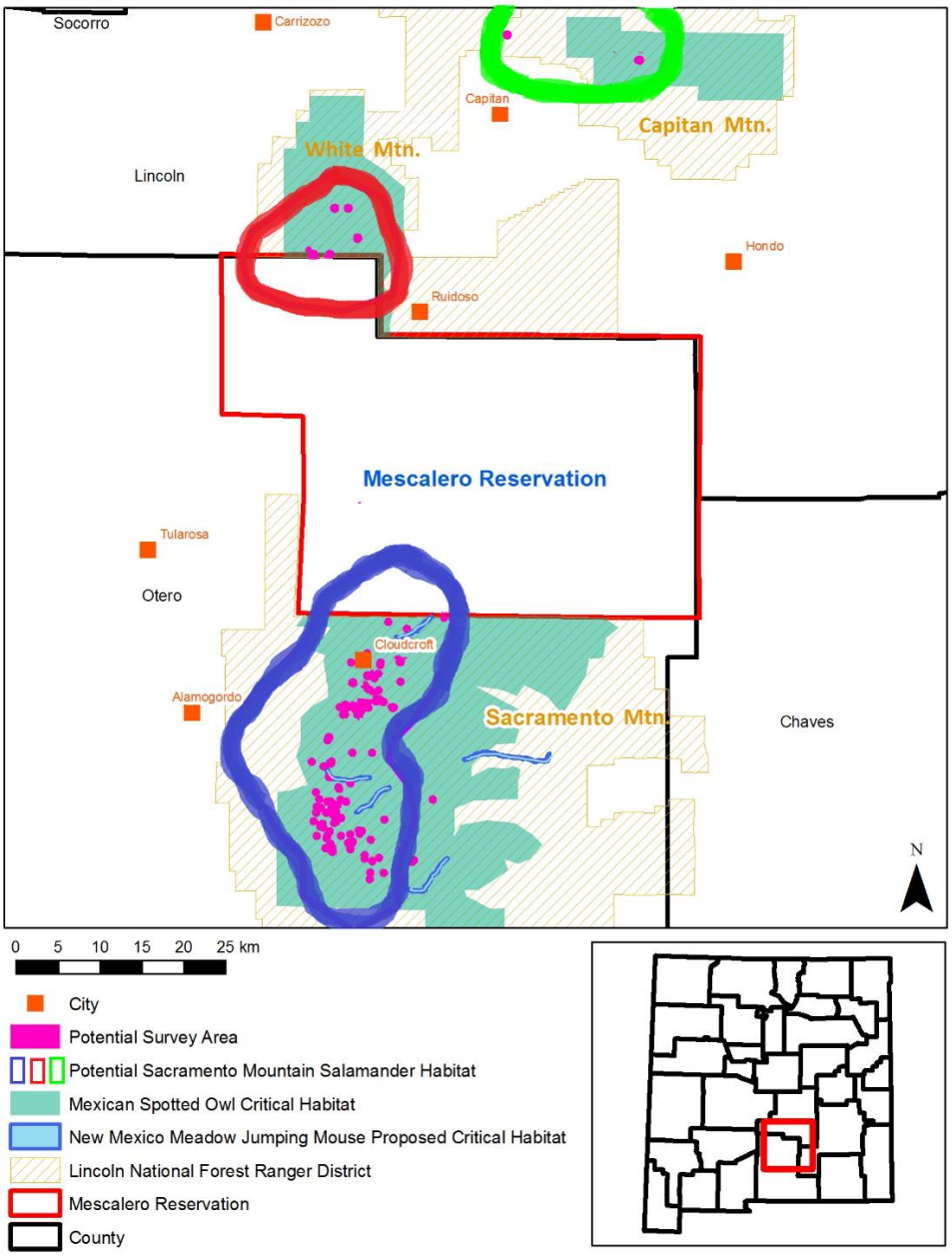

Figure 1. Map of sampling areas color coded by mountain top: blue is the Sacramento Mountain population, red is the White Mountain population, and green is the Capitan Mountain population. Pink dots indicate potential survey areas for *A. hardii*.

(Note: Adapted from a map made by NMDGF for this project)

Methods

Mitochondrial DNA Molecular Methods

We targeted two to four locations per mountain top for sampling that encompassed the range of *A. hardii* (Figure 1). When a specimen was encountered, the salamander was placed in a well-ventilated plastic bag with a moist paper towel for processing. We measured (in millimeters) the snout-vent length and total length. Lastly, we took a genetic sample utilizing non-lethal collection techniques and stored the sample in 95% ethanol (IACUC #13-100983-MC). We isolated DNA with standard proteinase-K digestion and phenol/chloroform isolation methods (Olmstead 1996). We amplified a 741 base pair segment of the mtDNA cytochrome b gene and 602 base pair segment of the ND4 gene with primers developed here from *A. hardii* GenBank sequences (Benson et al. 2013) using NCBI Primer-BLAST. Polymerase Chain Reaction (PCR) mixes of 30 μl total volume contained the following components: 3 μl template DNA, 1 μl Promega FlexiTAQ reaction buffer, 2 mM $MgCl_2$, 125 μM dNTPs, 0.5 μM of forward and reverse primers (ND4: Anhard_nd4F 5' GGTATGGAATTATTCGAGTAAC and Anhard_nd4R 5' CCTGARATTAACTCTGGTTTA; Cytochrome B: Anhard_cytbF 5' AGTACACATTTGCCGCGATG and Anhard_cytb1R 5' ACTGGTTGGCCTCCAATTCA) and 0.5 U of TAQ polymerase. For cytochrome b and ND4, PCR cycling conditions were: 90°C initial denaturation for 2 minutes followed by 30 cycles of 90°C for 30 seconds, 60°C, for 30 seconds and 72°C for 40 seconds and a final elongation step of 72 °C for 15 minutes. PCR products were purified using the OMEGA CyclePure Kit. DNA was sequenced using the Applied Biosystems BigDye Cycling Kit (Version 1.1) according to the manufacturer's instructions. Raw DNA

sequence reads were combined, edited, and aligned using the software program Sequencher® (version 5.4.6).

Microsatellites Molecular Methods

In *A. hardii*, we attempted to optimize microsatellites primers developed for other plethodontid salamanders. We assayed 84 microsatellite primer pairs obtained from the literature that represented eastern and western members of the genus Plethodon and an eastern species of the genus *Aneides* (DeGross 2004, Connors and Cabe 2003, Spatola et al. 2013, unpublished J.J. Apodaca). Of these, a single microsatellite, Plel111 (Degross 2004), amplified consistently and was sufficiently variable. Polymerase Chain Reaction mixes of 10 μl total volume contained the following components: 1 μl template DNA, 2 μl Promega FlexiTAQ reaction buffer, 1 μl $MgCl_2$, 0.8 μl dNTPs, 0.4 μl of forward primer labeled (5' GTATCACCCCACTCACTTTGCTA) and reverse primer (5' GTATGTCCACTGCTCGTCTTTCTT) and 0.1 μl of TAQ polymerase. PCR cycling conditions were: 90°C initial denaturation for 3 minutes followed by 25 cycles of 90°C for 30 seconds, 52°C, for 30 seconds and 72°C for 45 seconds. and a final elongation step of 72 °C for 15 minutes. Per sample, we mixed 10 μl formamide, 0.4 μl GeneScan 1000 Rox Size Standard, and 1.2 μl PCR product. Samples were denatured for 5 minutes at 90°C. Then the samples were run on an automated ABI3130 DNA sequencer and analyzed with ABI Genemapper software (ABI).

Genetic Diversity Statistical Analyses

Evaluation of sequence data from ND4 identified widespread, intra-individual polymorphism. Two tandem gene duplications within the mitochondrial DNA have been documented previously in *A. hardii* (Mueller et al. 2004; Mueller and Boore 2005). We

suspect that the intra-individual polymorphisms that we observed were most likely explained by a whole or partial duplication of ND4 in *A. hardii*. It is likely the additional copy is non-functional, as reported for other duplications in mitochondrial DNA genes in plethodontids (Mueller and Boore 2005). For this reason, we only report on the analysis of cytochrome b.

We determined the most appropriate model of DNA sequence evolution using the maximum likelihood model selection procedure as implemented in the program MEGA Version 6 (Kumar et al. 2016). Utilizing the model of sequence divergence, an uncorrected mean percent sequence divergence between mountain top populations by assigning aligned sequences to groups based on the mountain top where the sample was obtained. *Aneides hardii* cytochrome b haplotype networks were created in the program R using CRAN package to visualize the relationships between populations (R Core Team 2013). We divided the data in two ways: data group by mountain top for broad scale haplotype analysis and further partitioning of data within mountain-top samples to test for fine-scale population structure.

