INTERSPECIFIC HYBRIDIZATION ANALYSIS, POPULATION ESTIMATES, AND GENETIC RELATEDNESS OF COYOTE (CANIS LATRANS) CONSPECIFICS IN NORTHERN ALLEGANY COUNTY USING MITOCHONDRIAL DNA COLLECTED FROM HAIR SNARES

ROUNSVILLE

Interspecific Hybridization Analysis, Population Estimates, and Genetic Relatedness of Coyote (*Canis latrans*) Conspecifics in Northern Allegany County Using Mitochondrial DNA Collected from Hair Snares

by
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Abstract

The coyote is a species that only recently migrated to New York but is now firmly established, often as the keystone predator in some locations. Eastern coyotes are known to be much larger than their western relatives since eastern coyotes are considered to be a wolf-coyote hybrid. A barbed wire hair snare survey, specifically designed to maximize coyote capture rate while reducing other species by-catch, was conducted in Allegany County New York to collect DNA samples from free range coyotes of the local population. DNA was then isolated from these hair samples and mitochondrial DNA fragments were amplified using the PCR technique. These fragment sizes were then compared to the published sequences of the amplified fragments and compared to known coyote samples collected from western New York as positive controls. The comparison of these mitochondrial DNA fragments allowed for a better understanding of how coyotes entered western New York as well as the extent to which the eastern coyote has hybridized with wolves. Also, these data were used to calculate the relative abundance and a population size of coyotes nearby Houghton College of Houghton, New York. This study presents the results of the genetic analysis as well as a novel method for non-invasive hair snare surveys that specializes in collection of canid hair samples. Of the 74 samples collected during the entire hair collection period, only two revealed that they were of probable canid origin, particularly that of the coyote, due to the observed fragment sizes for samples 8 and 35 of 164bp for both, also indicating wolf-coyote hybridization.

LITERATURE REVIEW

Part I - Introduction to Coyote Characteristics and Hybridization

The coyote, *Canis latrans* Say, is a mammalian vertebrate that has only recently called western New York home. The coyote is very similar in body shape and stature to the gray wolf (*Canis lupus*), the red wolf (*Canis rufus* or *Canis lupus* ssp. *rufus*), and the common dog (*Canis familiaris*). Although coyotes are morphologically quite similar to the other species in the *Canis* genus, both the gray and red wolf is generally larger than coyotes which have a mean body length ranging from 1.0m to 1.35m (Bekoff 1977). Coyote males have an bodyweight range of 8kg – 20kg and coyote females have an bodyweight range of 7kg – 18kg. These bodyweights are such a large range because coyote bodyweight depends greatly on the varied resource availability of different environments, with coyotes in the northern United States having an average bodyweight of 18kg and their Mexican desert counterparts weighing an average of 11.5kg. The diet of a coyote is generally omnivorous, but vertebrate mammalian flesh compromises roughly 90% of the diet. Coyotes are social animals and a dominance hierarchy is often evident when individuals interact, but they often lack highly organized social structures outside of the main family unit. Coyotes may cooperate and hunt together in packs, but more frequently coyotes hunt and live alone. Coyotes are elusive mammals that have been studied extensively in recent history, but due to their secretive nature, still much about coyote behavior and life is unknown.

Coyotes are relatively new residents to western New York and have only called the area home since the second half of the 20th century. After the extirpation of the wolf in New York State, an unfilled niche for an upper tier predator became available (Kays et

al. 2009). Also, with the clearing of new lands for farming, the number of edge prey species, such as white-tailed deer (*Odocoileus virginianus*), eastern cottontail (*Sylvilagus floridanus*), ruffed grouse (*Bonasa umbellus*), and wild turkey (*Meleagris gallopavo*) became prevalent enough to attract new predators to the area, with the coyote, in particular, being one of these new species (Pilgrim et al. 1998). The increased human presence in New York State was also mainly incompatible with species like wolves that require large ranges and often stray away from human presence. Recent recolonization of wolves near areas of moderate human disturbance shows that wolves may be now learning how to adapt to human presence (Thiel et al. 1998). Coyotes are not shy of humans and have even been documented in and near major cities, such as on Manhattan in downtown New York City in the past five years (Stefan 2006). While the exact routes from which coyotes entered into western New York are currently extensively debated, (whether from Minnesota through the Canadian Shield into northern New York (Kays et al. 2009) or from Michigan across Ontario and into western New York (Wheeldon et al. 2010)) what is uncontested is that coyotes are now a major component of the regional ecosystem.

As found recently in the primary literature, due to the new developments in techniques of genetic analysis, the northeastern coyote is now considered to be a hybrid with wolves (Lehman et al. 1991), while the western coyote has not as significantly interbred with wolves (Pilgrim et al. 1998). Since coyotes, wolves, and even dogs are in the same genus, *Canis*, it is possible for these organisms to interbreed. The coyotes found in the eastern United States are much different from the western coyote, which is often considered to be the ancestor of the species. Northeastern coyotes have been shown to

both contain interspersed segments of wolf DNA, as well as possessing more wolf-like physical characteristics, such as having larger body mass (Bekoff 1977) and cranium size (Kays et al. 2009) than their relatives from the western United States. These wolf-like characteristics, combined with the ability of the coyote to live in close proximity of humanity allowed this organism to become a prominently established species in less than fifty years after their first arrival in the region due to the increased fitness brought about by the inclusion of wolf genes into the coyote genome (Kays et al. 2009).

In the Great Lakes region significant hybridization between coyotes and wolves has occurred and is well documented in the literature. As described in Pilgrim et al. (1998), wolf-coyote interbreeding is most likely to occur on the edge of the wolf's range where the amount of nearby conspecifics is much lower than the relative abundance of coyotes. Female wolves living on the fringe of their range where coyotes are present are much more likely to mate with a male coyote when the overall wolf density is low. The Great Lakes region is on the southern boundary of the gray wolf's range and is also an area where coyotes have much larger established populations. For this reason there are more instances of wolf coyote hybridization in this area than in the western United States where the ranges of the two animals do not overlap as significantly. It is even suggested that the coyotes colonizing the territory in the northeastern United States from which the wolf was eliminated more than a century ago succeeded in such rapid colonization because of this hybridization (Kays et al. 2009).

As discussed above, the exact route which the coyotes moved into the northeastern United States from the west has genetic implications. If the coyotes had moved into the northeastern United States strictly from the west, then these individuals

should show a more pure coyote DNA because coyote wolf hybridization only occurs extremely rare cases in west where the range of the coyote and the wolf overlap (Kays et al. 2009). Comparatively, if the coyote entered the northeast through a more northern route, where wolf populations were still present, the two had the opportunity to interbreed. The current research shows that the genetic diversity in coyotes located in the Midwest United States and the Northeast have variable mitochondrial DNA haplotypes. Coyotes from the Northeast exhibit less genetic variation than those from the western United States but northeastern coyotes have a large percentage of wolf genes in their mitochondrial genome, many more than those in the west. Kays (2009) hypothesizes that the coyotes that colonized much of the Northeast, from Maine south to eastern Pennsylvania, originated from a small number of individuals with little genetic diversity that crossed the St. Lawrence River from Canada and entered into far northern New York. From this location, the coyote-wolf hybrids are purported to have spread out until they reached the expanding line of coyotes from the west. These individuals then began to interbreed and much of western New York and western Pennsylvania coyote population contains remnants of both the coyote-wolf hybrid DNA as well as pure coyote lines from the west. In this area, the same area that this research project was conducted, the coyote populations exhibit both western coyote and wolf-coyote hybrid characteristics.

Although this research allows for a comparison of proposed migrational routes of coyotes, this research merely begins to understand the complex dynamic of populations at work in the Northeast. The samples that were tested in (Kays et al. 2009) contained only a fraction of each coyote's DNA, and of this fraction, it was only mitochondrial DNA.

The introgression of wolf DNA into the coyote genome cannot be completely measured by examining mitochondrial DNA since this DNA, as jokingly indicated by Kays in his discussion, is definitely not responsible for morphological alterations seen when comparisons of coyotes and wolves are conducted. Although it is not the best indicator, mitochondrial DNA serves as a tool to properly understand lineages of genetic transmission since mitochondrial haplotypes are passed down from mother to offspring without significant alteration, such that the haplotypes in a population are not likely to vary much over time. Further research should focus on exactly what genes are linked to these increases in size and more wolf-like physical appearances that have been endowed to coyotes as well as the consequences of wolf reintroduction into areas where large coyote populations are already established.

Part II - Coyote Management Techniques and Implications

The arrival and behavior of the coyote combine to make it a difficult animal to manage. Since the coyotes are not native species, one can consider them to be invasive and attempt to eradicate them because they can have a profound impact on the ecosystems in which they preside. Coyotes are viewed in many areas, particularly those with intensive agricultural use, as nuisances because of the predation of livestock (Wagner & Conover 1999). People cohabiting with coyotes in rural or suburban areas also often advocate for the destruction of the species because they fear for the safety of their children or their pets, especially cats, since some coyotes even specialize in capturing cats because they are easy prey (New York State Department of Environmental Conservation on Coyote Conflicts). In western New York, which is a valued white-tailed deer hunting area, hunters often speak adamantly about how coyotes are destroying the

potential deer harvest, even though this has never been scientifically proven. While it is known that the northeastern coyote has the ability and the stamina to kill and consume white-tailed deer, it is much more efficient for them to eat other prey items due to the high capture energy requirements (Bekoff 1977). Coyotes often only consume deer when other prey is not available or when the opportunity presents itself such as preying upon sickly or injured individuals, which would be deer that the hunters would not be interested in anyway. According the New York Department of Environmental Conservation, more than 4.1 bucks per square mile were harvested in Allegany County in 2008, which is indicative of the highest deer density levels in New York (New York State Department of Environmental Conservation 2009 Deer Hunting Forecast). The sheer abundance of deer in Allegany County has not been significantly reduced in recent history, even with the coyote being present.

Many different groups of people living in Allegany County push various solutions to "dealing" with the coyote's entrance into New York, often with significant legal backing. These groups may understand the way in which they seek to eliminate the problem of human and coyote conflict, but their standpoints on this issue usually lack crucial biological study that understands the ecological implications of enacting these plans. This situation introduces a perplexing situation to wildlife biologists and management officials due to the often biased, yet forceful views of the general public. Intelligent management decisions are made with the understanding that coyotes may be an invasive species, but that they serve an important role, often as the keystone predator. This niche was formerly held by the wolf, but since wolves are no longer present in New York, it must be filled in order to maintain an appropriate balance of prey species. One is

then faced with the decision of how to prevent the coyotes from being a burden to those that live near them, while at the same time allowing enough of a population to be present to perform their ecologically important role as predator. Since the natural balance of species in western New York has already been distorted as wolves, bears, and mountain lions, which were other native predators, have been historically removed, management techniques must be applied to ensure that massive extirpations and destruction of biodiversity do not occur as have in the recent past.

The first step in making management decisions for any organism is to establish a relative population size for a particular area. Knowledge of a population size in an area is crucial information because it can be used to determine the number of organisms in the area that are creating the particular situation evident in the local ecosystem (Henke and Knowlton 1995). Observations of reduced prey numbers may lead one to believe that it may be possible that the coyote density in one area may be more than the carrying capacity of that area, but since this type of a conclusion would require significantly more study, one could create an experiment to determine whether or not the coyotes were responsible for the prey shortage in the area. The population data can also be used to compare it to other areas and the effects that are seen in those locations. This comparison serves to pool as much data as possible into making management decisions related to regulating ecosystem stability. While the local population size of coyotes is the most basic form of knowledge about the organism in any one area, this number is also crucial because relative animal densities may be the factor that influences coyotes to travel to places with high human activity since there may be no space left. In order to conduct further study on the influence of coyotes in the Houghton College area in Allegany

County, the approximate size of the local population of coyotes needs to be determined. To determine this value, the researcher has a variety of methods available.

Since mammals, like coyotes, in particular, are highly mobile, many coyotes are constantly moving into and out of an area making determining an exact population impossible. While discovering the exact population of any organism in an area is impossible, there are varying degrees of specificity to which a population can be estimated. Tremendous manpower is required to determine the population of any organism other than a rough estimation. Due to the limitation of funding for technicians available to most biologists, a variety of survey methods that allow for a rough population estimate using low impact and cost effective methods have been developed. The current methods used to survey coyote and other animal populations range from photo documentation on trail cameras and video surveillance (Shivik & Gruver 2002), to direct and road kill counts (Gese 2001), animal tranquilization and documentation using live traps(mark and recapture studies) (Balser 1965), wildlife area visitor and public observation reports (Quinn 1995), aerial observation, trapping and hunting records (Clark and Andrews 1982), track and scat counts (Gese 2001), attendance at baiting stations (Shivik & Gruver 2002), and DNA sampling (Henke and Knowlton 1995). Of these methods, only a few are considered noninvasive, or methods that do not intrusively force the animal into a potentially stressful situation through direct human contact.

Compared to noninvasive techniques, invasive procedures for studying an organism are very effective in their sampling methodology, but these techniques are not practical for many studies due to logistical complications or the danger that it can pose to the study organism. Invasive sample techniques, such as tranquilization, can create

situations of extreme stress on an organism that can lead to a loss of fitness, paralysis, or even death in an adverse reaction to the medication administered or if and attack or predation occurs while the target organism is still sedated (L. M. Talbot & M. H. Talbot 1962). Other invasive techniques, such as trapping and hunting in order to gain understanding of populations are obviously directly fatal to the coyotes. Invasive techniques of wildlife sampling also place those collecting the data into direct contact with the organism being studied which can cause researchers to be put into a dangerous position. Also, invasive methods of sampling require the time and manpower to trap or track down the organism that is being studied and this type of sampling is not a feasible option for extremely rare or elusive organisms, particularly mammalian carnivores. As a biologist, it is crucial to not disturb that which is being studied because through inadvertent contact, one can destroy or greatly disturb exactly what is being sought out to be preserved initially. It was due to the lack of manpower, equipment, and the desire to preserve the integrity of the coyotes being studied that noninvasive techniques for sample collection were utilized in this study.

The noninvasive technique applied to this study is an application of recent and current population studies of grizzly bears in the western United States and Alaska that uses barbed wire corrals to collect hair samples (Mowat & Strobeck 2000), (Romain-Bondi et al. 2004). Barbed wire corrals, or exlcosures, as they are also called, are barbed wire structures constructed in wildlife habitats. The design is such that hair from passing organisms will be snagged on the wire for easy collection with the hope that the organism's DNA remains preserved in the hair sample captured. Barbed wire is a preferred method of hair collection since it requires minimal interference with the

organisms in the area being tested and it also allows for one researcher to collect samples over a large locality without being overextended. The ease of use, combined with the low cost and significant capture rate of such sampling methods make this method appealing to new population studies, especially of larger carnivores in remote areas where other techniques are not practical.

The goals of this hair collection, a population estimate and an analysis of coyote-wolf hybridizaiton, is achieved through the DNA analysis of the hair samples collected and the application of capture rate to determined formulas from past coyote research. While macroscopic techniques for hair analysis and speciation do exist (R. L. Harrison 2002), improper species identification can occur when samples from multiple species are collected on the same barb. Through the use of the polymerase chain reaction, or PCR, (Mullis et al. 1994), the minute amounts of DNA collected from the hair samples can be amplified for a desired region of DNA and then this fragment of DNA can be sequenced (Pilgrim et al. 1998) and (Kays et al. 2009) in order to determine the species from which the hair sample was taken. The use of DNA sequencing is also a much more accurate technique because it allows for an estimation of whether or not the hair collected is from the same coyote visiting multiple traps, or if there were many unique coyotes whose hair became snagged on the barbed wire exclosures.

