Population genetics of a plethodontid salamander endemic to New Mexico (Sacramento Mountain salamander, *Aneides hardii*)

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Executive Summary

Aneides hardii (Sacramento Mountain salamander) is found in cool, moist islands of montane forests, and talus rock at high elevation in Southern New Mexico. They have a fragmented distribution that spans approximately 160 kilometers encompassing three high elevation mountains ranges including the Capitan, White, and Sacramento Mountains. *Aneides hardii* is a species of conservation concern in New Mexico. The objectives of this study were to obtain population-level genetic data from *A. hardii*, including both mitochondrial and nuclear DNA loci, to describe the distribution of genetic variation across the range of the species. Our major findings were: (1) mitochondrial sequence data identified three genetically distinct lineages restricted to the three mountain ranges occupied by *A. hardii* (Capitan, White, and Sacramento Mountains), consistent with a previous study utilizing allozymes (Pope and Highton 1980). Preliminary microsatellite results were also consistent with this result. (2) Estimates of divergence times among these lineages suggest separation since the early Pleistocene, and there was no indication that these populations came into contact during more recent glacial periods. This result suggests that other landscape features may have served as barriers and/or life-history features of *A. hardii*, such as small home range, may have prevented secondary contact. (3) Genetic diversity differed among the lineages, with high diversity and deeper divergence among haplotypes restricted to the Capitan Mountains, very low diversity in the White Mountains, and high diversity with low divergence among haplotypes in the Sacramento Mountains lineage. These results suggest very different demographic histories within each lineage. Based on these results, we suggest that each of the lineages of *A. hardii* be designated as a distinct evolutionarily significant unit.

Introduction

Dramatic oscillations in climate produce significant changes in both fauna and flora. The last 700,000 years were dominated by ice ages occurring at approximately 100,000 year cycles. These were punctuated by shorter, warmer and wetter interglacial periods (reviewed in Hewitt 1996). The most recent ice age was 21,000 years ago and lasted until 11,500 year ago. During the interglacial periods, many montane specialists migrated upslope to cool climatic refugia. These have been referred to as "sky islands" (Warshall 1995). Sky islands are vertically stacked biotic communities which can support this type of clinal migration. These complexes can harbor greater species diversity, endemism, and clinal variation than comparable inland areas (Warshall 1999). Numerous studies have shown that the isolation of sky islands tends to create genetic structure in populations, and the degree of structure depends on how long local populations have been isolated on these habitat islands and can also depend on the size of local populations. 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 ultramarina*, McCormack et al. 2008). In plethodontid salamanders, population fragmentation can promote species diversity and rapid diversification, as seen in eastern Plethodontidae (Kozak et al. 2002). Conversely, isolation and small effective population size can erode genetic diversity, which has been documented in fire salamanders (Álvarez et al. 2015) and the long-toed salamander (*Ambystoma macrodactulym*) over ecological time (Giordano et al. 1999).

Aneides hardii (Sacramento Mountain salamander) is found on high-elevation sky islands in New Mexico mountains to the east of the Madrean sky island archipelago, which is between the southern Rocky Mountains and Sierra Madre Occidental (Warshall 1999). *Aneides hardii* depends on cool, moist islands of montane forests with talus rock. *Aneides hardii's* range is distributed across approximately 160 kilometers on three high elevation mountains ranges, Capitan, White, and Sacramento Mountains, in southern New Mexico (Degenhardt et al. 2005, Pope and Highton 1980). *Aneides hardii*, like most Plethodontidae, are long lived (at least 10 years) and have a small home range (Ramotnik 1997). Pope and Highton (1980) used alloymes to examine samples of *A. hardii* from Sacramento, White, and Capitan Mountains populations and they found evidence for genetic divergence among populations in different mountain ranges. However, sampling was limited to single sites representing each mountain range.

Populations are the basic unit for conservation and management (Waples and Gaggiotti 2006), so understanding the relationships of populations to one another is critical. For example, the strategies used to manage a species that is panmictic across its range are very different from how a series of isolated populations might be protected. The criteria used for delineating distinct evolutionary units have been much debated. For example, Ryder (1986) formulated the notion of the Evolutionarily Significant Unit (ESU), denoting genetically unique populations deserving protection. Recognition of distinct ESUs was based on concordance between two independent datasets. Moritz (1994) suggested that reciprocal monophyly in a mitochondrial gene tree, in addition to significant divergence among nuclear genes, be used as criteria for designating an ESU. Within ESUs, distinct management units (MUs) may also be defined. Management units are identified based on either divergent mtDNA or nuclear DNA (Moritz 1994) and are typically demographically independent, such that population growth is determined by local birth and death rates rather than on immigration (Palsboll et al. 2006).

The aim of this project is to determine the scale of population structure in *A. hardii*. We hypothesize that the main source of population fragmentation in *A. hardii* are low-elevation barriers between mountain top populations that have existed since the onset of the most recent interglacial period. Divergence dates older than this would suggest that gene flow was also restricted during glacial periods, when corridors for movement presumably existed. More recent anthropogenic fragmentation (e.g., roads) may also have curtailed movement between geographically close populations. We also describe genetic diversity within populations with respect to their demographic history.

