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*Landscape genomics and population viability analysis of the Flat-tailed Horned Lizard (*Phrynosoma mcallii*) in California and Arizona*

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Landscape genomics and population viability analysis of the Flat-tailed Horned Lizard (*Phrynosoma mcallii*) in California and Arizona

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ABSTRACT

The Flat-tailed Horned Lizard (*Phrynosoma mcallii*) is a species of special conservation concern in California, Arizona, and Mexico. Much of this lizard's range is found on public land including various military installations. Here, we analyzed thousands of nuclear loci collected from across the genomes of 41 individuals across the range of this species. We found that a one or two-population model is best supported by the data, corresponding to a major split on either side of the Colorado River, consistent with estimates of effective migration that show the lower Colorado River valley and the Salton Sea are the primary barriers on the landscape. We found that this species has unexpectedly high heterozygosity, possibly resulting from hybridization with neighboring divergent species of horned lizards, or a very large ancestral population size. Our phylogenetic results indicate that northwestern populations are nested within southeastern ones, consistent with our estimates of effective diversity which are highest on the Barry M. Goldwater Marine Corps Air Station and in Sonora, Mexico, and lowest in the Coachella Valley of southern California. Given the numerous anthropogenic barriers that now fragment remaining populations, these data indicate that a land management strategy focused on maintaining connectivity between management areas may be most effective. This research is ongoing as we continue working to analyze our genomic dataset in conjunction with an existing population viability analysis led by the Arizona Department of Game and Fish.

INTRODUCTION

Our study seeks to contribute to the substantial ongoing research on the Flat-Tailed Horned Lizard (FTHL), *Phrynosoma mcallii* (Figure 1). This species has the smallest range of any horned lizard (*Phrynosoma*) in the United States and is considered an at-risk species (Leavitt et al. 2015). *Phrynosoma mcallii* is restricted to sandy habitats in the Colorado Desert, ranging from the Coachella Valley in southern California, southeast to Laguna Salada in Baja California, Mexico, southwestern Arizona, and the Gran Desierto de Altar of northwestern Sonora, Mexico (Jones and Lovich 2009). This species is currently listed as a Species of Special Concern in California due to its sensitivity to urban, agricultural, and renewable energy development, habitat fragmentation (e.g. from highways and the international border fence), off-road vehicles, and invasive vegetation.

Figure 1. The Flat-tailed Horned Lizard (FTHL), *Phrynosoma mcallii*. Photo by the senior author.

Because the nuclear genome is directly involved with adaptation, and populations with higher genetic diversity and population connectivity should, in theory, have greater potential to adapt to changing environmental conditions, land managers may choose to prioritize the protection of intraspecific genetic diversity (Vandergast et al. 2013,

Gottscho et al. 2014). The most recent published genetic study of this species (Mulcahy et al. 2006) analyzed 781 base pairs of mitochondrial DNA (mtDNA) from 82 individuals of *P. mcallii* across their range. They found limited gene flow and significant population structure across the Colorado River, suggesting this barrier has historically been important in isolating populations, and that populations in California showed less genetic differentiation. However, because mtDNA is maternally inherited, it cannot track male-mediated gene flow. If males disperse more than females on average, this marker could be a misleading indicator of true migration rates. Perhaps more importantly, because all mitochondrial genes are linked into effectively single locus, this study effectively had a sample size of one marker. It is now widely appreciated that single-locus estimates of population history are subject to significant uncertainty due to stochastic demographic processes. Fortunately, the fields of genetics and genomics have advanced tremendously in the past decade since the study of Mulcahy et al. (2006), especially with the advent of next-generation sequencing (NGS) technology and novel statistical methods. NGS technology now allows for rapid collection of millions of base pairs of data for hundreds of individuals. These data can be mined for genome-wide single-nucleotide-polymorphisms (SNPs) using methods such as restriction-associated DNA sequencing and associated bioinformatics pipelines that perform *de novo* assemblies without reference genomes (Peterson et al. 2012, Eaton 2014, Gottscho et al. 2017).