To characterize population level genetic diversity in cytochrome b, we calculated standard measures of genetic diversity including number of haplotypes, haplotype diversity (*h*) and haplotype richness (H_R) for mitochondrial DNA cytochrome b sequences with the software program Contrib (Petit et al. 1998). The haplotype richness (H_R) statistic is calculated using a rarefaction approach that corrects for differences in sample size among collections (Hurlbert 1971). With the data, we created a table of haplotypes frequencies by locality (Table 1).

To assess microsatellite genetic diversity, we utilized the program FSTAT version 2.9.3.2 to calculate allelic richness (A_R) using a similar rarefaction approach as above, Nei's unbiased gene diversity (H_e) (Nei 1977), observed heterozygosity (H_o), and average inbreeding co-efficient (F_{IS}). We use A_R, H_e, and H_o to compare genetic diversity between mountain top populations (Nei 1977). Based on population genetic theory, we expected the inbreeding coefficient F_{IS} to approach 1.0 as the probability of identity by decent increases (Nei 1977). GENEPOP (Raymond and Rousett 1995) was used to test for departures from Hardy-Weinberg equilibrium (HWE), using the procedure of Guo and Thompson (1992).

Demographic History

Patterns of genetic variation of mitochondrial DNA are commonly used to explore population demographic history. For example, recent population expansion is reflected by a star-shaped phylogeny (Slatkin and Hudson 1991), an excess of rare mutations (Harpending and Rogers 2000), and a unimodal mismatch distribution (Rogers and Harpending 1992). We examined *A. hardii* sequences for signals of recent demographic expansion or population bottlenecks using Fu and Li's D^* (Fu and Li 1993) and Tajima's D (Tajima 1989) using DNAsp v5 (Librado and Rozas 2009). Tajima's D compares the number of segregating sites (Θ_w, Watterson 1975) to the mean number of differences between pairs of sequences (Θ, Tajima 1989). Fu and Li's D^* compares the number of number of singleton mutations and the total number of nucleotide variants. These statistics are affected by selection and the demographic process (Fu and Li 1993; Tajima 1989). Fu and Li's D* and Tajima's D values are zero under the null hypothesis and indicate a neutral locus or stable population (i.e., neither growing nor declining over

time). Significantly positive or negative values of Fu and Li's D^* and Tajima's D values lead to rejection of the null hypothesis and are interpreted to mean that variation at the locus of interest is affected by natural selection or the population is either expanding or declining (i.e., it is not stable). We obtained *P*-values and confidence intervals using for Fu and Li's D* and Tajima's D with 10,000 coalescent simulations implemented in DNAsp (Librado and Rozas 2009). Significant negative values are indicative of population expansion (or positive selection), and significant positive values are indicative of selective sweep or a population bottleneck (Librado and Rozas 2009). We also calculated Fu's $F_{S,}$ which compares the probability of the observed haplotypes versus the expected number of haplotypes under neutral conditions, and more observed haplotypes than expected results in negative values of Fu's F_S. Fu's Fs is a more sensitive neutrality test based on the results of simulation studies (Ramos-Onsins and Rozas 2002).

Population Structure

We used the software program Arlequin (version 3.0) (Excoffier et al. 2005) to determine intra- and inter-population dynamics with analysis of molecular variance (AMOVA) of mtDNA cytochrome b sequences (ϕ_{ST}) and the microsatellite (F_{ST}). The fixation index is a standard measure of population structure (Nei 1977). We grouped our molecular data by localities within a mountain top: Sacramento, White, and Capitan Mountains to assess the proportion of genetic variance attributable to differences within- and between-mountain localities as a measure of genetic distance. Specifically, we calculated the proportion of genetic variance attributed to differences among mountain top populations (ϕ_{CT}), among sites within a mountain top (ϕ_{SC}), and within sites (ϕ_{ST}). Additionally, pairwise ϕ_{ST} values were calculated between sampling localities. We used

similar methods to analyze our microsatellite in the program Arlequin to calculate AMOVA, pairwise F_{ST}, $F_{ST,}$ F_{CT}, and F_{SC} (Excoffier et al. 2005). Significance was evaluated using 1,000 bootstrap replicates implemented in Arlequin.

Lineage Divergence Estimates

We utilized the software program BEAUti and BEAST version 2.4.5 to calculate divergence time estimates between three lineages of *A. hardii*: Sacramento, White, and Capitan Mountains (Drummond et al. 2012). We included an outgroup (*Aneides lugubris*) to root the tree. For this reason, we truncated the *A. hardii* dataset for the BEAST analysis to match the 521 base pairs of sequence data available for the outgroup taxon. We employed the HKY model of sequence evolution, a lognormal, and relaxed clock with a mutation rate of 0.8 % per lineage per million years obtained in a previous study that utilized fossil calibration to estimate the divergence rate (Tan and Wake 1995). This rate was also used in another study of salamander phylogenenetics (e.g., Reilly et al. 2015). We used the constant coalescent prior. Three replicate runs in BEAST had a chain length of 1×10^7 trees and logged every $1{,}000^{th}$ tree. We used the program Tracer v.1.6 (Rambaut et al. 2014) to discard the first 10% of trees as burn-in and determine if the run had acceptable Effective Sample Size (ESS) values based on the distribution of trees and if they met expectations of a Markov chain. The programs in BEAST were used for the following: Logcombiner to combine the log files across three independent runs and TreeAnnotator to produce a maximum clade credibility tree of mean node heights (Drummond et al. 2012). Tree files were visualized with the software program Figtree v.1.4 (http://tree.bio.ed.ac.uk/software/figtree/) to determine if there was consistency between runs. Divergence time estimates were calculated to provide a framework for

understanding the timing of lineage divergence but should be viewed with an appropriate level of caution given that they are based on a single gene tree which may overestimate the age of lineage separation.

Results

Genetic Diversity Statistics

Data were obtained from 212 (cytochrome b) and 219 (microsatellite) individuals. Standard genetic diversity statistics differed by mountain top. Specifically, White Mountains had the fewest haplotypes, the lowest values for haplotype diversity, and haplotype richness (Table 2). In contrast, the haplotype richness was the highest in the Capitan population. There were also differences in haplotype frequencies among localities on each mountain top including haplotypes that were unique to some localities (Table 2). For example, there were five haplotypes that were identified only in the most southerly population (Timberon), and the West Capitan population had a unique haplotype in all six individuals that was not detected elsewhere.

The microsatellite (Ple111) was in Hardy-Weinberg equilibrium within each mountain top population. Genetic diversity metrics including A_R and H_e, were the highest in the Capitan Mountains population. The White Mountains inbreeding coefficient (F_{IS}) was indicative of inbreeding compared to the other populations.

Demographic History Statistics

Fu and Li's D* and Tajima's D were not significant for any of the mountain top populations (Table 3). Fu's Fs in the Sacramento Mountain was significantly negative and indicative of population expansion (Table 3).

Population Structure Statistics

The haplotype network revealed clusters of haplotypes that were unique to each mountain top (Figure 2), and there was substantial genetic divergence among them. The mean sequence divergence between mountain top populations were similar (2.5% between Sacramento Mountain and Capitan Mountain, 2.7% between Sacramento and White Mountain, and 3.0% between Capitan and White Mountain). AMOVA analysis revealed a significant proportion of variance could be attributed to differences between mountain tops (ϕ_{CT} = 0.90, *P*-value < 0.0001), as well as to differences between sampling localities within each mountain top (ϕ_{SC}= 0.53, *P*-value < 0.0001). Pairwise ϕ_{ST} values calculated between all sampling localities were all highly significant (Table 4). Likewise, pairwise F_{ST} values calculated from the microsatellite data were small but significantly different from zero in almost all cases (Table 4).