Technologies for DNA wildlife analysis also allow the researcher to trace potential hybridization of coyotes with other organisms based on a comparison of the derived genetic sequence with already known sequences. The ability to discern possible hybridization can allow for more informed management decisions since the extent to which a coyote shares wolf DNA can alter its physiology (Kolenosky 1971) and (Kays et

al. 2009). The exact area where coyotes that are now in western New York hybridized with is still intensely debated, but wolf DNA sequences can be easily isolated from a coyote sequence using mitochondrial DNA analysis. It is most likely that coyote-wolf hybridization occurred around the Great Lakes region as wolf females found coyote males at a higher frequency than members of their own species and then chose to mate with them (Kays et al. 2009). Mitochondrial DNA differs from nuclear DNA because mitochondrial DNA is almost always passed from mother to child unlike nuclear DNA that originates from both parents. Mitochondrial DNA is altered much more quickly from one generation to the next than normal genomic DNA, and thus is a much better tool for deciphering hybridization (Pilgrim et al. 1998). The current advances in DNA technology make it such that the researcher is no longer limited to just learning about the population size of the coyotes in any given area, but molecular techniques allow for the analysis of individual animals in order to gain a greater understanding of the local population dynamic.

Part III - DNA Extraction from Hair Samples, PCR, and Fragment Analysis

This section introduces, in detail, the methodology by which the mitochondrial DNA of the organisms whose hair samples were collected was isolated and then analyzed. As discussed previously, mitochondrial DNA is an extremely important resource when seeking to determine whether or not hybridization between species is occurring because mitochondrial DNA is inherited maternally. Mitochondrial DNA sequences and fragment sizes can also be used to differentiate individuals to determine whether or not the hair collected at one or multiple stations belonged to the same individual, allowing for the establishment of a population estimate. While this population

estimate is the goal of this study, the knowledge of the extent to which local coyotes are hybridized with wolves is also valuable information that can prove helpful in management. In the process of determining the number of unique individuals that visited the different exclosures, determining the hybridization of local coyotes is quite simple when the haplotypes of these individuals is compared to those of previous studies, as modeled by Kays et al. 2009.

Before the relative abundance of coyotes in the survey area could be ascertained, one must remember that it is with high probability that most of the samples collected are not from the desired species. While the methodology of this study attempted to eliminate as much contamination from species other than coyotes, cross-catch occurs in almost all hair snare studies (Kendall and McKelvey 2008). Due to the potential of multiple species or multiple coyote individuals' DNA being present in one hair sample, methodology of speciating the hair samples collected must first be used. In this study, the most common contaminant DNA is that of white-tailed deer, or *Odocoileus virginianus*, since the design of the exclosures put them at a height at which this would be one of the only other organisms in the Houghton area ecosystem tall enough to snag hair on the barbed wire. Many instances of white-tailed deer cross-catch were documented in this study, particularly when these deer destroyed the hair collecting barbed wire exclosures by becoming entangled in them. Species specific primers for members of the *Canis* genus were first used after mitochondrial DNA isolation to determine whether or not a significant amount of canid hair had been collected in the field before fragment analysis.

The first step in the eventual fragment analysis of the desired mitochondrial region of the samples of hair collected is the separation of the DNA from the cellular

material of the hair itself. Since the hair root is the only part of the hair that contains DNA, the rest of the hair must be separated from these DNA containing cells because this material can interfere with the Polymerase Chain Reaction and cause it to fail. Most authors of papers related to DNA isolation from hair samples from a variety of organisms elect to use a product from the DNEasy Kits line purchased from the Qiagen, Inc. of Valencia, California, due to their ease of use and easily reproducible results (Romain-Bondi et al. 2004), (Zielinski et al. 2006), (Bremner-Harrison et al. 2006), and (Kays et al. 2009). Due the limited funding available for this project, another method for DNA extraction from hair was used other than that of a DNA extraction kit.

The manila envelopes containing the hair samples were removed from the refrigerator where they had been stored at 4°C and sterilized forceps were used to remove up to ten individual hairs from each envelope. Additional hairs were returned to the envelope for later use if any were present. Some envelopes only contained single hairs from the previous collection and extra care was taken when handling these samples, but the maximum number of hairs available from each sample was used up to ten. Each of the hair samples were then trimmed to the root of the hair and were then placed into a microcentrifuge tube containing an alkaline solution, as adapted from a method similar to Graffy & Foran (2005) to assist in breaking down the cells.

The cell lysis step is important because its heated and basic conditions combine to rupture the cellular membranes such that the DNA of the cell becomes an entity no longer restricted to being inside the cell. Mixing using a pulse-vortex followed by heating enhance cell lysis and destruction. Following the completion of this cycle of steps, they were completed again to ensure that the DNA was removed entirely from the cellular

material and now freely available in solution. If the solution was not basic enough, or if enough heating had not occurred, the cellular membranes would remain intact and the next step of the reaction, the Polymerase Chain Reaction (PCR) (Mullis et al. 1994) would not succeed due to the unavailability of template DNA. The PCR reaction serves to copy a desired DNA sequence exponentially such that the DNA of even a single cell can be used to create millions of copies of a single desired DNA sequence. Once cell lysis was complete, Tris, or tris(hydroxymethyl)aminomethane at pH 7 was added to the hair and NaOH solution. Tris is an important buffer for nucleic acids and is important in ensuring that the pH of the solution stabilizes and this chemical also helps to preserve the porous nature of the already lysed cells. Once cell lysis was complete, the pH of the solutions was tested using Fisher Scientific Alkacid Test Paper to determine the relative pH of the solution to ensure that it was at the proper pH between 7.0 and 8.0. If the pH was not at the appropriate value, the DNA would not be as readily preserved and the success rate of the later PCR reaction could be reduced.

In the PCR reaction, the primers attach to the complementary strand of DNA already present and serve as the binding location for DNA polymerase, which synthesizes a new strand of DNA from the template strand already present. Primers will only bind to DNA sequences that are exactly or very similarly complementary to themselves in this reaction, so if primers are chosen that are able to bind to canid DNA sequence, but not the DNA of other species, then this technique will allow for the easy separation of hair extracts containing canid DNA from those samples that do not contain any canid DNA. The mDNA samples that were shown to contain canid DNA were subjected to fragment analysis to determine the presence of hybridization and also the species of the individual

bearing a particular DNA fragment based on the fragment sizes found in Pilgrim et al. (1998), for the amplified fragment size. Gray wolves (*Canis lupus*) have a 164bp fragment for this same primer pair in comparison to coyotes that have a 160bp fragment, and dogs (*Canis familiaris*) which have a fragment size of roughly 158bp.

MANUSCRIPT 1 – HAIR SAMPLING AND BAITING

Abstract

Recent advances in molecular genetics techniques have allowed for the technology of forensics to be applied to wildlife studies. Using baited barbed wire hair snare exclosures, hair samples, and the DNA that these samples contain, can be collected non-invasively from wildlife. Non-invasive methods of hair collection can provide a sample of organism's genomic and mitochondrial DNA without having to spend the resources actively track or trap the organism from which the samples are desired. Mitochondrial hair analysis can also be used to detect hybridization since the introgression of one species' mDNA into another is indicative of hybridization due strict maternal inheritance. Multiple studies have been designed to understand the hybridization between coyotes and wolves in the northeastern United States where a new study purports that these organisms have hybridized in these areas, including western New York, where this hair snare survey takes place. A hair snare survey was conducted to determine the efficacy of two baiting methods for the barbed wire corrals at collected coyote DNA for further analysis. The first baiting method is a novel method mimicking canid cache holes and the second used consumable food bait to attract coyotes. A total of 74 hair samples were collected from the barbed wire corrals in the area surrounding Houghton, Allegany County, New York for genetic testing and abundance analysis.

Introduction

The coyote (*Canis latrans*), a mid-sized mammalian carnivore, is a species that has rapidly expanded its range over the past century, initially from the western United States into the Northeast, into an available niche previously filled by wolves. Since the

wolves were extirpated by humans living in the Northeast more than one hundred years ago, most of these ecosystems lacked a keystone carnivore and had an abundance of prey due to the prevalence of edge habitats resulting from clearing of land for farming (Pilgrim et al. 1998). Coyotes from the western United States began migrating east to the newly available habitat after the elimination of the wolf in the early 20th century (Kays et al. 2009). This colonization rapidly proceeded, as recent research indicates, due to the hybridization between coyotes and wolves on the fringe of the wolf's range in areas of southern Canada surrounding the Great Lakes (Lehman et al. 1991). These Great Lakes Wolves interbred with the coyotes and produced viable hybrids that still had the stature of a coyote, but more wolf-like physical characteristics that allowed them to hunt deer, a readily available prey item, much more easily than the western Coyotes, which are much smaller (Kays et al. 2009). Studies examining the physical characteristics of coyotes have shown that coyotes from the eastern United States are much larger than those from the west (Pilgrim et al. 1998).

Current studies in coyote genetics seek to use mitochondrial DNA to determine the exact route of entrance of how coyotes migrated to the Northeast. These studies examine the mitochondrial DNA of coyotes in order to determine the level to which the coyotes of that particular area are hybridized with wolves. Northeastern coyotes have very little coyote genetic diversity, but have a large amount of wolf mDNA present, indicating almost all coyotes in the northeast are descendents of a small number coyote-wolf hybrids that entered the area (Kays et al. 2009). Midwestern coyotes exhibit much more diverse coyote mDNA haplotypes and have very little if any detectable wolf genetic sequences. The coyote population of the region in between these two areas, which

consists of western New York and Pennsylvania, has haplotypes that are a combination of the two "pure" groups. The exact routes from which coyotes entered into western New York are still debated, two main hypotheses exist that discuss whether the coyotes entered the Northeast from Minnesota through the Canadian Shield into Northern New York (Kays et al. 2009) or from Michigan across Ontario and into western New York (Wheeldon et al. 2010). Coyotes are now in some places a vital part of the ecosystem in which they live, serving as a major predator.

The wolf-coyote hybrids present in the northeastern United States pose a unique management situation since these animals are technically nonnative species and in some areas they can even be considered invasive. These animals are both supported and destroyed by many political and social groups seeking one agenda or another, but the coyote is an ecologically important species in many of the areas where it resides because it serves as a major predator. The first step to making any relevant management decision regarding an organism is to determine its relative abundance or population in a certain area (Henke et al. 1995). While a variety of methods for determine this type of value are available, very few are actually non-invasive and do not disrupt the life of the animal that is being researched. Invasive methods of research are usually less cost effective and can also be dangerous for the animal being studied as well as the researchers that have to be in close proximity to the animal. In this study, barbed wire hair snares, also known as exclosures, modeled after bear population surveys (Mowat & Strobeck 2000) and (Romain-Bondi et al. 2004), were constructed for the specific purpose of snaring coyote hair in order to use the root cells of this hair for DNA testing in order to make a population estimate of the local coyotes.

Various methodologies exist for evaluating relative coyote abundance, as is explained in a paper written on the topic by Henke and Knowlton (1995). One method of coyote abundance survey is based on visitation to scent-bait sites, similar to the exclosures constructed for the hair snare survey. The abundance levels given in these calculations are merely rough estimates that require further investigation to become more than just a comparison on the number of coyotes between different locations. The index of abundance can be calculated by this formula as given by Henke and Knowlton (1995), adapted originally from Linhart & Knowlton (1975), the number of bait stations with coyote visits divided by the number of stations or "station days" that were available is equal to the index of abundance. For this index one exclosure placed out for a single day counts as a "station day." The number of successfully analyzed coyote hair samples will be used later to determine the index of abundance for this site. This number could then be compared to other data from the surrounding area to determine if this value lies within what others have found for the area.

Hair snare exclosures consist of posts with barbed wire wrapped at a species specific height above the ground to ensure that the desired animal cannot climb over or move under the wire without snagging some of its hair on the wire (Kendall and McKelvey 2008). The center of the exclosure is then baited with a food or scent bait that encourages the targeted organism to enter the corral to investigate, thus snaring some of its hair on the wire in the process. Samples are to be collected from the wire using sterilized forceps with each barb counting as its own sampling point even though this may cause a species mixture to occur. Samples are then refrigerated in labeled manila envelopes until later use in DNA analysis if desired.

This hair snare survey seeks to collect coyote hair samples for genetic analysis from free ranging animals. The hair samples collected using either methodology of baiting the exclosures will be collected and tested for species and hybridization using mitochondrial DNA analysis. It is hypothesized that the cache hole method designed in the manner included in the literature review will snare a larger number of coyote hairs than the control runs of the exclosure with no baits and also the consumable food method for hair sample collection. Also, it is hypothesized that the cache hole method will achieve a lower level of other species-by catch due to the design of the baiting procedure that utilizes specific canid behavioral responses.

Methods

Five transects of 1km each with four barbed wire corrals located at 333m intervals on each transect were constructed for noninvasive coyote hair collection. The transect size and design used was scaled down and adapted from sampling methods as described in Kendall and McKelvey (2008), Henke and Knowlton (1995), and (Farry et al. 1998). These transects were constructed on a digital topographic map using GPS software covering all land available for research such that the exclosure points on each transect were randomly determined. Once the position of each exclosure along the transect was noted, a random number table was used to place the center of the corral a distance of 1m to 40m at a 90° angle from the central transect line with the odd numbered exclosures placed on the left hand side of the transect and the even numbered exclosures on the right. The positions of the map-selected exclosures were validated with field observation and any unsuitable locations were subjected to additional uses of the random number chart until a usable location was selected.

Before the construction of any of the exclosures, all of the equipment used in building the exclosures as well as any field observation tools were sterilized for scent removal to increase trap hair yield (Kendall and McKelvey 2008). The equipment was sterilized by being placed into a large metal drum (50 gallon) filled with 150L of water and the surface covered with freshly harvested staghorn sumac (*Rhus typhina*) fruit. A fire was constructed under the barrel and the contents were brought to an intense boil and then left to soak overnight. The equipment was then removed and placed in evergreen boughs (mainly *Tsuga Canadensis* and *Pinus strobus*) in a sealed metal container until use to prevent scent contamination.

At the locations which the exclosures were to be constructed, all of the vegetation within at least a three meter radius was cleared and the soil was overturned to eliminate the variable of vegetation from capture rate. It was determined from (Farry et al. 1998) that the visual cue of the removal of vegetation and raking of soil at the bait station does not negatively affect capture rates, but can actually increase them due to the coyote's natural investigative behaviors (Heffernan et al. 2007). Exclosures were constructed as an equilateral triangle of sides 1.5m around the central point with three 2.54cm x 5.08cm x 91.44cm (l x w x h) pointed wooden stakes serving as the edge points of the triangle after being hammered into the ground until stable. Fourteen gauge barbed wire with 10.0cm between barbs was strung around the stakes and fastened to them using flexible wire at a height of 45cm, as visualized in Figure 1 and Figure 2, which are included in the attached figures section.

Once the corrals were constructed, a one week control period was established with hair collection occurring from 10/23/09 to 10/30/09, which ended the week after

construction. All hair snagged on one barb of the wire was counted as a single sample by convention (Kendall and McKelvey 2008) and each hair sample was collected using sterilized forceps and placed into a labeled manila envelope for storage. Following the control period, two different baiting strategies were used to noninvasively collect coyote hair samples with the first being Kishel's Coyote Getter, a nonconsumable food scent bait and the consumable dog food bait Purina® Alpo® Homestyle Prime Slices with Chicken in Chicken Gravy.

For the first strategy, two mimic canid cache holes were dug 20cm deep at 40° to the horizontal per exclosure using a trapper's trowel shovel. One hole was dug in the exact center of the exclosure and the other outside of the exclosure, exactly 1.5m to the south of the center, as diagrammed in Figure 2. The hole outside of the exclosure was baited with .3ml of Kishel's Coyote Getter dispensed from a volumetric pipette into the bottom of the hole. Hair samples were collected over a two week period from 10/30/09 to 11/13/09, with hair samples being collected on 11/6/09 and 11/13/09, and exclosure rebaiting occurring on 11/6/09. When the second week of collection using the cache hole method ceased the cache hole were filled in and, another control period was run from 11/14/09 to 11/21/09. Next, $10cm^2$ birch plywood feeding platforms were baited with 200g of Purina® Alpo® Homestyle Prime Slices with Chicken in Chicken Gravy was placed in the center of each exclosure. Hair collection occurred over a two week period from 11/29/09 to 12/13/09, with collection occurring on 12/6/09 and 12/13/09, and rebaiting and unused bait removal on 12/6/09. All collected hair samples were stored at 4°C until used later for genetic testing using the protocol as described in manuscript II, which is attached to this paper.

