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# Assessment of the Genetic Diversity of the Abilene Christian University Feral Cat Population Through the Use of Mitochondrial Genes

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Assessment of the Genetic Diversity of the Abilene Christian University Feral Cat  
Population Through the Use of Mitochondrial Genes

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by

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This Project Thesis, directed and approved by the candidate's committee,  
has been accepted by the Honors College of Abilene Christian University  
in partial fulfillment of the requirements for the distinction

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## **ABSTRACT**

The Abilene Christian University feral cat population has been the subject of many different studies throughout the years, especially those that focus on the presence and spread of diseases in the population that have potential to be zoonotic. The ACU Trap-Neuter-Return (TNR) program captures the feral cats to be neutered and also draws blood samples from each. Extracting and purifying of the DNA from these samples allows for disease testing and sequencing of specific genes. In a preliminary study, we used gene sequences from the mitochondrial D-loop to test the effectiveness of the TNR program maintained by the school and found lower levels of genetic diversity in the ACU population than in randomly sampled cats from the greater Abilene region. Expanding on this, sequences for the mitochondrial enzyme ATPase 8/6 will allow for increased sensitivity in the analysis of genetic diversity of the animals in the population. Comparison of the genetic differences or similarities among the cats using DNA haplotype charts can help determine the effectiveness of the TNR program by assessing its impact on inbreeding and migration within the ACU feral cat population.

## TABLE OF CONTENTS

Acknowledgements	Pg. 5
Cover Page	Pg. 6
Introduction	Pg. 7-11
Figure 1	Pg. 12
Materials and Methods	Pg. 12-16
Table1	Pg. 14
Figure 2	Pg. 15
Figure 3	Pg. 17-19
Results	Pg. 19
Discussion	Pg. 20
Conclusion	Pg. 21
Literature Cited	Pg. 21-23

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# Assessment of the Genetic Diversity of the Abilene Christian University Feral Cat Population Through the Use of Mitochondrial Genes



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## INTRODUCTION

The Abilene Christian University feral cat population has been the subject of many studies over the years, mainly looking at disease and parasite prevalence in the population. In the last few years, ACU pre-veterinary students have partnered with adjunct professor Dr. Dale Hembree and the ACU grounds manager Gayenell Rainwater to create a Trap-Neuter-Return (TNR) program on campus. Through the TNR program, over 100 cats have been neutered, tested for diseases and parasites, and given a rabies vaccine. Using the blood drawn to test these cats for feline leukemia and feline immunodeficiency virus, a genetic assessment of these cats was made in comparison to a random sample of owned and shelter cats in the Abilene area. Genetic studies of this type look primarily at determining if the TNR program has successfully created a population that is well controlled or if the feral cats are continuing through inbreeding or migration into the colony, as well as if it has produced a feral cat population that is genetically different from other Abilene cat populations. If the TNR program is successful in reducing the amount of breeding cats, the genetic diversity of the population should decrease, especially in comparison to other cats in the Abilene area.

Feral cat populations have recently been the subject of many studies because it was suspected that these colonies were harming local environments and killing off several wild bird and mammal species. In America alone these cats have been found to kill up to 3.7 and 20.7 billion birds and small mammals respectively (Loss et. al. 2013). Along with their effect on other animal species, these cats are also carriers for various diseases and parasites. Up to 5-10% of all feral cats trapped and tested showed a positive result for feline leukemia virus and/or feline immunodeficiency virus. Both of these viruses can

easily be spread to other cats, such as owned cats, and also to other wildlife. It has also been found that up to 90% of these feral cats carry fleas, 37% carry ear mites, and 45% carry parasites such as roundworms. These cats can also spread protozoal diseases that other cats and even humans can get and they can infect human water systems with fecal matter and pathogens (Longcore et. al. 2009). Effects such as these make managing feral cat colonies on Abilene Christian University important. By testing these cats for diseases and removing those cats that carry or are infected with the illnesses, the health of the colony will improve and the incidence of humans catching diseases from these cats will decrease. Correct monitoring, control, and feeding of the colony can also help lower the amount of wild animals that are killed by these cats.

