HISTORIC GENETIC VARIATION OF THE TEXAS HORNED LIZARD (*PHRYNOSOMA CORNUTUM)* IN THE DALLAS/FORT WORTH AREA

By:

Kristin Scoggin

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Project Approved:

Supervising Professor: Dean Williams, Ph.D.

Department of Biology

Matthew Hale, Ph.D.

Department of Biology

Darren Middleton, Ph.D.

Department of Religion

ABSTRACT

The historical genetic variation of a population has been a mystery for a long time. With the advent of new methodologies, scientists now have the unique opportunity to sequence DNA from museum specimens. Past studies have shown that low genetic diversity often precedes extinction events. The Texas horned lizard (*Phrynosoma cornutum*) has been extinct in the DFW area since the early to mid-1970s. Using museum samples of the Texas horned lizard that were collected between 1959 and 1961, we wanted to determine if there had been a loss of genetic diversity before the DFW population's extinction. In addition, we tested for unique haplotypes compared to modern populations in other parts of Texas. We found 20 haplotypes in our sample of 32 horned lizards, 5 which were also found in modern populations, and 15 that were unique to the historic DFW population. The historic DFW population had higher haplotype diversity than any modern population. These results suggest that there has been a loss of diversity with the decline of the horned lizard, but that loss of genetic diversity did not precede the extinction of the DFW population.

Introduction

Species can go extinct for many reasons. A primary cause, such as habitat loss, loss of food, or invasive species, does not always result in immediate extinction. If this happens, these primary extinction causes can lead to secondary processes and lead to what is more commonly known as the extinction vortex (Brook et al. 2008). Often, these processes working together can create a synergetic relationship that ultimately causes the extinction of a species. These factors lead to small, fragmented populations, which causes a loss of genetic diversity and makes the population more likely to become inbred. This in turn reduces the adaptability of the population and lowers its fitness making the population more susceptible to demographic stochasticity (random reproductive outputs), environmental variation (e.g. different amount of rain each year), and catastrophes (e.g. hurricane) (Frankham, et al. 2007). These events further reduce the population size, which feeds back into the loop, until the population ultimately collapses and goes extinct (Brook et al. 2008).

Museum samples give us a unique look into the history of a species, and allow us to see if any sort of genetic event contributed to the population's decline. For example, Bruniche-Olsen, et al. (2014) used historic museum samples and modern samples to look at the genetic history of the Tasmanian devil (*Sarcophilus harrisii*). The researchers found evidence for a bottleneck in the population and are concerned about the future of the species due to the low genetic diversity it now has. Johnson and Dunn (2006) also used historic museum samples to look at the now extinct heath hen (*Tympanuchus cupido cupido*). They found that the heath hen had low genetic diversity about 30 years before its extinction suggesting the loss of genetic diversity may have contributed to its extinction.

With the invention of better DNA extraction methods and sequencing techniques, scientist are now able to use DNA extracted from museum specimens. Unfortunately, to preserve museum specimens, the DNA integrity is often compromised (Moraes-Barros et al., 2007). Even with new methodology, clean whole sequences are still difficult to get from these samples. Generally, mitochondrial DNA (mtDNA) is used over nuclear DNA in museum specimens, because the age of the samples and the way in which they were preserved can affect the quality and quantity of the DNA (Lounsberry, et al. 2014). Since there are more copies of mtDNA in a cell and the mitochondrial DNA is shorter, the chances of extracting a sequence that is not disrupted is greater. Formalin, a common preservative, has been found to cross-link DNA and degrade DNA if it is not buffered properly (Campos and Gilbert 2012). Another challenge to sequencing is due to cross-contamination. Shokralla et al. (2010), using the larvae of the agave butterfly (*Hypopta agavis*), found that a specimen can be sequenced from the liquid it is stored in, so if specimens are stored together, there is a high probability of cross contamination.

