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Population Genetics of the Western Toad (Bufo boreas) in the Central Valley of California

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POPULATION GENETICS OF THE WESTERN TOAD (*BUFO BOREAS*) IN THE CENTRAL VALLEY OF CALIFORNIA

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By

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ABSTRACT

A worldwide decline in amphibian populations has intensified the need for data comparing the influence of habitats on population dynamics and the potential for local extinction. From a conservation perspective it is important to understand the connections between ecology, geography, and genetics across landscapes that are increasingly affected by human influences and other uncontrollable environmental events such as climate change. The purpose of this study is to examine the landscape-level genetic patterns of Western toads, *Bufo (Anaxyrus) boreas*, and to conclude if gene flow is occurring between ponds. This will allow conservation practitioners to understand geographic features that might impede connectivity among ponds, and increase the risk of extinction. For this study, 143 individuals from 12 ponds in the Central Valley of California were genotyped using 10 microsatellite loci. Population genetic software (e.g., GENEPOP, STRUCTURE) was utilized to describe the genetic diversity of populations and to evaluate hypotheses relating to population structure. Results indicate ample genetic variation across the study landscape, high levels of gene flow between ponds, and a lack of a strong distance effect on genetic structure. The data suggest that over short timescales (one breeding season) western toads are resilient to low levels of disturbance to habitat adjacent to breeding ponds. These data are important, considering the potential for increasing anthropogenic stress faced by these populations.

Keywords: *Bufo boreas,* Western toad, population genetics, amphibian metapopulations, landscape genetics, gene flow, population structure

Dedicated to mom and dad.

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VITA

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Major Field: Biology

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CHAPTER ONE

INTRODUCTION

Recent research has demonstrated that there has been a steep global decline in amphibian populations in the past 20 years (Houlahan *et al.* 2000, *Stuart et al.* 2004, Adams *et al.* 2013). This pattern continues to be accelerating and conservation biologists project this to have dramatic effects on amphibian population size in the future (Adams *et al.* 2013). This observation has raised concern regarding the potential for dramatic reductions in amphibian biodiversity. It has been estimated that over one third of amphibians are threatened with extinction, and that amphibians are continuously declining more rapidly than either mammals or birds (Stuart *et al.* 2004). Many biologists consider amphibians to be prime indicators of environmental change and contamination (Blaustein 1994) because they rely on aquatic as well as terrestrial environments. Therefore, their rapid decline is alarming in the field of conservation biology.

Declines in amphibian populations are of immediate concern because many of them are even occurring in areas that remain relatively undisturbed by humans, such as national parks, conservation areas, and rural areas (Blaustein *et al.* 2007). Why is this happening? What is causing the declines? How can we reverse the trend and prevent the loss of amphibian species diversity? Finding the answers to these questions has occupied scientists since the first reports of the enigmatic amphibian decline phenomenon. Many hypotheses have been proposed, including habitat modification or destruction,

environmental pollutants, UV radiation, viral, fungal, or parasitic infection, and introduced species (Nichols *et al.* 2001, Bosch *et al.* 2007; Olson *et al.* 2013).

On a global scale, no single cause appears responsible for every observed decline in amphibian populations. However, a general consensus has emerged: Habitat loss is a persistent threat to populations, but in areas in which habitat loss is not a factor, emerging diseases, climate change, and other ecological processes that limit connectivity are the most severe threats to amphibians.

The purpose of this study is to contribute to continued efforts to understand the effects of habitat modification, a major factor in the decline phenomenon. Habitat loss or modification can result in the extinction of local populations, and contribute to regional declines by inhibiting movements between populations that typically can "rescue" declining populations or recolonize extirpated sites. Conservation genetics in this context involves making connections between ecology, geography, and genetics across landscapes. This study uses a landscape genetic framework to understand the role of habitat in affecting movements between populations. Landscape genetics is an interdisciplinary field that evaluates the connectivity of alternative habitat types in a metapopulation framework (Manel 2003).

Metapopulations are a series of discrete populations that are connected by dispersing individuals (Hanski and Gilpin 1991). Understanding the interactions among populations is important from a conservation genetic perspective because gene flow helps to maintain genetic diversity and buffers against local extinctions (Cushman *et al.* 2006). Overall, populations with high genetic diversity are better suited to respond to deterministic environmental perturbations (Cushman *et al.* 2006), such as habitat

modification driven by climate change or urbanization. High genetic diversity means that a population has more raw materials to muster an evolutionary response to contemporary circumstances that might otherwise cause the population to go extinct. In order for genetic diversity to be maintained, individuals within the landscape must not be prevented from dispersing to neighboring ponds and sharing novel variation that arises via mutation. Factors that could inhibit dispersal frequency include natural geographic barriers (e.g., rivers, mountains), and man-made structures (e.g., roads, buildings).

This study will use multilocus genetic data to infer patterns of movements among populations and, in conjunction with geographic information, draw conclusions as to which populations are interbreeding and what may be inhibiting others from doing so. This is vital to future research that monitors the growth/decline in these populations and attempts to ultimately inhibit future amphibian declines (Semlitsch 2000, Jenkins *et al.* 2006). Specifically, the goal of this study is to evaluate the pattern of genetic structure in the Western Toad, *Bufo boreas*, with respect to geographic isolation and ultimately, to specific habitat discontinuities.