The combination of large genomic datasets (at least two orders of magnitude larger than single-locus mtDNA studies) and new computer models allow for cutting-edge conservation science to be performed. For example, with large genomic datasets we can more accurately and precisely address questions such as: how do levels of genetic diversity vary, and how do genes flow, across the landscape? How many distinct populations exist, and how are they related to each other? It is already known that FTHLs are widely distributed across the Salton Sink (= geologic region surrounding Salton Sea), and that they tend to favor lowland sandy areas and badlands and avoid steep rocky areas. However, it is not known if they tend to use discrete corridors for migration between populations and if so, how important these corridors may be in maintaining healthy levels of genetic diversity. This knowledge is critical if we want to keep these corridors safe as

large renewable energy projects and other developments fragment increasingly large areas of the Colorado Desert outside of military lands. If populations become too small and isolated, inbreeding and stochastic population fluctuations could potentially result in local extinction. In the case of the DoD, this type of data could be used to help minimize the impact of habitat fragmentation on the connectivity of sensitive FTHL populations, without compromising military readiness, at relatively low cost.

STUDY OBJECTIVES

The objectives of this study were to address the following questions:

1. Which populations of *P. mcallii* have the highest genetic diversity, and how do these levels compare to other benchmark species?

2. How many populations are there, and how are they related to each other?

3. What are the main natural barriers to gene flow (migration) across the range of this species?

4. How can we incorporate intraspecific patterns of genomic diversity and gene flow into predicting the effects of renewable energy projects and other manageable impacts in the Colorado Desert region on long-term population viability?

BACKGROUND

The military is a signatory to the Range-wide Management Strategy (RMS; FTHL ICC 1997, FTHL ICC 2003) for the FTHL since 1996. NAVFAC Southwest, NAF El Centro, and MCAS Yuma are three of the 13 federal and state agencies that are signatories to the RMS. These agencies created the RMS as a basis for a conservation agreement for the species, modeled after USFWS Recovery Plans. Since 1993 the FTHL was first proposed for listing under ESA, and two decades and three trips through the federal courts later, via voluntary management of the species via the RMS, the FTHL has not been listed. This project is a direct outcome of the RMS, and will directly enable better management of the species both on military lands at NAF El Centro and MCAS

Yuma (BMGR), as well as on all of the other state and federal stakeholder lands covered under the RMS. It will directly support the mission through pro-active management of the species.

From 1993 to 2011, *P. mcallii* was scrutinized for protection under the federal Endangered Species Act, but in a series of court cases, the U.S. Fish & Wildlife Service (USFWS) withdrew the proposed 1993 rule four times. In 1997, multiple state and federal agencies with populations of *P. mcallii* on their lands signed a voluntary Interagency Conservation Agreement (ICA) to implement the Flat-tailed Horned Lizard Rangewide Management Strategy (FTHL RMS). One of the major goals of the ICA is to "encourage and support research to promote conservation of [*P. mcallii*] and desert ecosystems" (FTHL RMS 8), specifically to "determine genetic variation among populations and effects of barriers" (FTHL RMS 8.6).

In 2011, the USFWS decided against listing *P. mcallii* because "the effects to the species associated with the implied meaning of fragmentation… are not likely to constitute a substantial threat to the species now or within the foreseeable future" and because "the conservation efforts implemented by signatories of the [ICA] and associated [FTHL RMS] reduce the impact of existing threats in the United States and promote actions that benefit the Flat-tailed Horned Lizard throughout its range, including Mexico" (76 FR 14210; 14252-53). However, in June 2014 the Center for Biological Diversity submitted a petition to list *P. mcallii* as endangered throughout their range in California under the California Endangered Species Act (California Fish and Game Code §§ 2050 et seq., "CESA"). In October 2014, the California Department of Fish & Wildlife decided that the petition had merit and in February of 2015 proceeded with the mandatory one-year scientific review. In March 2017, the Department decided not to list this species, but will re-examine its status again in five years. This study was undertaken in an effort to better understand the genetic diversity found in the FTHL, and also to use that information to support land use and management decisions.

