The use of genetic techniques to facilitate noninvasive monitoring and conservation of wildlife
Mitochondrial DNA for species-level discrimination

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Abstract

There are a wide variety of ways researchers can monitor wildlife. Some methods are more involved than others and require a certain amount of physical interaction with the animal (radio-collaring, blood collection…). These methods are considered invasive because they directly engage the researcher with the animal. This interaction can lead to unintended consequences by inducing unhealthy stress in the animal, which may lead to a condition known as capture myopathy. Monitoring techniques that enable the researcher to collect the same or very similar data whilst minimizing the risk to both the researcher and the animal are considered to be more favorable alternatives. Several techniques, known collectively as noninvasive monitoring techniques, such as camera trapping, hair-snaring, and scat analyses have become increasingly useful and less costly alternatives to more invasive techniques.

Scat analysis is a noninvasive technique used to facilitate wildlife monitoring efforts and has been gaining increased use given its relative efficiency, low cost, and increased accuracy. I examined the use of molecular techniques to distinguish among felid samples using mitochondrial DNA isolated from scat. Two mitochondrial genes (16s rRNA and ATP6) were found to be effective in discriminating among the scats of three Neotropical felids: jaguars *(Panthera onca),* pumas *(Puma concolor),* and ocelots *(Leopardus pardalis)* at the species-level. Base pair differences were identified in the 16s rRNA and ATP6 regions at multiple nucleotide loci for all three species. This study also sequenced the subunit 6 region of the ATP gene for ocelots, previously unsequenced on NCBI.

Key words: Neotropical felids, jaguar, puma, ocelot, noninvasive wildlife monitoring, scat analysis, mtDNA, ATP6, 16s rRNA, species identification

Introduction

Noninvasive wildlife monitoring methods, such as camera trapping (Silver *et al.* 2004), hair-snaring (Beier *et al.* 2005), and scat analyses (Chaves *et al.* 2010; Kitano *et al.* 2007), have become increasingly useful and less costly alternatives to more invasive techniques (Waits and Paetkau 2005). Invasive monitoring methods such as, radio-collaring or blood/tissue collection requires physical detention of the animal, which can sometimes induce capture myopathy (Beringer *et al.* 1996; Conner *et al.* 1987). Patterson (in West *et al.* 2007) describes capture myopathy as a non-infectious condition where induced stress (i.e., capture, handling, restraint, pursuit, etc…) causes muscle necrosis, myoglobinuria, and metabolic acidosis. Capture myopathy affects a wide variety of animals, both domestic and wild (Patterson in West *et al.* 2007). While not all animals captured for invasive monitoring suffer from capture myopathy, other injuries (i.e., broken legs, strained muscles…) can be sustained, which could affect survivability of the animal being studied (Patterson in West *et al.* 2007). Research methods that reduce the risk to both the researcher and the animal should be considered when designing studies requiring the monitoring of wildlife.

One method of noninvasive wildlife monitoring utilizes fecal matter, or scat, left behind by animals. Scat, which is readily deposited by an animal, can be collected by researchers and transported with relative ease, which minimizes potential risks to the researcher and animal. Animal scat contains a significant amount of genetic information (Chaves *et al.* 2012), which can be used to identify samples based on a wide variety of criteria. With the proper training, scat samples from organisms can be identified and categorized by species, individuals, gender, diet, and even population dynamics if a large amount of samples are available or collected (Chaves *et al.* 2010; Kitano *et al.* 2007).

Habitat fragmentation is often cited as a primary cause resulting in global losses of biodiversity (Dirzo and Raven 2003). Fragmentation, direct persecution, and depletion of prey, which are due to direct conflict with humans, are the primary factors that have led to the decline for many large carnivore species

(Caso *et al.* 2008). Biological hotspots, such as tropical forests, are areas that contain disproportionately high level of species diversity in comparatively small areas (Myers *et al.* 2000), and particularly sensitive to habitat fragmentation and biodiversity loss (Dirzo and Raven 2003). Neotropical felids, such as the jaguar (*Panthera onca*), puma (*Puma concolor*), and ocelot (*Leopardus pardalis*) are solitary carnivores, typically present in low-densities, which can make monitoring of these organisms challenging (Silver *et al.* 2004). Wildlife biologists may rely on noninvasive survey methods to assist in the monitoring of species persisting at low-densities.

