# GENETIC ANALYSIS OF MOUNTAIN LION (*Puma concolor*) FECES FROM KOFA NATIONAL WILDLIFE REFUGE, ARIZONA

by

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I dedicate this work to my parents for helping me begin the climb up the coconut tree, for only now have I begun to obtain its fruit.

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## ABSTRACT

Investigations on recent records of mountain lions (*Puma concolor*) and concurrent declines in desert bighorn sheep (*Ovis canadensis mexicana*) on Kofa National Wildlife Refuge have necessitated the determination of the number of mountain lions and their diet on the refuge. Using genetic analysis, we identified mountain lion feces/scats (n=53) from the Kofa and Castle Dome Mountains in southwestern Arizona. We identified 11 individual mountain lions that included at least 6 males and 2 females. We also identified prey species from bone and connective tissue remains inside the mountain lion scats. Our data suggest that a majority of mountain lion diet (62 %) on the refuge is mule deer (*Odocoileus hemionus*). These estimates greatly enhance our knowledge of mountain lions in an area where, historically, their presence was considered transient.

Additionally, recognizing the need for reliable species identification and to improve species identification from non-invasive samples, we developed a Polymerase Chain Reaction (PCR) primer set that would enable the amplification of the complete cytochrome *b* gene from a large number of mammalian species. DNA sequence information obtained from the use of this primer set can be used for the development of mammalian species' databases and referencing.

Overall, this project demonstrates the efficacy of genetic techniques and their potential to provide reliable and necessary information on elusive species to wildlife managers.

# CHAPTER 1: INTRODUCTION

The Kofa National Wildlife Refuge comprises two major mountain complexes, the Kofa Mountains and the Castle Dome Mountains, and spans 2,693 sq. km. of the Sonoran desert in southwestern Arizona. Established in 1939 as a reserve to protect desert bighorn sheep (*Ovis canadensis mexicana*), the number of bighorn sheep on the refuge has been actively monitored by the U.S. Fish and Wildlife Service (USFWS) and the Arizona Game and Fish Department (AGFD) since 1955. Between 2000 and 2006, bighorn sheep numbers declined from approximately 800 individuals in 2000-2002 to approximately 400 individuals in 2006-2008 (USFWS 2009). This prompted an investigation into potential factors responsible for the decline. Potential mortality factors include drought, disease, hunting and predation by mountain lions (*Puma concolor couguar*, also known as American puma or cougar). Predation by mountain lions, however, was initially considered of least concern because mountain lions were thought to be transient in southwestern Arizona and on the refuge. Since the last verifiable record of a hunter-killed mountain lion in 1944, the first sighting of a mountain lion took place in the Kofa Mountains in 2003 during an aerial survey by the AGFD. However, further investigation between 2006 and 2008 via camera-trap efforts by refuge personnel revealed the presence of at least 5 individual mountain lions (three adults and two kittens) using the refuge. This documentation supported an alternative hypothesis that predation by mountain lions was occurring and could be contributing to the decline of bighorn sheep numbers. The USFWS and AGFD jointly tracked 4 mountain lions using GPS-telemetry on the refuge between 2007 and 2009, and concluded that the refuge is

providing habitat to mountain lions with large inter-mountain movements ranging between 751 sq. km. and 2297 sq. km. (Minimum Convex Polygon area estimates; USFWS, unpublished data). The study also documented predation on desert bighorn sheep and mule deer (*Odocoileus hemionus*). Although this information provided evidence to model predation risk on the Kofa bighorn sheep herd (USFWS, unpublished data), more information on the mountain lion population size and diet is necessary to model their impact on bighorn sheep. We collected and genetically analyzed puma feces between 2007 and 2009 to identify the minimum number, sex and diet of pumas on the refuge.