Samples were collected from three adjacent protected areas – Blue Oak Ranch Reserve, Rancho Cañada de Pala, and Joseph D. Grant County Park in Santa Clara County, California (Fig. 1). *Bufo boreas* was chosen due to recent data indicating that this species is declining in parts of its range, specifically the Rocky Mountain areas (Addis 2013). What are the potential causes of the decline of this species? Researchers believe the fungal pathogen *Batrachochytrium dendrobatidis* is contributing to this decline (Olson *et al.* 2013), but human impact is another potential contributing factor. The development of land for roads and infrastructure over areas where amphibians live or

migrate affects the probability of successful dispersal between sites. Habitat modification such as draining of wetlands, clear-cutting of forests and vegetation removal are all examples of modifications that appear to be affecting Western Toad populations (Hammerson *et al.* 2004). This study will determine the status of Western Toads in California's Central Valley, a region with a set of anthropogenic pressures that potentially overlaps with declining populations in the Rocky Mountains.

CHAPTER TWO

METHODS

Study Species

Bufo boreas is classified as a sensitive species and is a candidate for listing under the Endangered Species Act (Keniath *et al.* 2005). Therefore, conservation research on this species is currently of high importance. In addition, *B. boreas* toads are an ideal species to test for landscape effects on population connectivity because of their ability to thrive at a wide range of environmental conditions (Hammerson *et al*. 2004). Their dry, rough epidermis allows them to prosper in terrestrial environments in proximity to bodies of water for breeding (Bailey *et al.* 2005). The prime breeding season of *B. boreas* is from February to April immediately following winter hibernation. Their movements tend to be terrestrial and limited after breeding season (Schmetterling *et al.* 2008). Females breed in only one pond per breeding season, so sampling of tadpoles represents the yearly partitioning of genetic diversity across ponds. The larval period is typically three months and tadpoles metamorphose in early summer (Bailey *et al.* 2005).

Study Sites and Sample Collection

Samples were collected from 12 ponds at Rancho Cañada de Pala, Blue Oak Ranch Reserve, and Joseph D. Grant County Park in Santa Clara County, California (Fig. 1). Ponds were visited during the spring subsequent to reproduction and prior to

metamorphosis. Tadpoles were collected via seine or dip net and placed into small containers of ~95% ethanol. Upon returning to the lab, samples were placed in individual 2.0 ml microcentrifuge tubes with fresh ethanol and assigned a unique number.

DNA isolation and PCR amplification

During transfer to microcentrifuge tubes, tail tissue (~10mg) was excised from up to 12 tadpoles from each pond. Tissue samples were manually macerated and then placed in a 1.5 mL microcentrifuge tube containing 300 mL of cell lysis solution and 1.5 μL of Proteinase K (20mg/mL). The contents of the tube were vortexed and incubated 3-6 hours at 55^{o}C. Then, 0.6 μL of RNAase A (10mg/mL) was added and the solution was incubated again for 30-45 minutes at 37^{o}C. Once the sample was cooled to room temperature, 100 μL of protein precipitation solution was added to the cell lysate mixture followed by centrifugation (Eppendorf 5415 R) at 1300 rpm for 5 minutes, resulting in a tight pellet formation at the bottom of the tube. After pouring off the supernatent (leaving the pellet behind), 300 μL of 100% isopropanol was added and centrifuged. Then, 300 μL of 70% ethanol was added and centrifuged. The isolated pellet was allowed to air-dry overnight. DNA was rehydrated using 100 μL of 1XTE buffer and incubated at 65^{o}C for one hour. DNA content was determined using NanoDrop© NC-1000 Spectrophotometer. Samples that contained less than 2ng/ul were excluded from the study.

One hundred forty three samples from 12 ponds were extracted and amplified using 10 microsatellite loci developed for the study species (Table 1). Microsatellites are short sequences of nucleotide repeats (Morgante and Olivieri 1993) used in genetic studies for reasons such as higher mutation rate (Li *et al.* 2004) and putative selective

neutrality (Coughlan 2008). Overall, high variation is expected in microsatellite alleles at the landscape level.

The polymerase chain reaction (PCR) was used to amplify the microsatellite loci from genomic DNA of each individual. All PCR reactions followed a recipe of: 5.0 μL of GoTaq MasterMix, 3.4 μL of dnase-free water, 0.2 μL of the forward primer, 0.2 μL of the reverse primer, and 0.2 μL of the fluorescent dye primer per tube. Each locus was labeled with one of four fluorescent dyes: NED (yellow), PET (red), FAM (blue), or VIC (green). Colors were assigned based on expected allele sizes from the literature (Simandle *et al.* 2006). Our objective was to multiplex many loci during downstream applications (e.g., Genescan, see below), therefore assignment of particular fluorescent dyes to specific loci was important. Loci that have very distant size ranges were assigned the same color. This was acceptable because in the analysis, like colors would not overlap. Using this method, several loci were multiplexed into one submission plate, improving the efficiency (in terms of speed and cost) of results.

The PCR protocol was as follows: initial denaturation at 96 ^{o}C for 1 min, 35 cycles of 1) DNA denaturation at 94^{o}C for 1 min, 2) primer annealing at 55^{o}C for 30 seconds, and 3) new strand extension at 72^{o}C for 45 sec, followed by a final elongation at 72C for 5 minutes. The successful amplification of primer pairs was assessed for each locus using ~1% agarose gel electrophoresis. Based on band strength, post-PCR DNA was diluted, allocated to a submission plate, and dried. Submission plates were either shipped to the University of Georgia Genome Facility or run at the WKU Biotechnology Center for genotyping using Genescan®. The genotyping process produces the

multilocus genotypes of samples and allows us to evaluate the differences between individuals, ponds, or genetic clusters.