Historically, the Texas horned lizard (*Phrynosoma cornutum*) was abundant throughout Texas with the exception of the Piney Woods. They have been on the decline for a while now, and in recent decades have virtually disappeared from East Texas, roughly along a North-South line extending eastward from Fort Worth to Corpus Christi (Price 1990, Donaldson et al. 1994). Increased urbanization, the introduction of invasive red fire ants (*Solenopsis invicta*), and the loss of their native food source the harvester ant (*Pogonomyrmex spp.)* are the factors believed to have caused the decline of Texas horned lizards (Price 1990, Donaldson et al. 1994, Dixon 2000, Henke 2003). The Texas horned lizard has been functionally extinct in the Fort

Worth/Dallas (DFW) area since the early to mid-1970s (Glenn Kroh pers. comm). The species is still present in other areas of Texas, especially south and western Texas. In particular, we wanted to see how the historic DFW population compared to the modern-day populations from the rest of Texas. Using museum specimens, we compared the genetic variation and haplotypes of the historical DFW population to that of present day Texas horned lizards. Our first objective was to see if genetic diversity was low before the DFW extinction, which would indicate that genetic factors may have contributed to their decline. Our second objective was to see if there were any unique genetic types (haplotypes) in the DFW area that are not found in modern populations.

Methods

Our specimens were found in a jar housed in the Fort Worth Museum of Science and History and were collected between 1959 and 1961, and 1983 (Table 1). There were 14 samples collected in 1959, 6 in 1960, 13 in 1961, and 1 in 1983. We had 21 females, 11 males and 2 of unknown sex. It is not clear whether the one lizard collected in 1983 was part of some remnant population in the DFW area or if this may have been a pet. If this lizard was from elsewhere in Texas then one of the genetic variants we report on below may not be from the DFW area.

We extracted DNA from each preserved individual (n=34) by removing a toe, then soaking the toe in 1 ml of 10 mM Tris, pH 8.5 for one day and changing the liquid 3X times during the course of the day. The toe was then put in 300 µl of lysis buffer (1% SDS, 20 mM EDTA, 10 mM Tris) and 20 µl Proteinase K (20mg/ml), incubated with shaking overnight at 55^{o}C, and then vortexed the next day. We then added ½ volume of 7.5M Ammonium Acetate,

vortexed the samples and put them on ice for 10 minutes. The samples were then centrifuged to pellet the proteins. The supernatant was put into a new tube and 0.7 volumes of isopropanol was added and then votexed. The tube was centrifuged for 20 minutes to pellet the DNA. We then poured off the isopropanol and washed the DNA pellet with 70% ethanol. We removed all of the ethanol and resuspended the DNA in 100 µl of 10mM Tris, pH of 8.5.

We then amplified 454 base pairs of the d-loop region in the mitochondrial DNA using PCR (polymerase chain reaction) using the primers HLCR_F: 5'-CTTATGATGGCGGGTTGCT-3' and HLCR_R: 5'-GGCTGTTAAATTTATCCTCTGGTG-3'. Polymerase chain reactions (20µL) contained 10-50 ng DNA, 0.2 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM $MgCl_2$ pH8.7, and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were 1 cycle at 95°C for 15 min, followed by 40 cycles of 30s at 94°C, 90s at 55°C, 90s at 70°C, then a final extension at 70°C for 5 min. PCR reactions were set up in a PCR dedicated AirClean® 600 PCR workstation. Negative controls were used in all PCR reaction batches.

All of the specimens were stored in the same jar, so cross-contamination was very likely. We therefore cloned the PCR products to obtain clean single sequences. PCR products were gel purified and inserted into pGEM T-vectors and cloned using JM109 competent *E. coli*. The cloned d-loop was then amplified using pGEM primers. To do this, we created our master mix, aliquoted it into PCR tubes, and used a pipette tip to "pick" about 8 colonies per plate and then swished the tip around in the PCR tubes. Amplification products were checked on an agarose gel to ensure they amplified properly.