Monitoring of this program is challenging as it is difficult to know which cats have been captured and neutered and how many have not. As a result, it is helpful when monitoring a TNR program to have some idea of the genetics of the population. Knowing the genetics of the population not only allows for a deeper understanding of how the population is moving from one generation to another, but it also allows for a look into potential genetic diseases or afflictions that the animals might carry from one generation to another. Looking at the genome of feral cats in a TNR program can also help determine if the colony is currently growing through inbreeding which can cause a huge increase in homozygosity at potentially harmful or lethal alleles if continued for long periods of time. Gene sequencing can also help determine the lineage of the cats which can help determine which cats in the population still need to be fixed and also which cats are responsible for most of the population (Farias et. al. 2017). In TNR situations complete genome sequencing would be ideal to compare similarities in sequences between the cats and also

to look for potential genetic diseases. Full genome sequences are difficult and costly to analyze and the data is difficult to compile. However, studying genomes can also be accomplished using individual genes or small sequences of mitochondrial DNA.

Mitochondrial DNA is commonly used in studies such as this because it is easily accessible in both blood and hair samples of cats (Tarditi et. al. 2011) (Wesselinka et al. 2015). Mitochondrial DNA has been used in various studies to help determine how closely related different species of cats are. Mitochondrial DNA, especially 525 base pairs in the RS2 gene region, has been used to compare how related wild African cheetahs, ocelots, and domestic cats are. Based on similarities and differences in the sequences, several different charts that show the differences between the species at this haplotype can be created (Freeman et. al. 2001). These charts can be understood by the way they group specific sequences based on how related they are in circles that show which species, or subspecies, is which. In this case, the individual sequence is assigned to a specific species based purely on sequence differences. Using this method, a clear view of how related different individuals are at specific sequences can be shown (Randi et. al. 2001). A similar method was used on the initial feral cat genetic research and it was discovered that the feral cat population differed slightly from a random population of owned cats in Abilene, Texas (Hankins et. al. 2017).