Genotype Scoring

Raw data generated by Genescan were imported into GeneMapper (Applied Biosystems, Foster City, California), which visualizes peaks of fluorescence that represent the sizes of microsatellite alleles. Peaks were automatically scored and placed into bins, and were manually reviewed, and edited. Locus BBR16 was removed from the study at this point due to insufficient raw data. A data table of raw genotypic information for each individual was exported from GeneMapper and used for all subsequent population genetic applications.

Null Alleles

Microchecker (Oosterhout *et al.* 2004) is a software package that determines if null alleles are present in the data set. Nonamplified (null) alleles are alleles that fail to amplify during PCR. Null alleles may be common when using microsatellite DNA markers (Dakin *et al.* 2004). It is useful to determine the presence of null alleles because they may cause individuals to be scored as homozygous instead of heterozygous (Callen *et al.* 1993). Unfortunately our data matrix contained too many missing values to fully implement the Microchecker software. However, recent evidence suggests that when performing genetic assignment tests (using STRUCTURE for example, see below), null alleles are not detrimental to the overall outcome of the project (Chapuis *et al.* 2007).

Therefore, analysis continued despite the potential for the presence of undetected null alleles in the data.

Evaluation of Hardy-Weinberg Equilibrium

GenAlEx (Peakall and Smouse 2008) is a Microsoft Excel add-in for working with population genetic data. GenAlEx was used to generate allele frequency data and to calculate geographic distances between sites based on decimal lat/lon data. Sampling sites were georeferenced in Google Earth (Google Inc, Santa Clara, California) and incorporated into our raw genotype data file in GenAlEx.

Genepop (Raymond *et al.* 1995) is another population genetics program that was used for three separate purposes: 1) To compute exact tests for Hardy-Weinberg equilibrium (HWE); 2) To compute estimates of differentiation between populations (Fst); and 3) To convert the input Genepop file to formats used by other programs (Raymond et al 1995). Analyzing samples for deviations from HWE is a recommended practice for population-based genetic studies (Chang *et al.* 2013). HWE states that genotype frequencies will match expectations derived from allele frequency masurements, and that allele frequencies will remain unchanged in a population throughout generations in the absence of evolutionary influences such as selection, genetic drift, mutation, and gene flow. Determining if our samples are in HWE will allow us to decide if our microsatellite loci or populations are under the influence of evolutionary forces. Our expectation is that loci will be in equilibrium, but that sampling sites may show deviations based on recent population histories.

Genetic Assignment Tests

The program STRUCTURE (Pritchard *et al.* 2000) was used to provide an unbiased grouping of individuals into clusters based on multilocus genetic composition. STRUCTURE assigns individuals into clusters assumed to be under HWE based on genotypes and allele frequencies (Pritchard *et al.* 2000). STRUCTURE was told which value of K (i.e., number of genetic clusters) to assume, and the program iteratively assigned each individual to a cluster using Markov Chain Monte Carlo (MCMC) methods. The MCMC was performed for 100,000 iterations, and replicated for each value of K (from K=1 to K=10) five times. The program provided bar graphs for different K values. The data from the STRUCTURE output is color coded to depict each genetic cluster (i.e., population), with each column representing an individual. The purpose of evaluating alternative values of K in this way is to detect patterns of genetic structure without any bias based on physical sampling site or other preconceptions regarding the data. By replicating STRUCTURE runs for each potential value of K, the most likely "true" value for K may be ascertained.

Next, STRUCTURE data were exported into HARVESTER (Earl and vonHolt 2012). HARVESTER is used to determine the optimal number of populations for a given data set. HARVESTER plots the mean log-likelihood values ($\pm$ SD) for the five replicate runs of 100,000 iterations for each K value. These data statistically support particular values of K by computing likelihood values and graphically representing the accuracy and consistency of numerous K values on a single plot. In addition, HARVESTER generated another graph that utilized the Evanno *et al.* (2005) method for detecting the most likely value of K.

Isolation by Distance

Geographic and genetic distance matrices created in GenAlEx, were imported into the program IBD (Isolation by Distance, Bohonak 1999, Jensen *et al.* 2005) to determine if there was a strong correlation between genetic distribution and geographical distribution. Genetic distance data consists of pairwise Fst values. These data provide estimates of gene flow between populations by comparing variance in allele frequencies of pairs of subpopulations to the variance observed in the total population (i.e., all subpopulations). When compared to geographic distances between ponds, Fst values can provide an overview of the relationship between geographic distance and gene flow.

IBD performs a Mantel test to calculate the correlation between genetic and geographic distance matrices and generate an R-value, which exemplifies the strength of the correlation.

CHAPTER THREE

RESULTS

Evaluation of Hardy-Weinberg Equilibrium

In this study, 141 individuals were successfully genotyped across 12 ponds using 10 microsatellite loci (Table 1). Raw data were input into Genepop, which calculated the probability of their consistency with HWE using Chi-squared tests (Table 2). The HWE expectations were not consistent with expected results. Only loci 86, 87b, and 281 appeared to be in HWE, and Ponds 1, 4, and 5 exhibited HWE values as well. Across all ponds and populations, our data do not appear to be in HWE.

Genetic Assignment Tests

From the determined K=2 value, sampling sites were "re grouped" into two large clusters (Fig. 2) based on genetic similarity, instead of viewing the 12 sampled ponds as discrete genetic entities. Ponds 1-7 and 9 belong to one cluster that appears to be in HWE while ponds 8,10,11, and 12 comprise a second cluster in HWE (Figure 2). The distinct coloration of the bar plots suggest that two distinct genetic clusters exist, one cluster represented by the "red" coloration and the second cluster represented by the "green" columns. Each cluster is comprised of several sampling sites that share similar genetic variation, suggesting that the individuals are dispersing between ponds within clusters regularly.