Before sequencing, we used ExoSap to clean up the extra primers and dNTPs that could interfere with the sequencing. After the ExoSap, we used the BigDye v3.1 kit to run our sequencing reaction. Originally, we ran both a forward and reverse reaction. However, the reverse primer was not as good as the forward, and we were not getting a lot of sequences, so then we only ran forward sequencing reactions. We ran the sequences on the ABI 3130XL Genetic Analyzer. We then trimmed the vector sequence and primers off of the sequences and checked the chromatographs using Sequencer v5.0 (Gene Codes USA) to assure that each base was called correctly. All trimmed sequences had quality scores >20 indicating they were of high quality. Sequences were then aligned in MEGA 6.0 (Tamura et al. 2013) using Muscle (Edgar 2004). Haplotypes were identified using GenAlEx v6.5 (Peakall and Smouse 2012).

We were able to get sequences for 32 of the 34 samples. We sequenced 8 clones per sample, but only about 4 worked for each sample. All individuals had multiple haplotypes indicating there had been cross-contamination in the jar. We found 73 unique haplotypes (unique d-loop DNA sequences) which is three times higher than the number of lizards in the jar. Heteroplasmy (presence of multiple types of mitochondria in a single individual) is unknown in this species and so the high number of haplotypes is most likely due to *Taq* polymerase error and cloning. To determine the most likely haplotypes, we generated a parsimony haplotype network for the museum samples. We used the *Taq*-error rate, which is ~$4X10^{-5}$ bp, to determine how likely we were to see an error in one sequence. The expected error rate per nucleotide = 4 X 10^{-5} x 40 PCR cycles x 454 nucleotides, which is about a 1 bp error in every two clones. We then used the following rules: A) if a haplotype matched a modern haplotype it was retained (n=5 haplotypes), B) if it was found in more than one individual it was retained (n=6

haplotypes), and C) if it was connected to A or B in the network by >2 bp it was retained (n=9 haplotypes). This reduced our original 73 haplotypes down to 20. Given the error rate calculated above, we did not expect to see the exact same error across multiple individuals (B) and it would also be unlikely to get more than two errors in the same sequence (C). All of the other haplotypes were connected to A, B, or C haplotypes by a single base and so these were then assigned the haplotype to which they were connected. This was probably a conservative estimate of the numbers of haplotypes in the 34 lizards and we may have missed one or more haplotypes that were one base different from the retained haplotypes. These 20 haplotypes were then aligned to other known modern Texas horned lizard haplotypes and a parsimony haplotype network was constructed at the 95% level using the program TCS (Templeton et al. 1992, Clement et al. 2000) to view relationships between the haplotypes.

Since we could not assign specific haplotypes to an individual (due to the cross contamination) we analyzed haplotype diversity it two separate ways and compared it to modern day populations of Texas horned lizards. We first calculated haplotype diversity by assuming that the number of times a sequence was cloned was related to the number of lizards that contained that haplotype. We calculated the relative proportion of each haplotype and multiplied that by our sample size (32) to get the number of lizards that contained a particular haplotype. Haplotype diversity was then calculated as $h = (1-\Sigma pi^2)(n/n-1)$, where pi is the frequency of the ith haplotype and n is the sample size (n = 32). Haplotype diversity is the probability that two individuals will be different. The second way we calculated diversity was simply by dividing the number of unique haplotypes by the sample size (i.e. for DFW 20/32 =

0.625) since this makes no assumptions about how the haplotypes are distributed among different numbers of individuals.

Results

Of the 20 unique haplotypes we found, 5 were also found in modern-day populations. These were: M17, M70, M80, M104, and M101. The remaining 15 were unique to the historical samples. Nine of those 15 were only found once in the historical population. The haplotypes that were most common were M70 with 41 clone sequences, M84 with 26 clone sequences, M54 with 23 clone sequences, and M104 with 11 clone sequences. Of these common haplotypes, M70 and M104 are found in modern-day horned lizards, while M84 and M54 are unique to the historic population. M80 was the predicted ancestral haplotype, or the "original" haplotype (Fig. 2). The DFW haplotypes do not form a single cluster and are instead found in multiple places in the network. The unique DFW haplotypes are also genetically similar to many modern day haplotypes (i.e. they are only a few base pairs different).