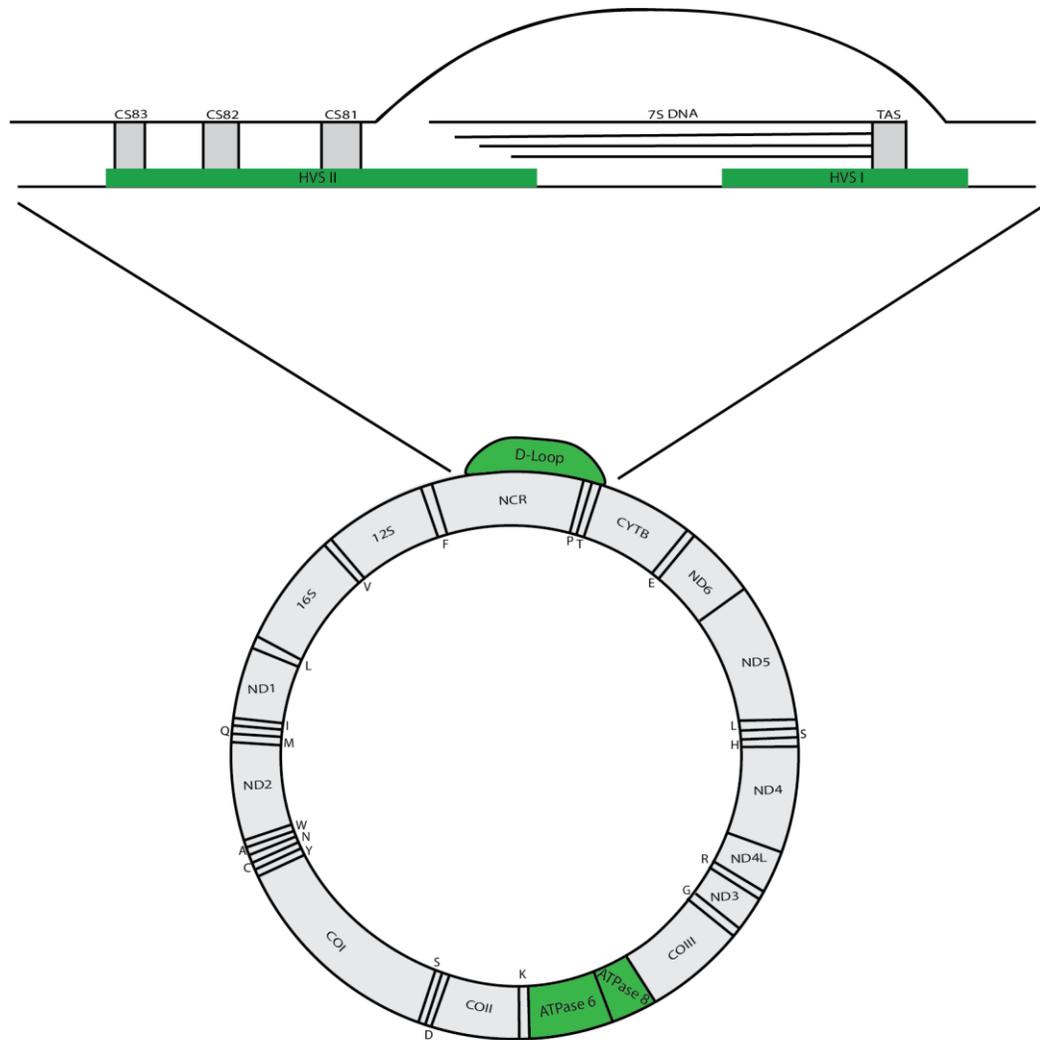
There are several sections of DNA found in blood that can be used to assess the genetic diversity of cat populations. In the mitochondrial genome, there is a section of DNA called the d-loop. This is a section of the circular mitochondrial genome where the genome has three strands of DNA and not just two. The exact reasoning behind why the d-loop is continuously synthesized is unknown, but it can gather mutations throughout

lineages and can be sequenced (Nicholls et. al. 2014). In felines, the mitochondrial d-loop can be broken down into several distinct sections known as hyper variable sequence one (HVS I) and hyper variable sequence two (HVS II). Comparing the sequences of HVS I and HVS II can help determine not only at that haplotype how differentiated these animals are within their species, but also how different they are from other species when the same sequences are analyzed. The primers used most often to sequence the HVS I are from cheetahs and are CH3F, which is the forward primer, and CH3R, which is the reverse primer (Cossios et. al. 2009). In the case of the ACU feral cat population, the cheetah primers could not find matching sequences in the mitochondrial d-loop of the feral cat samples and so other primers were used to test the HVS I sequence. The primers that were used for the ACU feral cats were Pampas cat primers FCB-Z for the forward primer and JHmtR3 for the reverse primer. For HVS II the primers of choice are also from Pampas cats and they consist of the forward primer JHmtF3 and the reverse primer JHmtR3.

Another gene in the mitochondrial genome that can be used is the gene for ATPase 8/6. This enzyme is commonly used in studying genetic diversity in different populations. Several studies have been done that look at this specific sequencing in Holstein cattle as it applies to milk production, and the genetic diversity of a popular food fish known as *Channa striata*. This gene sequence for these enzymes is commonly used because it is polymorphic, meaning that it is found in several different forms and that it can be passed from one generation to the next (Baisvar et. al. 2015). For the purposes of this study, the ATPase 8/6 enzyme mitochondrial gene sequence is being used to help determine the genetic diversity of this population. The primers used for this experiment,

ATP68H87cat and ATP68L94cat, are Pampas cat primers but have been found to match with some DNA in the elucidated DNA samples from the blood of the feral cats.