Kitano *et al.* (2007) developed a set of universal vertebrate primers capable of amplifying the mitochondrial deoxyribonucleic acid (mtDNA) of the 16s rRNA gene. Similarly, Chaves *et al.* (2012) developed a set of mitochondrial primers targeting the ATP subunit 6 gene (ATP6) capable of identifying the species of carnivore scat samples. Collectively, these primers could be used to extract and identify mtDNA from scat samples. The goal of this project was to test the ability to discriminate among jaguar (*Panthera onca*), puma (*Puma concolor*), and ocelot (*Leopardus pardalis*) scat using regions of the 16s rRNA and ATP 6 genes present in mitochondrial DNA, isolated from felid scat.

Materials and Methods

Noninvasive Sample Collection

Scat subsamples (smaller versions of collected samples) used in this project were obtained from the Global Felid Genetics Program at the American Museum of Natural History. Samples were collected in the wild from South America, dried using silica gel, and stored in a cool, dry place to allow for proper desiccation of scat samples. A total of 15 subsamples were obtained, representing three felid species; including six jaguar (*Panthera onca*) scat subsamples, six puma (*Puma concolor*) scat subsamples, and three ocelots (*Leopardus pardalis*) scat subsamples. Scat subsamples were stored in re-sealable plastic bags with silica beads before and after DNA extraction.

mtDNA Extraction

Mitochondrial DNA was extracted using the MP Biomedicals© FastDNA™ SPIN Kit for Feces. A surgical blade was used to scrape off the outer layer of fecal material and approximately 250µg of fecal material was weighed before being homogenized in a MT buffer and sodium phosphate buffer. Cells were lysed according to the MP Biomedicals © FastDNA™ SPIN Kit for Feces protocol. 250µl of PPS was added to clean 1.5ml tubes and the supernatant following cell lysis and centrifugation was added to the PPS tubes being careful not to suck up any of the pellet. Binding matrix (1ml) was added to 15ml tubes and vortexed prior to adding the scat samples. The mtDNA extraction was completed following the MP Biomedicals © FastDNA™ SPIN Kit for Feces protocol and dH_2O was used to elute the extracted DNA off of the column.

Primer Design, PCR Amplification, Purification, and Sequencing

Primers for the 16s rRNA gene were designed to capture vertebrate DNA and were used as described by Kitano *et al.* (2007). The forward and reverse primers developed by Kitano *et al.* (2007) were designed in highly conserved regions and are capable of amplifying short DNA fragments (e.g., 215 and 244bp, Table 1). Chaves *et al.* (2012) developed short mtDNA primer sequences for the ATP6 gene capable of amplifying and identifying carnivore DNA from scat samples (Table 1). I added T7 (on the forward primer) and SP6 (on the reverse primer) tags to aid in the analysis of sequencing results.

The expected mtDNA fragments were 244bp and 134bp for 16s RNA and ATP6, respectively (Chaves *et al.* 2012; Kitano *et al.* 2007). Contents of the PCR tubes were kept on ice until placed into the PCR unit. A master mix containing GoTaq Green, forward and reverse primers, and dH_2O was make and proper amounts were added to each tube. Extracted DNA was then added to each respective tube. PCR reactions were run with the parameters described in Table 2 (see Appendix). PCR products for 16s rRNA and ATP6 were visualized on a 1% Agarose gel (Figures 1 and 2) before PCR purification and sequencing (GENEWIZ, Inc. South Plainfield, NJ, U.S.A.). Purified PCR products were sequenced in

both directions using the T7 and SP6 tags for the forward and reverse primers, respectively. Forward and reverse sequences were aligned and compared using Nucleotide BLAST ® via NCBI. Furthermore, forward and reverse sequences were aligned and analyzed for single nucleotide loci indicating differences among species using Clustal Omega (EMBL-EBI©).