Methods

Samples (3-5 millimeters [mm] tail tips) were collected from 212 individuals encompassing the range of *A. hardii*, with multiple sampling sites per mountain top (i.e., mountain range) (Figure 1). We isolated DNA using a standard proteinase-K digestion and phenol/chloroform isolation method (Olmstead 1996). We amplified a 741-base pair segment of the mitochondrial cytochrome b gene and 602 base pair segment of the ND4 gene with primers developed in our lab from publically accessible *A. hardii* sequences available on GenBank. Polymerase Chain Reaction (PCR) mixes of 30 µl total volume contained the following: 3 µl template DNA, 1X Promega Flexi TAQ reaction buffer, 2 mM $MgCl_2$, 125 µM dNTPs, 0.5 µM of forward (ND4:

Anhard_nd4F 5' GGTATGGAATTATTCGAGTAAC and Anhard_nd4R 5' CCTGARATTAACTCTGGTTTA; Cytochrome b: Anhard cytbF 5' AGTACACATTTGCCGCGATG and Anhard_cytb1R 5' ACTGGTTGGCCTCCAATTCA) and reverse primer (ND4 or Cytochrome b), and 0.5 U of TAQ polymerase. For cytochrome b and ND4, PCR cycling conditions were: 90°C initial denaturation for 2 minutes (min) followed by 30 cycles of 90°C for 30 seconds (s), 60°C for 30 s, and 72°C for 40 s, plus a final elongation step of 72 °C for 15 min. PCR products were purified using the OMEGA CyclePure Kit. DNA was sequenced using the Applied Biosystems BigDye Cycle Sequencing Kit (Version 1.1) according to the manufacturer's instructions. PCR products were sequenced, and raw DNA sequence reads were edited and aligned using the software program Sequencher® (version 5.4.6).

For *A. hardii*, we attempted to use cross species amplification of microsatellites developed for other plethodontid salamanders. We selected 84 microsatellite loci from the literature representing east and west coast *Plethodons* and an east coast *Aneides* (Connors and Cabe 2003; De Gross 2004; Spatola et al. 2013; unpublished J.J. Apodaca). A single microsatellite, Plel111 (De Gross 2004), amplified consistently and was variable. The other primers either did not amplify, did not amplify consistently, were invariant, or did not appear to amplify a repetitive sequence in *A. hardii.* Polymerase chain reactions for Plel111 contained 10 µl total volume contained the following: 3 µl template DNA, 1X Promega FlexiTAQ reaction buffer, 2 mM $MgCl_2$, 125 µM dNTPs, 0.5 µM of forward primer labeled (5' GTATCACCCCACTCACTTTGCTA) and reverse primer (5' GTATGTCCACTGCTCGTCTTTCTT), and 0.5 U of *Taq* polymerase. PCR cycling conditions were: 90°C initial denaturation for 2 min followed by 30 cycles of 90°C for 30 s, 60°C for 30 s, and 72°C for 40 s, plus a final elongation step of 72 °C for 15 min. Fragment size analysis was conducted on an ABI3130 automated capillary sequencer by combining 1µl of PCR product with 10µl of formamide and 0. 4µl of HD1000 size standard, which was denatured at 93°C for 5 minutes. Genotype data were scored in GENEMAPPERVersion 4. 0 (Applied Biosystems).

Statistical Analysis- Genetic Diversity

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 that 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.

To determine the most appropriate model of DNA sequence evolution for cytochrome b we used the maximum likelihood approach implemented in MEGA which compares 24 different models (Kumar et al. 2016). We also used MEGA to calculate the mean, uncorrected percent sequence divergence within and between mountain top populations. We calculated standard measures of genetic diversity, including haplotype diversity (*h*) (Nei 1987), number of haplotypes and haplotype richness (H_R) for mitochondrial DNA cytochrome b sequences with the software program Contrib (Petit et al. 1998). Haplotype richness (H_R) is calculated using rarefaction to account for differences in sample size among collections. These statistics are reported for each mountain top population. We also report haplotype frequency by collection locality. We used DNAsp (Librado and Rozas 2009) to calculate nucleotide diversity (π, the average number of nucleotide substitutions per site between two sequences, Nei 1987), sequence diversity (*k*, the average number of nucleotide differences between two sequences, Tajima 1983), the number of segregating (polymorphic/variable) sites (*S*), and the number of mutations per site (θ, Nei 1987). MICROSATELLITETOOLKIT (add-in for Microsoft Excel, written by S. Park, available at http://animalgenomics. ucd. ie/sdepark/ms-toolkit/) was used to check the data for scoring errors and to estimate additional diversity statistics, including observed heterozygosity (H_o), Nei's unbiased gene diversity (H_e), and mean number of alleles (N_a). GENEPOP (Raymond and Rousett 1995) was used to test for departures from Hardy-Weinberg equilibrium (HWE), using the procedure of Guo and Thompson (1992). Average inbreeding coefficients (F_{IS}) and allelic richness (A_R) were obtained using FSTAT vers. 2.9.3.1 (Goudet 1995). The number of private alleles (P_A) (alleles unique to one population) per mountain range was calculated using GenAlEx vers 6.5 (Peakall and Smouse 2006).

Population Structure

To visualize the relationships among *A. hardii* cytochrome b sequences, we created haplotype networks. Weir and Cockerham's (1984) analysis of molecular variance (AMOVA), based on haplotype frequencies and implemented in ARLEQUIN vers. 3.5 (Excoffier et al. 2005), was used to examine the partitioning of genetic variance among mountain tops (ϕ_{CT}), among sites within a

mountain top (ϕ_{SC}), and among sites (ϕ_{ST}). We also calculated pairwise ϕ_{ST} and F_{ST} among sampling localities. Significance was assessed by 10,000 bootstrap replicates. The results from the analysis of microsatellite data should be viewed as preliminary as it is based on a single locus.