Based on literature finding, there are several ways to go about researching the genetic diversity of the ACU feral cat population. Currently little is known about the genetics of the population. However, studying different sequences in the mitochondrial DNA found in blood can help get a head start in determining the amount of inbreeding and migration occurring in the population. By first working to sequence the mitochondrial genome, the sequences can be compared to create a haplotype network that would allow for determination of differences within the feral cat population. The use of mitochondrial DNA can also help determine the relatedness of the feral cat population to other species of felines, such as pampas cats and/or cheetahs. The ease of access to mitochondrial DNA makes it easy to use for studies like this, and its polymorphism and small amounts of differentiation within species make it the perfect sequences to use for genetic analysis.



**Figure 1.** Illustration of the mitochondrial genome. HVS I, HVS II, and ATPase 8/6 can be seen highlighted in green.

## MATERIALS AND METHODS

### *Trap Neuter Return Program*

The feral cats were trapped in wire animal cages using canned cat food as an incentive to enter the cage. The cages are set out on Tuesday nights each week and any cats that are trapped are taken to Dearing Veterinary Clinic where they are spayed or neutered, given a rabies vaccine, tested for feline leukemia virus and feline

immunodeficiency virus, and one milliliter of blood is drawn for genetic research. Before they are woken up from sedation each cat has their ear tipped to signify that they have been spayed or neutered. Neutered male cats have their left ear tipped and spayed female cats have their right ear tipped. Once the procedure and the testing are done, the cats are returned to Abilene Christian University campus, where they are fed at various cat feeding stations throughout campus.

#### *Obtaining and Storage of Blood Samples*

One milliliter blood samples were drawn from each of the twenty-three feral cats that was spayed and neutered, as well as twenty shelter cats and one owned cat. This blood was drawn using three milliliter Covidien syringes with twenty-two gauge, three-fourths of an inch long needles. The blood was drawn from the jugular vein. Once drawn, the blood samples were then transferred into Becton Dickenson Vacutainer® plastic hematology tubes sprayed with K2EDTA, an anticoagulant. The samples were stored at six degrees Celsius in a laboratory refrigerator until DNA could be eluted.

#### *Eluting of DNA from Stored Blood Samples*

Whole blood samples were used to elute DNA. Blood samples were handled according to OMEGA® bio-tek E.Z.N.A. Tissue DNA kit protocol corresponding to whole blood samples. Eluted DNA samples were stored at six degrees Celsius in a laboratory refrigerator.

#### *Obtaining PCR Results*

Each eluted DNA sample is mixed with 15.25 µl of sterile water, 2.0 µl of 10X Standard Taq Reaction Buffer (New England BioLabs Inc.), 0.25 µl of 10 mM dNTPs, 1.0 µl of PCR primers, 0.2 µl of NEB Taq DNA Polymerase (New England BioLabs

Inc.). The primers used for each gene sequence as well as PCR protocol are listed in Table 1.

Gene	Forward Primer	Reverse Primer	PCR Procedure	
<b>Hyper Variable Sequence I</b>	CH3F (3' ctcctaagacttcaagg aag '5)	CH3R (3' catctggttcttactcagg '5)	94°C for 1 min 94°C for 1.5 min** 59°C for 0.5 min**	72°C for 1 min** 72°C for 10 min 6°C for hold
<b>Hyper Variable Sequence I</b>	FCB-Z (5' atgaatcggtggccaacc tgt 3')	JHmtR3 (3' gtcctgtggaacaatagg 5')	95°C for 15 min 94°C for 0.75 min** 58°C for 1.5 min**	72°C for 1 min** 72°C for 10 min 10°C for hold
<b>Hyper Variable Sequence II</b>	JHmtF3 (3' gatagtgcttaatcgtgc 5')	JHmtR3 (3' gtcctgtggaacaatagg 5')	94°C for 1 min 94°C for 1 min* 56°C for 1 min*	72°C for 1 min* 72°C for 10 min 4°C for hold
<b>ATPase 8/6</b>	ATP68H87 (5' ggctcaaaccatagcttc 3')	ATP68L94 (5' gcataggaattaggggga cagg 3')	94°C for 1 min 94°C for 0.5 min* 55°C for 0.5 min*	72°C for 1 min* 72°C for 10 min 4°C for hold

**Table 1.** This table shows the genes used for this study of genetic diversity as well as the primers used to study them and the PCR protocol for each gene. The CH3F and CH3R primers did not amplify DNA in the PCR and is therefore excluded from the rest of this study.