The HARVESTER results support the pattern that is obvious in the STRUCTURE plots (Fig. 3). The K=2 data point demonstrates a high mean log-likelihood value with very little variation among replicate runs (i.e., small error bars). Furthermore, the Evanno et al. (2005) Delta K method clearly demonstrates a distinct peak for K=2 (Fig. 4).

Isolation by Distance

Populations tend to become more differentiated from each other as geographic distance increases (i.e., IBD), and a strong positive correlation between genetic and geographic distance is typically expected. However, over small spatial scales or under circumstances when connectivity among populations is high, a significant isolating effect of distance might not be observed. We did not detect a significant effect of distance on genetic isolation between ponds (Fig. 5). From the Mantel test, we found R= 0.042 indicating a lack of fit to a strict IBD model, and no significant correlation between geography and genetic relatedness on this landscape.

However, pairwise distances were not uniformly low and high genetic distance did occur when comparisons were made between ponds in separate genetic clusters. Table 3 shows pairwise Fst values, the red columns representing the “red” cluster and the green columns representing the “green” cluster. In addition, tan areas indicate the ponds areas of overlap between the clusters. These tan areas were where all of the Fst values >0.1(indicated in bold) appeared to fall. The two clusters from STRUCTURE match up with the pattern observed in Fst value.

CHAPTER FOUR

DISCUSSION

Overall, the purpose of this study was to determine if gene flow is occurring between ponds in the selected area and interpret the movement patterns of *B. boreas* based on the observed genetic structure. Since gene flow plays a critical role in the longevity of a population and the success of a species, the results of this study are relevant to aiding in efforts to stop the ongoing amphibian decline. The integration of population genetics into the practice of conservation and management is in its infancy, therefore any data generated are useful in the pursuit of understanding why amphibian populations are declining and ultimately preventing future extinctions.

The loci used in this study showed numerous deviations from HWE. This is unexpected because microsatellites loci are supposed to be inherited as selectively neutral markers. The deviations could be due to one or more reasons. First, the microsatellite sequences of DNA used could be physically linked to protein coding loci that are under selection. Second, the data could suffer from abundant null alleles. Unfortunately Microchecker was unable to confirm or refute the existence of null alleles due to large amounts of missing data (see 'Future Directions' below). Either of these reasons (and potentially others) could lead to genotype and allele frequencies that deviate from HWE. Many populations also showed deviations from HWE. The reasons for this include non-

random mating (i.e., inbreeding), potentially resulting from recent population bottlenecks, or large fluctuations in effective population size. Furthermore, small effective population sizes can lead to large effects of genetic drift, which could also lead to deviation from HWE expectations. Taken together the observed deviations from HWE render it difficult to draw firm conclusions regarding the genetic structure of these populations. However, some patterns were very strong (e.g., the STRUCTURE data), and may prove consistent with future results after the HWE issues have been resolved.

The *a priori* expectation was that the studied landscape would be free from serious barriers to gene flow because it consists of protected natural areas. Therefore, as a null hypothesis the prediction was that the sample sites would comprise a single panmictic metapopulation with large amounts of gene flow between most pairs of ponds. Additionally, while strong barriers might not exist, as a general rule, with increasing distance between ponds, the amount of gene flow between them would decrease and some degree of genetic structure would be apparent on this landscape. Interestingly, the data generated proved to follow a strong, unexpected pattern, with two distinct genetic clusters emerging. These clusters are illustrated in Figure 2, the red columns representing one cluster of genetically similar individuals and the green cluster representing the second cluster. However, the two distinct genetic clusters do not coincide with what would be expected on the map of the landscape. The expectation is for the clusters that have similar genetic information (sites 1-7, 9) and (8, 10-12) to be in close proximity to one another, or perhaps for the clusters to be separated by some obvious physical barrier like a road or other anthropogenic feature. This does not prove to be the case geographically. It is possible unknown barriers or particular corridors of dispersal are

determining the boundaries of the clusters. Alternatively, the patterns observed could be related to the deviations from HWE discussed above.

Within each cluster, the data demonstrate that gene flow between most ponds is sufficient to prevent the formation of complicated patterns of genetic structure, and strict isolation due to geographic distance alone is not occurring. Other studies have shown that *B. boreas* is known to distantly breed at distances as great as 6 km (Muths *et al.* 2003). Ponds 8 and 12 are a prime example of this. The two ponds are geographically distant yet contain more similar genetic consistency than some of the ponds they border. These results are consistent, across several different analysis methods, including evaluation of pairwise Fst values and genetic assignment tests. Determination of these clusters is useful because it visualizes the connections between sites where *B. boreas* tends to breed each season. Since the two clusters exhibit strong genetic similarity, they appear to be mating within these clusters each breeding season.