Results

A total of 74 hair samples to be used in genetic analysis were collected from the twenty barbed wire exclosures during the different phases of the study period. Of these hair samples collected, a total of eight were collected for the control periods, with four being collected during each of the one week controls. From the cache hole method, a total of 46 hair samples were collected, as compared to the 20 samples that were collected using the consumable food bait method. When the results of the control periods are combined into a two week period, the two weeks of sampling with the cache hole method increased the capture rate 575% from that of the control and the consumable food bait method increased the capture rate 250% as compared to the control. For the first trial of the consumable food baiting method, 75% of the baits were partially or completely consumed, and for the second trial 45% of the baits were partially or completely consumed, which was classified as the bait being taken.

Exclosure #13 collected 20.3% of the total number of samples, which was the most of any of the exclosures, and was located within a white pine (*Pinus strobus* L.) forest fragment, within 10m of a major trail and less than 200m from nearby houses, a road, and soccer fields. From Exclosure #11, located in a small deciduous forest fragment within 100m of both the Genesee River and expansive cornfields and edge areas, 13.5% of the hair samples were collected, which is the second highest amount for any one exclosure. Overall, an average of 3.7 hair samples were collected from each exclosure, with only 15% of these exclosures yielding no hair samples at all. The majority of the exclosures yielding no samples were from interior forested locations. Throughout the length of the experiment five different exclosures were destroyed by collisions with

animals and had to be rebuilt. These collisions resulted in the collection of multiple samples from a single individual, which may have influenced the results.

Based on the genetic analysis of these 74 collected hair samples, only two of the many samples collected are likely to have been snagged by a coyote. Due to contamination in the analysis, the conclusion that two wolf-coyote hybrids were detected cannot be stated with much confidence. These two samples, sample 8 and sample 35, were both collected using the cache hole method, being collected from exclosures six and eleven respectively. If these were the only canid hair samples collected, then the cache hole method successfully collected a higher percentage of coyote hair as compared to the control or consumable food bait method of baiting. Also, if two cases of coyote hair were confirmed to have been collected, using the index of abundance formula as proposed by Henke and Knowlton (1995), a total of two coyote visits divided by the product of twenty exclosures operating for a total of six weeks, or 840 trap days, yields a fraction of 2.38×10^{-3}, multiplied by 1000 to give an index of abundance of 2.38.

Discussion

A direct comparison of the overall yield of hair samples of one baiting method versus another cannot be completed using the conditions under which the hair collection was made since the two methods had a number of extraneous variables operating on them. For instance, during the final week of the consumable food bait trials, extremely cold temperatures froze the bait and a snowstorm covered a majority of them, resulting in a reduced yield, but still more than the controls. A 30% drop in bait uptake was witnessed between the first week of data collection using the consumable bait method as compared to the second week using this same method in which there was inclement weather. This

loss in capture due to the weather is responsible for the failure of bait stations to properly estimate coyote abundance, as described in Henke and Knowlton (1995). Also, the rate at which corrals were destroyed can be correlated to the number of hair samples that were collected during any particular trial period because white-tailed deer (*Odocoileus virginianus*) colliding with the barbed wire exclosures resulted in a larger number of collections. This is because five hair samples or more were not uncommonly collected after one of these collisions. Using macroscopic methods of hair analysis, the primary by-catch of the exclosures was white-tailed deer, as predicted previously. Macroscopically, hair can be sepciated based on presence, absence or size of vacuoles, as well as other characteristics such as color, rigidity, and root tip structure (Harrison 2002).

This hair snare survey served to gather coyote hair for mitochondrial DNA analysis, which was used not only to determine the species of the samples collected, but to also research the possible hybridization with wolves present in these individuals. The cache hole method, as expected, produced more samples that were potentially coyote than the other two methods that failed to produce viable canid samples, either by test failure or by returning a negative result. Comparatively, the sheer number of samples collected during the cache hole method's trials that were not from coyotes brings forth the possibility that there may have been some attractant to deer also contained inside the Kischel's coyote getter. Another hypothesis for this large amount of by-catch is that at the period in time in which the cache hole trials were completed, the local deer were not yet habituated to the locations of the exclosures and simply ran into them. Once the deer learned the positions of the exclosures, they no longer collided with them, which can be witnessed in the fact that no corrals were destroyed during the consumable food

collection period. Further study and experimentation would allow for greater understanding of this phenomenon.

The overall purpose of the comparison of methodologies for hair collection was to determine if the cache hole method was a viable means to collect usable mDNA from coyotes that can be used to assist in making management decisions. After reading extensively and discovering all of the coyote survey methods available (Henke and Knowlton 1995), hair sample collection may not be the best or most accurate method for establishing an index of abundance. The index of abundance was calculated using the assumption that the two samples that are possibly coyote in nature are actually coyote. Since not every organism that visits the exclosure site actually yield hair samples, the number of visits is skewed because it is limited to encounters in which extractable DNA was collected. In reality, coyotes may visit the site repeatedly without ever leaving a hair sample, and thus this coyote would never be recorded. Retrospectively, using data collected from either hair snare technique to calculate an index of abundance does not seem that it would provide an actual, accurate assumption of the index of abundance due to the large number of visitations of coyotes at the exclosure stations that is never recorded.

Further study should be conducted using this new methodology of baiting known as the cache hole method. It has demonstrated by this study that this method is capable of capturing potential canid DNA, and with further variation, these exclosures should be able to further enhance the rate at which canid samples are collected while still reducing other species by-catch. Also, a direct comparison of the cache hole method to the food

bait method could be completed in order to evaluate the effectiveness of each in collecting a desired type of samples under controlled conditions.

In order to apply the index of abundance, its value has to be compared to the indexes of coyote abundance from other areas near Houghton, NY. Relative abundance is a relational term that compares the relative population size of one species and compares it to the population of the same species in another area Henke and Knowlton (19955). They purport this technique to be valuable in eventual coyote population density estimation, although admit that at times it can be divergent from actual coyote density data. Gompper et al. (2006) found that only rarely were abundance measures actually related to the measured densities when using non-invasive sampling methods. This was often due to the same individuals visiting multiple trap sites and skewing the data, unless the method of analysis was coupled with another method of analysis. This project combines data collected using non-invasive techniques with genetic analysis, but the index of abundance calculated for the local coyote population using this data still fails to account for coyotes that visit the exclosure site and yet do not leave a viable hair sample.

Based on the information contained in Henke and Knowlton (1995), a population density estimate or population size estimate can only be conducted following the comparison of relative abundance rates to an area with a known coyote density gathered using an alternative method. No recent coyote abundance data that was connected with population density near the Houghton, New York area could be found. Further study would be required to first determine the relative density of coyote individuals in locations in Allegany County, and from this data, the relative abundance rate discovered for

coyotes in the Houghton, New York can be used as a relation between the two to gather population data on the local coyotes.

Although no index of success exists for measuring the outcome of a hair snare survey specifically designed to sample from coyotes, a two in 75 samples ratio appears as a large amount of trap effort required for such a small response. In conversations with New York State Biologist, Dr. Roland Kays, the relatively low number of responses gathered when using hair snares to gather DNA from coyotes had caused him to change DNA collecting methodologies in the past. While it is uncertain whether or not the actual coyote response was low or not, it is possible that the exclosures were designed too high such that the target coyotes could enter and exit the exclosures without being snagged. It is possible that the one modification to the trap design to prevent by-catch may have also resulted in the capture of less hair samples to use for genetic analysis.

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Table 1- A comparison of the total number of samples collected for each baiting strategy. Number of baits accepted is the number of baits that were wholly or partially consumed followed by the number of corrals that were damaged beyond the ability to collect samples during each corresponding baiting strategy.

Baiting Strategy	**Number of Samples Collected**	**Number of Baits Accepted**	**Number of Corrals Destroyed During Each Trial**
Control #1	4	-	2
Control #2	4	-	1
Control Avg.	4	-	1.5
Cache Holes #1	24	-	1
Cache Holes #2	22	-	1
Cache Holes Avg.	23	-	1
Dog Food #1	14	1-5	0
Dog Food #2	6	9	0
Dog Food Avg	10	12	0

Table 2 – This table contains the data regarding the hair samples collected from each exclosure for each of the different trials. In the bait acceptance columns, a zero indicates that the bait was not disturbed by what appeared to be a mammalian carnivore during the time period which is was left out. The exclosures labeled with one indicate the bait was either partially or wholly consumed or the feeding platform was moved outside of the exclosure.

Exclosure Number	Control #1	Cache Holes #1	Cache Holes #2	Control #2	Food Bait #1	Food Bait #2	**Total Samples Per Exclosure**	Bait Acceptance Food #1	Bait Acceptance Food #2
1	0	0	0	0	1	0	**1**	1	0
2	0	0	0	1	0	1	**2**	0	1
3	0	1	2	0	0	0	**3**	1	0
4	0	1	0	0	0	1	**2**	0	0
5	0	0	0	0	0	0	**0**	1	0
6	1	4	0	1	1	0	**7**	1	0
7	1	1	1	0	2	0	**5**	1	0
8	0	1	0	0	1	0	**2**	1	0
9	0	0	0	0	0	1	**1**	0	1
10	0	4	0	0	0	1	**5**	1	1
11	0	0	4	2	3	1	**10**	1	1
12	0	0	0	0	0	0	**0**	0	0
13	0	10	2	0	3	0	**15**	1	1
14	0	0	2	0	0	0	**2**	1	1
15	2	1	0	0	1	0	**4**	1	0
16	0	1	3	0	0	0	**4**	0	0
17	0	0	2	0	1	1	**4**	1	1
18	0	0	1	0	0	0	**1**	1	1
19	0	0	0	0	0	0	**0**	1	1
20	0	0	5	0	1	0	**6**	1	0
Totals:	**4**	**24**	**22**	**4**	**14**	**6**	**74**	**15**	**9**

Table 3 – Table showing the dates and baiting method used when each particular sample was collected

Baiting Method	Date of Collection	Sample Number	Baiting Method	Date of Collection	Sample Number
Control #1	10/30/2009	1	Cache Holes #2	11/13/2009	38
Control #1	10/30/2009	2	Cache Holes #2	11/13/2009	39
Control #1	10/30/2009	3	Cache Holes #2	11/13/2009	40
Control #1	10/30/2009	4	Cache Holes #2	11/13/2009	41
Cache Holes #1	11/6/2009	5	Cache Holes #2	11/13/2009	42
Cache Holes #1	11/6/2009	6	Cache Holes #2	11/13/2009	43
Cache Holes #1	11/6/2009	7	Cache Holes #2	11/13/2009	44
Cache Holes #1	11/6/2009	8	Cache Holes #2	11/13/2009	45
Cache Holes #1	11/6/2009	9	Cache Holes #2	11/13/2009	46
Cache Holes #1	11/6/2009	10	Cache Holes #2	11/13/2009	47
Cache Holes #1	11/6/2009	11	Cache Holes #2	11/13/2009	48
Cache Holes #1	11/6/2009	12	Cache Holes #2	11/13/2009	49
Cache Holes #1	11/6/2009	13	Cache Holes #2	11/13/2009	50
Cache Holes #1	11/6/2009	14	Control #2	11/29/2009	51
Cache Holes #1	11/6/2009	15	Control #2	11/29/2009	52
Cache Holes #1	11/6/2009	16	Control #2	11/29/2009	53
Cache Holes #1	11/6/2009	17	Control #2	11/29/2009	54
Cache Holes #1	11/6/2009	18	Dog Food #1	12/6/2009	55
Cache Holes #1	11/6/2009	19	Dog Food #1	12/6/2009	56
Cache Holes #1	11/6/2009	20	Dog Food #1	12/6/2009	57
Cache Holes #1	11/6/2009	21	Dog Food #1	12/6/2009	58
Cache Holes #1	11/6/2009	22	Dog Food #1	12/6/2009	59
Cache Holes #1	11/6/2009	23	Dog Food #1	12/6/2009	60
Cache Holes #1	11/6/2009	24	Dog Food #1	12/6/2009	61
Cache Holes #1	11/6/2009	25	Dog Food #1	12/6/2009	62
Cache Holes #1	11/6/2009	26	Dog Food #1	12/6/2009	63
Cache Holes #1	11/6/2009	27	Dog Food #1	12/6/2009	64
Cache Holes #1	11/6/2009	28	Dog Food #1	12/6/2009	65
Cache Holes #2	11/13/2009	29	Dog Food #1	12/6/2009	66
Cache Holes #2	11/13/2009	30	Dog Food #1	12/6/2009	67
Cache Holes #2	11/13/2009	31	Dog Food #1	12/6/2009	68
Cache Holes #2	11/13/2009	32	Dog Food #2	12/13/2009	69
Cache Holes #2	11/13/2009	33	Dog Food #2	12/13/2009	70
Cache Holes #2	11/13/2009	34	Dog Food #2	12/13/2009	71
Cache Holes #2	11/13/2009	35	Dog Food #2	12/13/2009	72
Cache Holes #2	11/13/2009	36	Dog Food #2	12/13/2009	73
Cache Holes #2	11/13/2009	37	Dog Food #2	12/13/2009	74

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Figure 1 – This figure is top-down view of the construction of the barbed wire exclosure used to capture coyote hair. It consists of 14 gauge barbed wire of length 1.5m per side strung around three posts to form a triangle. For the cache hole method, two holes were dug at the exclosure site, with one in the direct center of the wire that received no bait and a second outside of the exclosure that was baited with a non-consumable scent bait. For trials completed with a food scent bait, a baited feeding platform was placed in the center of the wire.

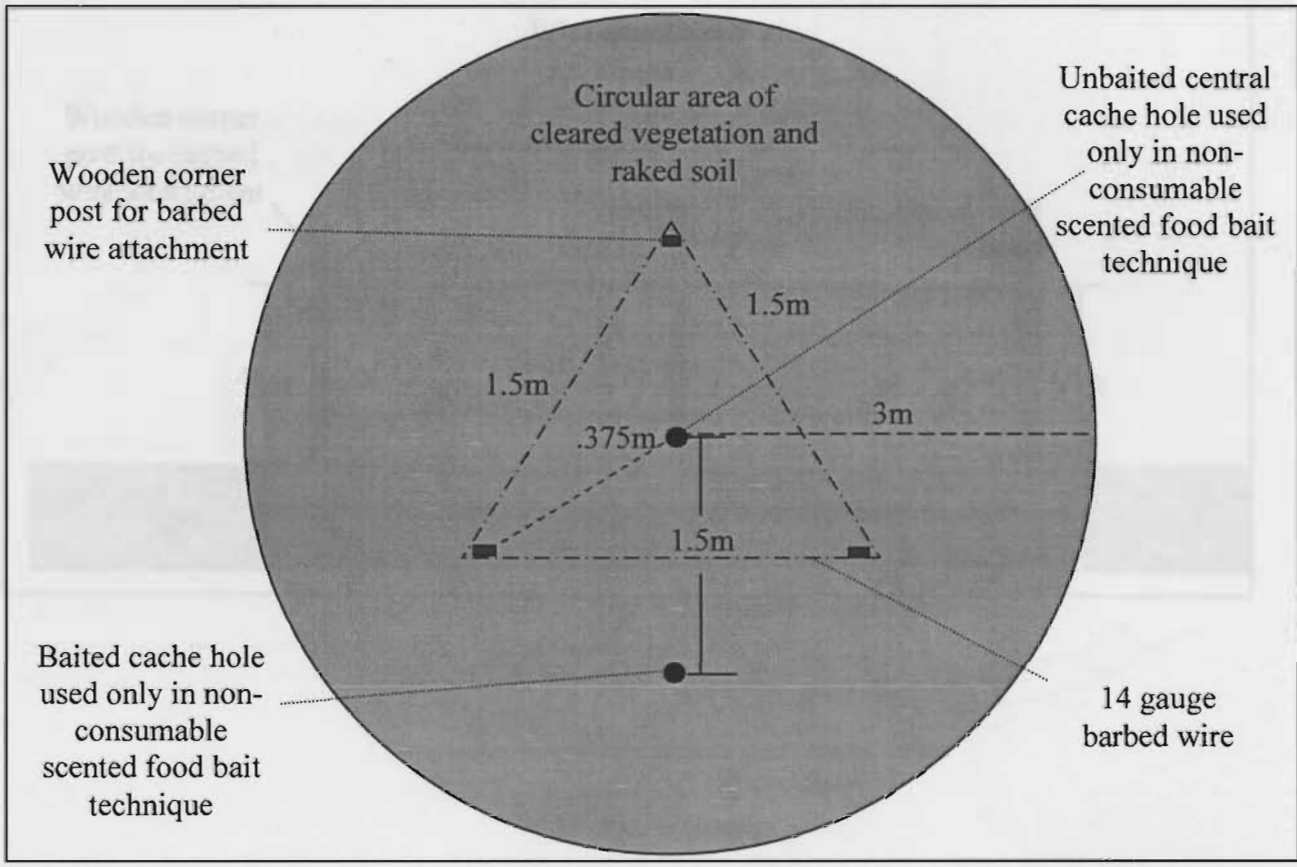

Figure 2 - This figure presents a side view of the same barbed wire exclosure used to capture coyote hair. The wire was placed .45m from the ground to ensure optimum coyote capture while maintaining minimum by-catch. Although the terrain at the exclosure often varied, the wire was bent such that the majority of the barbed wire was kept .45m from the ground to achieve the highest probability of snaring any coyote that entered the exclosure.