Demographic History

Patterns of genetic variation of mitochondrial DNA can be used to explore the demographic history of a population. 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). Fu and Li's D^* compares the number of number of singleton mutations and the total number of nucleotide variants. Tajima's D compares two estimates of θ obtained from empirical data; θ_w, which is based on the number of segregating sites (Watterson 1975) and θ_π, which is based on the mean number of differences between pairs of sequences (Tajima 1989). These statistics are affected by selection and demographic processes (Tajima 1989; Fu and Li 1993). Fu and Li's D* and Tajima's D values do not differ significantly from zero in stable populations and in the absence of selection. Significant negative values are indicative of population expansion (or positive selection), and statistically significant positive values are indicative of either a selective sweep (reduction of genetic diversity among nucleotides near a mutation due to strong selection for the beneficial allele) or a population bottleneck (Librado and Rozas 2009). We also calculated Fu's F_S, which compares the probability of the observed number of haplotypes versus the expected number of haplotypes under neutral conditions. More 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). These statistics were calculated for each mountain top population and significance was assessed using 10,000 coalescent simulations conducted in DNAsp (Librado and Rozas 2009). Examining the number of pairwise differences between sequences (mismatch analysis) can also provide information about the historical demography of a population. Specifically, the signature of recent population expansion is characterized by a unimodal distribution (Poisson distribution) (Slatkin and Hudson 1991). A ragged or multimodal distribution is indicative of population stability (Rogers and Harpending 1992). We used Arlequin 3.1.1 (Excoffier et al. 2005) to conduct mismatch analyses. To test for demographic expansion against the null model of population stability, we used the raggedness index (R_2) (Ramos-Onsins

and Rozas 2002). A significant R_2 value suggests population expansion. We also used the sum of squared deviations between the observed and expected mismatch distributions to test for the signature of population stability (significant values indicate stability). Significance of these test statistics were assessed with 1000 bootstrap replicates in Arlequin (Excoffier et al. 2005). Mismatch analysis also provides the value tau (τ), which is a moment estimator that represents a unit of mutational time (Schenekar and Weiss 2011). This can be used to calculate the time since population expansion occurred (t) using the equation $t = \tau/2u$ where u is the cumulative probability of substitution (Schenekar and Weiss 2011). We used the excel spreadsheet calculator (http://www.uni-graz.at/zoowww/mismatchcalc/index.php) to estimate the time since expansion using the sequence divergence rate of 1.6 % per million years (Tan and Wake 1995). The excel calculator converts this estimate into the cumulative number of substitutions per generation (assuming a generation time of 3 years) and provides an estimate of the time since expansion in years.

Lineage Divergence Estimates

We utilized the software program BEAST 2.4.5 (Brouckaert et al. 2014) to estimate the divergence times among the three lineages of *A. hardii*: Sacramento Mountains, White Mountains, and Capitan Mountains (Drummond et al. 2012). We included an outgroup (*A. lugubris*) to root the tree. For this reason, we truncated the *A. hardii* data for BEAST analysis to match the 521 base pairs of sequence available for the outgroup taxon. We employed a lognormal, 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 has also been used in several other studies of salamander phylogenenetics (e.g., Reilly et al. 2015). We used the HKY (identified as the most appropriate) and a coalescent constant prior. We conducted three independent runs with 50 million generations each, sampling every 5,000th tree. The first 10% of trees were discarded as burn-in. Log and tree files from multiple runs were combined using LogCombiner. We used the program Tracer (http://Beast.bio.ed.ac.uk/Tracer) to examine the log files and check for convergence of model parameters. TreeAnnotator vers. 2.1.2 was used to annotate the tree with mean heights and 95% posterior densities of divergence time estimates. Tree files were visualized with the software program Figtree v.1.4 (http://Beast.bio.ed.ac.uk/FigTree). Divergence time estimates were calculated to provide a framework for understanding the separation of lineages. However, these estimates 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

Cytochrome b data was obtained from 212 *A. hardii* individuals. The Sacramento Mountains population had the highest *h* and H_R (Table 1). The number of variable sites (*S*) was the same in the Capitan and Sacramento Mountains populations, *k* and π were higher in the Capitan population (Table 2), indicative of more divergent haplotypes. All measure of diversity calculated from cytochrome b were low in the White Mountains population (Table 1). Haplotype frequencies differed substantially among mountain ranges, with no haplotypes shared among them (Table 3). There were also substantial haplotype frequency differences among sites within mountains. West Capitan individuals (n=6) shared a single unique haplotype. The southern-most sampled locality (Timberon) had five haplotypes that were not detected elsewhere in the Sacramento Mountains (Table 3). Gene diversity calculated from microsatellite locus Ple111 was high for all populations (Table 1). The Capitan population has the highest allelic richness and number of private alleles. F_{IS} ranged from 0.015 (Sacramento) to 0.120 (White). The locus conformed to Hardy- Weinberg expectations.

Population Structure

Haplotype networks revealed groups of haplotypes that were unique to each mountain range (Figure 3-6). Divergence between them was 2.9% between Capitan and White, 2.6% between Capitan and Sacramento, and 2.8% between White and Sacramento. AMOVA analysis revealed that a significant proportion of variance could be attributed to differences between mountain tops ($\phi_{CT} = 0.131$, $p = 0.02$), as well as to differences between sampling localities within each mountain top ($\phi_{SC} = 0.360$, $p = 0.0001$). Pairwise ϕ_{ST} values calculated between all sampling localities were all highly significant (Table 5). Likewise, pairwise F_{ST} values calculated from the microsatellite data were small but significantly different from zero in most all cases (Table 6).

Demographic History and Divergence Time Estimates

Fu and Li's D* and Tajima's D did not differ significantly from zero for any of the mountain top populations (Table 4). Fu's F_s was significantly negative and R_2 was small and significant for the Sacramento Mountains population, an indication of historical population expansion. Comparisons of

observed mismatch distributions (Figure 2) to those expected under sudden demographic expansion were not significant. The estimated time since population expansion ranged from ~126,000-181,849 years ago for the Sacramento and White Mountains populations to ~400,000 years ago for Capitan Mountains. Estimated times of lineage divergence between mountain top populations date to the early Pleistocene, with the Capitan lineage diverging first, followed by White and Sacramento Mountains populations (Figure 7). It is important to note that these estimates should be viewed with an appropriate degree of skepticism as they are only based on a single gene and are largely dependent on the rate of sequence evolution used in their calculation.