MATERIALS AND METHODS

Genomic DNA Samples. We extracted genomic DNA from more than 100 tissue samples of *P. mcallii* collected throughout their known range, including those used in Mulcahy *et al*. (2006) and including DoD installation sites, using the PureLink Genomic DNA kit (ThermoFisher Scientific, Waltham, MA). Most of these samples represent tissue samples in the form of tail clips or toe clips that were collected by numerous individuals/agencies over the last 20 years (see Acknowledgements for list of collectors and permits) and were generally preserved in 96-100% ethanol and stored at -20º C or -80º C. We then screened these for quality, excluding those degraded samples with low DNA concentration and low molecular weight. In our final dataset, we included 41 high-quality samples of *P. mcallii* collected throughout their known range (Table 1, Figure 2), as well as one outgroup sample of the Desert Horned Lizard, *P. platyrhinos*.

Table 1. Localities and number of samples collected per locality. *Current (active) DoD installation. **Former DoD installation. ***From a land parcel directly bordering a DoD military installation.

Locality	State, Country	# Samples
Coachella Valley	California, USA	5
Dos Palmas	California, USA	1
Ocotillo Wells***	California, USA	5
Salton Sea**	California, USA	10
West Mesa*	California, USA	1
East Mesa	California, USA	1
Barry M. Goldwater Marine Corps Air Station*	Arizona, USA	6
Gran Desierto Biosphere Reserve***	Sonora, Mexico	5
El Pinacate Biosphere Reserve***	Sonora, Mexico	6
Laguna Salada	Baja California, Mexico	1

Figure 2. Sampling localities (shown as purple dots).

Library preparation and sequencing. We collected DNA sequence data using the double-digest Restriction-Associated-DNA sequencing (ddRADseq) method (Peterson et al. 2012). Genomic DNA samples were digested with the restriction enzymes *SbfI* and *MspI* (New England Biolabs, Ipswich, MA). After the ligation reaction, we selected fragments 415-515 base pairs (bp) long. Pooled libraries were amplified and Illumina indices were attached using the Polymerase Chain Reaction (PCR). The final libraries were sequenced (150 bp single-end reads) on a NextSeq (Illumina, San Diego, CA) at the Institute of Integrative Genome Biology (University of California, Riverside, CA).

Bioinformatics. The python pipeline pyRAD v3.0.6 (Eaton, 2014) was used for quality control, *de novo* assembly of RAD loci, genotype calling, and generating alignments of loci and SNPs. We also used the proportion of polymorphic sites (percent heterozygosity) as a metric of genetic diversity.

Population Structure.

Admixture v1.23 (Alexander et al. 2009) was used to analyze a dataset of unlinked biallelic SNPs (one per RAD locus) to estimate the number of populations,

assign individuals to those populations, and calculate the fixation index (F_{ST}), which ranges from 0 to 1. A zero value indicates a panmictic population; that is, that the two populations share all of their alleles. A value of one implies that the two populations do not share any alleles and indicates completely isolated populations. Admixture implements an underlying population genetic model similar to Structure (Pritchard et al. 2000). The cross-validation error (CVE) was the criterion used to select the optimal number of populations (K), testing values ranging from 2 to 10. We also used Discriminant Analysis of Principal Components (DAPC) implemented in the R package adegenet v2.0-0 (Jombart et al. 2010) to assign individuals to populations using the same dataset. First, we conducted the Principal Components Analysis (PCA), retained the first 41 PCs, and used the Bayesian Information Criterion (BIC) to select the optimal K value (2-10). Second, K-means clustering was implemented by retaining the first 13 PCs retaining more than 80% of the cumulative variance and the first two discriminant functions to assign individuals to clusters.

Phylogeny. In order to determine the evolutionary relationships among individuals, and gain insight into migration routes and barriers, a maximum likelihood phylogeny was estimated with RAxML v8.2.4 (Stamatakis 2014) using 500 rapid bootstrap replicates and a GTR+G substitution model.