\*Indicates a step that was repeated 34 times.

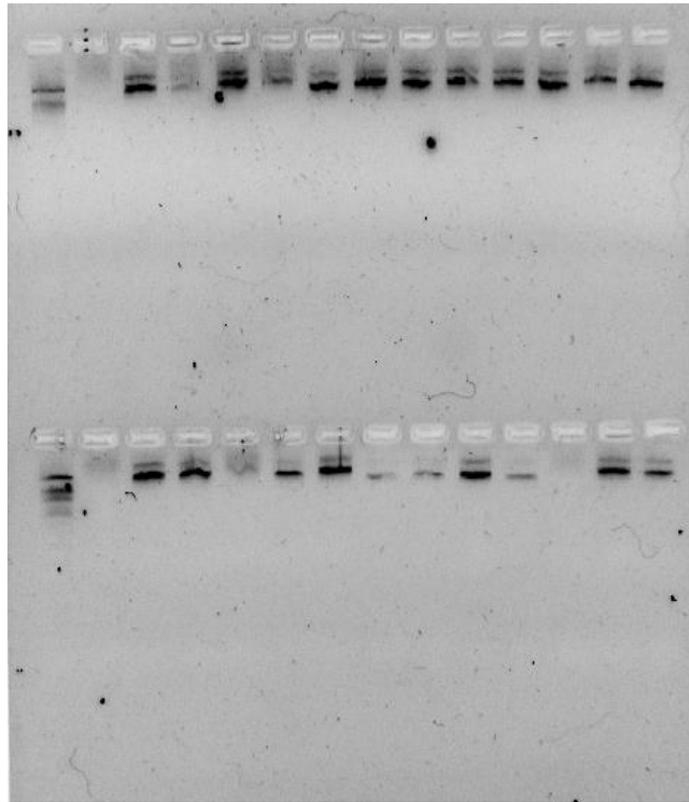
\*\*Indicated a step that was repeated 39 times.

### *Gel Electrophoresis of PCR Samples*

PCR samples were then put into gel in order to visualize if each sample has DNA in it. Gel electrophoresis was accomplished using SYBR green and blue loading dye in a one microliter to two microliter ratio respectively to create a three microliter mixture.

Three microliters of the PCR sample were added into each well with three microliters of the SYBR green/loading dye mixture. Gels were created using agarose powder rehydrated with TAE buffer. The liquid agarose was poured into a mold and a comb with the correct number of lanes was added as it solidified. Once the gels were solidified, the gels were

placed in an electrolyte bath in an electrophoresis rig. Each sample mixture was added into a lane in the gel, with the first lane on each row being a ladder for comparison purposes. The rig was set to 100 volts and allowed to run for 25 minutes. Once this time was over, the gels were viewed using computer imaging software. An example of these gels is shown in Figure 2.



**Figure 2.** This is an image of a gel electrophoresis for the Hyper Variable Sequence I gene. The first lane in each row shows the ladder, which is used for comparison. Each black line indicates where DNA ran down the lane. Each lighter band represents a different sequence of DNA that was found in smaller amounts in the samples.