Discussion

Mitochondrial sequence data presented here support earlier allozyme data (Pope and Highton 1990), which identified genetically distinct lineages restricted to the three mountain ranges occupied by *A. hardii*; the Capitan, White, and Sacramento Mountains. Preliminary microsatellite results (presence of private alleles in each mountain top population) from a highly variable locus are also consistent with this result. However, this result need to be verified by either additional microsatellites or DNA sequence data from nuclear loci. *Aneides hardii*, like many plethodonids, is a terrestrial breeder, mates in pairs, and has species-specific courtship displays, which can lead to highly differentiated mtDNA and nuclear loci (Zamudio and Savage 2003). Estimates of divergence times among these lineages suggest separation since the early Pleistocene. Furthermore, there is no indication that these populations came into secondary contact during more recent glacial periods. This is likely a result of the very small home ranges of these salamanders and possibly of landscape features that served as barriers. Genetic diversity also differed among the lineages, with high diversity and deeper divergence among haplotypes restricted to the Capitan Mountains, very low diversity in the White Mountains, and high diversity with low divergence among haplotypes in the Sacramento Mountains lineage. These results suggest very different demographic histories within each lineage.

Broad Scale Fragmentation

The degree of fragmentation observed among the mountain ranges suggests that *A. hardii* comprises three distinct, mountain top lineages. This is consistent with the idea that ‘sky islands’ provided isolated refugia during the warmer, interglacial periods. Likewise, Shepard and Burbrink (2009) reported significant divergence among montane populations of *Plethodon fourchensis* (2009), with

lineage divergence dating to the middle Pleistocene and likely caused by fragmentation of a widely-distributed ancestor. Their results suggested that *P. fourchensis* populations expanded during interglacial periods, coinciding with the expansion of deciduous forests, and contracted during glacial periods. This finding contrasts with environmental conditions of southwestern Madrean sky islands in which range contractions, and hence fragmentation of montane species, are associated with interglacial periods. Specifically, pine-oak forests contracted during these periods, thereby isolating populations on adjacent mountain tops (Smith and Farrell 2005; Masta 2000). We did not find evidence that populations of *A. hardii* came into contact during more recent glacial periods, when coniferous forests dominated (Davis 1983). This suggests that, although movement may have extended downslope during cooler periods, the mountain top populations remained isolated from one another. Barriers from other landscape features, such as rivers, or life-history characteristics that precluded movement sufficient to connect populations may explain this result. Other southwestern species have also been isolated on 'sky islands' (e.g., New Mexico ridge-nosed rattlesnake [Holycross and Douglas 2007] and the Mexican jay [McCormack et al. 2008]), but the timing of isolation differs substantially among species. Like *A. hardii*, Mexican jays are poor dispersers and divergence among populations also likely predates the extreme glacial-interglacial cycles in the past 700,000 years. In contrast, timings of divergences among New Mexico ridge-nosed rattlesnake populations were much more recent (i.e., Holocene). In the European alpine salamander (*Salamandra alta*), which, like *A. hardii*, is completely terrestrial and found in mixed coniferous forest, pre-Pleistocene divergence times were estimated among some populations (Ribon et al. 2001).

Divergence time estimates suggest that the Capitan population is the oldest, which is consistent with the presence of more divergent haplotypes in this population. In contrast, the Sacramento Mountains population has a star-like arrangement of haplotypes, which is consistent with a younger and expanding population. It has been shown in numerous taxa that colonizing lineages typically have lower genetic diversity due to the small number of founding organisms comprising only a subset of the haplotype variation found in the ancestral range (Templeton 1998). Grant and Bowen (1998) found that small, but rapidly expanding, founding populations had high gene diversity but low nucleotide diversity, as was observed for the Sacramento Mountains population. Nucleotide diversity is affected by the size of the ancestral population more severely than the number of variable sites (Tajima 1989). Several other statistics (Fu's F_s and R_2) also support historical expansion of the Sacramento population. Higher gene diversity in this population is also consistent

with the apparently higher abundance (and presumably higher genetic effective population size) of *A. hardii* in the Sacramento Mountains. Indications of haplotype frequency differences among sampling sites within the Sacramento Mountains suggests that further examination with microsatellites may reveal finer scale structure. Within the Capitan Mountains, genetic information suggests a distinct break between the East and West mountain populations. This is not surprising as West mountain is separated from the rest of the mountain range by the lower elevation Capitan Pass. Interestingly, the habitat occupied by *A. hardii* differs between the East and West mountain populations. Specifically, the East mountain samples were collected in the vicinity of talus slopes and most often from under rocks and this kind of habitat was not available at West Capitan. Although only relatively few samples were collected from the West mountain population, they were all monomorphic for a single haplotype. This suggests reduced diversity and possibly low effective population size in this population. Further sampling in this area is warranted to monitor the status of the population and to further characterize diversity. In contrast, samples from the East mountain were collected from a single talus slope and surrounding forest, yet numerous divergent haplotypes were identified. Additional surveys on East mountain may also be warranted to characterize diversity at additional locations, if other survey sites exist.

The White Mountains population had low levels of genetic diversity. Tajima (1989b) found that the number of variable sites is influenced more strongly by the current size of the population, than is the sequence diversity. Also, preliminary data from a microsatellite locus had a higher value for the inbreeding co-efficient (F_{IS}) for the White Mountains population than either the Sacramento or Capitan Mountains populations. This result was somewhat surprising as at least one locality (Ski Apache) had high local abundance of *A. hardii*. Reduced genetic diversity and relatively high local abundance in this population may reflect a recent population bottleneck and subsequent population recovery. The high severity Little Bear Fire in 2012 affected salamander habitat and perhaps reduced salamander abundances in the White Mountains.