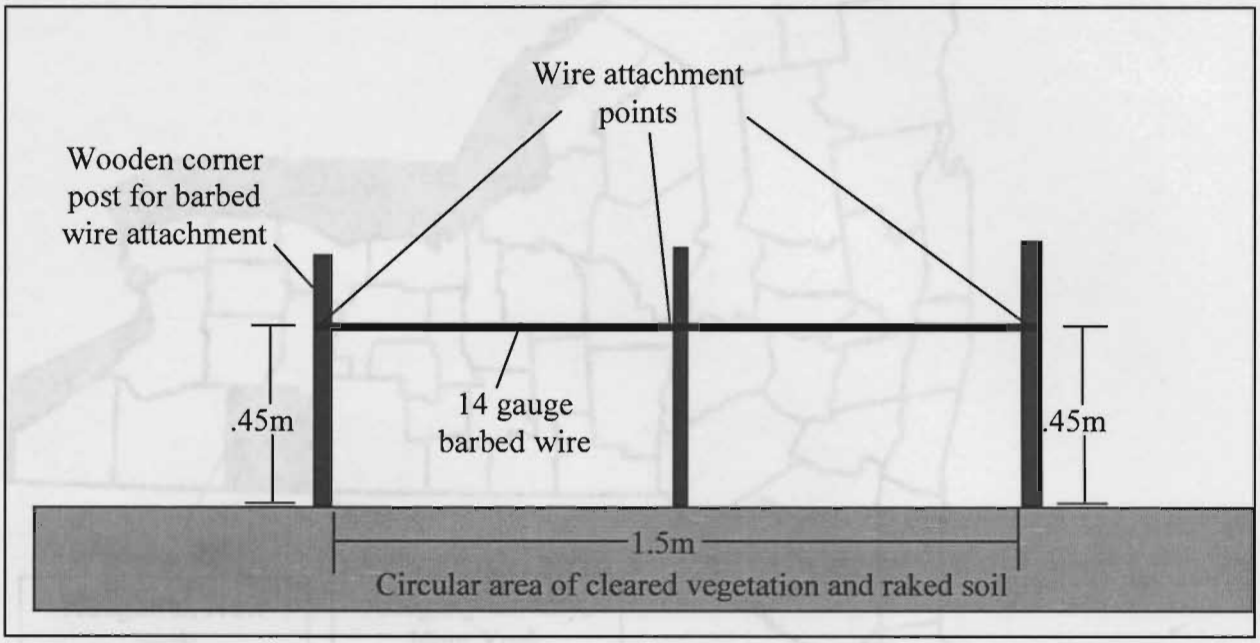

Figure 3 – A Map of New York State with Allegany County, the location of the study marked, as well as the location of Houghton, New York.

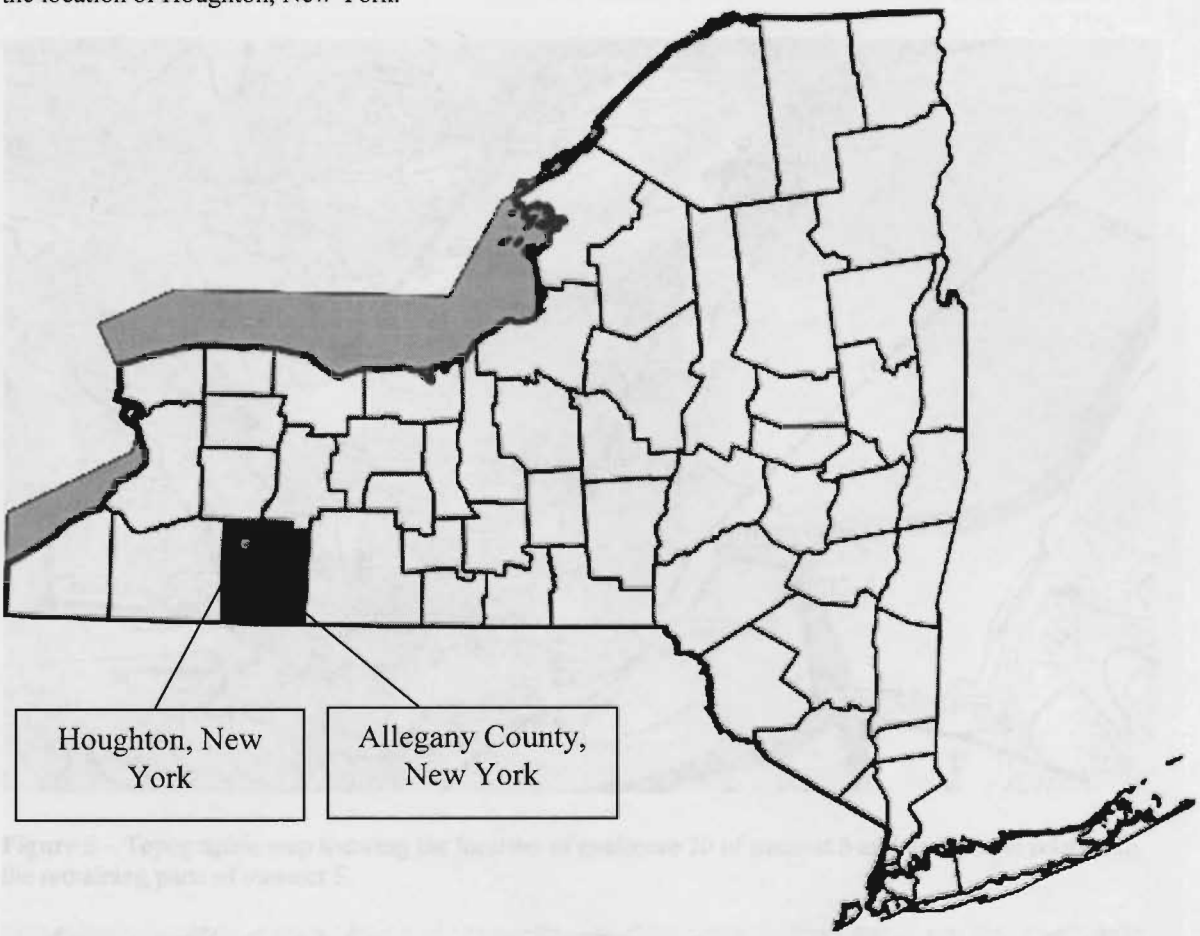

Figure 4 – Topographic map of the Houghton, NY area showing the locations of exclosures 1-5 in relation to one another.

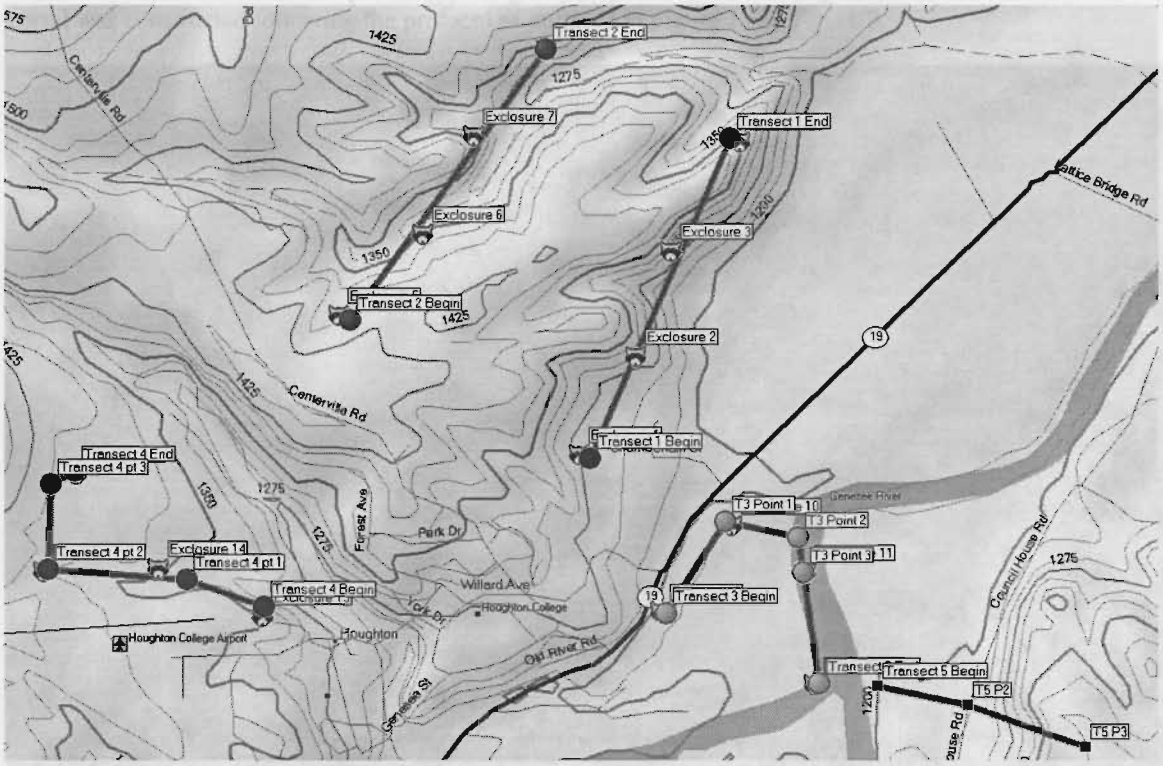

Figure 5 – Topographic map showing the location of exclosure 20 of transect 5 and its location relative to the remaining parts of transect 5.

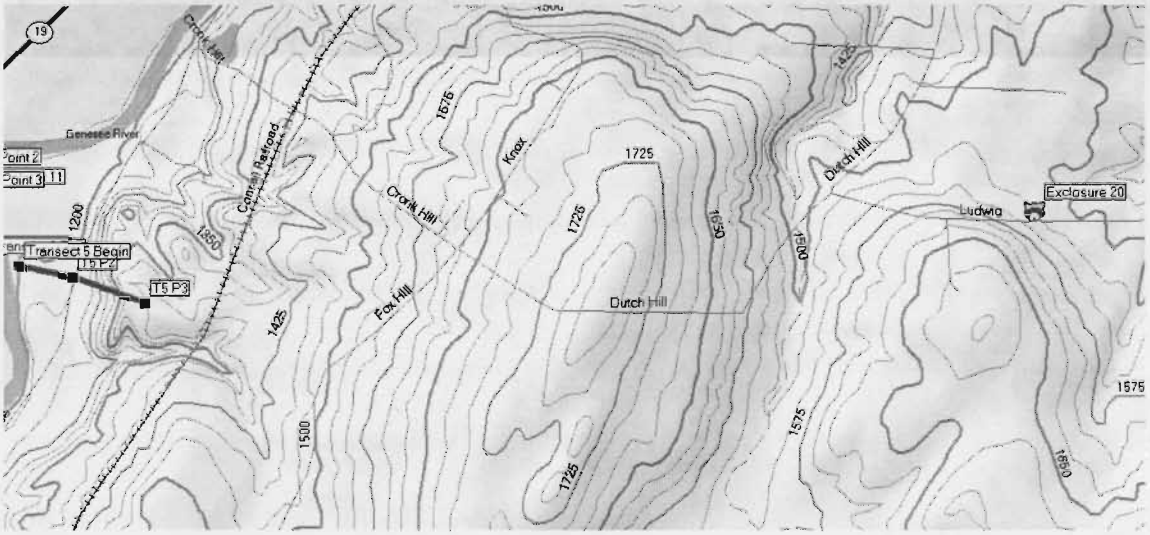

Figure 6 – A photograph of exclosure number nine taken the spring after the study. Note the cleared circle of vegetation where the new goldenrods are sprouting in comparison to the surrounding dead vegetation. When this exclosure was in use previously the ground was raked after all vegetation was removed. This corral was constructed following the protocol as outlined in Figure 1 and Figure 2.

Appendix – Methodology of Hair Sampling and Exclosure Baiting Techniques

To noninvasively collect hair samples from local coyotes, barbed wire corrals were constructed along transect lines in the vicinity of Houghton College, in Allegany County, New York. In the layout of these transects, a variety of potential habitats was chosen, but the main limiting factor in deciding the locations of the transects was the availability of space, particularly an unbroken stretch that remained in wilderness or relatively wild areas such that human tampering with the study could be limited. The manner of transect construction and density of bait stations per square kilometer were modeled after Farry et al. (1998), with a scaling down of the transect sizes based on the available space to conduct this study. Around each exclosure, an area of flat, undisturbed soil was created with raking to allow for the quick detection of animal entry into the corral or the immediate vicinity around it.

The vegetation around each corral was cleared for two purposes, with first being the utilization of the natural curiosity of the coyote. Coyotes are naturally attracted by their curiosity to an area that was suddenly disturbed without any significant human presence at the time of exploration, which would enhance the probability of capture (Heffernan et al. 2007). The second purpose in clearing the vegetation was to eliminate the variable that vegetation surrounding the corral could have on whether or not a coyote would enter the exclosure and thus skew the ability to evaluate potential effect of the local environment on the success of each corral, and this is a procedure frequently carried out in other studies of a similar nature (Farry et al. 1998).

Each corral was constructed by the following parameters, based on those as described in Kendall and McKelvey (2008). They recommend the use of trees as posts for

barbed wire when constructing exclosures, but since the manner in which this study selected its points for corrals, this type of corral construction became problematic. Some of the habitats in which corrals were to be constructed included fields and wetland areas, and since trees were not readily available in all of these locations, as they were randomly selected, a new methodology was needed specifically for the scope of this research. Due to the diversity of habitats used in this study, each of the corrals was constructed using a combination of both wooden stakes and barbed wire. The corrals were initially designed to be circular, but after extensive difficulty in physically constructing the corrals, they were redesigned to be triangular in nature. Each corral was then designed to be an equilateral triangle with sides of length 1.5m.

Scent pollution is a major cause of trap failure when attempting to attract canids, whose olfactory senses are far superior to those of humans. Something as simple as a scent trail near a corral could make it such that a coyote would be too timid to explore the area due to the detection of a potential predator (Kendall and McKelvey 2008). To avoid such situations and maximize the collection of hair samples from local coyotes, multiple scent reduction techniques were used to minimize the scent impact of researchers on the results of the study. Before any of the corrals were constructed, all of the stakes, barbed wire, and tools to be used while constructing or visiting the corrals in the future were sterilized using a method traditional to coyote trappers, which is boiling all of the submerged equipment in staghorn sumac (*Rhus typina*) fruits overnight. This sterilization method is also used by coyote trappers to instill a more natural and less standoffish color to the wire and the stakes without adding strange scents by painting them. After sterilization was completed, the equipment was removed using unscented vinyl gauntlets

and the materials used to construct the corrals were stored in eastern hemlock (*Tsuga Canadensis* L.) and white pine (*Pinus strobus* L.) boughs to prevent further scent contamination.