Table 1: Primer designs used to amplify mtDNA from scat samples

Primer	*Sequence*	*Source*
L2513	5′-GCCTGTTTACCAAAAACATCAC-3′	Kitano *et al.* 2007
H2714	5′-CTCCATAGGGTCTTCTCGTCTT-3′	
L7987	5′-AACGAAAATCTATTCGCCTCT-3′	Chaves *et al.* 2012
H8114	5′-CCAGTATTTGTTTTGATGTTAGTTG-3′	

Table 2: PCR Parameters

Temp	*Time (min)*	*Process*
95°C	3:00	Initial denaturation
94°C	0:30	Denaturation
58°C	0:30	Annealing
72°C	0:30	Extension
72°C	2:00	Final Extension
4°C	∞	Hold

Results

DNA Amplification for 16s rRNA and ATP6

PCR products were loaded onto a 1% Agarose gel using a 1:10 dilution factor. DNA was successfully amplified from PCR products of the 16s rRNA gene (Figure 1). A band was not observed for one of the jaguar samples, J6 (Figure 1). Bands of PCR products that were successfully amplified were purified and sequenced (GENEWIZ, Inc. South Plainfield, NJ, U.S.A.).

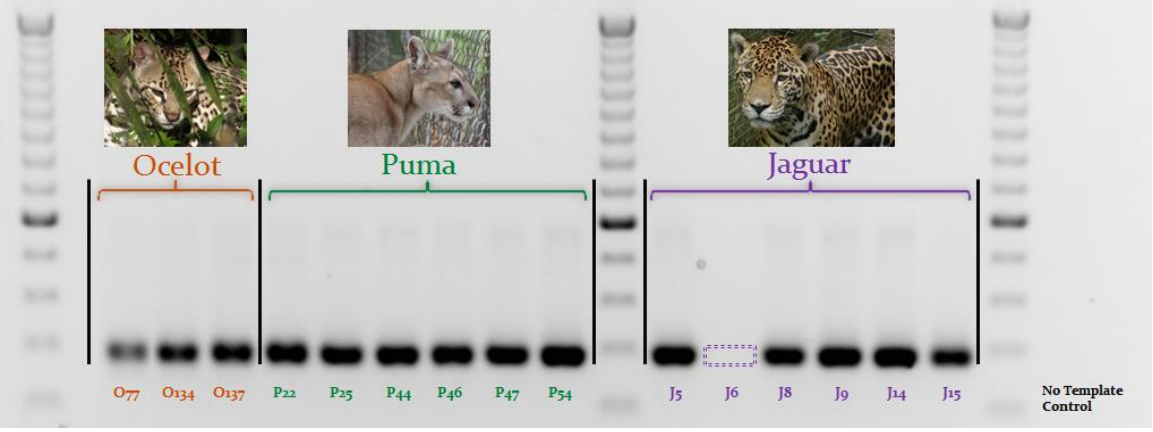

Figure 1: Amplification of PCR Product from 16s rRNA gene using a 1:10 dilution factor

PCR products for the ATP6 gene were visualized on a 1% Agarose gel using two concentrations: 1:10 and 1:100 dilution factors (Figure 2). A band was not observed at the 1:100 concentration of one of the puma samples (P44), or at either concentration (1:10 or 1:100) for one of the ocelot samples (O77, Figure 2). DNA was successfully amplified in all other samples and dilutions for ATP6 gene. Bands of PCR products that were successfully amplified were purified and sequenced (GENEWIZ, Inc. South Plainfield, NJ, U.S.A.).