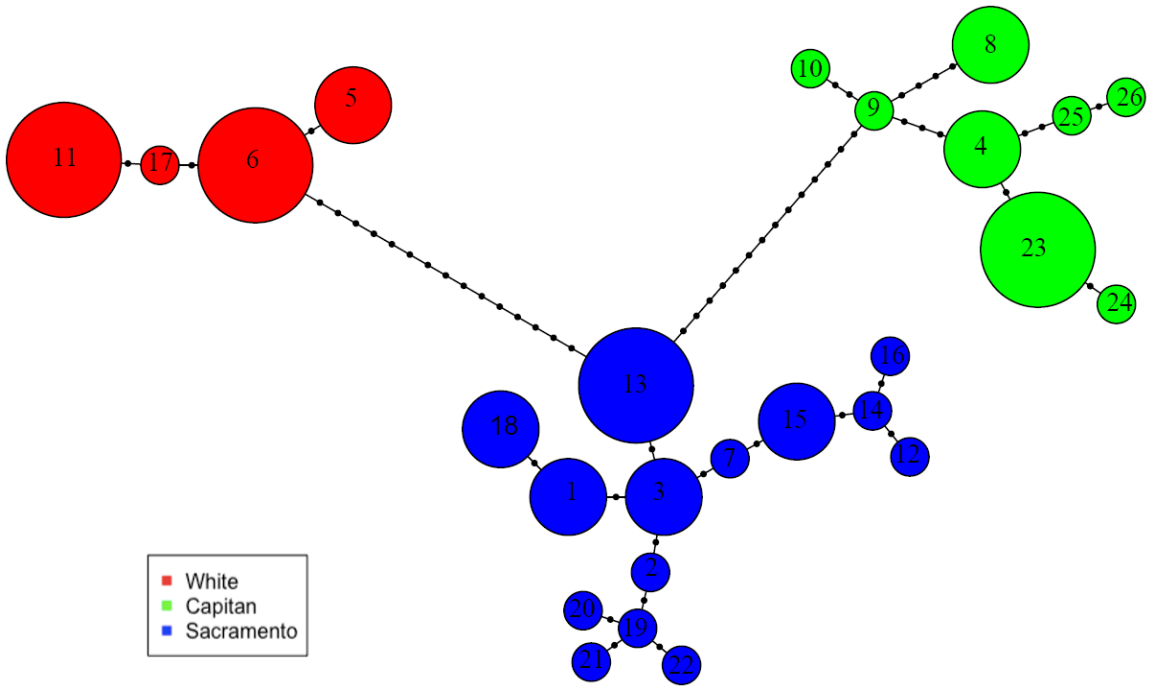

Figure 2. Haplotype network by mountain top (Sacramento Mountains in blue, White Mountains in red, and Capitan Mountains in green). The size of circle indicates the approximate frequency of haplotype and the number within the circle is the haplotype number (each unique haplotype was assigned a unique haplotype number between 1 and 26; see Table 1 for details).

Table 1. *Aneides hardii* cytochrome b haplotype frequencies by locality in New Mexico with unique haplotypes to a locality indicated by *. Raw numbers of individuals of each haplotype is in parentheses, and the total number (n) is indicated next to the locality.

	CAPITAN MOUNTAIN		WHITE MOUNTAIN		SACRAMENTO MOUNTAIN			
	East Mountain n= 31	West Mountain n= 6	Big Bear n= 25	Ski Apache n= 43	Observatory n= 29	Rio Penasco Road n= 26	Russia Canyon n= 28	Timberon n= 25
Hap01						0.58* (15)		
Hap02						0.12* (3)		
Hap03					0.03 (1)		0.36 (10)	
Hap04	0.26* (8)							
Hap05			0.40* (10)					
Hap06			0.60 (15)	0.44 (19)				
Hap07								0.04* (1)
Hap08		1.00* (6)						
Hap09	0.03* (1)							
Hap10	0.03* (1)							
Hap11				0.51* (22)				
Hap12								0.04* (1)
Hap13					0.83 (24)		0.50 (14)	0.20 (5)
Hap14								0.16* (4)
Hap15								0.52* (13)
Hap16								0.04* (1)
Hap17				0.05* (2)				
Hap18					0.03 (1)	0.19 (5)		
Hap19						0.12 (3)	0.04 (1)	
Hap20					0.10* (3)			
Hap21							0.07* (2)	
Hap22							0.04* (1)	
Hap23	0.52* (16)							
Hap24	0.06* (2)							
Hap25	0.06* (2)							
Hap26	0.03* (1)							

Table 2. *Aneides hardii* genetic diversity statistics by mountain top location in New Mexico. Reported mitochondrial DNA (in blue) values include: sample size, number of haplotypes, haplotype richness, and haplotype diversity. Reported microsatellite (in green) values include: allelic richness (A_R), Nei's unbiased gene diversity (H_e), observed heterozygosity (H_o), and average inbreeding co-efficient (F_{IS}).

MITOCHONDRIAL DNA				
	Sample size	# Haplotypes	Haplotype richness	Haplotype diversity
Capitan	36	8	7.0	0.740
White	68	4	2.8	0.632
Sacramento	108	14	8.8	0.797
MICROSATELLITE				
	A_R	H_e	H_o	F_{IS}
Capitan	41.00	0.983	0.921	0.064
White	26.31	0.957	0.851	0.112
Sacramento	29.14	0.952	0.939	0.015

Table 3. Fu and Li's D*, Tajima's D, and Fu's F_S of mitochondrial DNA in blue by mountain-top location: Capitan, White, or Sacramento Mountains. Significant *P*-value is indicated by *.

	Fu and Li's D*	*P*-value	Tajima's D	*P*-value	Fu's Fs	*P*-value
Capitan	-0.294	0.363	-0.161	0.489	-0.499	0.450
White	0.863	0.737	1.678	0.949	1.616	0.816
Sacramento	-0.769	0.237	-0.546	0.337	-5.581	0.020*

Table 4. F-statistics: Mitochondrial DNA is in blue (ϕ_{CT}, ϕ_{SC}, and ϕ_{ST}) and microsatellite is in green (F_{CT}, F_{SC}, and F_{ST}). Significant *P*-values are indicated by *.

MITOCHONDRIAL DNA		
ϕ_{CT}= 0.90	Among mountain top	*P* < 0.0001*
ϕ_{SC}= 0.53	Among sites within mountain top	*P* < 0.0001*
ϕ_{ST}= 0.95	Within sites	*P* < 0.0001*
MICROSATELLITE		
F_{CT} = 0.00	Among mountain top	*P* = 0.67
F_{SC} = 0.03	Among sites within mountain top	*P* < 0.0001*
F_{ST} = 0.02	Within sites	*P* < 0.0001*

Table 5. Pairwise F_{ST} of mitochondrial DNA in blue and microsatellite in green by locality. Significant *P*-values are indicated by *.

	East Mountain	*West Mountain*	*Big Bear*	*Ski Apache*	*Observatory*	*Rio Penasco Rd.*	*Russia Canyon*	*Timberon*
East Mountain	0.00	0.02	0.02*	0.02*	0.02*	0.02*	0.03*	0.01*
West Mountain	0.76*	0.00	0.03	0.04*	0.02	0.02	0.06*	0.01
Big Bear	0.95*	0.98*	0.00	0.03*	0.02*	0.02*	0.05*	0.00
Ski Apache	0.94*	0.96*	0.46*	0.00	0.02*	0.02*	0.02*	0.03*
Observatory	0.94*	0.97*	0.97*	0.96*	0.00	0.00	0.03*	0.02*
Rio Penasco Rd.	0.93*	0.95*	0.96*	0.95*	0.54*	0.00	0.02*	0.02*
Russia Canyon	0.93*	0.96*	0.96*	0.95*	0.12*	0.39*	0.00	0.04*
Timberon	0.93*	0.96*	0.96*	0.95*	0.49*	0.62*	0.46*	0.00