Further elimination of scent contamination by handling was achieved by the wearing of rubber gloves during the construction procedure. When the corrals were constructed the stakes were also positioned such that they were exactly 150cm away from each other, indicating the creation of an equilateral triangle of sides 1.5m. Based on the average body size of the coyote, the center of the corral could not be reached from any side by a coyote without first snagging on the barbed wire since coyotes have an average body length of 1.0m to 1.35m (Bekoff 1977). The shortest distance from the outer edge of the exclosure to the center of it was roughly .45m. For studies with the goal of collecting bear hair, Kendall and McKelvey 2008 recommended that the bait in the center of the corral be placed at least 2m from any side to ensure capture of bears entering the area. Since bears are proportionally larger than coyotes, this recommended distance can be scaled down since any coyote investigating the corral would become snagged on the wire before it would be able to move its head completely into the corral to investigate. When the stakes holding the barbed wire were finally hammered into the ground, no standard depth was used because of the varying soil moisture contents in different areas that were used for corral construction. In forested locations with fairly stable soil, much less soil was needed to keep the stakes stable as compared to wetland areas that required a greater depth to achieve the sample level of stability.

Once the stakes were inserted into the ground as a stable point of attachment for the barbed wire, the wire was attached to the stakes by bending additional 12 gauge metal

wire around the post in the barbed wire and then twisting it multiple times with wire clippers. This method of attachment was used on each stake of the corral once the height of the barbed wire from the ground had been measured to be 45cm. Since the wire is suspended off of the ground for distances of 1.5m at a time, it is inevitable that some of the wire deviated in either direction of the desired value of 45cm. It was crucial to ensure hair capture of any coyote that entered the corral that this wire height is kept as close as possible to the standard. Frequent adjustments had to be made to the corral to ensure that this optimum height was maintained, sometimes with the ground height being altered (Kendall and McKelvey 2008). This height was chosen relative to the body size of the coyote such that the coyote had to either step over the wire to access the center of the corral and have some become snagged or if the coyote chose to access the center of the corral by going under the wire, that the wire was low enough still such that some hair was also collected.

Bycatch is a frequent occurrence when using hair corrals for the sampling of wildlife. Bycatch is when hair of an undesired species is caught instead of the target species, as described in Kendall and McKelvey 2008. In order to avoid by-catch and specialize in the species that was desired to be collected, the author decided to change the height of the wire that was chosen to make it slightly higher than is normally be used for canids Kendall and McKelvey (2008), but still low enough to be effective. This height, although higher at 45cm, is still valid for coyote hair capture. Adjustment was made to the average height used due to the relative abundance of other animals in the vicinity of Houghton, such as skunk, raccoon, and domestic cat, such that these animals could simply pass underneath the wire and not be caught. The populations of some of these

species locally is so great that the hair shed by these organisms on the barbed wire could have significantly diluted and contaminated the coyote DNA present on the wire, making DNA analysis much more difficult. The potential loss of some samples due to the wire being too high was worth the risk since DNA contamination from these other species would result in a greater loss of coyote samples available.

Once each of the corrals was constructed according to specification, each corral was left alone to sit fallow with no bait for one week to allow for a gathering of control data. A period of one week for each test was chosen mainly due to the logistical constraints of preventing DNA degradation (Kendall and McKelvey 2008), the availability of the researcher to collect samples, and to allow significant time for visitation by the coyotes, rather than strictly coyote behavioral reasoning. Once one week had elapsed, each of the corrals was visited by a single researcher wearing both rubber gauntlets and rubber boots and carrying a previously sterilized plastic bucket in order to prevent as much scent contamination as possible. Hair samples were collected from the control run by using the procedure for hair collection and storage as outlined in Kendall and McKelvey (2008). This procedure states that each barb of the barbed wire is treated as one unique sample, even though this may not be the case. Each barb that had hair snagged on it could contain only one or multiple hairs which could be from multiple individuals of the same species, from the same individual, or from entirely different species, but since no researcher was present to actually see what the hairs on the barb belonged to, the hair on each barb is considered one sample by convention (Romain-Bondi et al. 2004) and (Kendall and McKelvey 2008).

The first baiting procedure that was tested used a two-fold technique to entice coyotes to enter into the exclosure and snare their hair on the barbed wire. This technique was adapted into a scientifically reproducible procedure from those used by a local trapper, Robert Smalley, to successfully trap coyotes. After a lengthy literature search, this is possibly the first application of such a technique to collecting hair samples from coyotes for scientific purposes, while at the same time greatly reducing by-catch and animal habituation to the trap site. The first technique used to attract coyotes to the area is the visual stimulus of a cache hole. Cache holes are frequently used by canids and other carnivores to store surplus food in varying distributional strategies for later use in the ground such that other animals will not immediately discover and consume that which was stored (Phillips et al. 1991). Any canid entering the area that saw the hole would recognize it as a cache hole and would immediately associate this stimulus with a free meal waiting for them to consume since the hole was not marked with a urine scent. It has been demonstrated that wolves, coyotes, and foxes use a 'bookkeeping system' of urination on cache holes to indicate to the individual that created the hole that all of the food was removed such that time is not wasted excavating it again for no reason (Harrington 1981). Two cache holes were dug in the vicinity of the corral, with one being in the center of the corral and a second hole being 1.5m to the south of the hole in the center of the corral. These cache holes were dug using a specifically designed trapper's trowel for such a purpose. The holes were dug at a 40° angle to the horizontal and were made 20cm deep with respect to the plane of the ground, as per the recommendation of the trapper, Robert Smalley.

The second attractant used in this technique was a food scent bait specifically manufactured for trapping coyotes called Coyote Getter, which is manufactured by the Kishel's Scents company of Franklinville, NY. Into the cache hole outside of the exclosure 0.3mL of Coyote Getter was added using a volumetric pipette, as per the recommendations on the bottle for how much bait should be used per set. This type of bait is also a necessity to this project since it is not a consumable food object. Thus, coyotes will not immediately associate the exclosure site with a consumable food item and then return later at a higher than would be natural rate due to habituation to the bait site (Kendall and McKelvey 2008). Eventually, coyotes would also exhibit trap bias to not return to the same corrals due to being habituated to the lack of food associated with a particular scent (Kendall and McKelvey 2008), thus multiple baits were used overall in this sampling such that trap bias did not have time to develop. Scent attractants are a very helpful tool to attracting coyotes to an area due to the extensively developed canid olfactory senses and is currently used as a baiting tool for management (Turkowski et al. 1983). Although scent baits are advantageous to use, environmental conditions such as inclement weather and nearby human activity can factor significantly into the success of such methods (Henke and Knowlton 1995).

No bait was added into the cache hole dug in the center of the exclosure such that the scent attractant would not directly draw any organisms into the barbed wire corral. Only the canids would know to search both cache holes for food, whereas other species would be just attracted to the outer hole and would not enter the corral. It was thought that by using this method species by-catch could be greatly reduced through using the natural behavior of canids involving searching of cache holes. Other organisms, without

this behavioral stimulus, would find no food in the first cache hole and would leave since the second hole bore no attractant.

The hair collection was undertaken using two distinct processes of baiting, the cache hole method and the food bait method. The food bait method arouses the coyote's olfactory senses and then both the visual and olfactory sensation of the food itself serves as a method to convince coyotes to enter into the corral and get snared by the barbed wire and thus leaving their hair for collection. Wet dog food has been shown by Farry et al. (1998) to be successful in the baiting of coyotes. The particular type of dog food chosen for this experiment was Purina® Alpo® Homestyle Prime Slices with Chicken in Chicken Gravy. This particular dog food type was chosen because it was the most closely available flavor to flesh that a coyote would naturally consume since grouse (Bekoff 1977), which is similar to chicken, is a frequent food item for coyotes to keep the baiting situation as natural as possible.

Feeding platforms were constructed from Birch plywood and were boiled in staghorn sumac fruits and water (Rhus typhina) as described above to sterilize, remove scents, and naturally stain the platforms. Feeding platforms were used to ensure that the food was placed the same way in each transect such that results were optimal and influenced by as few variables as possible. Also, it has been shown that for the first six days of any baited collection period that the visitation rate of mammals at cleared bait stations with feeding platforms and groomed ground were significantly higher than those that were simply placed in the environment with no alterations (Farry et al. 1998).

MANUSCRIPT II – GENETIC ANALYSIS OF HAIR SAMPLES

Abstract

Mitochondrial DNA, found in the mitochondria of any eukaryotic organism is a powerful tool for detecting hybridization because it is maternally inherited. The presence of one species' mitochondrial DNA in the genotype of another species allows for a conclusion to be made that those two species successfully hybridized and produced offspring. Current research shows that coyotes found in the northeastern United States are hybrids of coyotes and wolves based on studies of mitochondrial DNA(Kays et al. 2009), (Pilgrim et al. 1998), and (Lehman et al. 1991). This genetic analysis serves to determine the presence of wolf-coyote hybridization in hair samples collected from a barbed wire hair snare survey in western New York—a location where wolf-coyote hybrids are thought to reside (Kays et al. 2009). Of the 74 hair samples tested, only two samples tested showed a probable positive identification of canid DNA. Due to contamination of samples, this conclusion cannot be made with too much certainty, but it would appear that samples 8 and 35, with fragment sizes of 164bp and peaks of 1800 and 750, respectively were collected from coyotes that contain wolf mitochondrial DNA, indicating the presence of hybridization.

Introduction

Many current methods exist to examine the DNA of an organism, mostly due to the new developments in molecular genetics. Wildlife genetics combines these new molecular techniques with DNA samples collected either invasively or non-invasively from wildlife. Since mitochondrial DNA is transferred directly from mother to offspring, it is used frequently in research as a tool to detect hybridization using both fragment

analysis and DNA sequencing techniques (Kays et al. 2009), (Pilgrim et al. 1998), (Lehman et al. 1991). Once the DNA is extracted from the cells of an organism, DNA primer sequences can be used to amplify any desired segment of mitochondrial DNA, providing the sequence is already known using the polymerase chain reaction (Mullis et al. 1994). This amplification yields specific products of the desired size based on the construction of the primers used in the reaction. In this analysis, mitochondrial DNA was isolated and amplified from hair samples collected by hair snare exclosures to analyze wolf-coyote hybridization and provide data for a coyote population analysis. To analyze these sequences, genus-specific primers and species-specific fragment sizes were used to isolate DNA from the *Canis* genus from the other hair samples collected.

Based on recent mitochondrial DNA research, coyotes in the northeastern United States and the Great Lakes region have been confirmed as wolf-coyote hybrids (Kays et al. 2009), (Wheeldon et al. 2010), and (Lehman et al. 1991). These hybridization events result in organisms that carry mitochondrial DNA from their mother that is an alternate species than their father. Thus, the detection of another species' mitochondrial DNA sequences in the genome of a separate species would indicate that hybridization has occurred, as is described by Pilgrim et al. (1998), which has yet to occur in the Rocky Mountains region of the United States. This same premise was applied in this analysis to mDNA isolated from hair samples of canids taken from western New York, more specifically from Houghton, of Allegany County, where wolf-coyote hybrids are purported to reside (Kays et al. 2009).

Hair samples provide a source of DNA that is not used as often in the literature due to the often diluted and poor condition of the DNA from the hair samples that are

collected, as compared to DNA collected from other methods (Kendall and McKelvey 2008). While this may be true, the samples used in this analysis were derived from hair snare exclosures, which is a relatively new method for isolating DNA samples from organisms that would otherwise be very difficult to sample (Mowat & Strobeck 2000), (Ruell & Crooks 2007), (Romain-Bondi et al. 2004), and (Kendall and McKelvey 2008). In order to gain usable DNA samples from hair, the unneeded cellular material must be separated from the root cells of the hair (Kendall and McKelvey 2008). This is done because hair shafts do not contain DNA and excess material may deter the polymerase chain reaction from proper functionality. Current methodology for hair sample DNA analysis comes from the forensic sciences for use in crime investigation. While many methods for the isolation of this DNA exist, (Graffy & Foran 2005) demonstrate that the largest amount of usable DNA for the PCR reaction from hair samples results when using an alkaline digestion, particularly of sodium hydroxide. Since hair samples collected non-invasively from wildlife are often limited in nature, the processes that yield the maximum amount of usable DNA must be used to ensure that no collected samples are wasted.

It is expected that canid DNA will be successfully amplified from the collected samples using canid specific primers, providing canid DNA was collected. These primers are designed to amplify the desired segments of mitochondrial DNA that will allow for speciation of the mother of the organism from which the hair samples were collected. If canid mDNA is present in the samples, this methodology should detect the mDNA and provide conclusions of the presence or lack of hybridization witnessed among individuals from which hair samples were collected using the barbed wire hair snare survey.

Methods

The hair samples used in this mDNA isolation and analysis were collected using the methodology described above in manuscript 1. Up to ten hair samples, or the maximum number available, were taken from each manila envelope stored at 4°C. These hair samples were trimmed to 0.5cm measured from the root and were placed into a 1.5mL microcentrifuge tube containing 100μL of 50mM NaOH. The sample tubes were heated to 95°C and then mixed using a pulse-vortex for 30 seconds followed by an additional cycle of both steps. Once cell lysis was complete, 15μL of 1M Tris at pH 7 was added to the hair extract solution and Fisher Scientific Alkacid Test Paper was used to ensure pH between 7.0 and 8.0. Once extracted, the DNA samples were stored at minus 25°C until later use.

Extracted DNA samples were analyzed for fragment size after amplification using the primers of sequence 5'-GAAGAAGCTCTTGCTCCA-3' and fluorescently labeled primer of sequence 5'-CAAACCATTAATGCACGACG-3' adapted from (Pilgrim et al. 1998) using an ABI-310 genetic analyzer. The DNA was first amplified using the polymerase chain reaction with a stock solution of 2μL of template DNA, 2μL of 10 μM forward primer, 2μL of 10 μM reverse primer, 4μL of distilled water, and 10μL of Bioline's 2x Biomix™ used in each reaction. The amount of reactants required for each reaction was measured out using the stock solution method. The stock solution contained 1.1 times each necessary component for the PCR reaction times the number of reactions that were to be completed. The stock solution was then thoroughly mixed via tapping and shaking and 18μL of this solution was added to each PCR tube. To each prepared tube 2μL of the desired template DNA was then added to complete the preparation of the PCR reaction.

PCR was completed first by the samples being heated to 94°C for three minutes followed by 35 cycles of 94°C for one minute, 55°C for one minute, and 72°C for thirty seconds. 10μL of PCR product from each sample were examined by gel electrophoresis on a 2% agarose gel in 1X TBE buffer to ensure the success of the PCR reaction before fragment analysis, in the form of a 158-164bp visible fragment. Once verified successful, the PCR reaction was completed again under the exact same conditions as above except that only 21 cycles were completed. Throughout the course of the study, varying PCR conditions were used based on the application of the PCR products. 2μL of each PCR product were added to a 15μL of a stock solution containing 16μL of HiDi™ formamide and 0.25μL of the molecular size standard LIZ 500. The samples were mixed using a pulse-vortex, centrifuged down, and heated to 95°C for three minutes, followed by repeating the mixing and centrifugation steps. The samples were then loaded into an ABI-310 genetic analyzer which uses capillary electrophoresis combined with a laser to determine the fragment sizes of the fluorescently labeled fragments of DNA. The raw data was then analyzed using the Applied Biosystems GeneMapper Version 4.0 software which produced visual readouts of the data collected.

Results

Due to the limited nature of the samples collected, a source of contamination entered the samples which could not be removed by using alternate samples because no additional copies of the same sample were available. This source of contamination was canid in nature and made the mDNA analysis extremely complicated. The initial technique of gel electrophoresis that was used to screen samples for the presence of potential canid DNA first revealed the presence of contamination. This contamination

was realized when negative controls tested positive and when the results of one gel electrophoresis were inconsistent with the results of other gels. An example of the occurrence of this contamination can be visualized between the PCR products isolated on the gels in Figure 8 as compared to those in Figure 9. In Figure 8, a negative control of a deer DNA sample is used and tests negative for canid DNA. In the gel attached as Figure 9, this same negative control is used and it tested positive for canid DNA even when none of the reaction conditions had been varied. Due to the inconsistency of this method of screening results, fragment analysis was conducted on any DNA sample that tested positive for canid DNA on a gel, even though many of these results were known to be false positives.