### *Sequencing of DNA Samples*

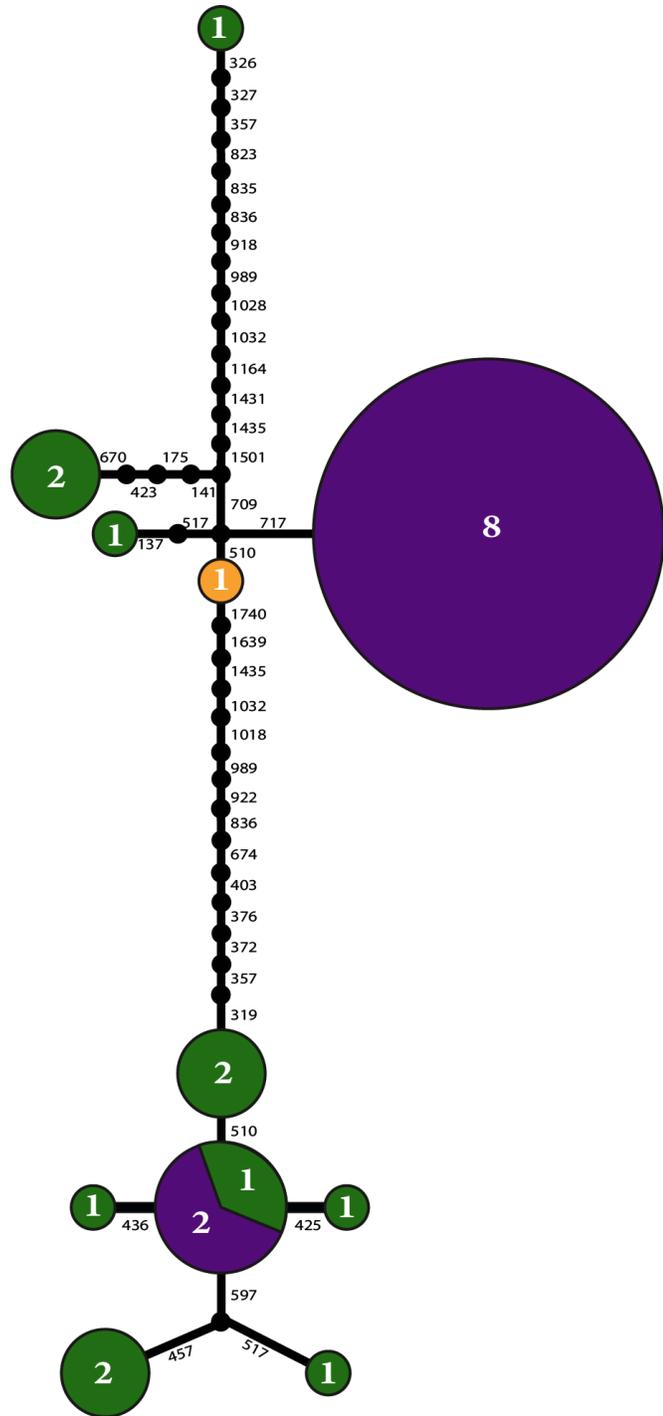
Once the DNA in the samples had been amplified using the PCR and confirmed as present in the sample in a gel, the samples were tested in the Nanodrop™. The

Nanodrop™ technology shines light through a thin string of the sample in order to determine how many nucleotide bases are in a sample. This allows for determination of how much DNA is in the sample regardless of whether or not it is the DNA in question. Once these Nanodrop™ values are determined, the samples are purified once again and reagents are added according to the standards of the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT). These samples were then sent to the DNA Analysis Facility and were sequenced.

#### *Creation of Haplotype Networks*

DNA sequences were compared to each other using Sequencher software. Nucleotide sequences that had less than ninety percent accuracy were removed from the sequencing before they were used to form haplotype networks. These networks were created using TCS version 1.21. Once the rudimentary network was created, it was recreated in Adobe Illustrator® to create a finished product.