Management Implications

Phylogenetic information can identify genetically distinct units that may warrant protection and as such are useful for making informed conservation decisions. Based on the data presented here, we recommend that *A. hardii* be recognized provisionally as three evolutionarily significant units corresponding to each of the inhabited mountain tops (Capitan, White, and Sacramento). This

recommendation is based on several lines of evidence including: (1) substantial divergence and reciprocal monophyly of a mitochondrial gene, which suggests isolation of lineages for 1-2 million years with no evidence of gene flow; (2) nuclear data (from allozymes [Pope and Highton 1980] and a microsatellite locus) that also suggest separation; and (3) a disjunct distribution, which means that population dynamics within each unit are completely independent of those in other units. The degree of divergence between populations is similar to that observed between other *Aneides* sp. populations that have also been designated as ESUs (e.g., Reilly et al. 2012). However, additional data from nuclear markers would be informative. For example, there are some cases of discordance between nuclear and mitochondrial markers on the location of breaks between populations. In black salamanders (*Aneides flavipunctatus*), data suggested that the mitochondrial boundary between two populations remained stable while there was nuclear gene flow from south to north. This pattern would occur if there was male-biased northward dispersal (i.e., males dispersed further than females), which would carry new nuclear genes into a region but not new mitochondrial haplotypes (e.g., Reilly et al. 2012). *Ensatina* sp. salamander males have also been shown to disperse nearly twice as far as females (Staub et al. 1995). It may also be prudent to conduct a geometric morphometric analysis of museum specimens collected from across the range of *A. hardii* to determine if there are any differences among them. However, it has also been shown that in plethodontid salamanders, there can be extreme morphological stasis, such that the same morphology has been maintained over long periods of time encompassing dramatic climatic change (e.g., Wake 1983). Larson (1984, 1989) also demonstrated that in numerous salamanders, speciation had been decoupled from morphological evolution, specifically there have been more speciation events than morphological innovations (Highton 1990). Hence, absence of morphological divergence would not negate the recommendation to treat *A. hardii* as three distinct ESUs.

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Table 1. Genetic diversity statistics for mtDNA cytochrome b and the microsatellite Plel111 by mountaintop.

	MtDNA-Cytb							**Microsatellite-Plel111**				
	n	N_{haps}	H_R	*h*	π	*k*	*S*	H_e	H_o	A_R	F_{IS}	P_A
Capitan	36	8	7.0	0.740	0.003	2.284	10	0.983	0.921	41	0.064	10
White	68	4	2.8	0.632	0.002	1.162	3	0.958	0.851	29.42	0.12	7
Sacramento	108	14	8.8	0.797	0.002	1.492	10	0.953	0.939	26.31	0.015	3

Sample size (n), number of haplotypes (N_{haps}), haplotype richness (H_R), haplotype diversity (*h*), nucleotide diversity (π), mean pairwise differences between sequences in the sample (*k*), number of segregating (i.e., variable) sites (*S*), gene diversity (H_e), observed heterozygosity (H_o), allelic richness (A_R), inbreeding co-efficient (F_{IS}) and number of private alleles (P_A).

Table 2. Population expansion statistics (and associated p-values) calculated from cytochrome b among mountain ranges. Asterisks denote significant values.

	Sudden demographic expansion		Spatial Expansion	
	τ	R_2	SSD	R_2
Capitan	4.742	0.1	0.041	0.100
p-value		0.325	0.331	0.585
White	2.156	0.115	0.019	0.115
p-value		0.321	0.348	0.593
Sacramento	1.492	0.058	0.008	0.058
p-value		0.158	0.102	0.027*

Tau (τ); Ramos and Onsins and Rozas (2002) raggedness statistic (R_2) (significant values are indicative of population expansion); and sum of squared deviations (SSD) between the observed mismatch distribution and the expected distribution under a sudden demographic expansion model. Significant results support population stability.

Table 3. Sample size (n) and haplotype frequencies by collection locality.

	CAPITAN		WHITE		SACRAMENTO			
	West	East	Big Bear	Ski Apache	Observatory	Rio Penasco Rd	Russia Canyon	Timberon
Haplotype	n=6	n=30	n=25	n=43	n=29	n=26	n=28	n=25
1	-	-	-	-	-	0.577	-	-
2	-	-	-	-	-	0.115	-	-
3	-	-	-	-	0.034	-	0.357	-
4	-	0.267	-	-	-	-	-	-
5	-	-	0.400	-	-	-	-	-
6	-	-	0.600	0.442	-	-	-	-
7	-	-	-	-	-	-	-	0.040
8	1.000	-	-	-	-	-	-	-
9	-	0.033	-	-	-	-	-	-
10	-	0.033	-	-	-	-	-	-
11	-	-	-	0.512	-	-	-	-
12	-	-	-	-	-	-	-	0.040
13	-	-	-	-	0.828	-	0.500	0.200
14	-	-	-	-	-	-	-	0.160
15	-	-	-	-	-	-	-	0.520
16	-	-	-	-	-	-	-	0.040
17	-	-	-	0.047	-	-	-	-
18	-	-	-	-	0.034	0.192	-	-
19	-	-	-	-	-	0.115	0.036	-
20	-	-	-	-	0.103	-	-	-
21	-	-	-	-	-	-	0.071	-
22	-	-	-	-	-	-	0.036	-
23	-	0.533	-	-	-	-	-	-
24	-	0.067	-	-	-	-	-	-
25	-	0.067	-	-	-	-	-	-
26	-	0.033	-	-	-	-	-	-

Table 4. Fu and Li's D*, Tajima's D, and Fu's F_S and associated p-values. Asterisks denote significant p-values ($p < 0.05$).

	Fu and Li's D*	p	Tajima's D	p	Fu's Fs	p
Capitan	-0.294	0.371	-0.161	0.494	-0.499	0.45
White	0.861	0.746	1.668	0.944	1.603	0.816
Sacramento	-0.769	0.237	-0.546	0.337	-6.605	0.005*

Table 5. Pairwise ϕ_{ST} among collection sites calculated based on haplotype frequencies. All values were highly significant (p=0. 00001); asterisk indicates a p-value of 0.002.