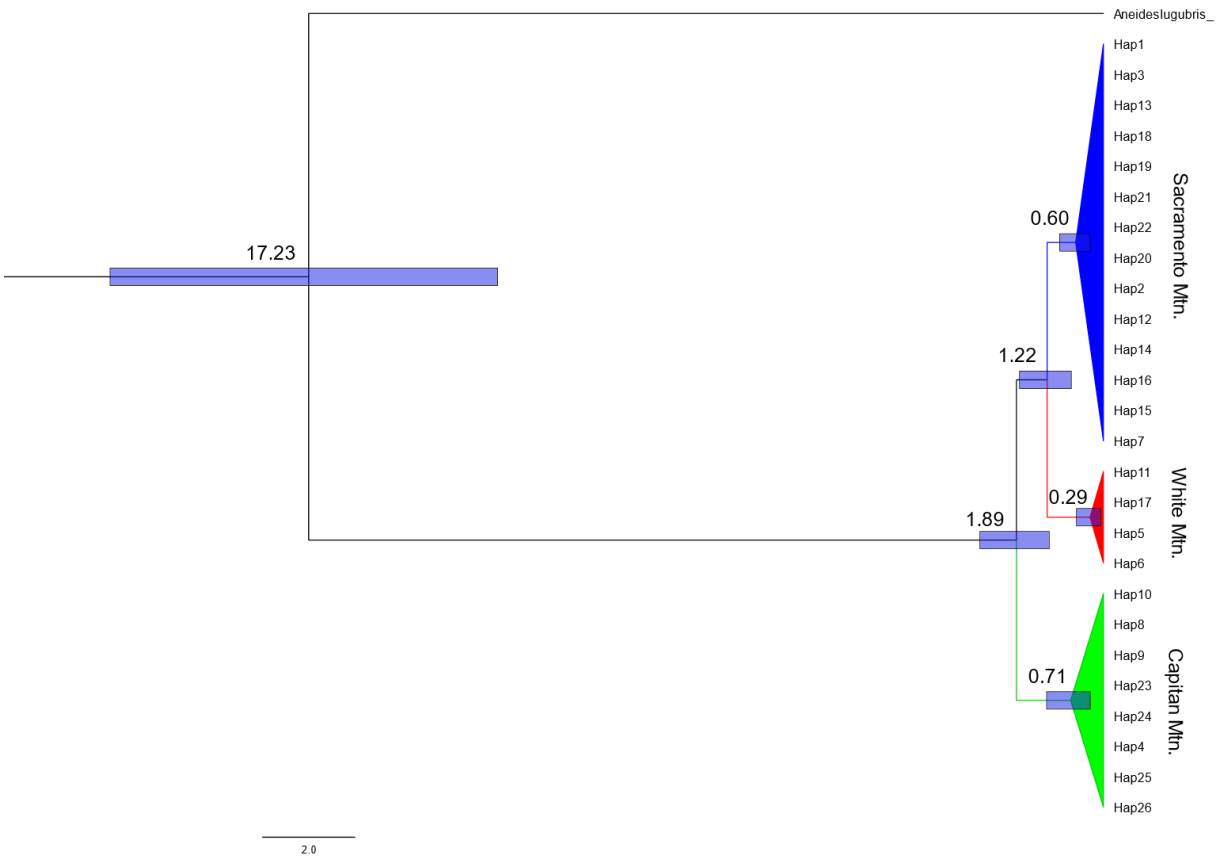

Figure 3. *Aneides hardii* divergence time between mitochondrial lineages (Sacramento Mountain in blue, White Mountain in red, and Capitan Mountain in green) with an outgroup *Aneides lugubris*. The numbers at the node indicate the divergence age in million years with a scale at the bottom left indicating the size of 2.0 million years. Blue bars at the node indicate the 95% confidence interval of the divergence age from oldest to youngest: *A. lugubris* divergence age interval of 13.13 to 21.53 Mya, *A. hardii* lineages divergence age interval of 1.17 to 2.67 Mya, Capitan Mountain divergence age interval of 0.29 to 1.23 Mya, Sacramento and White Mountain divergence age interval of 0.29-0.95 Mya, Sacramento Mountain divergence age interval of 0.29 to 0.94 Mya, and White Mountain divergence age interval of 0.06 to 1.02 Mya.

Lineage Divergence Estimate

In BEAST, the estimated time since sequence divergence between mountain top lineages dated to the Pleistocene at 1.89 Mya with a 95% confidence interval of 1.17 to 2.67 Mya (Figure 3). The node lengths vary by mountain top. The White Mountain has the most recent divergence age of 0.29 Mya with a 95% confidence interval of 0.06 to 1.02 Mya, and Capitan Mountain has the oldest divergence age of 0.71 Mya with a 95% confidence interval of 0.29 to 1.23 Mya (Figure 3). Although, it is important to note the overlapping or close divergence times of the 95% confidence intervals between nodes.

Discussion

Summary of Main Findings

In this study, we assessed the scale at which populations were fragmented and the genetic diversity within each of the mountain top populations. We found substantial divergence among the Capitan, White, and Sacramento Mountains populations. Divergence of these lineages dated to the early Pleistocene. Patterns of genetic diversity were variable between mountain tops with high diversity in the Sacramento Mountains and east Capitan Mountains, and low genetic diversity in the White Mountains and west Capitan Mountains (Table 1). Each mountain top should be considered separately in the overall conservation plan for *A. hardii* because of the significant scale fragmentation and variability in genetic diversity between mountain top populations.

Historical Context of Broad Scale Fragmentation

The degree of fragmentation supports three distinct mountain top lineages, which is congruent with allozyme data (Pope and Highton 1980). Furthermore, highly structured

populations support the idea that 'sky island' habitats have created long-term separation of *A. hardii* lineages, and Pleistocene divergence between lineages is comparable to other salamander species, e.g. *Aneides flavipunctatus* (Reilly et al. 2015), *Plethodon ouachitae* (Shepard and Burbrink 2008), *Plethodon fourchensis* (Shepard and Burbrink 2009), *Plethodon serratus* (Thesing et al. 2016), *Ensatina eschscholtzii* (Kuchta et al. 2009), and *Batrachoseps* (Jockusch et al. 2009). *Aneides flavipunctatus*, *P. ouachitae*, and *P. fourchensis* are all montane species, and display similar genetic patterns to *A. hardii*, e.g. highly structured populations, high elevation montane habitat with low elevation barriers, and fragmentation that dates to the Pleistocene (Reilly et al. 2015, Shepard and Burbrink 2008, Shepard and Burbrink 2009). Shepard and Burbrink (2009) proposed that *P. fourchensis* populations would have expanded during periods that promote forest growth because *P. fourchensis* is associated with deciduous forest. We would also expect in *A. hardii* to show similar patterns of expansion due to periods of coniferous forest habitat expansion. Limited dispersal capability, habitat fragmentation by intervening low-elevation barriers, habitat expansion and contraction, and overall Pleistocene climatic changes played a significant role in the genetic connectivity of *A. hardii* and many plethodontids in the United States.

Pleistocene glaciation was a historic process that greatly influenced the present-day population structure in *A. hardii* mitochondrial DNA and microsatellite data (Figure 2, Figure 3). The mountains that comprise the Lincoln National Forest formed during the Rio Grande rifting that began in the late Oligocene (Chapin and Cather 1994). The genus *Aneides* diverged 28 Mya (Shen et al. 2015) and subsequently expanded into the southwest, including *A. hardii*, following the formation the Capitan, White, and

Sacramento Mountains. The formation of the high-elevation mountain tops laid the foundation for mixed conifer forests and *A. hardii* persistence in the Southwest over millions of years.

The Pleistocene was characterized by glacial and interglacial cycles in which periods of glaciation were interspersed with warmer and drier periods that included mega-drought within New Mexico (Fawcett et al. 2011). These cycles presumably influenced the present-day distribution of *A. hardii*. The divergence time estimates among *A. hardii* lineages are consistent with this scenario (Figure 3). The cooler, wetter periods of the Pleistocene would have promoted contiguous coniferous forests at lower elevations that connected the mountains in our study area with suitable *A. hardii* habitat (Fawcett et al. 2011). Although, it is important to remember eastern and western members of the genus Plethodon divergence and the subsequent divergence of the genus Aneides occurred over millions of years (Shen et al. 2015). If the cooler, wetter periods that lasted for tens of thousands of years then were followed by drought, perhaps the stability of the climate was either not long enough for mixing of *A. hardii* populations or the mixed populations were extirpated during extended periods of drought. Our estimates of early Pleistocene divergence differ from the early estimates of late Pleistocene divergence based on allozymes (Pope and Highton 1980).

Fu and Li's D* and Tajima's D did not detect significant population expansion in any of the *A. hardii* populations (Table 3). The more sensitive Fu's F_S did detect significant population expansion in the Sacramento Mountains population, which is also supported by the star-shaped phylogeny detected in the haplotype network of the Sacramento Mountains (Figure 2, Slatkin and Hudson 1991). Population expansion in the

Sacramento Mountains is supported by the high number of haplotypes and abundance during survey. We conclude the White and Capitan Mountains populations are neither expanding nor contracting and follow the neutral population model (Tajima 1989).

Fine Scale Fragmentation

The mitochondrial DNA and microsatellite showed similar patterns with varying degrees of genetic divergence between *A. hardii* lineages. Mitochondrial DNA was an order of magnitude more divergent than nuclear DNA (Table 5), and it could be an indication sex biased dispersal (Reilly et a. 2012). Sex based dispersal would be an indication that females are more divergent due to minimal dispersal (i.e. small home range) compared to males in the population. We see differences between mitochondrial and nuclear DNA because mitochondrial DNA is maternally inherited. Additionally, mitochondrial DNA evolves at a faster rate than nuclear DNA, so lower divergence is predicted in nuclear DNA. Therefore, we suggest further sequencing of nuclear genes to further address sex based dispersal in *A. hardii*.

Genetic diversity statistics suggest limited movement between sites. Thus, limited movement between localities is indicative of population structure shown by mitochondrial and allozyme data (Pope and Highton 1980). Fine scale population structure could be indicative of life-history features, e.g. small home range.