Estimated Effective Migration. We used the program EEMS (Estimated Effective Migration Surfaces; Petkova et al. 2016) to model the relationship between genetics and geography. This approach inputs a genetic distance matrix and a geographic distance matrix to ascertain deviations from a null isolation-by-distance model. It does this by overlaying a triangular grid across the landscape, where vertices are demes and lines are edges, and assigns individuals to their nearest deme (local population). The genetic variance is decomposed into within-population effective diversity (q) and among-population effective migration (m) parameters. Deviations from the isolation-by-distance model can thus be visualized in the form of a spatial heat map displaying barriers and corridors for migration and local diversity hotspots. We calculated a Euclidian distance matrix of 41 individuals at 1,414 unlinked SNPs in adegenet v2.0-0. We overlaid a grid of 300 demes in the shape of the approximate range of this species. We ran the Markov Chain Monte Carlo (MCMC) chain for 20 million steps, discarding the first two million,

and sampling every 1,000 steps. Multiple runs with differing random number seeds were performed and the results of the MCMC chains were assessed for convergence following the authors' recommendations.

RESULTS

DNA sequence data. We obtained a total of 76.03 million reads (an average of 1.81 million per lizard), of which 75% (57.14 million) passed pyRAD's initial quality filter. These were assembled into an average of 111,642 loci per individual, of which an average of 8,275 passed the coverage threshold (10x). On average, we examined 627,732 nucleotide sites per lizard, of which 2,602 were polymorphic on average.

Population Structure and Phylogeny. The results of Admixture and DAPC indicate that a one or two population model best fits the data (Table 2). Increasing the number of populations beyond two results in a poorer fit of the model to the data. In Figure 3 we show the Admixture and DAPC clustering results for K=2 and K=3 aligned with the phylogeny produced with RAxML. Under K=2, both DAPC and Admixture agree in splitting *P. mcallii* into an eastern population and western population, roughly divided by the Colorado River. F_{ST} values between populations were low – under the two-population model, F_{ST} was 0.115, while under the three-population model, it was 0.11 between the eastern and western populations, 0.26 between the eastern and Coachella populations, and 0.22 between the western and Coachella populations. These F_{ST} values indicated that the majority of genetic variation is shared between these populations. The phylogeny is poorly resolved, indicating a lack of deep genealogical lineages within *P. mcallii*. Northwestern populations (eg Coachella Valley) are more deeply nested than southeastern populations in Arizona and Sonora.

Table 2. Cross Validation Errors (CVE) from Admixture and the Bayesian Information Criteria (BIC) from Discriminant Analysis of Principal Components. K is the number of populations. Lowest numbers indicate the best-fitting models, in this case, one or two populations best explains the data.

K	CVE	BIC
1	**0.438**	181.78
2	0.446	**181.54**
3	0.450	182.09
4	0.484	183.63
5	0.556	185.83
6	0.601	188.06
7	0.636	190.26
8	0.712	192.53
9	0.772	194.64
10	0.793	196.74