The historical DFW population showed more haplotype diversity (h) than any modern-day population. The max h possible would be 1.0, and the DFW population had an h of 0.95, which is extremely high. In modern populations, the highest h was 0.88, and some only had an h of 0.0, which means they had a single haplotype (Fig. 3). With the exception of Yoakum Dunes WMA and the Rolling Plains Quail Research Ranch (RQRR), protected areas in Texas such as the WMAs (wildlife management areas) had relatively high haplotype diversity while populations found in towns (Kenedy, Karnes City, Rockdale, Bastrop, Tinker AFB) and an island (Matagorda) had lower haplotype diversity. Our simpler calculation of h, which is just dividing the number of

haplotypes by the number of individuals in the population also showed that the historic DFW population had more diversity than modern day populations (Fig. 4).

Discussion

Our results allow us to conclude that low genetic diversity did not precede the extinction of the DFW population in the early 1970s. Instead, this extinction must have been due to other factors besides inbreeding and loss of diversity. Most likely, the culprits were urbanization and the invasion of fire ants. Urbanization causes loss of habitat, while fire ants outcompete the food source of the horned lizard (harvester ants) and kill baby horned lizards (Carpenter, et al. 2014). Similar to the Lounsberry (2014) study of buff-breasted sandpipers (*Calidris subruficollis*), we found that the DFW population seemed to maintain genetic diversity despite population declines and ultimately extinction.

We are also able to conclude that there was historically high genetic diversity, which suggests a very large population in the area. This claim is also supported by anecdotal evidence; anyone who grew up in the DFW area will tell you that they collected buckets of horned lizards when they were young! Unfortunately, this also means that there has probably been a loss of diversity, since 15 of our haplotypes have not been found in modern Texas horned lizards.

Another concerning observation that we made was that some modern populations have very low haplotype diversity. For example, Karnes City and Matagorda Island have an h of 0.0, which means there is only one haplotype in those populations. This very low diversity may indicate that these population are at risk for inbreeding. While inbreeding and loss of diversity did not precede the extinction of the DFW area, it can increase the risk of extinction in

populations (Frankham et al. 2007). Currently, the Karnes City population is steady and does not appear to be having negative side-effects from its low genetic diversity. In order to prevent populations like this from going extinct, we must continue to monitor their numbers and potentially intervene if they start to decrease rapidly in these areas.

As for reintroduction efforts in the DFW area, it seems highly likely that the horned lizard could be successful in this area. However, this would be dependent on finding suitable habitats and food sources, while controlling for the fire ant populations (Carpenter et al. 2014). Even though we found unique haplotypes that are no longer found in modern populations, the haplotypes were still very similar to modern haplotypes. Ideally, the five haplotypes that were found in modern and historic populations would be the haplotypes we would want to reintroduce into the area. If proper habitat and food sources can be found for the horned lizard in the DFW area, a new population should be able to be established.

Table 1: Jar of Horned Lizards from the DFW area (n=34) found at the Fort Worth Museum of Science and History

Horned Lizard number	Sex	Year Collected	Location	Length (mm)	Sequenced?
1	female	1959	Fort Worth	75	yes
2	female	1959	Fort Worth	89	yes
3	male	1959	Fort Worth	71	yes
4	female	1961	Tarrant	59	yes
5	male	1959	Fort Worth	65	yes
6	female	1959	Fort Worth	73	yes
7	male	1961	Tarrant	66	yes
8	male	1960	Fort Worth	71	yes
9	male	1960	Fort Worth	69	yes
10	female	1959	Fort Worth	77	yes
11	female	1959	Fort Worth	44	yes
12	female	1959	Fort Worth	72	yes
13	male	1959	Fort Worth	70	yes
14	female	1960	Fort Worth	78	yes
15	female	1961	Fort Worth	79	yes
16	female	1961	Tarrant	81	yes
17	female	1961	Tarrant	64	no
18	male	1961	Tarrant	62	no
19	female	1961	Tarrant	59	yes
20	male	1959	Fort Worth	61	yes
B1	male	1959	Parker	71	yes
B2	female	1959	Fort Worth	60	yes
B3	female	1959	Fort Worth	44	yes
B4	female	1961	Tarrant	62	yes
B5	male	1961	Tarrant	50	yes
B6	unknown	1983	Tarrant	unknown	yes
B7	female	1960	Fort Worth	73	yes
B8	female	1961	Tarrant	82	yes
B9	female	1959	Fort Worth	73	yes
B10	female	1960	Parker	76	yes
B11	male	1960	Fort Worth	72	yes
B12	female	1961	Tarrant	89	yes
B13	female	1961	Tarrant	45	yes
B14	unknown	unknown	unknown	52	yes