3A





**Figure 3.** Haplotype networks. Each larger coloured circle represents a different genotype. The large white number in the circle represents the number of cats that have that genotype. The green represents a shelter cat, the purple represents a feral cat, and the orange represents an owned cat. Each small black dot represents a place in the sequence where a mutation occurs from one genotype to another. The numbers in between these dots represent the number of nucleotide bases between each mutation. **3A.** This haplotype network shows the results for HVS I, HVS II, and ATPase 8/6 sequences combined. **3B.** This haplotype network shows the results for the HVS I gene. **3C.** This haplotype network shows the results for the HVS II gene. **3D.** This haplotype network shows the results for the ATPase 8/6 gene.

## RESULTS

Based on the haplotype networks shown in figure 3, there does appear to be a difference in the genotype for the feral cats versus the shelter cats and owned cats. In the haplotype network seen in figure 3B for HVS I there are thirteen haplotypes and there are feral cats found in only four of them. In the haplotype network for HVS II (Figure 3C) there are six haplotypes and feral cats are found in four of those six. In the haplotype network for ATPase 8/6 (Figure 3D), there are five haplotypes and feral cats are found in four of those five.

Figure 3A shows a combination of HVS I, HVS II, and ATPase 8/6 genes. There are a total of eleven genotypes in this haplotype. Of the eleven haplotypes present, the feral cat population exhibited only two of those haplotypes, with only one feral cat sharing a haplotype with a shelter cat. Most of the feral cats appear to share a similar haplotype for these genes.

The original cheetah primers used to sequence HVS I, CH3F and CH3R, did not show any DNA when the PCR samples were run through a gel. As a result, those primers were not used for sequencing purposes. Since the cheetah primers did not work for this sequence, Pampas cat primers, FCB-Z and JHmtR3, had to be used to sequence HVS I. Since the cheetah primers were unable to amplify any DNA in the domestic cat samples, it is likely that the cats we tested were significantly genetically different at this haplotype.

## DISCUSSION

From the data it appears as though the feral cat population has fewer haplotypes than the shelter cat population for these three combined haplotypes. Analysis of the individual genes shows that the feral cats sampled have consistently fewer haplotypes than the shelter cats sampled and higher tendency to be more closely grouped than the shelter cats. While this cannot be used to describe the entire genome of the feral cats, it can be inferred that for these haplotypes the ACU feral cat population shows significantly less genetic diversity than other cat populations in the Abilene area.

Understanding the genetic diversity of the feral cats at these haplotypes can help determine the effectiveness of the TNR program. Based on the current haplotypes, the population appears to be relatively genetically similar, especially when compared to the sample of shelter and owned cats, but that assumption can only be made for the three haplotypes that have been sequenced. If the TNR program is successful in controlling the population, a decrease in the genetic diversity of the feral cats would be expected. Based on the current results, for these three haplotypes it appears as though the TNR program is successfully reducing the genetic diversity of the feral cat population.

Of the forty-three cats that we sampled, there was only DNA sequenced in thirty-six cats for the HVS I sequence, thirty-five cats for the HVS II sequence, and thirty-four cats for the ATPase 8/6 sequence. There were only twenty-three cats that had sequences for HVS I, HVS II, and ATPase 8/6 genes. The inability to sequence all forty-three samples for each gene causes an inability to be able to view and discuss the complete picture.

## CONCLUSION

The ACU feral cat population does appear to be genetically diverse from Abilene shelter cats at these three haplotypes. The lack of genetic diversity within the feral cat population at these haplotypes appears to suggest that the TNR program may be successfully controlling the feral cat population. More genetic testing would be required to further understand the genetic diversity of the ACU feral cats. Continuing to try and get sequences from samples that have not conclusively had DNA in them at this point will be an important part of getting a conclusive view of the feral cat population. Continuing testing would require looking at more mitochondrial sequences, including microsatellites. In addition, more feral cats are sampled every week by Dr. Dale Hembree and so a more conclusive view of the genetic diversity of this population can be seen as the sample size grows larger. By further observations of the growth of the different ACU colonies as well as monitoring of migration into and out of the population, a more complete view of the genetic diversity of this population can be understood as it applies to the effectiveness of the TNR program.

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