Figure 3. Maximum likelihood phylogeny produced with RAxML aligned with clustering results from Admixture and DAPC under K=2 and K=3. Red indicates an eastern population in Sonora and Arizona, blue indicates a western population in California, and green indicates the Coachella Valley population in California. DGM482, from Laguna Salada, is west of the Colorado River but groups with the eastern population. Bootstrap support values are shown on nodes.
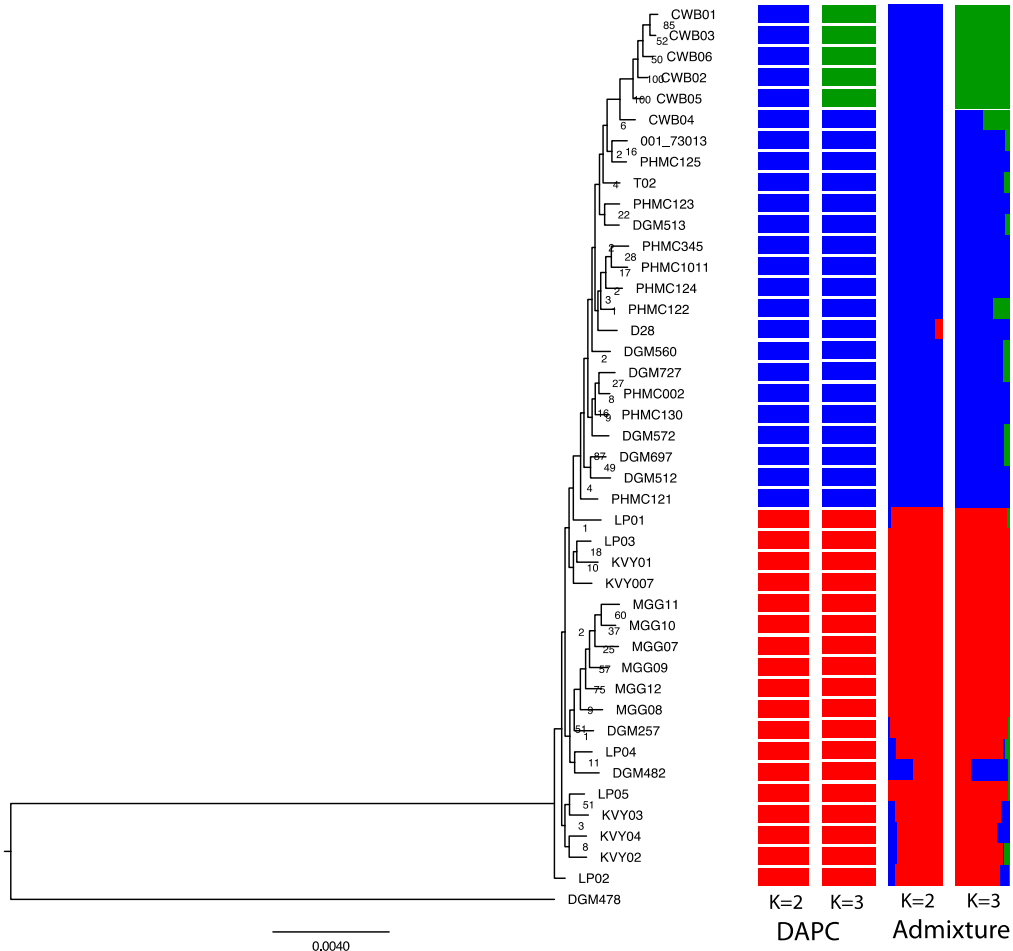

Heterozygosity. Surprisingly, *P. mcallii* had high levels of heterozygosity (ranging from 0.6% – 0.1%). There was no significant trend with latitude across the range (Figure 4). These values are higher than those reported for other sensitive co-distributed species, such as *Uma notata*, as well as other common desert lizards in the region, including *Callisaurus*, *Petrosaurus*, *Urosaurus*, and *Sceloporus* (ADG, unpublished data).