# CHAPTER 2: GENETIC ANALYSIS OF SCATS REVEALS MINIMUM NUMBER AND SEX OF MOUNTAIN LIONS ON KOFA NATIONAL WILDLIFE REFUGE, ARIZONA

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**Genetic analysis of scats reveals minimum number and sex of mountain lions on Kofa National Wildlife Refuge, Arizona**

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## ABSTRACT

**Recent records of mountain lions (*Puma concolor*) and concurrent declines in number of desert bighorn sheep (*Ovis canadensis mexicana*) on Kofa National Wildlife Refuge in Arizona have prompted investigations to estimate number and sex of mountain lions using the refuge. Using genetic techniques, we performed species, individual and sex identification analyses on mountain lion scats (n=53) collected from the Kofa and Castle Dome Mountains in the refuge. We identified 11 individual mountain lions including 6 males and 2 females. The sex of 3 individuals remained unidentified, due to the lack of amplifiable DNA from scats. This estimate**

**augments the number of mountain lions previously recorded using other methods, and greatly enhances our knowledge of mountain lion ecology in an area where this species was previously considered transient. We demonstrate the efficacy of non-invasive genetic techniques and their potential to provide reliable information on elusive species to wildlife managers.**

## INTRODUCTION

Predation by mountain lions (*Puma concolor*) on declining desert bighorn sheep (*Ovis canadensis mexicana*) populations in southwestern Arizona, especially on Kofa National Wildlife Refuge (Kofa NWR, Figure 1), has become a major concern to state and federal wildlife managers. Mountain lions were previously thought to be largely transient in southwestern Arizona (Shaw et al. 1988; Germaine et al. 2000) along with belief over the existence of an elusive subspecies, the Yuma puma (*Puma concolor brownii*), until Culver et al. (2000) established the existence of a single subspecies in North America. After the only verifiable record of a hunter-killed mountain lion in 1944, direct sightings, detection of tracks and an extensive camera-trap survey between 2003 and 2008 revealed the presence of at least 5 individual mountain lions occupying the Kofa and Castle Dome Mountains (Smythe 2008). This documentation, along with a declining population of desert bighorn sheep (U.S. Fish and Wildlife Service [USFWS] 2009), resulted in the implementation of an adaptive, site-specific predator management plan directed at removing any mountain lion responsible for more than 1 bighorn sheep kill during any 6-month period (Arizona Game and Fish Department [AGFD] 2007). Limited GPS

movement data of 4 radio-collared mountain lions also indicated that Kofa NWR is providing habitat to mountain lions with large home ranges including movements between 5 different mountain ranges (USFWS and AGFD, unpublished data). These preliminary findings, along with the concern over the declining numbers of desert bighorn sheep, have prompted further investigation into determining the minimum number and sex of mountain lions on the refuge.

## STUDY AREA

Kofa NWR is located in the Yuma and La Paz counties of Arizona and spans 2,693 sq. km. of the Sonoran desert. The refuge encompasses two major block-faulted mountain ranges (Kofa and Castle Dome Mountains, and portions of the Little Horn, Tank, and New Water Mountains) and predominantly shallow, stony soils and rock outcrops in the mountainous and steep slope areas. The refuge hosts more than 400 taxa of flora, 193 species of birds, 49 species of mammals, and 41 species of reptiles and amphibians (USDI 2007).