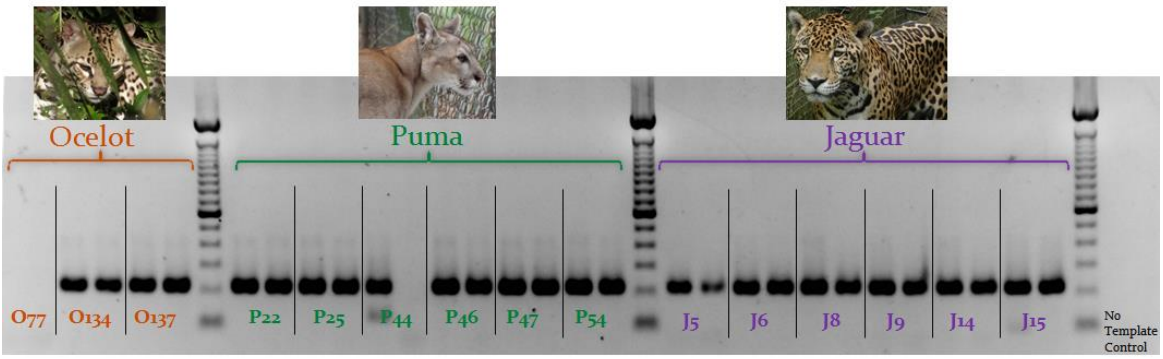

Figure 2: Amplification of PCR Product from ATP6 gene using 1:10 and 1:100 dilution factors

The forward and reverse sequences for 16s rRNA and ATP6 were aligned and analyzed using Clustal Omega (EMBL-EBI©). I highlighted single nucleotide loci differences in both the forward and

reverse sequences for both genes (Figures 3 and 4). Differences found in the forward sequences were confirmed in the reverse sequences for both genes (see excerpts below). Asterisks indicate identical base in all samples for that nucleotide loci. Differences at nucleotide loci were found throughout the sequenced gene, but an excerpt is provided below for simplicity.

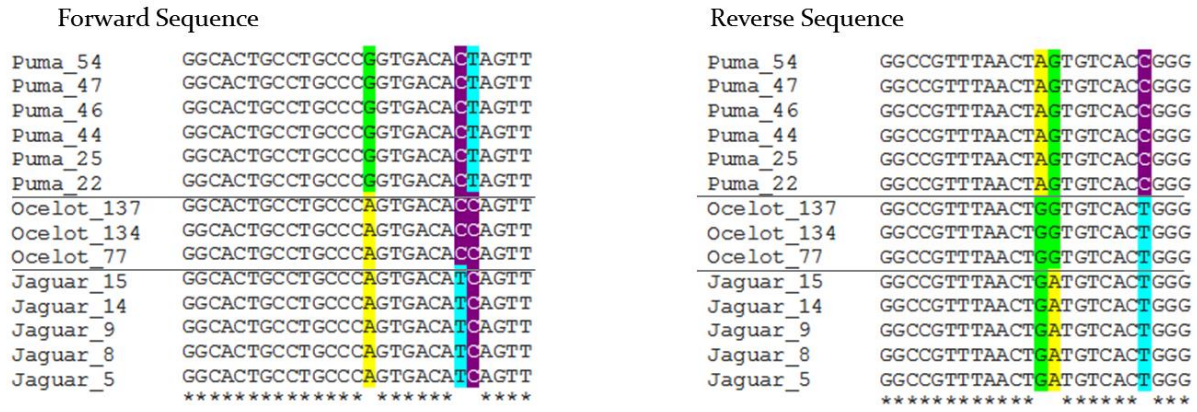

Figure 3: Single nucleotide base differences in the 16s rRNA gene among felid samples