Figure 4. Heterozygosity vs latitude. Adjusted R-squared: 0.095. F-statistic: 5.209, p-value: 0.028.
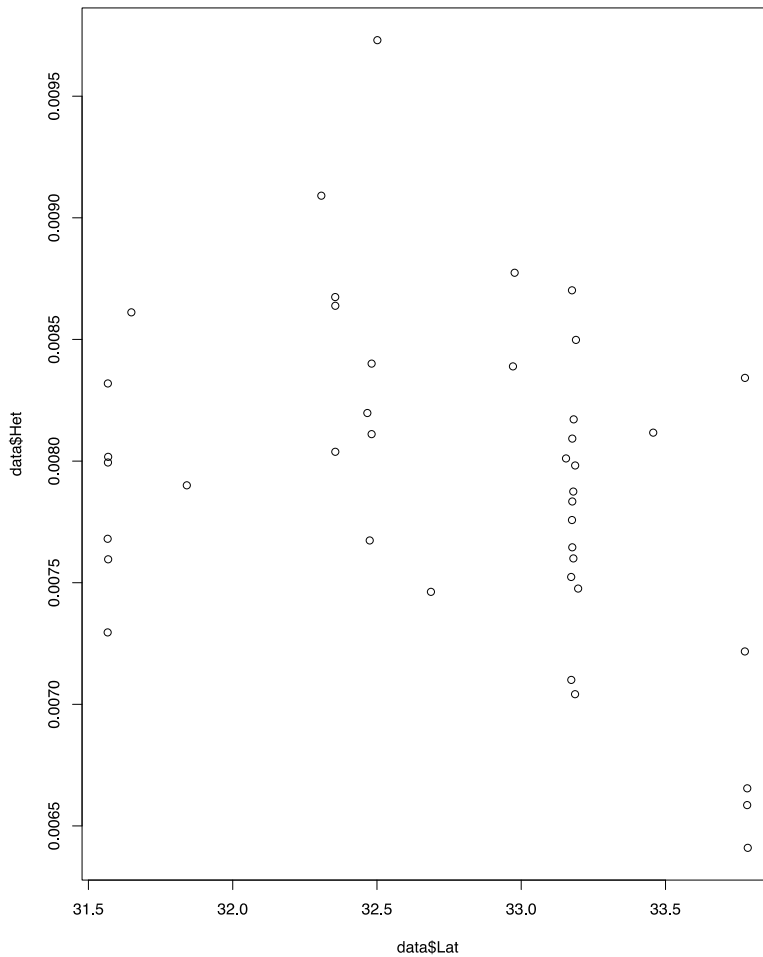
Estimated Effective Migration. The results of the EEMS analysis show that local effective diversity is highest in the southeastern part of the range and lowest in the northwest (Figure 5, top left). The only statistically significant areas of elevated diversity are in the vicinity of the Barry M Goldwater Marine Corps Air Station in southwestern Arizona and the El Pinacate Biosphere Reserve of Sonora, Mexico, and of decreased effective diversity in the Coachella Valley of southern California (Figure 5, top right). Interpretation of the raw effective migration heat map (Figure 5, bottom left) shows that the lower Colorado River Valley and the Salton Sea represent the only geographic barriers, but only the Salton Sea shows any statistical significance (Figure 5, bottom right).

Figure 5. Estimated parameter surfaces under the Estimated Effective Migration Surfaces model. The top two plots represent the diversity parameter (q), representing divergence *within* demes (local populations), while the bottom two blots represent the migration parameter (m), representing divergence *among* demes. Plots on the left show rates color coded on the log10 scale, with cool and warm colors representing values lower or higher than what is expected under the null isolation by distance model; plots on the right show only those parameters with high statistical confidence measured by posterior probability.
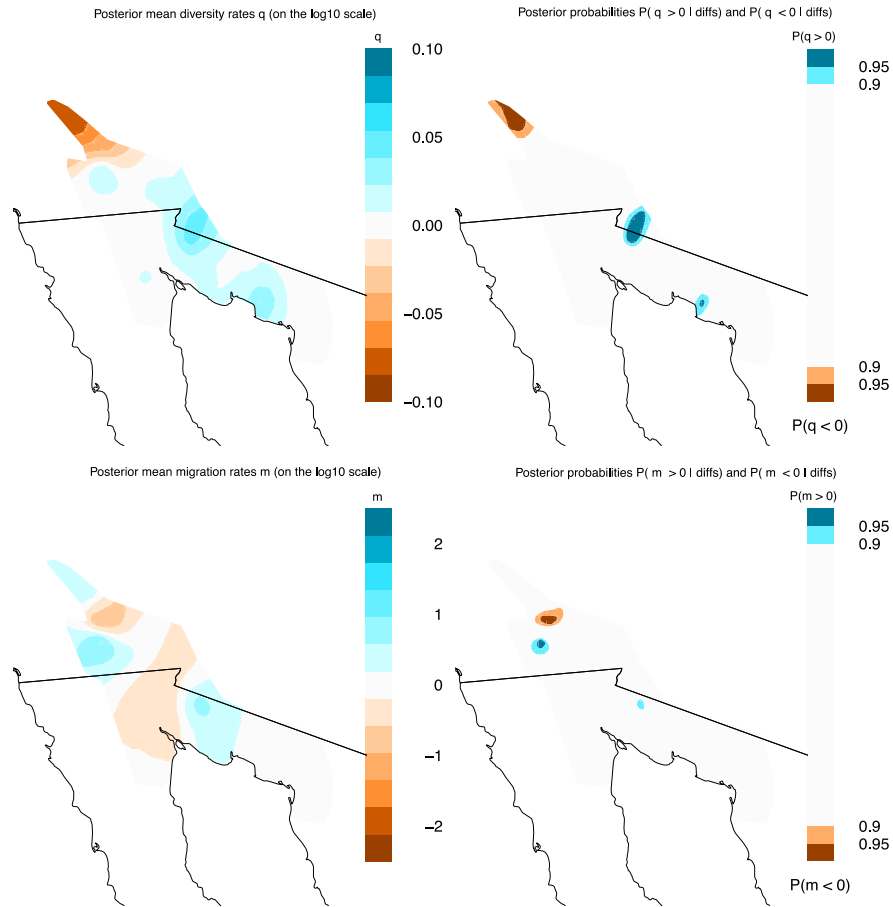
DISCUSSION