Of all the samples analyzed, only two tested positive for canid DNA above the level of background contamination when verification using fragment analysis on the ABI-310 genetic analyzer was utilized, but the varying levels of background contamination make this conclusion one that cannot be made with much certainty. The background levels of contamination were observed in negative controls where the addition of template DNA to the PCR reaction was replaced with water. Out of 74 initial samples, only two, sample 8 and sample 35, are likely to contain actual coyote DNA samples. Before an analysis of fragments is completed for samples 8 and 35, the two samples were examined using gel electrophoresis on the gel attached as Figure 9. While contamination is present in that the negative control tested positive for canid DNA, samples 8 and 35 produce darker bands, and hence more PCR product, when compared to any of the other samples. Two different reaction conditions were used in the fragment analysis of samples 8 and 35 because they were analyzed at different time periods, and

these differences are reflected on the attached electropherograms of each due to different reaction conditions. On the electropherograms, the size of each sample in base pairs (bp) represents the size of the particular DNA fragment, while the size of the peaks for each fragment reflects the amount of DNA of that particular size that passed through the capillary.

The electropherogram of the negative water control that was run using the same PCR stock as sample 8 is included as Figure 1. The PCR reaction that includes both this negative water control and sample 8 proceeded exactly as was listed in the methods except that 23 cycles of PCR were used. This negative control has peaks at 160bp and 164bp with sizes of 850 and 150 respectively, showing a mixture of contamination from different DNA sources since only one distinct peak should be visualized. For sample 8, which was collected during the cache-hole method hair snare exclosure survey as outlined in manuscript 1, the main fragment sizes were about 160bp and 164bp, with peak sizes of roughly 500 and 1800, respectively. The electropherogram of this sample is attached as figure 2 and can be compared directly to the water control peaks as described above. The peak for sample 8 at 164bp is more than 10 times larger than the water control, while the water contamination peak is visible in the 160bp range at a height of roughly 500.

The electropherogram of the negative water control that was run using the same PCR stock as sample 35 is included as Figure 3. This PCR that was carried out differed from the PCR of sample 8 and its corresponding water control in since 21 cycles, as described in the methods, was used in this reaction. This negative water control has peaks at 160bp and 164bp with peak sizes of roughly 280 and 340 respectively. Sample 35,

which is the second sample likely to contain coyote DNA, and was also collected using the cache hole method for hair snaring as described in manuscript 1, had fragment sizes of 160bp and 164bp with peak sizes of 475 and 750 respectively. The electropherogram of sample 35 is included below as Figure 4. The peak of sample number 35 at 164bp is more than twice that of the water control, indicating some other source of DNA than the background contamination, while the peak at 160bp is more indicative of contamination since the peak size is not even twice that of the water control.

As a method of comparison, a positive control was run with each sample to determine the expected magnitude of a positive result. The positive control used from the same PCR stock and reaction conditions as sample 8 failed due to be visualized due to an issue with the sizing standard, but the electropherogram of the sample of positive coyote DNA run with the same PCR conditions and stock solution as sample 35 is attached as figure 7. This sample has a two fragment sizes of roughly 159bp and 164bp, with peak sizes of 1600 and 250, respectively. Thus, for sample 35, a positive result should expect, at best, a fragment intensity of roughly 1600, but sample 35 achieved only half this result, possibly indicating the diluted nature of the DNA in sample 35 as compared to the positive control Also included in this analysis is one sample representing a failed reaction and another representing a negative result or failed reaction. The electropherogram that presents a negative result or failed reaction is for sample 34 and is attached as Figure 5. This electropherogram fails to show any peaks, but the orange sizing peak is readily visible, indicating a potentially failed reaction or a negative result. The electropherogram that presents a second type of negative result or failed reaction observed and is for sample 40, which is attached as Figure 6. This electropherogram shows two fragment peaks, one

at 160bp and the second at 164bp, with peak sizes of about 180 and 240 respectively. The fragment peaks that are present fall within the range and size of the contamination peaks present in the water control run for sample 35, which serves as a baseline for analysis for this sample also since it was run with the same PCR stock and under the same PCR conditions as sample 35.

Any sample that yielded multiple peaks revealed evidence of DNA contamination present. The level of contamination in the samples varied, especially as the number of PCR cycles that were carried out in a given reaction was varied. The inconsistency of fragment and peak sizes between one negative control sample and another negative control at slightly different reaction conditions reveals a complex situation of contamination that varied greatly with PCR. Overall, only the samples of 8 and 35 had particular peak or fragment sizes that caused them to stand out from the background contamination. Many other fragment analyses were conducted, but the resulting electropherograms were eliminated from being potential coyote DNA sources using the same methodology as was used in drawing conclusions with the electropherograms included as Figure 5 and Figure 6.

Discussion

If the two analyzed fragments that appear to contain canid DNA isolated from hair samples actually do contain canid mDNA instead of contamination, both samples would indicate that they are from coyotes that hybridized with wolves, due to the 164bp fragment size of the amplified mDNA. Again, the electropherograms for samples 8 and 35 are attached to this manuscript as Figures 2 and 4, respectively and the electropherogram of the negative control for sample 8 is attached as Figure 1 and the

electropherogram of the negative control for sample 35 is attached as Figure 3.

Mitochondrial DNA provides relevant information on the mother of the organism from which the DNA sample was collected, but unless other factors are known, it reveals little about the actual organism from which the DNA sample was collected. Genomic DNA serves as a much better reference for DNA characteristics that would allow for individualization. Based on the analysis completed by Pilgrim et al. (1998), if an amplified canid specific fragment using the previously specified primers was found to be 158bp, then the mother of the canid organism that was snared on the exclosure could be concluded as a common dog (*Canis familiaris*) with most certainty. This conclusion is based on the fact that coyote-dog hybrids, or coydogs, are very rare organisms (Kays et al. 2009).

If the amplified fragment is of size roughly 160bp, then this fragment can be considered of coyote in origin and that the mother of the organism snared was coyote. Male coyotes are much more likely to mate with female wolves than the opposite (Pilgrim et al. 1998), and the prevalence of coydogs is quite low, such that the most likely identity of an organism snared with this base pair size is coyote. If a 164bp fragment is found, then this indicates that the mother of the animal in question carries wolf mitochondrial DNA. This information does not allow for a conclusion on the species of the mother of the animal though. While it can be concluded that the mother has wolf mitochondrial DNA, whether or not this individual is a wolf or coyote cannot be ascertained. Based on the long ranges required for wolves to migrate into the western New York area, the appearance of a wolf mother is a phenomenon that would be extremely rare. Wolves have been extirpated from and currently do not range in western

New York, and thus a maternal lineage resulting from a wolf-coyote mating in the past is the most probably reason that a canid in this area would be bearing wolf mitochondrial DNA. It is possible that this DNA is from a dog-wolf hybrid, but based on the location of this study, far from the range of the wolf, this situation is also quite unlikely. If these observed DNA fragments have truly been isolated from the collected samples, instead of brought about by contamination, then these fragment sizes would serve as evidence of wolf-coyote hybridization.

It is probable that of all the hair samples collected that only two of them are canid, but it is also likely that additional canid samples were collected that did not amplify or visualize properly during fragment analysis. As described by Graffy & Foran (2005) in the field of forensic hair analysis, even the best DNA isolation techniques from hair do not have success rates higher than roughly 90%. This success rate is still a best-case scenario because these samples were collected from a controlled environment as opposed to the conditions in which the wildlife hair samples were collected which resulted in less than optimal DNA condition. Even if an actual success rate of 90% were achieved for the hair extraction, roughly eight samples out of the 74 collected would fail to be extracted properly even though they may have contained canid DNA.

Using the available method for analysis, the difference between a negative result and a failed reaction cannot be determined. Even though other PCR reactions in from the same products may have successfully amplified DNA, the absence of peaks does not simply mean that the sample contained no canid mDNA. The lack of peaks in a sample could be for a variety of reasons, ranging from failed PCR in one tube to the lack of a high quality mDNA template to be amplified. Also, the reaction conditions may not have

been proper for the reaction to proceed. The results of the mDNA analysis of each of the samples needs to be verified before a proper conclusion can be made, but the time for this crucial verification was not available, and thus much of the data requires further study and verification.

Retrospectively, mitochondrial DNA analysis of collected hair samples requires more extraction steps then conventional methods of DNA isolated from tissues, and thus more chances for contamination, as was witnessed in this research, is possible. Much difficulty was encountered when searching for literature regarding mDNA fragment analysis or sequencing of DNA isolated from hair samples due to the trouble with getting the analysis to work properly. Grizzly bear (*Ursus arctos horribilis*) population research projects are the main current usage of barbed wire hair snare exclosures (Mowat & Strobeck 2000) and (Romain-Bondi et al. 2004), but grizzly bears are shown from research to leave very large clumps of testable hair at exclosures that they enter (Kendall and McKelvey 2008). This was not the case for the canid DNA isolated from the exclosures, since for many samples, only single hairs were collected in the field to be utilized in research. Another difficulty of using the canid specific primers was the issue of the mDNA of multiple individuals being contained in one amplified fragment. By convention (Kendall and McKelvey 2008), all hair samples found on one barb of a hair snare are counted as one sample, since no observer was present to see if multiple individuals became snagged on the same barb. From this initial assumption, DNA contamination can occur from the onset of research because it is likely that two individuals can become snared on the same barb leading to a difficult conclusion from the resulting DNA fragment analysis.

For further research on this same topic using the same canid specific primers, it is recommended that a multiplicity of steps is taken to avoid contamination of samples. Once DNA is successfully extracted from hair, each sample should be divided and placed in multiple individual tubes to ensure that contaminated samples can simply be removed if an issue of contamination arises. During this research it was discovered that the number of PCR cycles cited in the literature by the originators of the canid specific primers (Pilgrim et al 1998) for the mDNA fragment being studied created vast amounts of PCR products, much more than is actually needed. The PCR products of 35 cycles are so concentrated that extremely small amounts of these products can cause large amounts of contamination, so any handling of such large PCR products should be avoided. In this paper the number of PCR cycles was reduced to 21, and the amount of contamination witnessed in the samples was reduced significantly because at 35 cycles, any background DNA that is amplified is visualized at such high product concentration, leading to multi-formed peaks instead of the single peaks that would be expected in an mDNA fragment analysis.

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Table 1 – List of samples tested for canid mDNA, date collected, and the results of the testing

Date of Collection	Sample Number	mDNA Analysis Result	Date of Collection	Sample Number	mDNA Analysis Result
10/30/2009	1	Negative / Failure	11/13/2009	38	Negative / Failure
10/30/2009	2	Negative / Failure	11/13/2009	39	Negative / Failure
10/30/2009	3	Negative / Failure	11/13/2009	40	Negative / Failure
10/30/2009	4	Negative / Failure	11/13/2009	41	Negative / Failure
11/6/2009	5	Negative / Failure	11/13/2009	42	Negative / Failure
11/6/2009	6	Negative / Failure	11/13/2009	43	Negative / Failure
11/6/2009	7	Negative / Failure	11/13/2009	44	Negative / Failure
11/6/2009	8	Potential Positive	11/13/2009	45	Negative / Failure
11/6/2009	9	Negative / Failure	11/13/2009	46	Negative / Failure
11/6/2009	10	Negative / Failure	11/13/2009	47	Negative / Failure
11/6/2009	11	Negative / Failure	11/13/2009	48	Negative / Failure
11/6/2009	12	Negative / Failure	11/13/2009	49	Negative / Failure
11/6/2009	13	Negative / Failure	11/13/2009	50	Negative / Failure
11/6/2009	14	Negative / Failure	11/29/2009	51	Negative / Failure
11/6/2009	15	Negative / Failure	11/29/2009	52	Negative / Failure
11/6/2009	16	Negative / Failure	11/29/2009	53	Negative / Failure
11/6/2009	17	Negative / Failure	11/29/2009	54	Negative / Failure
11/6/2009	18	Negative / Failure	12/6/2009	55	Negative / Failure
11/6/2009	19	Negative / Failure	12/6/2009	56	Negative / Failure
11/6/2009	20	Negative / Failure	12/6/2009	57	Negative / Failure
11/6/2009	21	Negative / Failure	12/6/2009	58	Negative / Failure
11/6/2009	22	Negative / Failure	12/6/2009	59	Negative / Failure
11/6/2009	23	Negative / Failure	12/6/2009	60	Negative / Failure
11/6/2009	24	Negative / Failure	12/6/2009	61	Negative / Failure
11/6/2009	25	Negative / Failure	12/6/2009	62	Negative / Failure
11/6/2009	26	Negative / Failure	12/6/2009	63	Negative / Failure
11/6/2009	27	Negative / Failure	12/6/2009	64	Negative / Failure
11/6/2009	28	Negative / Failure	12/6/2009	65	Negative / Failure
11/13/2009	29	Negative / Failure	12/6/2009	66	Negative / Failure
11/13/2009	30	Negative / Failure	12/6/2009	67	Negative / Failure
11/13/2009	31	Negative / Failure	12/6/2009	68	Negative / Failure
11/13/2009	32	Negative / Failure	12/13/2009	69	Negative / Failure
11/13/2009	33	Negative / Failure	12/13/2009	70	Negative / Failure
11/13/2009	34	Negative / Failure	12/13/2009	71	Negative / Failure
11/13/2009	35	Potential Positive	12/13/2009	72	Negative / Failure
11/13/2009	36	Negative / Failure	12/13/2009	73	Negative / Failure
11/13/2009	37	Negative / Failure	12/13/2009	74	Negative / Failure

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Figure 1 - Electropherogram of pure water sample (negative control) run on 4-18-10, under the prescribed PCR conditions except that a total of 23 cycles were run. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram shows peaks at roughly 160bp and 164bp with intensities of 850 and 150, respectively.

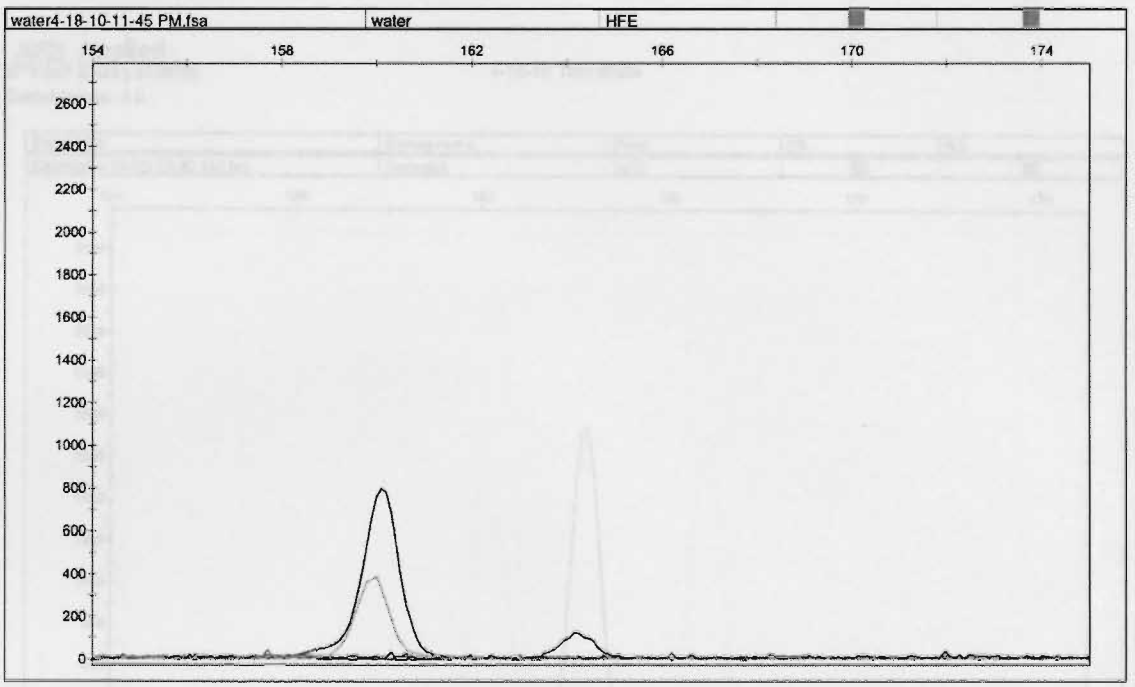


Figure 2 - Electropherogram of sample 8, run using the same PCR stock as the negative control in Figure 1, and also using a total of 23 cycles of the aforementioned PCR procedure. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram shows peaks of roughly 160bp and 164bp, with peak sizes of roughly 500 and 1800, respectively, indicating the presence of potential canid mDNA, particularly at the wolf fragment size.