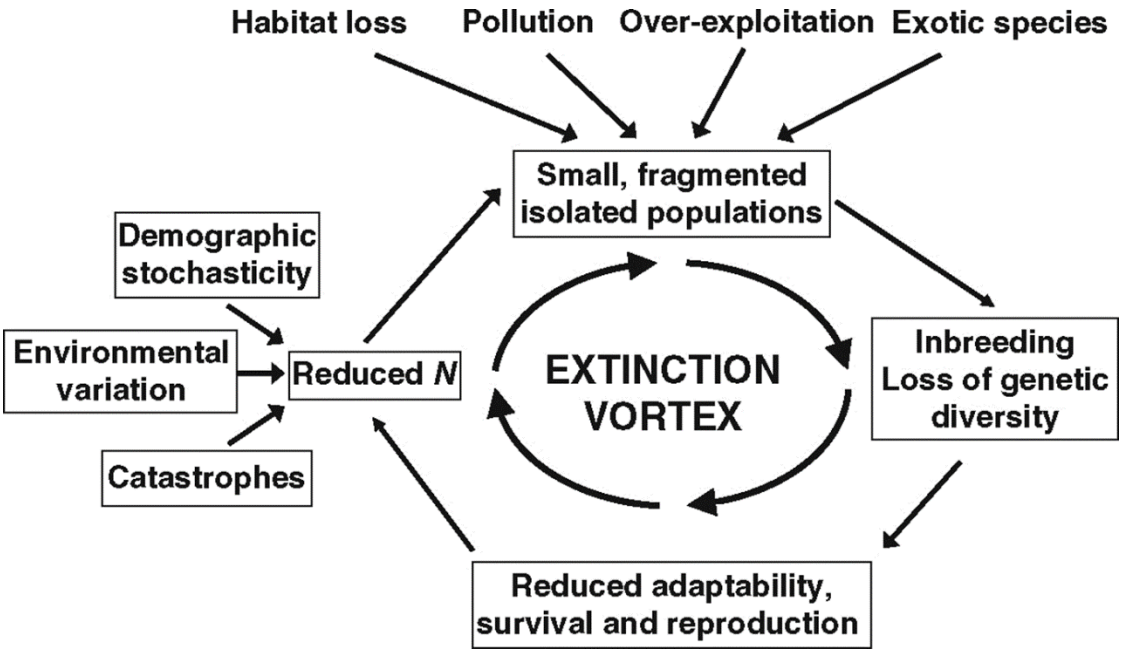

Figure 1: The Extinction Vortex. This figure shows common causes of extinction and show how they work together to cause extinction (From Frankham et al. 2007).