	East Capitan	West Capitan	Big Bear	Ski Apache	Observatory	Rio Penasco	Russia Canyon	Timberon
East Capitan	-							
West Capitan	0. 514	-						
Big Bear	0. 407	0. 634	-					
Ski Apache	0. 389	0. 580	0. 280	-				
Observatory	0. 502	0. 756	0. 598	0. 553	-			
Rio Penasco	0. 347	0. 552	0. 436	0. 414	0. 532	-		
Russia Canyon	0. 342	0. 543	0. 429	0. 408	0. 173*	0. 365	-	
Timberon	0. 318	0. 518	0. 407	0. 388	0. 409	0. 343	0. 265	-

Table 6. Pairwise F_{ST} calculated from microsatellite data between mountain tops (lower left diagonal); p-values are shown on the upper right diagonal.

	East Capitan	**West Capitan**	**Big Bear**	**Ski Apache**	**Observatory**	**Rio Penasco**	**Russia Canyon**	**Timberon**
East Capitan	-	0.057	0.002	0.001	0.000	0.000	0.000	0.003
West Capitan	0.018	-	0.087	0.017	0.142	0.004	0.159	0.177
Big Bear	0.019	0.028	-	0.000	0.008	0.000	0.009	0.115
Ski Apache	0.016	0.044	0.034	-	0.000	0.008	0.000	0.000
Observatory	0.022	0.016	0.020	0.024	-	0.001	0.060	0.003
Rio Penasco	0.031	0.060	0.047	0.018	0.026	-	0.011	0.000
Russia Canyon	0.020	0.017	0.020	0.023	0.009	0.019	-	0.000
Timberon	0.020	0.017	0.021	0.023	0.009	0.039	0.024	-

Figure 1. Map of sampling areas color coded by mountain top: Green is the Capitan Mountains population, Red is the White Mountains population, and Blue is the Sacramento Mountains population. Pink dots indicate potential survey areas for *A. hardii*.

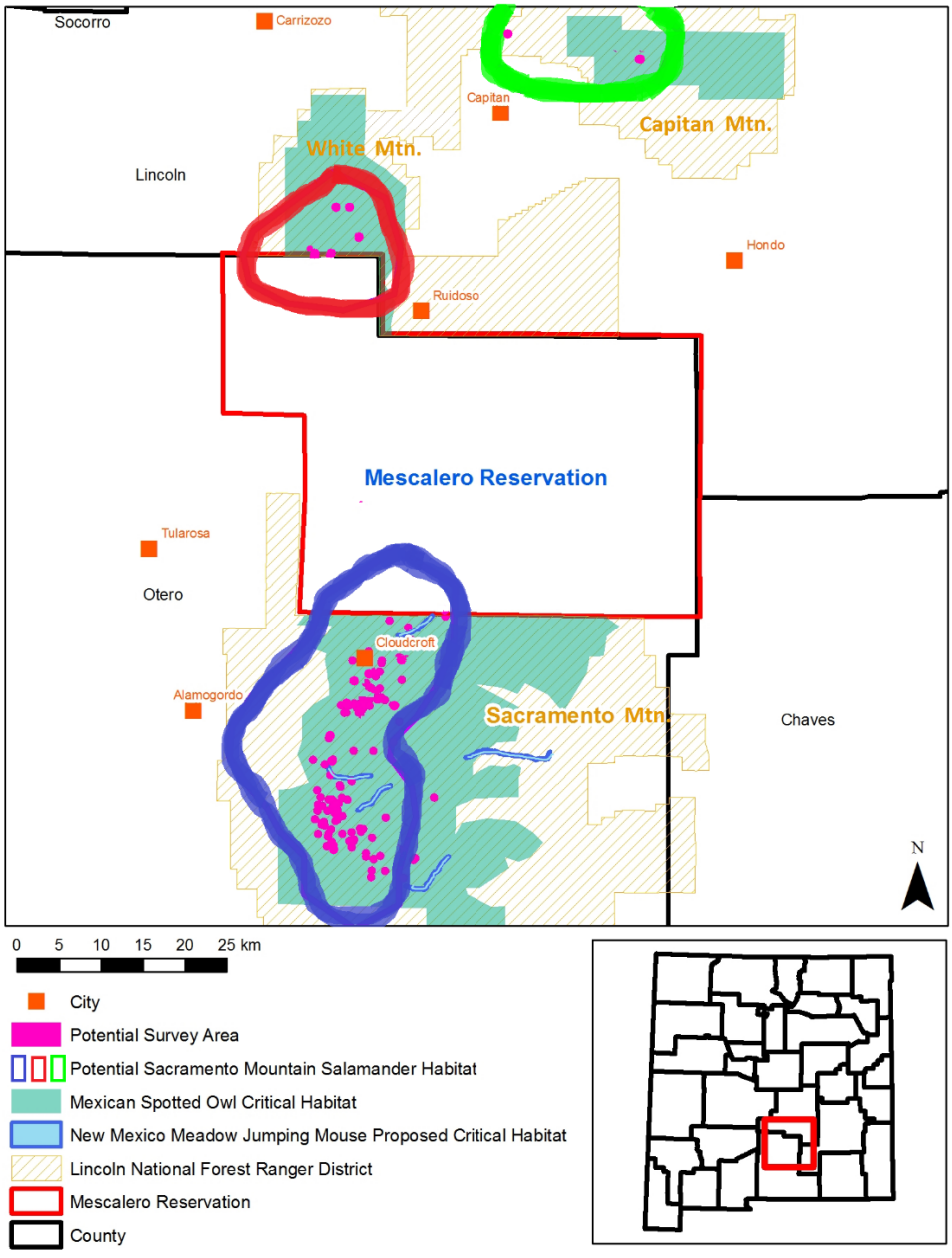

Figure 2. Frequency distribution of pairwise number of differences between individual sequences. A. All populations, B. Capitan, C. White, and D. Sacramento Mountains. Smooth line is the model fitted to the data and the dashed line with circles is the observed data.