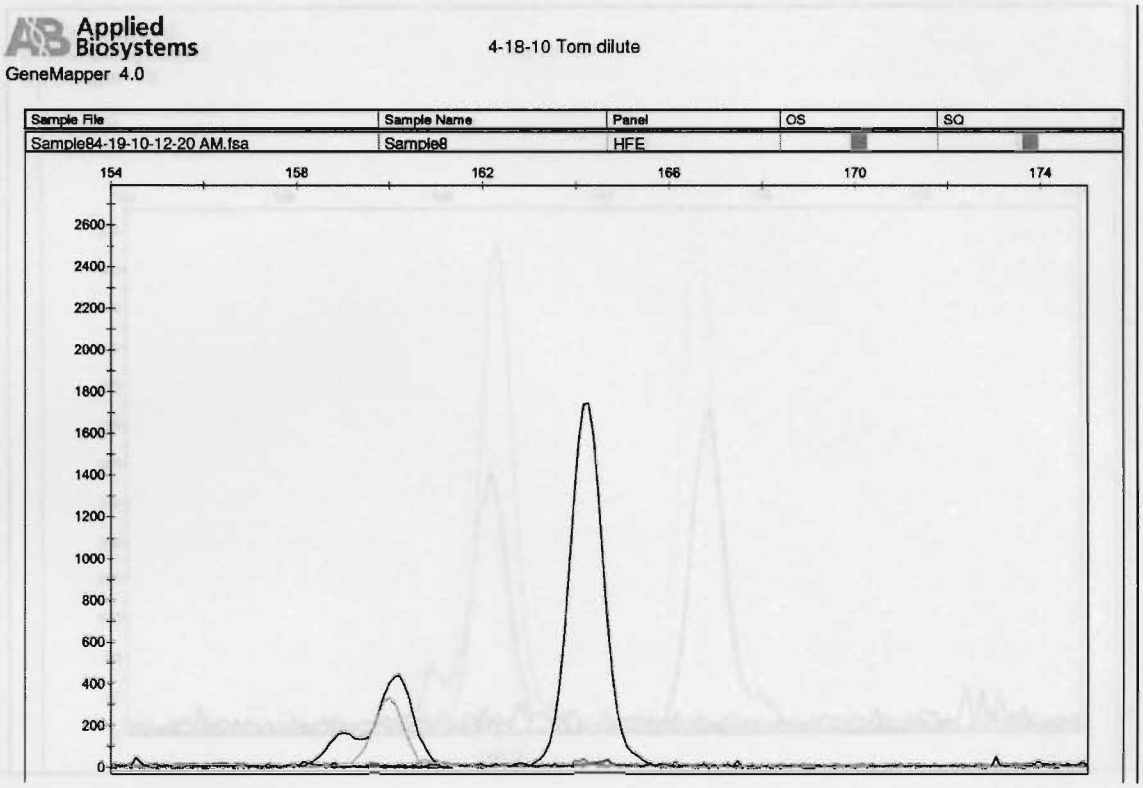


Figure 3 - Electropherogram of a pure water sample (negative control) run on 4-20-10, using 21 cycles of PCR. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram shows peaks of roughly 160bp and 164bp, with peak sizes of roughly 280 and 340, respectively.

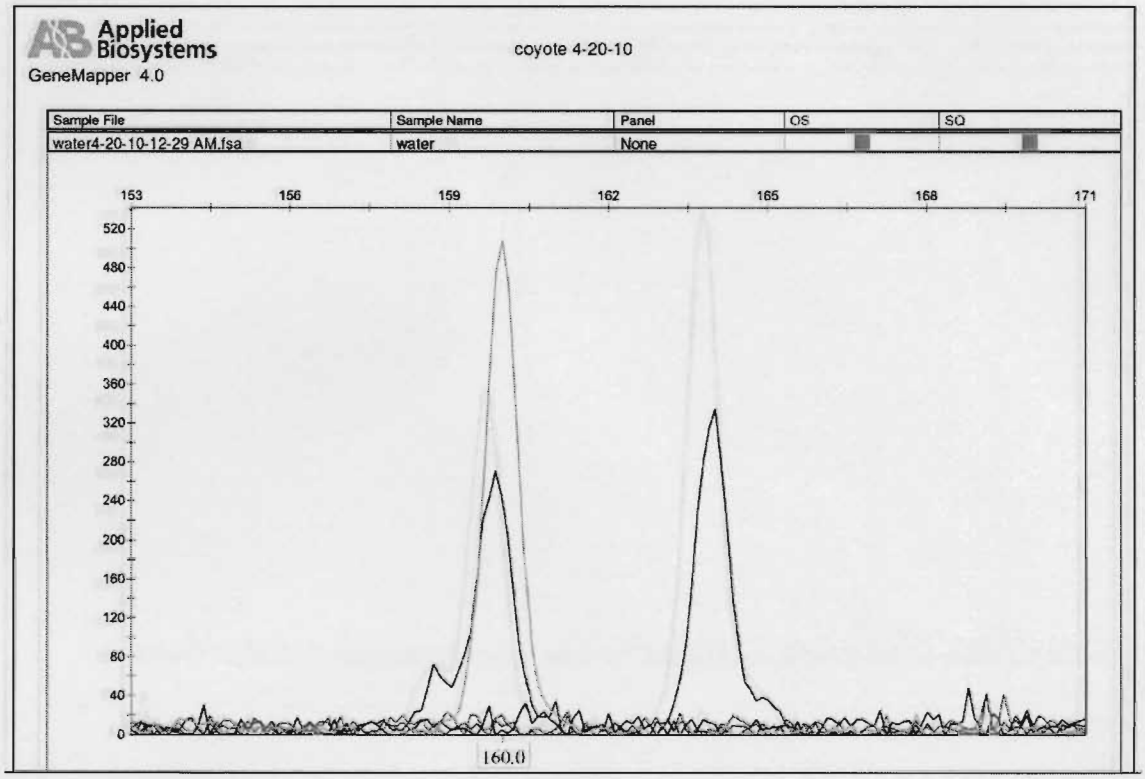


Figure 4 - Electropherogram of sample 35 run on 4-20-10, using the same PCR stock and conditions of 21 cycles as the negative control in Figure 3. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram shows peaks of roughly 160bp and 164bp with sizes of roughly 475 and 750, respectively, which represents the probable presence of canid DNA, particularly at the wolf fragment size.

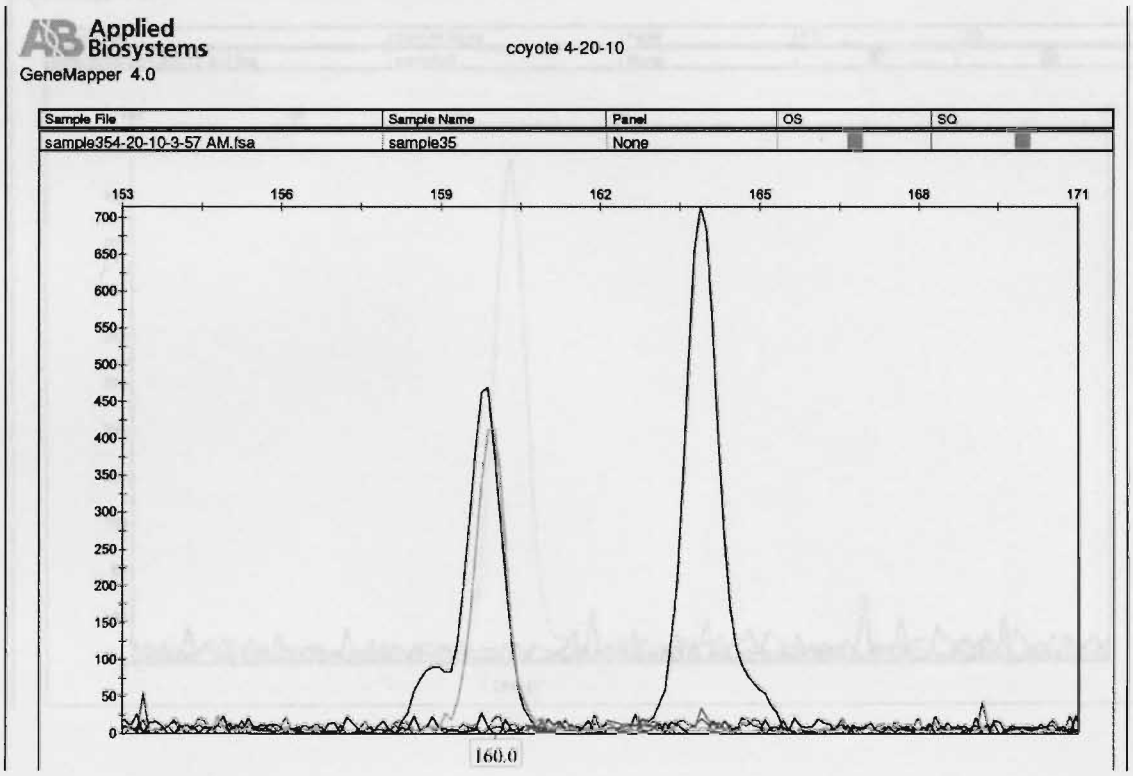


Figure 5 - Electropherogram of sample 34 run on 4-20-10 using 21 cycles of PCR and the same stock solution as Figures 3 and 4. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The lack of blue peaks represents the there were no detected mDNA fragments in the sample, either from a failed reaction, or from a negative result. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis.

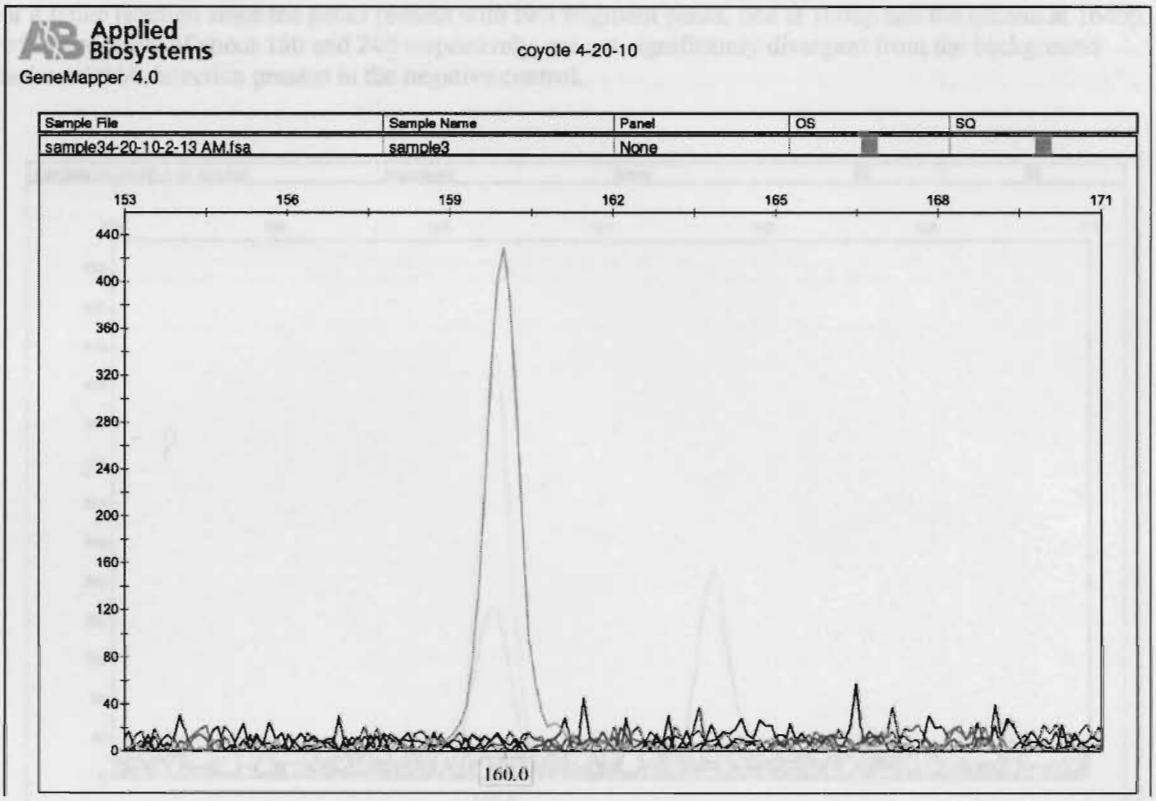


Figure 6 - Electropherogram of sample 40, run on 4-20-10 using the same PCR stock and conditions (21 cycles) as Figures 3, 4, and 5. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram represents a negative result or a failed reaction since the peaks present with two fragment peaks, one at 160bp and the second at 164bp, with peak sizes of about 180 and 240 respectively, are not significantly divergent from the background noise mDNA detection present in the negative control.

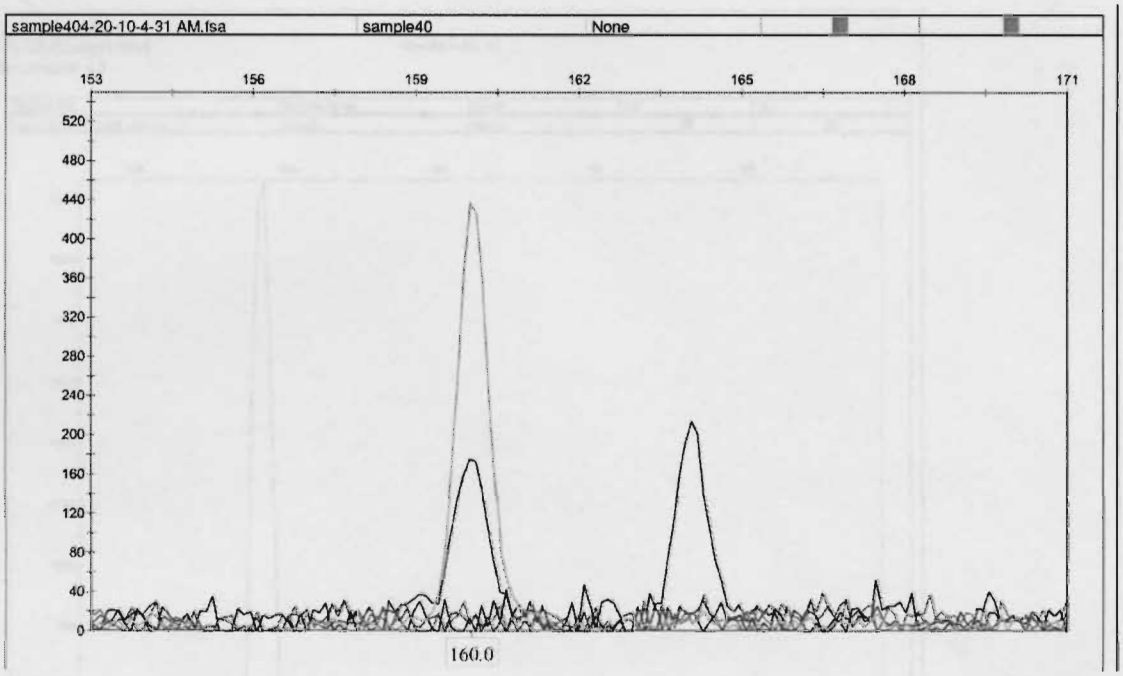


Figure 7 - Electropherogram of sample Kays' coyote #3, a positive coyote DNA control, run on 4-20-10 using the same PCR stock and conditions (21 cycles) as Figures 3, 4, 5, and 6. On this electropherogram, the orange peak represents the sizing standard used by the genetic analyzer to know how to analyze the raw data. The blue peaks represent the detected mDNA fragments which are represented as blue because the canid-specific primers that are used in the PCR reaction and are analyzed by the laser were marked with this color dye. Along the upper x-axis, this numbering indicates the fragment size in base pairs of the particular peak that is measured in base pairs (bp). Along the y-axis, these degradations represent the intensity, or amount of mDNA that was absorbed at the particular fragment size as represented on the x-axis. This electropherogram shows what a positive result would look like in a best-case scenario for the reaction conditions because this DNA sample is more concentrated, and thus showing larger peaks, than the DNA extracted from hair samples. This sample has a two fragment sizes of roughly 159bp and 164bp, with peak sizes of 1600 and 250, respectively.