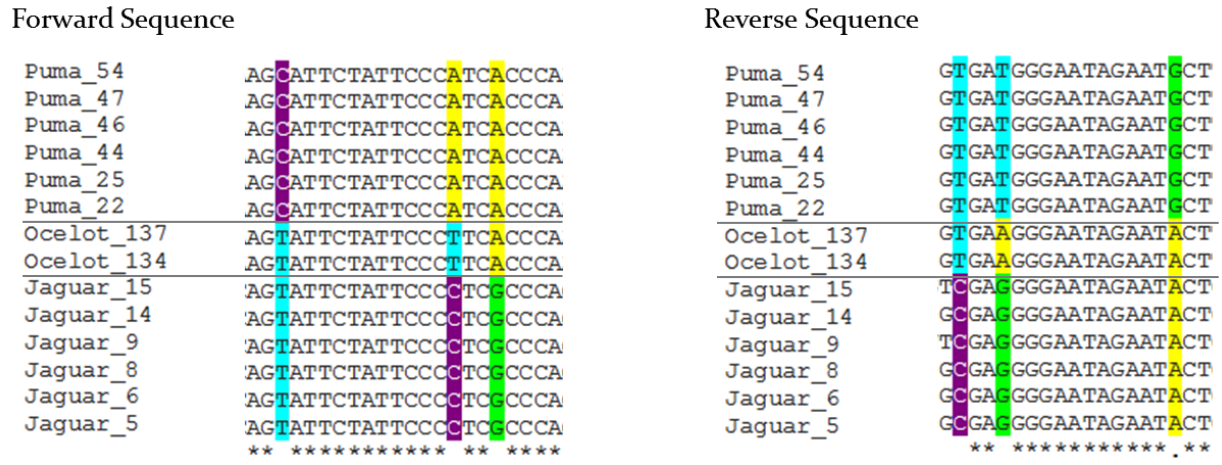

Figure 4: Single nucleotide base differences in the ATP6 gene among felid samples

Discussion

Failure to amplify one of the jaguar samples (J6) in the 16s rRNA mitochondrial gene was likely due to a small amount of starting material. Homogenization of fecal sample in the buffer prior to cell lysis called for ~250µg, of which only ~125µg was able to be extracted. This was due to the large quantity of hair in the digested fecal material of the sample. Failure to amplify one of the 1:100 concentration of one of the puma samples (P44) of the ATP6 mitochondrial gene was likely due to human error. Given that both concentrations of nearly all other samples were successfully amplified (Figure 2), it is likely that the DNA for P44 may have never been put into the PCR tube before amplification. Similarly, a failure to amplify O77 (ocelot) at either concentration could have been due to an absence of DNA.

I was able to successfully extract and purify mtDNA from scat samples using the 16s rRNA and ATP6 mitochondrial genes. Gene regions were sequenced and I was able to determine species-level identification from all successfully amplified scat samples. Various regions throughout the 16s rRNA and ATP6 mitochondrial genes were identified in jaguar (*Panthera onca*), puma (*Puma concolor*), and ocelot (*Leopardus pardalis*) suitable for species-level discrimination from scat samples (Figures 3 and 4). The 16s rRNA and ATP6 genes both identified nucleotide loci sites depicting differences across all three species, and the ATP6 mitochondrial identified three separate sites capable of distinguishing among all three species at a single nucleotide loci. In summary, the 16s rRNA and ATP6 mitochondrial genes were found to be suitable for discrimination among jaguar, puma, and ocelot scat samples.

All three species were able to be matched for the 16s rRNA mitochondrial gene to sequences available on NCBI. Both the puma and jaguar were able to be matched for the ATP6 mitochondrial gene but sequences are not available to match ocelots for subunit 6 of the ATP mitochondrial gene. Sequencing of the subunit 6 of the ATP mitochondrial gene for ocelots (*Leopardus pardalis*) is novel to this study, has not previously been sequenced, and is not available on NCBI.

Additionally, a subsample of tissue from the liver of a male snow leopard (*Panthera uncial*) was obtained from Amanda Makkay of Fordham University for use in gender-typing of scat samples. Preliminary gender-typing analyses were conducted but not completed prior to the time of this report. Regions of the male-linked SRY gene were used to assign a suspected gender to the scat samples using the provided male positive control. Three individuals reported a band in the SRY region and were confirmed on NCBI, but a lack of a female positive control indicated that further testing is required. To cross-reference the SRY results, another gene region present on both the X- and Y-chromosomes will be used. This gene, Amelogenin, described by Pilgrim *et al.* (2005) takes advantage of Y-chromosome deletions resulting in different size bands when visualized on 1% Agarose gel. A gel depicting a female should display a single band at 214bp, whereas the Y-chromosome deletions should display two bands for a suspected male, a band at 214bp and one band at 194bp. Further analysis is required using the methodology presented in Pilgrim *et al.* 2005 to the gender of the felid from which the scat samples were obtained.

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