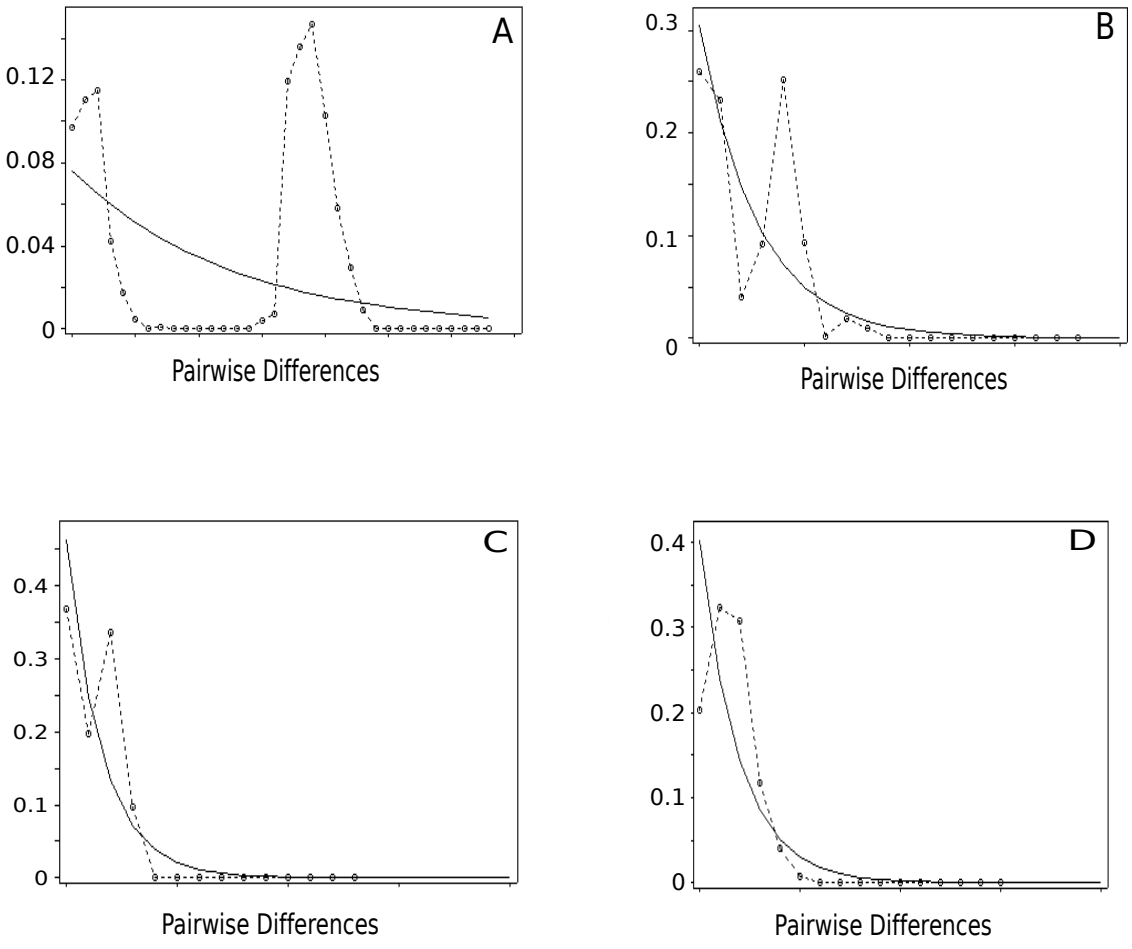

Figure 3. Haplotype network by mountaintop (Green- Capitan Mountains, Red- White Mountains, Blue- Sacramento Mountains). Dots between large circles represent nucleotide substitutions. Numbers within the large circles refer to the haplotype number (Table 3). Circle size reflects haplotype frequency. The numbers by the lines between mountain top lineages refers to the number of nucleotide substitutions. Different shades of gray represent distinct sampling sites (identified in Figures 4-6).