The subjects collected for this experiment were obtained from a protected area with minimal human disturbance and are believed to be traversing great distances across the landscape. Human impact, in the form of land development, agricultural practices, and pollutants are all suspects of the decline in other areas. When comparing the current results of this study to that of previous projects carried out in a similar fashion, the results appear to differ between experiments. In a *B. boreas* population genetics study performed by Addis (2009) in Glacier National Park, all of the loci used (which were identical to the ones in this study) showed no consistent deviations from HWE. In addition, there was a correlation between pairwise Fst values and distance in the Addis (2009) study, whereas no significant correlation was present in this study. For the Addis (2009) study, the two

study areas each exhibited a K=2 value, corresponding to high and low elevation groups. It was determined that the clusters were a result of elevation differences, a possibility that could be explored on my study landscape. Another experiment of amphibians in Alaska (Moore *et al.* 2011) demonstrated that *B. boreas* exhibit resilience to warming climate trends due to their ability to disperse across variable habitats and recolonize. This wide range of movements and selective clustering is also what was discovered in this study. However, in a study performed in Lassen County, CA (Manier *et al.* 2006), *B. boreas* exhibited low migration rates and limited gene flow. This opposes the results of my study, and the studies of Addis (2009) and Moore *et al.* (2011). Overall, the gene flow patterns of *B. boreas* appear to be dependent upon the area in which the toads were studied and the variation presented between studies is due to the different locations of study and the landscapes that make them up. Ultimately, it is important to conduct population genetics studies in a variety of landscapes so that general patterns regarding topographical features that may prohibit or encourage gene flow can be identified.

The results of this study are also useful for the conservation managers at the study sites in Santa Clara County, CA to raise awareness about where exactly gene flow is occurring. This is vital to the sustainability of the *B. boreas* population in this area. If the reserve chooses to pave an area, for example, it would be beneficial to select an area that would not be detrimental to the toad's expected migratory breeding patterns. In addition, future work would enable conservation biologists to monitor breeding of all amphibians in the area.

Future Directions

This research is a segment of a larger population genetics project that encompasses monitoring the gene flow of several different amphibian species. It is valuable to compare these data with other species to determine if there is any correlation between the movements of different groups in this area. Possible subjects include testing *Taricha torosa* and *Pseudacris regilla* on this landscape. These results can lead to two main conclusions from this entire population genetics project: 1. A unique pattern of breeding for the Western toad may be demonstrated that involves distant breeding between ponds or each season that differs from other amphibians in the area; 2. All amphibians in the area take part in very similar breeding patterns between ponds. It is vital, however, that future investigations of *B. boreas* occur in this area to determine a reason behind their distant breeding patterns before continuing to apply this to a larger scale.

In addition, further investigation of the study landscape is still critical to drawing a reasonable explanation for the unexpected breeding patterns exhibited in the results. Exploring geographical barriers that may be present could inhibit gene flow in certain areas, making long distances favorable for breeding. For example, there may be some geographical barrier that prevents neighboring ponds from breeding but clear paths, hazard free, that enable successful breeding in distant ponds. Other landscape features, such as hills, mountains, rivers, and rough terrain may also have an impact our results. Additionally, the geohydrology of each individual pond should be determined to ascertain if they dry up at certain points of the year and features such as their size, shape, common inhibitors, and clarity should be determined.

Since this study had considerable missing data, re-runs are still required to confidently conclude this particular experiment. The initial step of checking for null alleles was ineffective due to insufficient data. Thus, the presence of null alleles could potentially have had impacts on the results. By filling in missing data, checking for null alleles, and accounting for null alleles from in data, the loci used may have been more congruent with HWE and ultimately produce more reliable results. In addition, eliminating ponds and loci that continued to fail in their consistency with HWE would lead to further validating the results of this study. Much laboratory work, genetic analyses, and landscape application is still required before final completion of this project.

On a global scale, since *B. boreas* is able to effectively breed between distant ponds in our study area, thus increase their larval dispersal great distances, this research would be valuable to conservation biologists around the world to combat the amphibian decline. If we can modify environments to better enable amphibian breeding across the landscape and breed, then it is possible to apply this knowledge to preserving amphibians in areas where they are experiencing drastic declines. Carrying out similar population genetics studies on various amphibian species will present a big picture of breeding patterns specific to the studied area. Tracing the genetic patterns of other species will allow further conservation efforts. In addition, regulations and laws could be enforced in an effort toward population conservation if breeding patterns are clearly determined. Ultimately, this knowledge can aid in awareness and preservation of amphibian populations.

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APPENDIX OF FIGURES

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Table 1: Listing of microsatellite loci for *B. boreas* utilized in this experiment (from Simandle et al. 2005). Loci designated with a * were purchased with the forward primer attached, therefore fluorescent dye was not manually added to the recipe.

Locus	Motif	Expected Range (bp)
BBR17	[AAT]10	121-142
BBR29	[AAT]8	88-101
BBR36*	[TAGA]15	177-221
BBR86*	[AAT]8	149-165
BBR87b	[ATCT]13	134-255
BBR201	[AAT]8	106-148
BBR233*	[ATT]9	211-223
BBR281	[AAT]10	139-156
BBR292*	[AAT]8NAT[AAT]7	107-144
BBR293*	[AAT]10	145-167
BBR297*	[AAT]2AAATA[AAT]11	107-129

*Denotes use of incorporated fluorescent primer

Table 2: Determination of Hardy-Weinberg Equilibrium A) per locus and B) per pond using chi square tests. If $P < 0.05$ it was considered to be inconsistent with *HWE*.