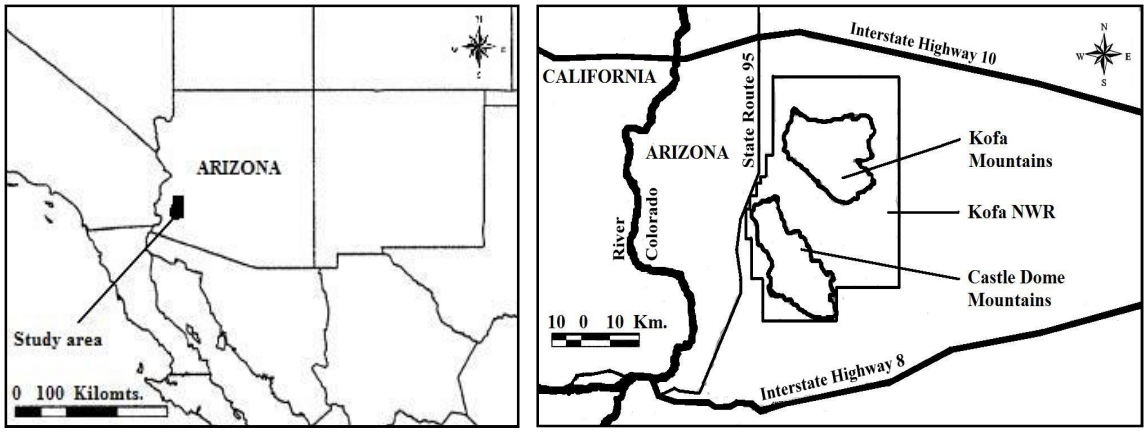


**Figure 1** Location of study area – Kofa NWR in southwestern Arizona, USA.

## METHODS

*Sample Collection and DNA Extraction*

We collected 105 scat samples suspected to be mountain lion in origin between December 2006 and August 2009 from the Kofa and Castle Dome mountains in Kofa NWR. To prevent degradation of DNA in these samples after collection, we ensured no exposure to sunlight or moisture by storing samples in paper bags and transferring samples into sealable plastic bags containing desiccant silica. Within 1 week of collection, we transported samples to the Conservation Genetics Laboratory at the University of Arizona (Tucson, Arizona) for cold-storage at -20 $^{o}$C. In the laboratory, we scraped the surface of each sample to obtain sloughed epithelial cells from the predator (i.e., cells from the large intestine of the depositor or owner of the scat sample). We used 0.1 – 0.3 mg of the scrapings for DNA extraction. We used QIAamp DNA Stool Mini Kits (Qiagen, Valencia, CA, USA) and followed the manufacturer's protocol for DNA extraction and purification.

*PCR Amplification and DNA Sequencing*

We amplified DNA from the mitochondrial cytochrome b (*cytb*) gene region using the 'universal' PCR primers, *mcb398* and *mcb869*, designed for mammalian species identification (Verma and Singh 2003). We subjected DNA that did not amplify to a second set of PCR primers for the same region – *Cytb-1 and Cytb-2* (Janczewski et al. 1995). We performed PCR amplifications in a 20 µL reaction volume with the following final concentration: 1 X PCR Buffer (Qiagen), 1 mM $MgCl_2$ (Qiagen), 0.2 mM dNTPs (Qiagen), 0.05 % BSA (Sigma-Aldrich, St. Louis, MO, USA), 0.5 U of *Taq* DNA Polymerase (Qiagen), 0.5 µM each of forward and reverse primers, and 4 µL of template DNA. PCR conditions for both reactions consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 51 °C for 1 min, extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. We used *Mastercycler* PCR machines (Eppendorf, Westbury, NY) for all PCRs. We prepared positive PCR products for sequencing with the *ExoSAP-IT PCR Clean-up* kit (USB Corporation, Cleveland, OH, USA) using manufacturer's recommendations. We submitted cleaned PCR products to the University of Arizona Genetics Core (http://uagc.arl.arizona.edu/) for sequencing on a 3730 Automated DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

*Species, Individual and Sex Identification*

We used the program BLAST (Altschul et al. 1990, http://www.ncbi.nlm.nih.gov) to query DNA sequences and identified species of origin of based on best matches obtained

with reference sequences in GenBank (http://www.ncbi.nlm.nih.gov). To be confident about the species identity, we set an E-value cut-off of 0.0 and maximum identity ≥ 95 %. We excluded sequence matches that were outside these values from our data set.