Habitat characteristics and anthropogenic fragmentation are contemporary processes that may explain the high diversity population in the Sacramento Mountains and low diversity populations in West Mountains (Table 1). For example, Sacramento Mountains have high abundance during surveys and the largest contiguous *A. hardii* habitat. It was not surprising to see high diversity in the Sacramento Mountains and

potentially higher genetic effective population size. However, the east and west Capitan Mountains have populations have small disconnect populations due to fragmentation caused by a geographic low-elevation barrier (Figure 1). The west Capitan Mountains has the smallest amount of available habitat and reduced genetic diversity. In the White Mountains, the Little Bear Fire in 2012 was a high severity fire in *A. hardii* habitat. Additionally, the White Mountains have significant anthropogenic influence due to urbanization. The White Mountains, specifically the Ski Apache location, have a ski recreation area that is heavily influenced by land use, e.g. ski lifts, ski trails, mountain bike trails, roads, and buildings. Despite a recent fire and anthropogenic influence, this population has a relatively high abundance (S. Cordova, personal observation). The reduced diversity and high local abundance in at Ski Apache may reflect a historic population bottleneck and subsequent population recovery at this site.

Management Implications

The identification of substantial divergence between mountain top lineages has important implications for *A. hardii* conservation. Evolutionary significant units (ESUs) and management units (MUs) prioritize species management (Moritz 1994). ESUs have reciprocal monophyly of nuclear and/or mitochondrial DNA, and they conserve historically distinct populations rather than recent adaptations based on genetic divergence (Moritz 1994). ESUs can be composed of multiple MUs. MUs are genetically divergent and typically demographically independent, such that population growth is determined by birth and death rates rather than immigration (Palsbøll et al. 2007). We suggest managing each population separately as ESUs based on genetic diversity statistics (Table 2). ESUs would be beneficial to *A. hardii* because each

historic lineage is evolutionarily significant.

Management should focus resources on populations that are identified here as potentially 'at risk' from genetic factors, e.g. low diversity populations, because stochastic factors (i.e. disease and climate change events) have the potential to eradicate entire evolutionary significant lineages. The White Mountains and west Capitan Mountains have low genetic diversity and low abundance (Table 2). These vulnerable ESUs should be prioritized for conservation with yearly monitoring. However, the Sacramento and east Capitan Mountains have high genetic diversity and abundance and should be monitored before or after ground disturbing activities as necessary, e.g. road construction, urban development, logging, wildfire, and prescribed burns.

Conclusions

Aneides hardii requires moisture above all else because of physiological constraints, e.g. lungless breathing. The overall *A. hardii* population ecology is tied to a moist microhabitat. Based on Pleistocene refugia, we would expect climate to push *A. hardii* higher in elevation under warmer, drier conditions and lower in elevation in wetter, cooler conditions. Additionally, Bd and Bsal are fungal diseases that should be of high concern because fungal disease and climate change are substantial threats to plethodontid persistence (Rollins-Smith 2017). *Aneides hardii* has shown the ability to persist through extreme climatic event like the Pleistocene, but disease adds an additional complication. Managing *A. hardii* lineages as ESUs could promote genetic diversity, abundance, and persistence by tailoring monitoring/conservation efforts based on quantitative genetic data.

Chapter 3

Terrestrial Environmental DNA of the *Aneides hardii*

Introduction

Genetic material obtained from environmental samples (e.g. soil or water) without signs of biological source material is defined as environmental DNA (eDNA) (Thomsen and Willerslev 2015). Many organisms continually leave DNA in their environment, and eDNA may come from urine/feces (Valiere and Taberlet 2000), skin, and/or from dead individuals. Both mitochondrial and nuclear DNA are present in the environment. DNA persistence and preservation time in the environment is highly variable from weeks in water (e.g. Thomsen et al. 2012) to hundreds of thousands of years in cold, dry permafrost (e.g. Willerslev et al. 2003). The species composition of an eDNA sample is detected using PCR with either taxon-specific primers or using a multispecies approach in which generic PCR primers are used for the focal group.

Environmental DNA can be degraded into small fragments and may occur at low abundance. Therefore, PCR primers that amplify short DNA fragments and high copy-number genes, e.g. mitochondrial DNA, are ideal targets of eDNA detection methods (Andersen et al. 2012). Detection of rare taxa from water samples has been widely used (e.g. Jerde et al. 2011; Olson et al. 2012; Goldberg et al. 2011), but few studies have utilized soil samples for detecting rare terrestrial vertebrates. However, Andersen et al. (2012) showed that short mitochondrial DNA fragments from large vertebrates could be recovered successfully from soil in temperate climatic regions. Taberlet et al. (2012) also showed that extracellular DNA could be isolated from soil using methods that assess

(usually microbial) biodiversity via high-throughput DNA sequencing. Developing eDNA methods for detecting rare and cryptic terrestrial species, like our study on endemic salamanders of New Mexico, would provide a useful monitoring tool for these species.

The Nature Conservancy (TNC) partnered with the University of Washington's (UW) Conservation Canines Program to conduct surveys of *Plethodon neomexicanus* (Jemez Mountains Salamander) and *Aneides hardii* (Sacramento Mountain Salamander) in 2013-2014 field seasons. Both salamander species can be captured above ground during the summer monsoon season (Degenhardt et al. 2005). Conservation canines were trained to target species using scent and scat cues in the environment. However, the canine detection team's main challenge was low detection rates and "positive hits" that could not be verified by the dog handler. Following the 2014 field season, UW's Conservation Canines suggested using eDNA to verify salamander presence when a positive hit cannot be visually confirmed.

In this study, we describe the development of eDNA methodology for detection of a captive surrogate, *A. hardii*. We used *A. hardii* because there is a captive population held at the Albuquerque BioPark. This allowed for controlled sampling conditions so that we could determine the parameters under which eDNA detection is possible, e.g. soil type/quantity and persistence time of DNA. Our aim was to determine the efficacy of eDNA methodology and to examine the sampling parameters under which eDNA can be detected.

Methods

Genetic sampling

Primer3 software package (Untergasser et al. 2012) was used to develop species specific PCR primers based in the *A. hardii* ND4 sequences obtained in Chapter 2. We designed primers to targeted short DNA fragments (<120 base pairs) to increase the likelihood of detecting degraded DNA in environmental samples (Dejean et al. 2011). Primer pairs that targeted annealing sites with genetic variation among *A. hardii* individuals were discarded. Our primers, AnHard114f (forward) and AnHard114r (reverse), amplified a 114 base pair fragment. We used the National Center for Biotechnology Information (NCBI) Primer-BLAST (Ye et al. 2012) to further limit the suite of primers identified by Primer3 to species specific targets (Wilcox et al. 2013).

Primers were optimized with high quality *A. hardii* DNA extracted from tissue samples. Primers were tested using 10-fold serial dilutions of DNA (20 ng/µl-2x10-8 ng/µl) to identify those primer pairs that yielded the most amplification, particularly at the lowest DNA concentrations that are characteristic of soil samples. Reactions for eDNA primer testing contained: template DNA, 1X Promega FlexiTAQ reaction buffer, 2 µM MgCl2, 125 µM dNTPs, 0.5 µM of forward (AnHard114f) and reverse primer (AnHard114r) and 0.5 U of TAQ DNA polymerase. PCR cycling conditions were: 90°C initial denaturation for 2 minutes followed by 45 cycles of 90°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds and a final elongation step of 72°C for 15 minutes. PCR products were visualized on a 1.2% ethidium stained agarose gel.

DNA isolation from soil samples

Soil DNA isolations were conducted in a clean DNA laboratory using a PCR Workstation with eDNA-dedicated pipettors. Our clean laboratory is separate from the laboratory where salamander tissue DNA isolations were conducted. All equipment was cleaned with 10% bleach solution prior to eDNA isolations to prevent contamination.

DNA was extracted from soil using the MoBio PowerSoil® DNA Isolation Kit with an added lysis step to enable isolation of vertebrate DNA. Soil samples were weighed and added to the PowerBead tubes, then vortexed. MoBio PowerSoil® solution C1 (60 µl) and 20 µl of proteinase K (20 mg/ml) were added to each tube, vortexed and incubated at 65°C for 30 minutes. After this step, the manufacturer's protocol was followed. We tested seven different soil amounts: 0.025 grams (g), 0.05 g, 0.1 g, 0.2 g, 0.3 g, 0.4 g, 0.5 g to determine the optimal amount of soil for isolations. *Aneides hardii*, microbial and non-target species DNA was quantified using the NanoDrop spectrophotometric system.

Environmental DNA primers were tested using three dilutions of DNA template isolated from soil: undiluted, 0.1% *A. hardii* DNA dilution, and 0.2% *A. hardii* DNA dilution. PCR conditions described above were used. We checked for the presence of *A. hardii* eDNA PCR products on a 1.2% agarose gel and verified positive bands. DNA was sequenced using the Applied Biosystems BigDye Cycling Kit (Version 1.1) according to the manufacturer's instructions. PCR products were sequenced, and raw DNA sequence reads were combined and aligned using the software program Sequencher® (version 5.4.6). For all PCRs, multiple positive and negative controls were included to detect contamination and to verify that the correct size PCR product was amplified.