A) Locus	P value	Consistent with HWE?
16	<0.001	No
17	0.6494	Yes
36	<0.001	No
86	0.0142	No
201	0.9997	Yes
233	0.2365	Yes
281	0.0001	No
292	<0.001	No
293	0.1599	Yes
297	0.8630	Yes
87b	<0.001	No

B) Pond	P value	Consistent with HWE?
1	0.0299	No
2	0.8593	Yes
3	0.0475	No
4	0.0004	No
5	0.0003	No
6	<0.001	No
7	<0.001	No
8	0.1925	Yes
9	0.0827	Yes
10	0.0352	No
11	0.1894	Yes
12	0.2812	Yes

Table 3: Pairwise Fst values (below diagonal) and log-transformed Euclidian geographic distances (m, above the diagonal). Comparisons between ponds within the "red" cluster (see Fig. 2) are filled red, comparisons within the "green" cluster are filled green, and comparisons between clusters are filled tan. Fst values >0.1 are indicated in bold.

pop	1	2	3	4	5	6	7	8	9	10	11	12
1	--	2.00	1.73	1.81	0.95	1.35	1.32	1.88	1.45	1.88	2.17	1.76
2	0.022	--	2.28	2.42	2.18	2.17	1.53	2.44	2.27	0.80	0.89	2.27
3	0.003	0.001	--	1.05	1.58	1.04	1.90	1.09	1.09	2.26	2.41	0.23
4	0.024	0.031	0.000	--	1.57	1.26	2.07	0.36	1.05	2.39	2.54	1.12
5	0.000	0.003	0.000	0.000	--	1.13	1.65	1.66	1.12	2.09	2.32	1.62
6	0.000	0.000	0.000	0.000	0.000	--	1.68	1.36	0.66	2.13	2.32	1.10
7	0.011	0.000	0.000	0.022	0.000	0.000	--	2.11	1.82	1.39	1.80	1.91
8	**0.189**	**0.180**	**0.164**	**0.171**	**0.167**	**0.148**	**0.196**	--	1.18	2.42	2.56	1.15
9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.177**	--	2.22	2.41	1.17
10	**0.134**	**0.153**	**0.164**	**0.168**	**0.125**	**0.105**	**0.167**	0.098	**0.152**	--	1.18	2.26
11	0.072	0.089	0.074	0.083	0.055	0.054	0.097	0.065	0.071	0.045	--	2.41
12	0.081	**0.101**	**0.105**	**0.107**	0.080	0.071	**0.106**	0.077	0.099	0.003	0.000	--

Figure 1: Sample Site and pond distribution for this study. Samples were collected from 12 ponds at Rancho Cañada de Pala (white), Blue Oak Ranch Reserve (red), and Joseph D. Grant County Park (green) in Santa Clara County, California. Note: red and green borders are for illustrative purposes only, and do not correspond to colors used in Fig. 2.