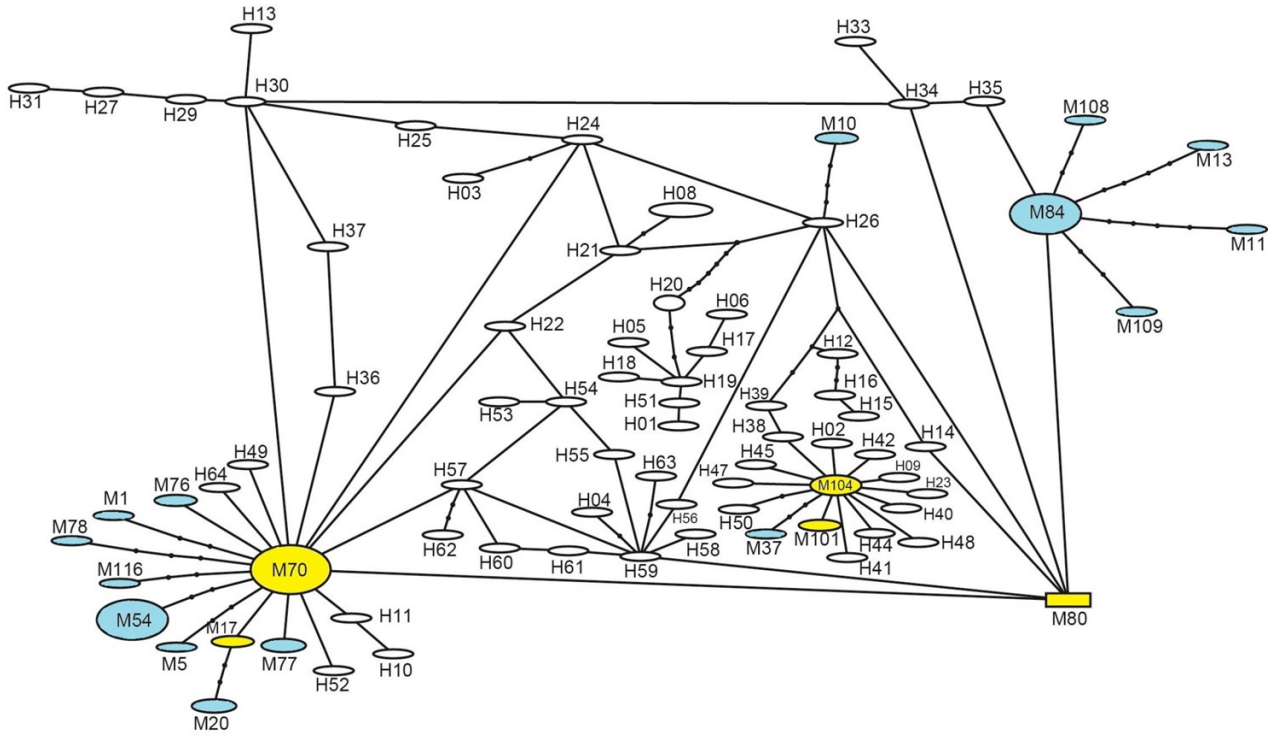

Figure 2: Parsimony haplotypes network of historical DFW horned lizards and modern Texas horned lizards. Each oval is a haplotype (unique mtDNA sequence) and the sizes of the ovals found in the DFW population are proportional to the number of clones containing that sequence. Each line (regardless of length) is a single base difference between haplotypes, small black circles are inferred haplotypes that were not sampled.