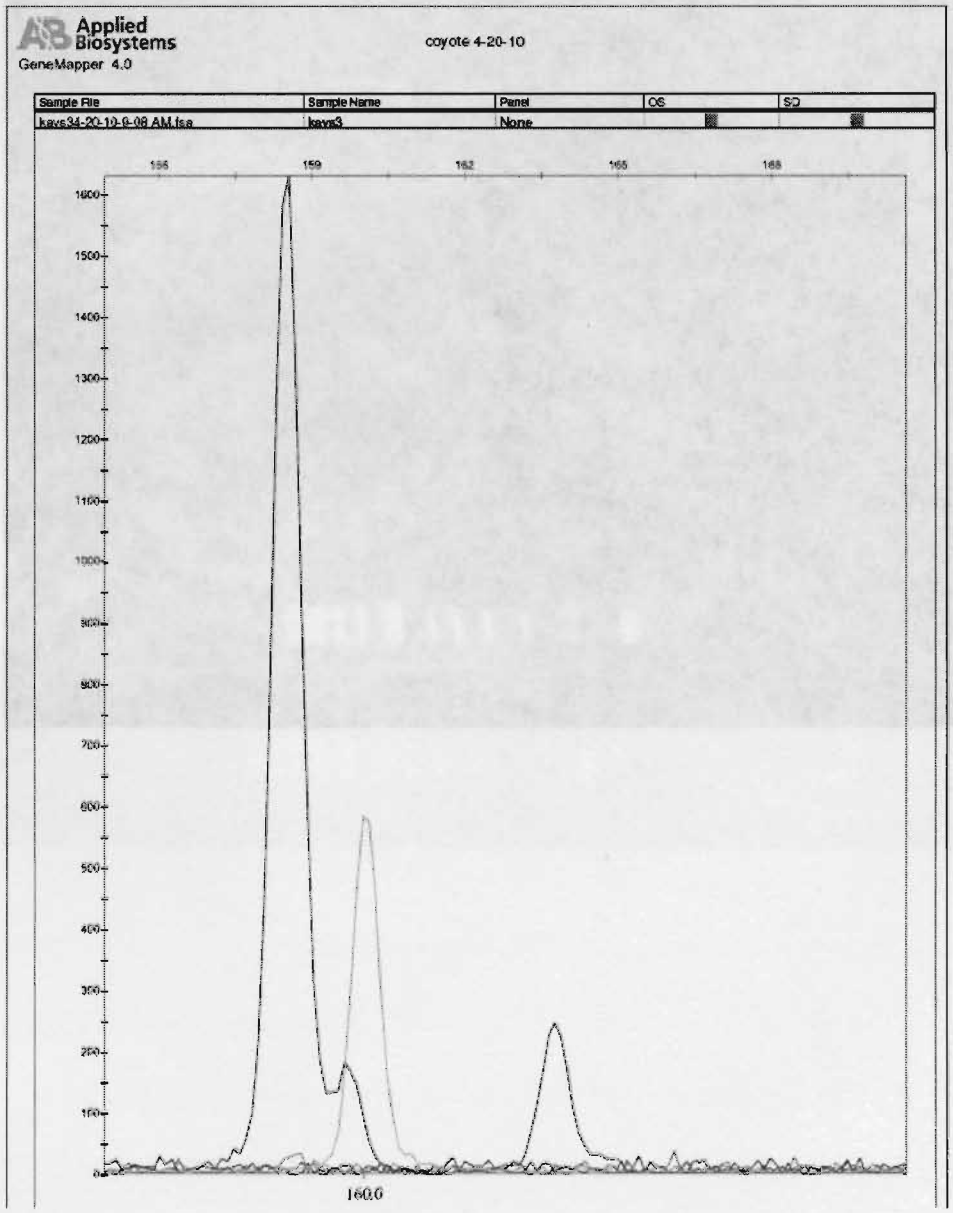


Figure 8 – Photograph of separation for 35 cycle PCR products visualized on a 2% agarose gel using gel electrophoresis. The samples were separated by running an electric current across the gel with the positive lead being on the right-hand side of the gel, causing the DNA fragments to migrate. Bioline's Hyperladder II was used as the molecular weight marker for this gel and can be visualized on both the very top and bottom reaction wells. Each sample that was run on the gel is labeled on the left hand side for the well in which that particular sample was run. A positive result is interpreted as a visible band of mDNA fragments at the desired fragment size of roughly 160bp. In this gel, all of the samples except for 7, 20, 27, and the Deer negative control give a positive result for the presence of canid DNA.

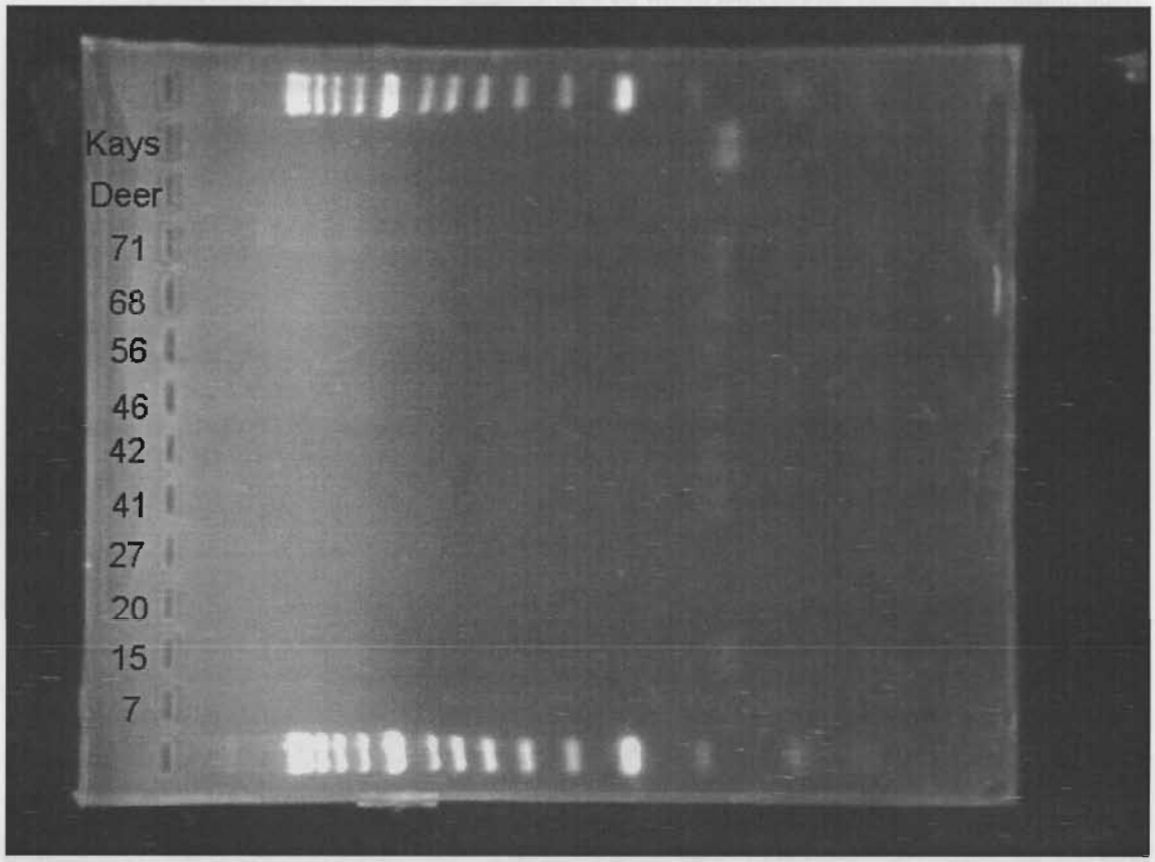
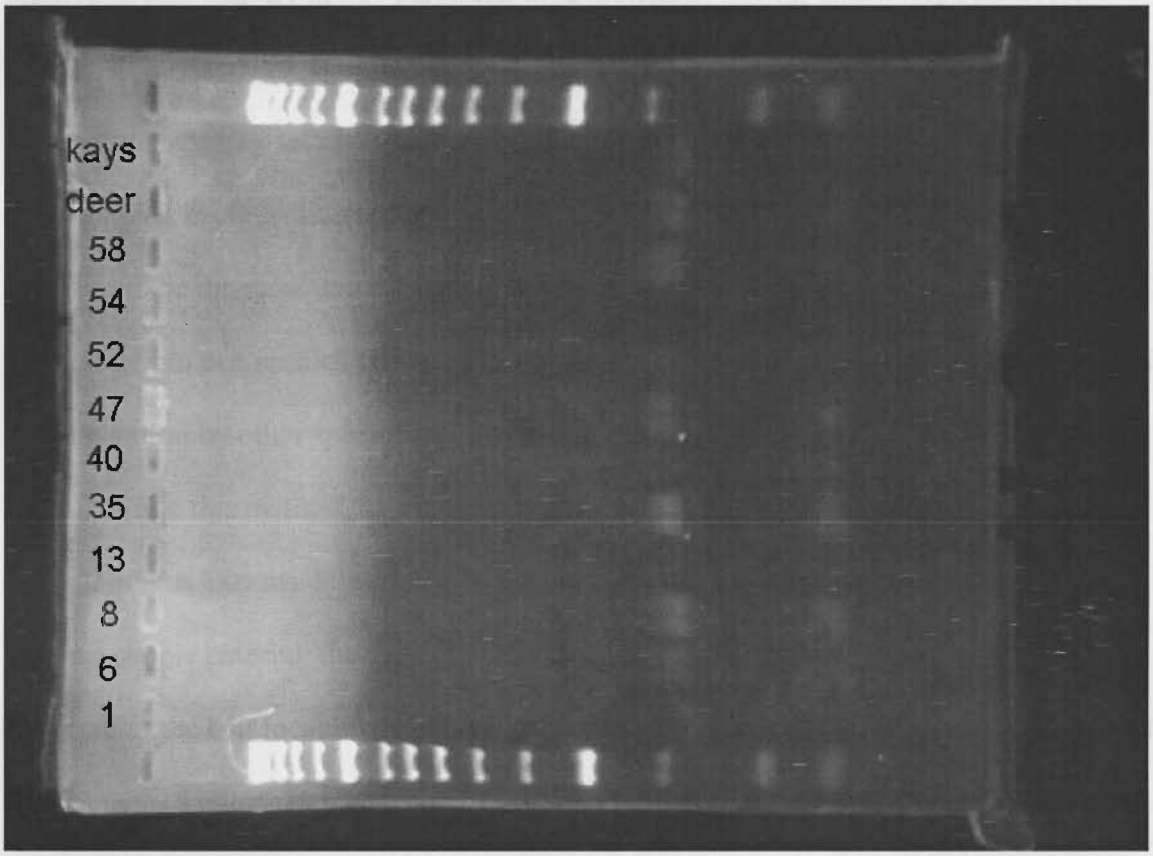


Figure 9 – Photograph of separation for 35 cycle PCR products visualized on a 2% agarose gel using gel electrophoresis. The samples were separated by running an electric current across the gel with the positive lead being on the right-hand side of the gel, causing the DNA fragments to migrate. Bioline's Hyperladder II was used as the molecular weight marker for this gel and can be visualized in both the very top and bottom wells of the gel. Each sample that was run on the gel is labeled on the left hand side for the well in which that particular sample was run. A positive result is interpreted as a visible band of mDNA fragments at the desired fragment size of roughly 160bp. In this gel, all of the samples except for sample 54 would be counted as a positive result, including the negative control, which indicates the presence of contamination. Note that the bands for samples 8 and 35, which were thought to contain canid mDNA after fragment analysis have brighter bands than the other samples present, indicating a greater concentration of PCR products possibly from greater amounts template material.



Summary and Conclusion

The potential for success of this noninvasive method of barbed wire exclosures to collect hair samples from local coyotes with viable DNA demonstrates the practical applications this method of sample collection has to management. Baiting stations like these can be set up in many locations and can be monitored weekly to determine the relative coyote activity in any particular area instead of having to actively search for coyotes or coyote sign. Barbed wire exclosures allow for research over a large scale that can be conducted by relatively few researchers, which can be an attractive method due to the reduction in expenditures for data collection. The DNA analysis of this collected hair provides to be the most powerful tool, as indicated by the ability to discern the samples collected from one species versus another because using this method one limitation is that contamination by other species is unavoidable.

While this method may be helpful for detecting coyote populations on public lands, obvious hazards do exist. Exposed barbed wire can cause extensive damage to unwary people entering the research area as well as pets, especially dogs, which are attracted to the bait locations and wonder into the exclosures. In areas of extensive human traffic, these exclosures would surely be tampered with, as well as the opportunity exists for injury to those using the premises. Due to the behavior of the coyote, signs located near the wire to warn of its presence have also been known to deter coyotes from even coming near the exclosure (Heffernan et al. 2007). Also the nearby scent of humans to the exclosure itself will surely alert the olfactory senses of any canid and may have an influence on catch rates. For that reason, exclosure collection methods are not

recommended to research areas that have frequent human interaction due to the ready possibility of injury, scent contamination, and exclosure tampering.

While in this study there was no direct comparison to traps contaminated with human scent, coyotes are known to stray away from baits if they detect strange scents that they feel do not belong in a certain environment. The usage of sterilization and wearing of gloves and boots throughout the entirety of the experiment ensured that the scent of the researcher was not added as a variable to this project. Also, the cache hole method of coyote baiting successfully captured probable coyote hair even though there was no bait located in the center of the exclosures. This project simply served as a means to experiment with the success of such a method for collection. The fact that the coyotes entered the exclosure based on visual cues alone, once lured there by a scent bait, validates that coyotes are stimulated to a search behavior when confronted with a cache hole, even if it is unbaited. While it is possible that a coyote became snared on the exclosure as it was coming to investigate the scent, this should not account for all of the hair samples found on traps baited with this method because the first cache hole can be approached without encountering the exclosure from roughly all directions. This method was not found through in the literature and its usage has great potential once further research on its viability has been completed in the many manners as described in other sections of this paper.

Throughout the length of the experiment five different exclosures were destroyed by collisions with animals. White-Tailed Deer (*Odocoileus virginianus)* sign found nearby each of the destroyed exclosures allows for the conclusion that deer colliding with the support stakes or the barbed wire was the most likely cause of making the exclosures

unable to collect data. Curiously, more exclosures were destroyed during the first trials of the experiment as compared to the latter trials, possibly indicating that the local deer eventually became habituated to the positions of the exclosures and then no longer ran into them. It is suggested that in further study using the barbed wire exclosure method in areas of high deer density that a waiting period of more than four weeks be completed before running trials such that the deer will be able to learn the locations of the exclosures and will be much less likely to destroy them during a collection and potentially skew the results.

Based on the difficulties incurred during the mDNA analysis, the procedure of fragment size analysis must be completed with significant care. Isolation of DNA from hair samples requires additional steps that can lead to additional chances for contamination that make positive identification of fragment sizes extremely difficult. It is recommended that in further studies of this nature that DNA samples are split into multiple tubes once extracted such that contamination of all samples will not occur. As low of a level as possible of PCR cycles should be used while still maintaining sufficient resolution of products, such that concentrated PCR products do not pose a contamination risk to other samples when being absorbed into a pipette on the nanoliter scale. Also, some of the hair samples yielded poor quality DNA, as was evidenced when analysis were carried out when no contamination was present.

Finally, it would appear that the cache hole method of hair collection has viability in future study and may be modified to become very efficient at snaring hair from coyotes for analysis. The method for determining hybridization used in this paper is a functional one if contamination can be avoided, which hundreds of hours of lab work

were spent diagnosing and troubleshooting. Wildlife genetics serves as an important method to analyze the organisms in a particular area on a scale that has only recently become available due to the low cost of these genetic analyses.

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