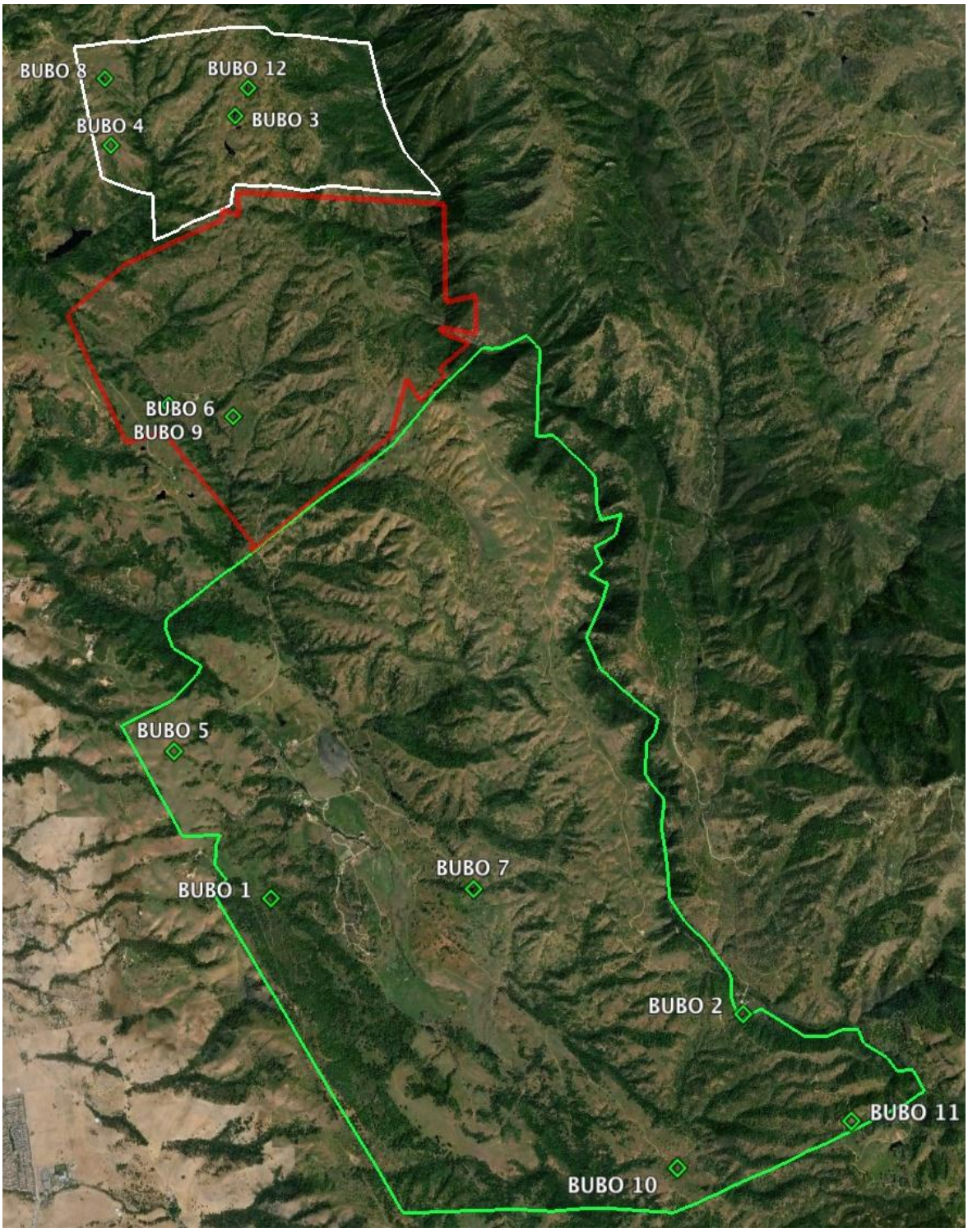

Figure 2: Bar plots from STRUCTURE (Pritchard et al. 2000). The data from the STRUCTURE output is color coded to depict each genetic cluster (i.e., population), with each numbered column representing a sampling site, and each sub-column representing individuals. The three graphs presented represent three potential K values: A) K=2 B) K=3 C) K=4.

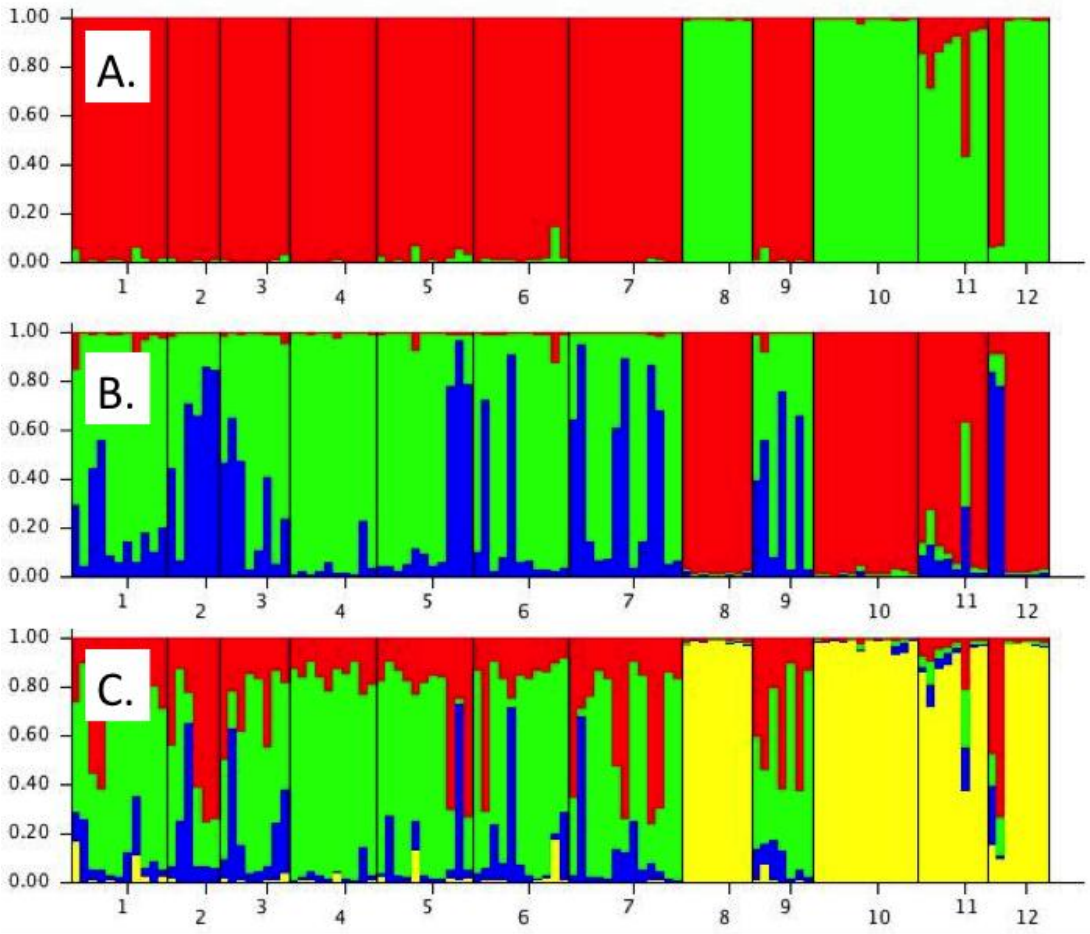

Figure 3: Mean log-likelihood values (± SD) for five replicate runs of 100,000 iterations for each K value. Favorable K values have a high mean log-likelihood value (blue) with little standard deviation (vertical gray lines). From this plot, K=1 and K=2 exhibit these two parameters.