Experiment 1: eDNA of a Captive Surrogate

In the first experiment, we developed eDNA methods using a captive population of *A. hardii* as a surrogate. We collected 10 grams of soil each from 10 *A. hardii* enclosures (true positive salamander soil), and 500 grams from the potting soil bag (true negative salamander soil). Soils used in this protocol were SunGro Metro-Mix 350: Professional Growing Mix. Nine enclosures contained a male and female *A. hardii*. One enclosure had a single individual. Conditions (temperature, moisture, and soil) within the enclosures were similar across all 10 replicate enclosures. Samples were placed in plastic bags and stored at -40˚ C for three months until DNA-isolation and PCR amplification methods were optimized. Soil samples were homogenized individually by hand for 10 minutes prior to DNA isolation, then weighed and partitioned into small centrifuge tubes for isolation (100 samples of 0.4 grams= 40 grams with 10 grams of soil leftover). DNA was isolated and quantified from 0.4 grams of soil from each enclosure and PCR was conducted as described above.

In this experiment, two negative salamander soil controls were tested per enclosure (100 salamander positive samples, 20 salamander true negative samples, and total of 120 samples). We could isolate up to 24 samples at a time (limited by the protocols horizontal vortex adaptor), so 20 samples of salamander positive soil and two samples of salamander negative soil were isolated at the same time to ensure there was no cross contamination of salamander DNA during the soil DNA isolation step.

Experiment 2: DNA Persistence Time of eDNA

We aimed to determine how long eDNA persists in soil when exposed to full sun compared to under cover objects (i.e., shade) at ambient temperature. This experiment is relevant because plethodontid salamanders spend considerable time under cover objects. Shaded conditions are likely to reflect field sampling conditions (i.e. under rocks, logs, and debris). DNA is likely to degrade over time (Dejean et al. 2011), therefore we predict that the highest yield of DNA will be at the initial time point. Sunlight and associated UV radiation may also speed DNA degradation, and we expected that eDNA would persist longer in shade treatments.

In experiment 2, we resampled unused soil samples from experiment 1 enclosures one through five, and placed five grams of soil in a petri dish with tape on half (shade treatment to simulate cover object) and no tape on the other half (sun treatment to simulate soil exposed to UV). Soils were separated by cardstock and tape to prevent contamination between treatments. Five enclosures were used as replicates and each had three petri dishes to sample each enclosure at one, two, and four weeks post treatment. We limited the time to week-long periods and a total of four weeks based on previous eDNA persistence experiments (Dejean et al. 2011). Experiment 2 was discontinued following week two because salamander DNA did not amplify in any of the soils across treatments.

Data Analysis Methods

Experiment 1 and 2 data were analyzed using the Receiver Operating Characteristics (ROC) analytical framework (Wright 2005, Table 6). ROC is advantageous over linear regression because it detects sensitivity and specificity of the methods being tested. The ROC is the rate of classifying binary data in each experiment. In this circumstance, binary categorical data are partitioned into: true positive and true negative (meaning it is known if there is or is not salamander DNA prior to running the experiments). Following the experiment, the results were then classified as true positive, true negative, false positive, and false negative for salamander DNA isolations. Captive surrogate *A. hardii* is advantageous because biotic conditions are idealized under captive conditions.

Table 6. Calculations for ROC statistics

True Positive Rate (TPR)	= Σ Experimental Result True Positive / Σ Total Condition Positive
False Negative Rate (FNR)	= Σ Experimental Result False Negative / Σ Total Condition Positive
False Positive Rate (FPR)	= Σ Experimental Result False Positive / Σ Total Condition Negative
True Negative Rate (TNR)	= Σ Experimental Result True Negative /Σ Total Condition Negative
Accuracy	= (Σ Exp. Result True Positive + Σ Exp. Result True Negative) / Σ Total Population

Results

Experiment 1: eDNA of a Captive Surrogate

Experimental data were partitioned by initial positive or negative salamander DNA soil treatment designations. Following PCR and gel characterization, each of 10 DNA samples from 10 enclosures (100 samples total) were classified as true positive, false negative, false positive, true negative (Table 7). We verified that these were A. hardii by comparing the DNA sequences to Genbank reference sequence. We detected *A. hardii* DNA in one out of four true positive samples or 25% true positive detection rate, the false negative rate was 0.75, and a true negative rate of 100% (TPR= 0.25, FNR=0.75, TNR=1; Table 7). Additionally, there was no amplification of negative controls (soils with no salamanders) and indicates no cross-contamination in experiment 1 (FPR = 0, Table 7).

Table 7. Detection rates calculated for experiment 1 using ROC statistics

	Detection Rate
True Positive Rate (TPR)	0.25
False Negative Rate (FNR)	0.75
True Negative Rate (TNR)	1
False Positive Rate (FPR)	0

Experiment 2: DNA Persistence Time of eDNA

A. hardii DNA amplification was detected in week one, but not in subsequent samples. In the week one sun treatment, two out of five captive *A. hardii* soil samples tested positive for salamander mitochondrial DNA following sun treatment and one out of five samples tested positive in the shade treatment (Table 8). PCR amplification success was unsuccessful in week two sun and shade treatments and amplified in one out of five positive freezer control samples (Table 8). Negative freezer controls did not test positive for *A. hardii*, and there was no contamination in week one or two soil isolations. We suspended experiment two prior to the week four sampling event because sun/cover treatment samples (true positive) did not amplify salamander DNA during week two of the experiment.

Table 8. Experiment 2 summary: true positive results per number of replicates

	Week 1	**Week 2**
Treatment	*True Positives/Total Replicates*	*True Positive/Total Replicates*
Sun	2/5	0/5
Shade	1/5	0/5
Control		
Negative Freezer Control	0/5	0/5
Positive Freezer Control	2/5	1/5

Discussion

Environmental DNA studies are prolific in rare and endangered aquatic species (Dejean et al. 2011, Jerde et al. 2011), but there are relatively few studies that utilize eDNA from terrestrial vertebrates. Development of methodology for detection of terrestrial eDNA would be a useful conservation tool because small amounts of DNA can be detected even in the absence of the target species. Terrestrial environments pose significant differences in sampling conditions, i.e. less soil processed and soil contaminants, compared to aquatic eDNA studies (Jerde et al. 2011). Overall, we developed eDNA methodology and successfully isolated *A. hardii* DNA from soil samples.

For both experiments, the most notable limiting factor in our eDNA methodology was the amount of soil (0.4 grams) we could process in one isolation. In aquatic studies, the environmental water samples are processed through a filter. Aquatic eDNA methods process 1 liter of environmental water samples compared to our 0.4 grams of environmental soil samples (Dejean et al. 2011, Jerde et al. 2011). An environmental DNA study in *Plethodon cinerus* had similar methodological constraints as far as the amount of soil that could be processed (Walker et al. 2017). Also, soils may inhibit PCR because humic acids and other soil constituents may affect and reduce enzymatic action of Taq DNA polymerase or alter the efficiency of primer binding to template DNA (Lakay et al. 2007).

In experiment 2, the highest yield of *A. hardii* DNA is at the initial sampling point. Although, the treatments seemed to have negligible effect on the outcome of experiment 2. Perhaps, the shade treatment did not work as we had anticipated or the

methodology could not consistently detect patchily distributed DNA. We detected eDNA one week following treatments, and aquatic eDNA persisted less than a month in another study (Dejean et al. 2011). Our species and terrestrial study system differs significantly from aquatic species because of environmental conditions that degrade DNA, e.g. UV light. Additionally, our soil was frozen before use, and fresh (unfrozen) soil could have yielded higher quality DNA during the extraction process.

Experiment 1 and 2 results indicated DNA is patchily distributed in the environmental samples. In our methods, we utilized multiple isolates and PCR amplifications from a single soil sample enhance probabilities of detection from a sample. In the future, we suggest developing methodology to further improve the true positive rate from 25% to >50%. An eDNA study in *Plethodon cinerus* had comparable detection rates of 33% (Walker et al. 2017). In ROC, binary classification rates above 50% are said to be greater than the chance of flipping a coin (Wright 2005). Methodology with the capability to process more soil could improve accuracy and increase the ROC binary classifications rate about the threshold of 50%. There are some alternative DNA isolation methods designed to reduce the effect of local heterogeneity of DNA distribution in the soil by processing substantial amounts of soil at once (Taberlet et al. 2012). In the future, implementing an additional saturated phosphate buffer (Na2HPO4; 0.12 m; pH ≈8) soil wash step would eliminate weighing soil and test more soil per DNA isolation (Taberlet et al. 2012).