We subjected mountain lion scat DNA samples (along with 4 male mountain lion tissue samples collected as reference samples during radio-collaring efforts by the AGFD and USFWS on Kofa NWR) to a genotyping assay employing 3 microsatellite loci – *FCA057*, *FCA090* and *FCA043*. These loci were chosen from the domestic cat genetic linkage map (Menotti-Raymond et al. 1999) and known to be polymorphic in mountain lion populations (e.g., Sinclair et al. 2001, Ernest et al. 2003 and McRae et al. 2005). We amplified the microsatellites using conditions described in Menotti-Raymond et al. (1999). We submitted PCR products to the University of Arizona Genetics Core for fragment analysis via electrophoresis using an ABI 3730 analyzer (Applied Biosystems). We analyzed fragment size data using GENOTYPER v3.7 (Applied Biosystems). We repeated microsatellite PCR amplification and analysis 3 times on all samples to check for allelic dropout and false alleles. We excluded samples that resulted with inconsistent genotypes within the 3 genotyping attempts. We identified individuals based on a unique genotype from all the 3 loci.

To identify sexes of the individuals, we used a felid sex-identification PCR designed by Pilgrim et al. (2005) for non-invasive genetic samples. This system distinguishes females from males based on y-chromosome deletions in the *zinc-finger* and *amelogenin* regions. We used primers targeting the *amelogenin* locus to obtain a single band on an agarose gel sized 214 bp for females versus two bands sized 214 bp

and 194 bp for males. We used tissue DNA samples from the 4 known male mountain lions and 1 additional female mountain lion (radio-collared by AGFD near Sabino Canyon, Tucson, Arizona) as positive controls.

*Contamination Precautions*

We used sterile latex gloves to collect scat samples and stored each scat sample in fresh bags. We performed DNA extractions and post-PCR analysis in separate laboratories, in separate buildings. To maintain sample integrity, we placed equipment that directly contacted samples (trays, spatulas, forceps, etc.) in a 20 % sodium-hypochlorite bath for at least 10 min and used *DNA-OFF* decontaminating solution (Takara Bio, Madison, WI, USA) to wipe all equipment between each use. We controlled for contamination by including DNA extraction controls (blanks) and PCR controls (negatives) by substituting samples with PCR grade water. We used *TipOne* sterile filter tips (USA Scientific, Ocala, FL, USA) for pipetting during all manipulations. We wore protective face-masks while scraping scat samples and wore sterile latex gloves during all stages of laboratory work.

**RESULTS**

From the 105 scats, we identified 53 mountain lion, 12 bobcat (*Lynx rufus*) and 3 coyote (*Canis latrans*) scats. The remaining 37 scats either failed to yield PCR products or yielded poor quality sequence, thus were not identified after the DNA sequencing stage. Nearly 65 % of scat samples were identified to species level.

All 3 microsatellite loci (*FCA057*, *FCA090* and *FCA043*) consistently amplified in the 4 reference mountain lion samples and 21 of 53 mountain lion scats. All 3 loci were polymorphic and displayed 4, 6 and 6 alleles respectively with a combined probability of identity, P (id) value, of $2.65 \times 10^{-3}$ (GENALEX v6.1, Peakall and Smouse 2005). Among the total of 25 genotypes obtained, we identified 11 unique genotypes (individuals). We confirmed that 13 scats matched with 3 of the 4 known male controls from Kofa NWR. In addition to these 4 males, we identified 2 individual female and 2 individual male mountain lions from scat DNA. We were unable to identify the sex of 3 individuals because DNA from their scat samples failed to amplify with the sexing PCR assay.

Overall, the individual mountain lions identified from scat DNA included at least 2 females and 5 males (Figure 2). One additional male, identified from a reference tissue sample genotype, did not match any of the scat DNA genotypes.