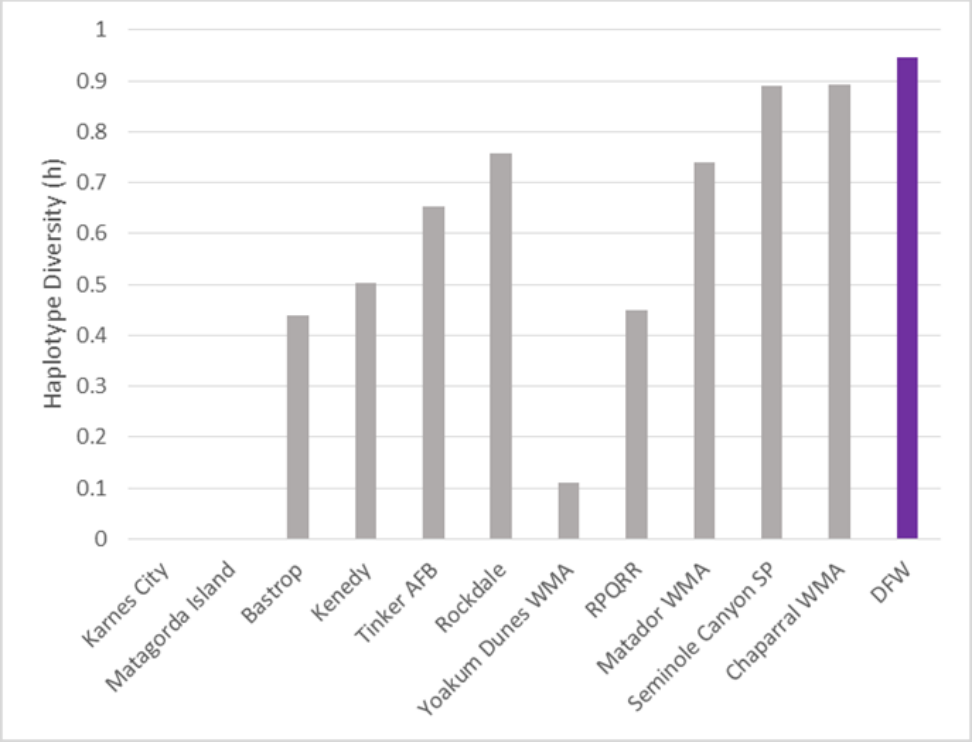

Figure 3: Comparison of the haplotypes diversities (h) between modern populations (grey bars) and the historical DFW population (purple bar).

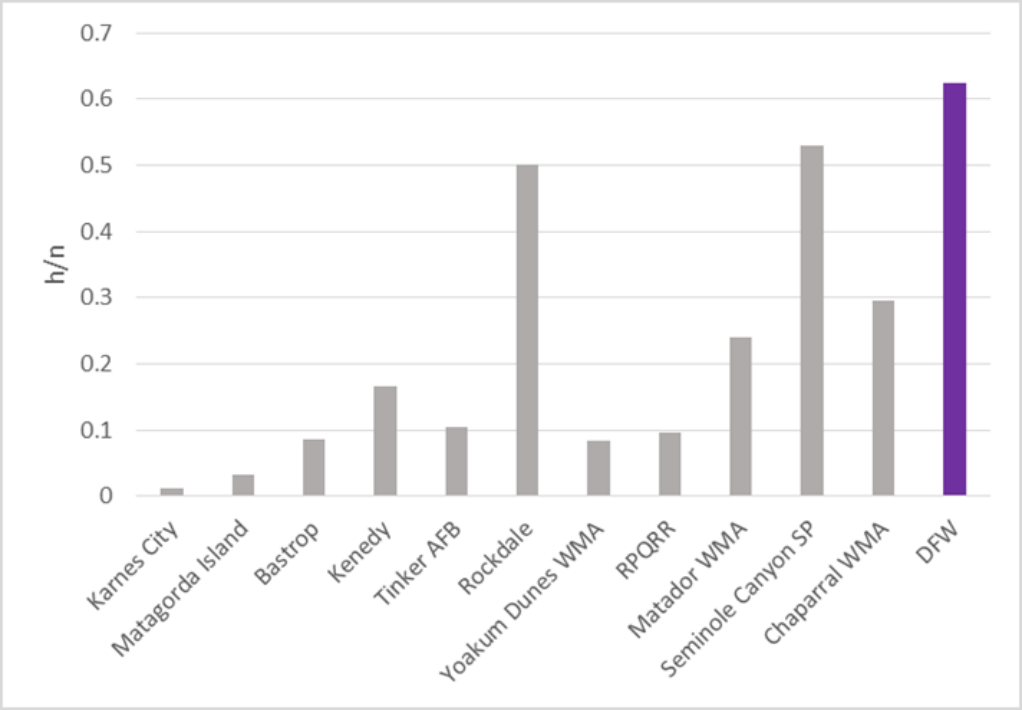

Figure 4: Comparison of the number of haplotypes/number of individuals between modern populations (grey bars) and the historical DFW population (purple bar).

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