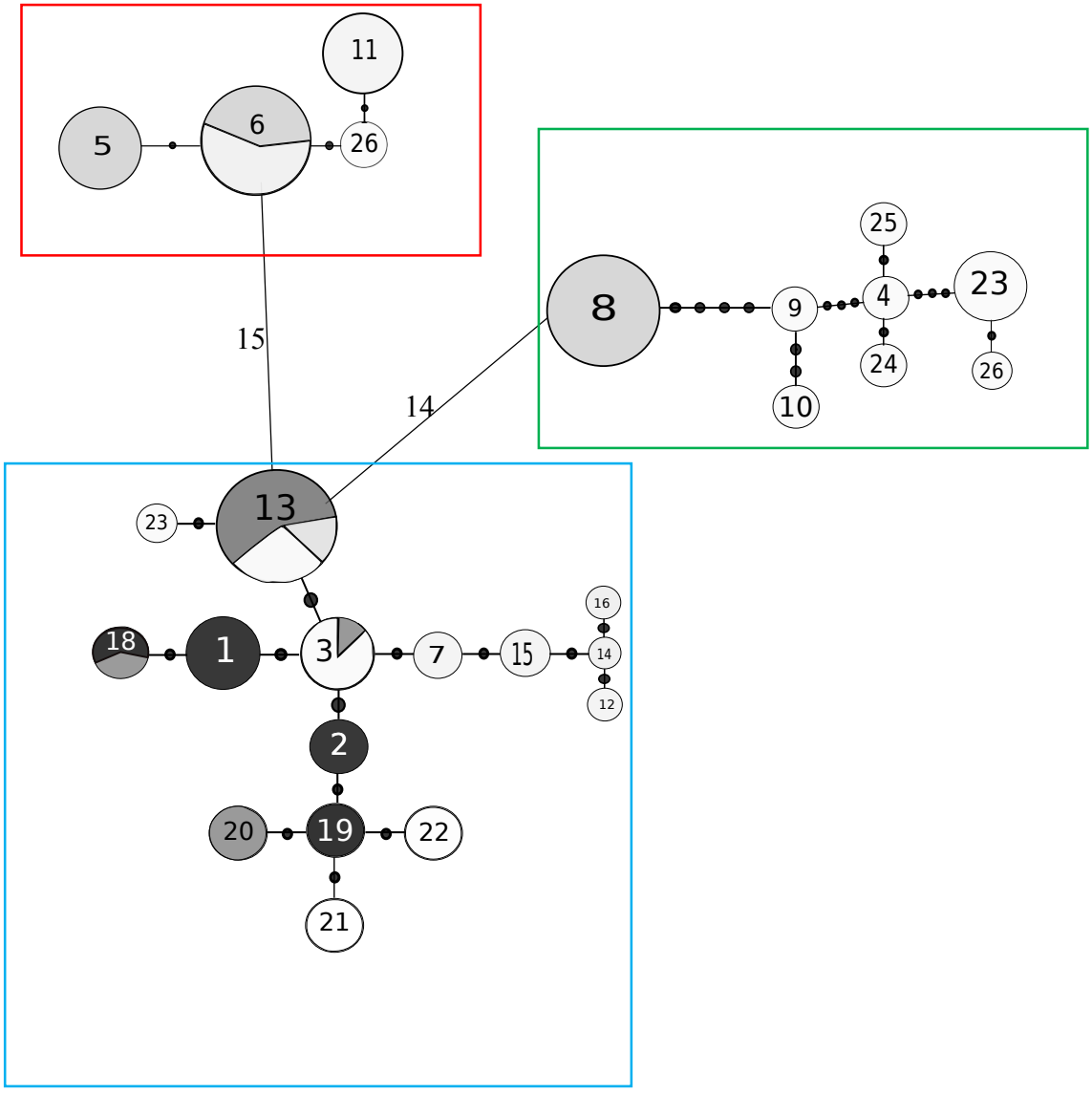

Figure 4. Capitan Mountains haplotype network color coded by sampling locality. Dots between large circles represent nucleotide substitutions. Numbers within the large circles refer to the haplotype number. Circle size reflects haplotype frequency.

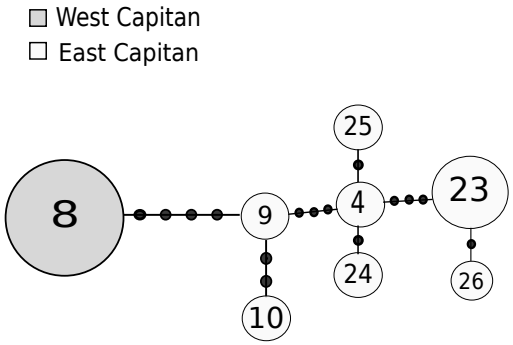

Figure 5. White Mountains haplotype network color coded by sampling locality. Dots between large circles represent nucleotide substitutions. Numbers within the large circles refer to the haplotype number. Circle size reflects haplotype frequency.

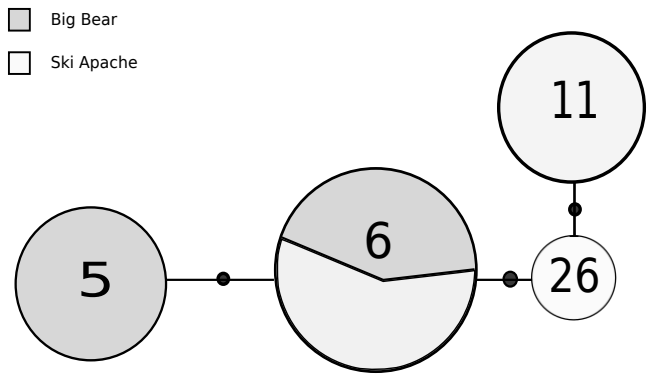

Figure 6. Sacramento Mountains haplotype network color coded by sampling locality. Dots between large circles represent nucleotide substitutions. Numbers within the large circles refer to the haplotype number. Circle size reflects haplotype frequency.

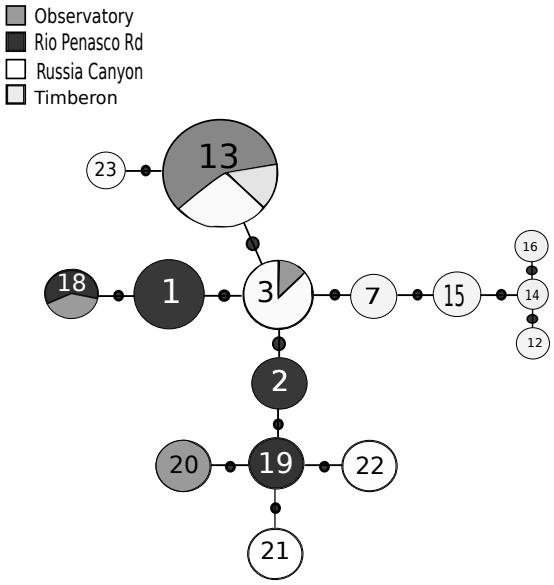

Figure 7. *Aneides hardii* divergence time (in millions of years) between mitochondrial lineages (Capitan Mountains- Green, White Mountains- Red, and Sacramento Mountains- Blue) with 95% confidence intervals shown by the branch nodes. Haplotype numbers are also shown. The outgroup is not shown on the figure.