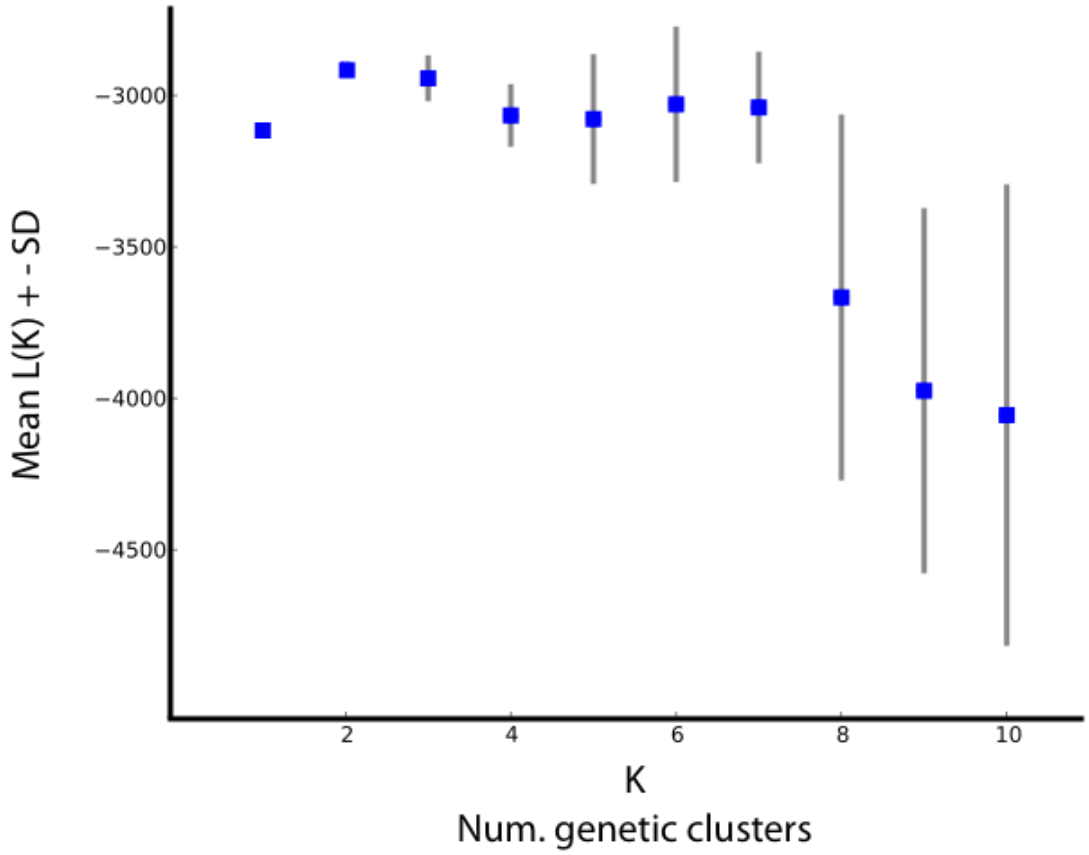

Figure 4: Evanno's (2005) Delta K for assessing the most appropriate K value for the given data set. Delta K takes the mean log-likelihood as well as the standard deviation into account. The high Delta K for K=2 demonstrates that it has a high likelihood and small SD when compared to K=1 or K=3.

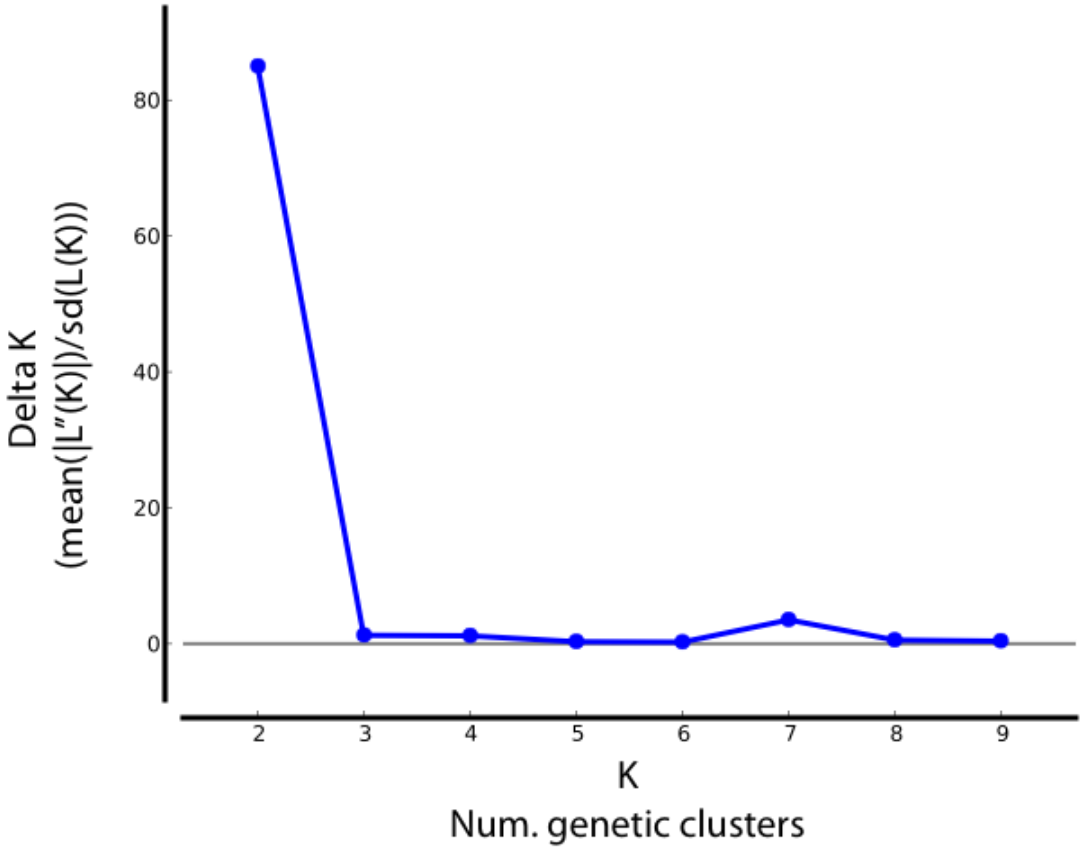

Figure 5: Isolation by distance (IBD) was plotted to determine if any significant correlation was present between geographic vs. genetic difference between ponds. From this plot, it is inferred that no strong correlation exists between the two matrices, which supports the random pond distribution on the study landscape.