Conclusion

In conclusion, eDNA is a viable method for verifying salamander presence, and we successfully detected salamander DNA from environmental samples for up to one week. It will be necessary to field test the methodology developed, but our results conclusively demonstrate it is possible to isolate terrestrial environmental DNA of *A. hardii* from soil samples under captive conditions. Although, it is important to note the limitations of terrestrial eDNA because the data are presence/absence and our methods have a detection rate of 25%. We believe our terrestrial eDNA methodology is a viable option to detect terrestrial salamanders (*A. hardii*) and could be applied to endangered species (*P. neomexicanus*).

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Appendix A

Table 1. Cytochrome b sequence haplotype are numbered 1-26 with unique numbers assigned to each.

Haplotype	Cytochrome b Sequence
1	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ACTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATTCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
2	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ACTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTTTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
3	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ACTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
4	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACGTAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT

	ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATCTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGAGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
5	ATCTGCATCTATATACACATCGGACGAGGTATCTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAACCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGGGGGGGCTTTTCTGTAGACAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCCAACCCCCTAGTCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATTTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTTCTAGCCTCTATTATAATT CTAATATTAATCCCCCTACTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAATAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
6	ATCTGCATCTATATACACATCGGACGAGGTATCTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAACCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGGGGCTTTTCTGTAGACAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCCAACCCCCTAGTCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATTTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTTCTAGCCTCTATTATAATT CTAATATTAATCCCCCTACTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAATAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
7	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACTTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ACTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC

	GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
8	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACGTAT ACAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATACCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
9	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGAGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
10	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACGTAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGAGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
11	ATCTGCATCTATATACACATCGGACGAAGTATCTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAACCTCCTTTCAGCAATCCCCTATATAGGAAATATGCTTG TACAATGAATTTGAGGGGGCTTTTCTGTAGACAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTATCTCTATTTTCTCCAAA

	CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCCAACCCCCTAGTCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATTTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTTCTAGCCTCTATTATAATT CTAATATTAATCCCCCTACTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAATAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
12	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACTTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGGGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAGGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
13	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
14	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACTTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGGGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
15	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACTTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT

	TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
16	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACTTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGGGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGATGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
17	ATCTGCATCTATATACACATCGGACGAGGTATCTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAACCTCCTTTCAGCAATCCCCTATATAGGAAATATGCTTG TACAATGAATTTGAGGGGGCTTTTCTGTAGACAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCCAACCCCCTAGTCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATTTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTTCTAGCCTCTATTATAATT CTAATATTAATCCCCCTACTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAATAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
18	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTCTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATTCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT

19	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTTTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
20	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTTTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
21	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTTTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATTACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
22	ATCTGCATCTACATACACATCGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTATTATTTTATTATTTTTAGTGATGGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTTG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATTTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGGCTTCTAATTTTAATTTTACTATTAACAATACTGTCTATATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCAGCAAACCCCCTAATCACCCCG CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGCTC

	TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT TTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GTCCAACATCACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
23	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACGTAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATCTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
24	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACGTAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTTATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATCTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATCCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGGGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
25	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGAGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATCTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGAGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT
26	ATCTGCATCTACATACACATTGGACGAGGTATTTACTACGGATCATACATAT ATAAAGAGACCTGAAATATTGGTGTTATTTTATTATTTTTAGTGATAGCAAC AGCATTTGTAGGATATGTATTACCTTGAGGACAAATATCATTCTGAGGTGCT ACTGTTATTACCAATCTCCTTTCAGCAATCCCCTATATAGGAGATATGCTCG TACAATGAATTTGGGGAGGCTTTTCTGTAGATAAGGCTACTCTTACCCGATT TTTTGCTTTTCACTTCATTTTACCATTTATTATCTCCGGCGCCAGTATTGTCC ATTTATTATTTCTTCATGAAACAGGATCAAATAACCCAATAGGACTAAACTC

	TAACCCTGACAAAATCTCATTCCACCCATACTTTTCACTTAAAGACTTACTA GGACTTCTAATTTTAATTCTCCTATTAACAATACTATCTCTATTTTCTCCAAA CCTTCTTGGTGACCCAGAAAATTTTACCCCGGCAAACCCCCTAGTCACCCCA CCTCACATTCAACCAGAATGATATTTTTTATTTGCCTACGCAATCTTACGTTC TATTCCAAATAAGCTAGGAGGAGTAATAGCCCTCCTAGCCTCTATTATAATT CTAATATTAATCCCCCTATTACATACATCTAAACACCGAAGTTTAATATTCC GCCCAACAACACAAATATTATTTTGATCATTAATTGCAAACACACTTGTATT AACTTGAATT

Table 2. *Aneides hardii* sample number, locality, microsatellite in blue (Plel111), and cytochrome b haplotype in green (numbered 1-26) data for a total of 238 individuals. No data is indicated by "-".

Sample number	Locality	Microsatellite (Plel111)		Cytochrome b haplotype number
BB_42_Sb	Big Bear	572	594	5
BB_43_Sb	Big Bear	588	604	6
BB_44_Sb	Big Bear	580	580	6
BB_45_Sb	Big Bear	586	580	6
BB_46_Sb	Big Bear	584	678	6
BB_47_Sb	Big Bear	594	598	5
BB_48_Sb	Big Bear	590	604	5
BB_49_Sb	Big Bear	-	-	6
BB_50_Sb	Big Bear	672	378	6
BB_51_Sb	Big Bear	588	594	5
BB_52_Sb	Big Bear	590	590	-
BB_53_Sb	Big Bear	584	678	6
BB_54_Sb	Big Bear	594	598	5
BB_55_Sb	Big Bear	584	594	6
BB_SB_10	Big Bear	552	556	6
BB_SB_11	Big Bear	556	600	6
BB_SB_12	Big Bear	594	608	6
BB_SB_13	Big Bear	600	604	6
BB_SB_14	Big Bear	594	598	6
BB_SB_15	Big Bear	556	584	5
BB_SB_16	Big Bear	-	-	5
BB_SB_17	Big Bear	600	662	6
BB_SB_9	Big Bear	594	594	-
BB_SMS_96	Big Bear	580	584	5
BB_SMS_97	Big Bear	572	594	5
BB_SMS_98	Big Bear	560	602	5
BB_SMS_99	Big Bear	502	604	6
EC_SB_6	East Mountain	588	622	9
EC_SB_7	East Mountain	712	720	24
EC_SB_8	East Mountain	572	712	10
EC_SMS_166	East Mountain	628	658	23
EC_SMS_167	East Mountain	540	648	23
EC_SMS_168	East Mountain	576	696	23
EC_SMS_169	East Mountain	666	696	23

EC_SMS_170	East Mountain	560	658	23
EC_SMS_171	East Mountain	580	662	23
EC_SMS_172	East Mountain	606	620	23
EC_SMS_173	East Mountain	540	584	23
EC_SMS_174	East Mountain	570	576	24
EC_SMS_175	East Mountain	556	590	23
EC_SMS_176	East Mountain	556	640	23
EC_SMS_177	East Mountain	612	642	23
EC_SMS_178	East Mountain	570	672	23
EC_SMS_179	East Mountain	594	738	23
EC_SMS_180	East Mountain	580	662	23
EC_SMS_181	East Mountain	570	628	25
EC_SMS_182	East Mountain	638	638	26
EC_SMS_183	East Mountain	542	678	25
EC_SMS_184	East Mountain	598	666	23
EC_SMS_185	East Mountain	634	710	23
EC_SMS_186	East Mountain	638	666	4
EC_SMS_89	East Mountain	598	606	4
EC_SMS_90	East Mountain	556	566	4
EC_SMS_91	East Mountain	598	612	4
EC_SMS_92	East Mountain	580	594	4
EC_SMS_93	East Mountain	624	652	4
EC_SMS_94	East Mountain	646	652	4
EC_SMS_95	East Mountain	602	648	4
OBS_SMS_121	Observatory	604	646	13
OBS_SMS_122	Observatory	566	646	13
OBS_SMS_123	Observatory	580	622	13
OBS_SMS_124	Observatory	586	590	13
OBS_SMS_125	Observatory	580	622	13
OBS_SMS_126	Observatory	590	600	13
OBS_SMS_127	Observatory	566	610	13
OBS_SMS_128	Observatory	552	624	20
OBS_SMS_129	Observatory	572	590	13
OBS_SMS_130	Observatory	552	584	13
OBS_SMS_131	Observatory	626	634	20
OBS_SMS_132	Observatory	584	638	13
OBS_SMS_133	Observatory	604	604	13
OBS_SMS_134	Observatory	584	596	13
OBS_SMS_135	Observatory	552	556	13