Here, we present the results of the first population genomic study of the Flat-tailed Horned Lizard, *Phrynosoma mcallii*. This dataset of thousands of nuclear loci represents two orders of magnitude more data than the previous study published by Mulcahy et al. (2006), reflecting the tremendous advance in DNA sequencing technology over the past decade. With this influx of new data, we were able to gain further insights into the biology of this species that were not possible using the old mitochondrial dataset. For example, it was impossible to measure heterozygosity with the mtDNA dataset of Mulcahy et al. (2006), because mitochondrial data is haploid. Thus, the fact that P. mcallii has high heterozygosity represents a new discovery of this study.

The first question we asked was, which populations of *P. mcallii* have the highest genetic diversity, and how do these levels compare to other benchmark species? We found that although our EEMS results showed decreased local *effective* diversity in the Coachella Valley and increased diversity in the vicinity of the Barry M. Goldwater Marine Corps Air Station (Figure 5), there was no clear pattern of heterozygosity across the range (Figure 4). Surprisingly, heterozygosity levels were much higher than expected. In fact, they were even higher than many common desert lizards with much larger ranges, such as the zebra-tailed lizard, *Callisaurus draconoides*. What could account for this discrepancy? Given that this species has the smallest range out of any horned lizard in the United States, we would expect it to have less diversity, like the co-distributed fringe-toed lizards (*Uma notata* complex). One hypothesis is that there could be some hybridization between neighboring sympatric species of *Phrynosoma*, such as *P. goodei* or *P. platyrhinos*. This hypothesis is supported by at least three hybrids that have been found, and our sequence data (not presented here) confirm that these are real hybrids. Another possibility is that the ancestral population was much greater than today. This explanation is plausible, especially considering that urbanization of the greater Palm Springs area, and vast agricultural operations in the Coachella and Imperial Valleys, have resulted in considerable loss of habitat for this species. As we work towards preparing a manuscript for peer review, we shall continue analyzing our data to distinguish between these two hypotheses.

The second question we asked was, how many populations are there, and how are they related to each other? Our analyses support a one or two population model, as subdividing the dataset past this results in a poorer fit of the model to the data. Our phylogenetic analyses cannot resolve the relationships of these populations with confidence, but it appears that northwestern populations are descended from southeastern ones. This is consistent with the estimated effective diversity being highest in the Barry M. Goldwater Marine Corps Air Station in southwestern Arizona and the El Pinacate Biosphere Reserve in Sonora, and lowest in the Coachella Valley of southern California (Figure 5), which is consistent with the pattern observed in the co-distributed fringe-toed lizard (Gottscho et al. 2017).

The third question we asked is, what are the main natural barriers to gene flow (migration) across the range of this species? Our results, especially effective migration rates, point to the Colorado River as the primary divide between the eastern and western populations (Figure 5, bottom left), although the statistical power of this divide is weak (Figure 5, bottom right). Otherwise, we detected no significant deviation from isolation-by-distance within the range of *P. mcallii*. There is also some statistical support for splitting out Coachella Valley as a separate population (Figure 3) – this population is isolated by the Salton Sea and urbanized or agricultural lands.