OBS_SMS_136	Observatory	560	600	13
OBS_SMS_137	Observatory	580	584	13
OBS_SMS_138	Observatory	590	598	13
OBS_SMS_139	Observatory	576	602	20
OBS_SMS_140	Observatory	566	604	13
OBS_SMS_141	Observatory	552	566	13
OBS_SMS_142	Observatory	552	584	13
OBS_SMS_143	Observatory	590	600	13
OBS_SMS_144	Observatory	552	604	13
OBS_SMS_145	Observatory	584	590	13
OBS_SMS_146	Observatory	604	620	3
OBS_SMS_42	Observatory	590	618	13
OBS_SMS_43	Observatory	580	610	13
OBS_SMS_44	Observatory	604	610	18
RC_SMS_147	Russia Canyon	572	664	21
RC_SMS_148	Russia Canyon	584	602	19
RC_SMS_149	Russia Canyon	548	584	13
RC_SMS_150	Russia Canyon	584	590	13
RC_SMS_151	Russia Canyon	618	664	13
RC_SMS_152	Russia Canyon	548	618	13
RC_SMS_153	Russia Canyon	552	616	13
RC_SMS_154	Russia Canyon	584	616	13
RC_SMS_155	Russia Canyon	608	616	22
RC_SMS_156	Russia Canyon	572	640	13
RC_SMS_157	Russia Canyon	572	594	13
RC_SMS_158	Russia Canyon	556	612	21
RC_SMS_159	Russia Canyon	532	576	13
RC_SMS_160	Russia Canyon	566	612	13
RC_SMS_161	Russia Canyon	552	612	13
RC_SMS_162	Russia Canyon	572	598	-
RC_SMS_163	Russia Canyon	552	552	13
RC_SMS_164	Russia Canyon	620	624	13
RC_SMS_165	Russia Canyon	572	608	13
RC_SMS_75	Russia Canyon	556	580	3
RC_SMS_76	Russia Canyon	584	656	3
RC_SMS_77	Russia Canyon	-	-	3
RC_SMS_78	Russia Canyon	560	584	3
RC_SMS_79	Russia Canyon	556	594	3
RC_SMS_80	Russia Canyon	590	590	3

RC_SMS_81	Russia Canyon	552	598	-
RC_SMS_82	Russia Canyon	566	584	3
RC_SMS_83	Russia Canyon	584	590	3
RC_SMS_84	Russia Canyon	584	590	3
RC_SMS_85	Russia Canyon	552	618	3
RPR_SMS_45	Rio Penasco Road	528	544	19
RPR_SMS_46	Rio Penasco Road	552	584	18
RPR_SMS_47	Rio Penasco Road	566	612	18
RPR_SMS_48	Rio Penasco Road	556	566	19
RPR_SMS_49	Rio Penasco Road	566	612	18
RPR_SMS_50	Rio Penasco Road	610	618	18
RPR_SMS_51	Rio Penasco Road	552	610	18
RPR_SMS_52	Rio Penasco Road	560	618	19
RPR_SMS_53	Rio Penasco Road	560	576	1
RPR_SMS_54	Rio Penasco Road	556	584	1
RPR_SMS_55	Rio Penasco Road	556	560	-
RPR_SMS_56	Rio Penasco Road	556	584	1
RPR_SMS_57	Rio Penasco Road	576	604	1
RPR_SMS_58	Rio Penasco Road	548	548	-
RPR_SMS_59	Rio Penasco Road	556	566	1
RPR_SMS_60	Rio Penasco Road	580	612	1
RPR_SMS_61	Rio Penasco Road	552	556	2
RPR_SMS_62	Rio Penasco Road	552	604	1
RPR_SMS_63	Rio Penasco Road	544	576	1
RPR_SMS_64	Rio Penasco Road	556	584	1
RPR_SMS_65	Rio Penasco Road	556	618	1
RPR_SMS_66	Rio Penasco Road	548	618	2
RPR_SMS_67	Rio Penasco Road	552	552	2
RPR_SMS_68	Rio Penasco Road	556	560	1
RPR_SMS_69	Rio Penasco Road	556	560	1
RPR_SMS_70	Rio Penasco Road	560	576	1
RPR_SMS_71	Rio Penasco Road	528	604	-
RPR_SMS_72	Rio Penasco Road	552	552	1
RPR_SMS_73	Rio Penasco Road	560	612	1
SA1_56_Sb	Ski Apache 1	524	524	6
SA1_57_Sb	Ski Apache 1	598	616	11
SA1_58_Sb	Ski Apache 1	556	556	11
SA1_59_Sb	Ski Apache 1	556	570	11
SA1_SMS_3	Ski Apache 1	548	556	6

SA1_SMS_4	Ski Apache 1	532	584	6
SA1_SMS_5	Ski Apache 1	580	618	11
SA1_SMS_6	Ski Apache 1	560	560	11
SA2_SMS_10	Ski Apache 2	532	572	11
SA2_SMS_11	Ski Apache 2	580	596	11
SA2_SMS_12	Ski Apache 2	566	580	6
SA2_SMS_13	Ski Apache 2	536	566	6
SA2_SMS_14	Ski Apache 2	-	-	6
SA2_SMS_15	Ski Apache 2	590	610	-
SA2_SMS_16	Ski Apache 2	560	566	11
SA2_SMS_17	Ski Apache 2	572	588	6
SA2_SMS_18	Ski Apache 2	540	566	-
SA2_SMS_19	Ski Apache 2	556	556	11
SA2_SMS_20	Ski Apache 2	566	580	6
SA2_SMS_21	Ski Apache 2	572	572	11
SA2_SMS_22	Ski Apache 2	-	-	11
SA2_SMS_23	Ski Apache 2	528	540	11
SA2_SMS_24	Ski Apache 2	584	618	6
SA2_SMS_25	Ski Apache 2	560	526	6
SA2_SMS_26	Ski Apache 2	520	678	11
SA2_SMS_27	Ski Apache 2	556	604	17
SA2_SMS_28	Ski Apache 2	532	552	6
SA2_SMS_29	Ski Apache 2	560	604	11
SA2_SMS_30	Ski Apache 2	566	566	11
SA2_SMS_31	Ski Apache 2	566	588	6
SA2_SMS_32	Ski Apache 2	540	572	6
SA2_SMS_33	Ski Apache 2	560	566	11
SA2_SMS_34	Ski Apache 2	540	688	11
SA2_SMS_35	Ski Apache 2	572	572	11
SA2_SMS_36	Ski Apache 2	520	678	11
SA2_SMS_37	Ski Apache 2	560	576	17
SA2_SMS_38	Ski Apache 2	560	626	6
SA2_SMS_39	Ski Apache 2	580	590	11
SA2_SMS_40	Ski Apache 2	-	-	11
SA2_SMS_7	Ski Apache 2	548	560	6
SA2_SMS_8	Ski Apache 2	528	560	6
SA2_SMS_9	Ski Apache 2	560	588	6
SA3_SMS_86	Ski Apache 3	566	570	11
SA3_SMS_87	Ski Apache 3	548	580	6

SA3_SMS_88	Ski Apache 3	524	590	6
TIM_Sac_1	Timberon	594	606	12
TIM_Sac_10	Timberon	566	590	15
TIM_Sac_11	Timberon	576	602	13
TIM_Sac_12	Timberon	566	576	15
TIM_Sac_13	Timberon	594	594	16
TIM_Sac_14	Timberon	580	610	14
TIM_Sac_15	Timberon	544	624	13
TIM_Sac_16	Timberon	556	624	15
TIM_Sac_17	Timberon	548	594	-
TIM_Sac_18	Timberon	580	606	-
TIM_Sac_19	Timberon	580	590	15
TIM_Sac_2	Timberon	580	604	13
TIM_Sac_20	Timberon	552	580	15
TIM_Sac_21	Timberon	590	624	14
TIM_Sac_22	Timberon	576	584	15
TIM_Sac_23	Timberon	556	594	15
TIM_Sac_24	Timberon	548	580	15
TIM_Sac_25	Timberon	604	612	15
TIM_Sac_26	Timberon	556	606	-
TIM_Sac_27	Timberon	584	610	-
TIM_Sac_28	Timberon	580	602	-
TIM_Sac_29	Timberon	598	620	14
TIM_Sac_3	Timberon	-	-	14
TIM_Sac_30	Timberon	576	616	15
TIM_Sac_4	Timberon	-	-	13
TIM_Sac_5	Timberon	580	606	7
TIM_Sac_6	Timberon	584	594	13
TIM_Sac_7	Timberon	580	590	15
TIM_Sac_8	Timberon	-	-	15
TIM_Sac_9	Timberon	576	594	15
WC_SB_2	West Mountain	620	708	-
WC_SB_3	West Mountain	576	620	8
WC_SB_4	West Mountain	590	590	8
WC_SB_5	West Mountain	584	590	8
WC_SMS_187	West Mountain	594	630	8
WC_SMS_188	West Mountain	624	624	8
WC_SMS_189	West Mountain	628	632	8