The final question we asked is, how can we incorporate intraspecific patterns of genomic diversity and gene flow into predicting the effects of renewable energy projects and other manageable impacts in the Colorado Desert region on long-term population viability? To this end, we are currently working on integrating our genomic data (in the form of a population pedigree or studbook file containing genotypes for each individual) with the population viability analyses conducted by the Arizona Department of Game and Fish (Grimsley et al. 2017; Grimsley et al. *in prep*). The first population viability analysis (Grimsley et al. 2017) focused on the Yuma Desert Management Area, primarily located on the Barry M. Goldwater Marine Corps Air Station in southwest Arizona. The second analysis is range-wide and is currently in progress. We are integrating out data using the program Vortex 10 (Lacy and Pollok 2017), which simulates the probably of extinction given a set of demographic parameters. In addition, this program can simulate allele frequencies through time using the starting genotypes provided by this study. When

complete, we hope that these analyses will yield further insight into the demography of *P. mcallii* on military lands.

MANAGEMENT IMPLICATIONS

It is important to note that these results are first analyzed and reported herein, have not yet been subjected to scientific peer review. Nevertheless, the finding that *P. mcallii* has high genetic diversity (heterozygosity) represents an important and exciting discovery and establishes a baseline for future management efforts, but more work is needed to confirm this finding. The lack of population structure between populations in Arizona, USA and Sonora, Mexico indicates that the border fence separating these populations could be contributing to attrition of migration. In order to maintain this high genetic diversity, it may be necessary to maintain corridors of gene flow between Sonora and Arizona, and the East and West Mesas. This project is still a work in progress, we are continuing to analyze our data to better understand these migration routes and the most cost-effective way to maintain them without disturbing the military readiness mission. The results of this study are a powerful genetic lens through which to see how this species has continued to evolve given past and present land use. It will be mined for its utility to management and land-use decisions.

Our research also enhances strategic planning and management of DoD's natural resources by developing a modern genetic fingerprint of extant FTHL populations. We have uncovered phylogenetic relationships and historic corridors/barriers to gene flow that will help in protecting processes crucial to survival of the species into the foreseeable future. One of the important findings of the study for the military is that unique haplotypes, or even species, have not been uncovered within FTHL. High levels of heterozygosity are also resolved, and this is a good outcome for land use, versus a finding where there are unique haplotypes that would potentially require differential management and/or added protections. The different Management Areas, and single Research Area are large expanses of lands with continuous habitat for FTHL, and now we have a baseline of genetic information and diversity with which to compare and maintain while managing

the species in perpetuity. This information will aid resource managers at installations in assessing project level impacts to the species. It will directly support the mission through pro-active management of the species, and the enhanced understanding of the genetics of the species will support project-specific impacts analyses, maintenance of gene flow and evolutionary corridors for movements, etc.

Ultimately, this study provides an excellent example of the utility of modern genetic research for at-risk species. Ideally, this sort of information and research would be applied to all at-risk species across the DoD landscape, both CONUS and OCONUS. The reality however, is that it takes a great deal of time, effort, and funding in order to accomplish this, and it simply is not available for all species. The authors would encourage military services to consider not only this sort of multi-stakeholder, voluntary partnership for at-risk species on installations, but also prioritizing the pursuit of genetic information for all the benefits described herein.

FUTURE DIRECTIONS

This project has opened up many exciting possible research directions. We are currently continuing to analyze this RADseq dataset as we prepare a manuscript for publication in a peer-reviewed journal. For example, we are implementing demographic models that use coalescent theory to model alternative hypotheses regarding migration rates and changes in effective population size. Another direction is to use a parallel dataset (collected on many of the same samples) of genomic ultra-conserved elements to examine whether the high heterozygosity is real or the result of large-scale copy number variation in the genome. Ultimately, the next obvious step is to sequence and assemble *de novo* a high-quality reference genome. This reference genome could be used to map the RADseq loci against to ensure that they are orthologous. Furthermore, loci linked to important protein-coding genes could be identified, which is important because some of these genes may be under directional selection. For these and many other reasons, obtaining a high-quality reference genome will undoubtedly be a valuable resource for

future management efforts of this sensitive species and represents the most obvious next step. Fortunately, the cost of doing so is rapidly decreasing.

ACKNOWLEDGEMENTS

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