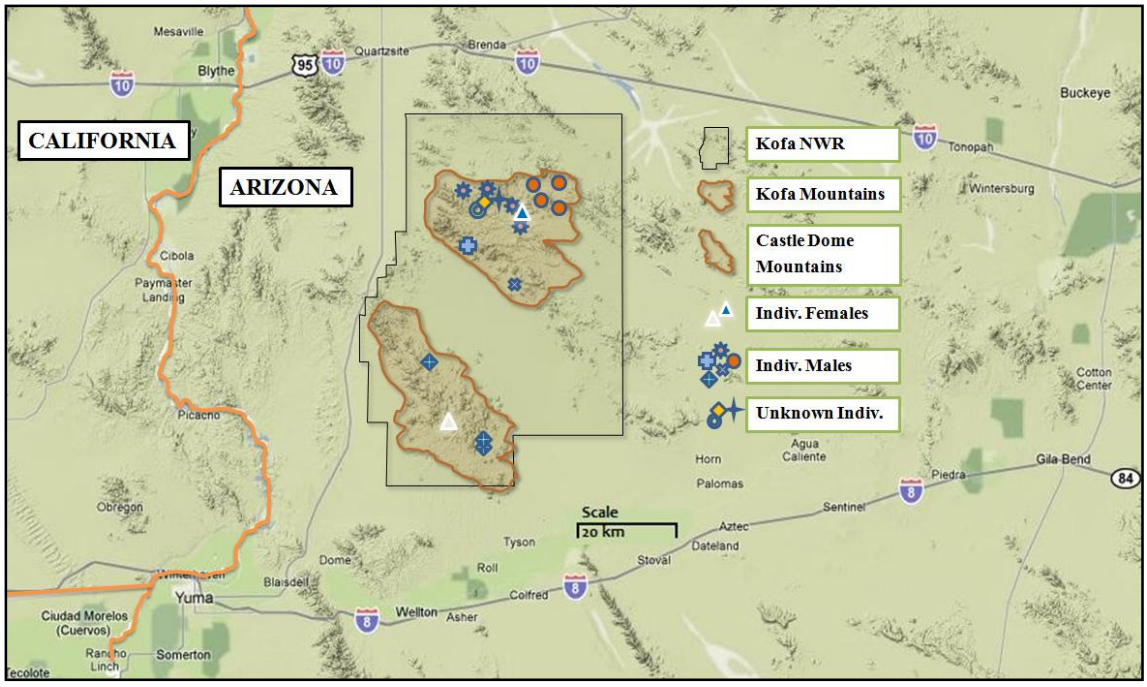


**Figure 2** Individual male and female mountain lion scat locations on Kofa NWR, Arizona.

## DISCUSSION

We documented presence of mountain lions in Kofa NWR in 2007, 2008, and 2009 from scats and concluded that a minimum of 11 individual mountain lions, including 6 males and 2 females, used Kofa NWR between 2007 and 2009. The sex of three individuals is unidentified due to lack of amplifiable DNA from their scats. We identified 8 of the 11 individuals in 2007, 5 in 2008 and 3 in 2009. We genetically captured 2 individuals in all three years, 1 individual in 2007 and 2009, and all other individuals in only one of the three years. These estimates supplement GPS-tracking and camera-trap data on mountain lions using the Kofa NWR between 2006 and 2008, as presented by Smythe (2008).

Although very little can be said about the residency or transiency of these individuals, we are continuing to sample the area. Analyses using these data as a baseline for using mark-recapture may further elucidate residency on the refuge.

We hypothesize success in identifying, genotyping and sexing mountain lion scats (105 scats collected, 53 identified as mountain lion, 21 genotyped and 4 sexed) can be limited by DNA degradation caused by exposure of scats to environmental factors prior to collection, high temperatures (Hájková et al. 2006), age of scats (Santini et al. 2007) and high concentration of PCR inhibitors in scat DNA (Waits and Paetkau 2005). Our success rate resembles previous non-invasive genetic research using scat DNA samples (reviewed by Taberlet et al. 1999, Waits and Paetkau 2005).

Initially, we tested a total of 6 microsatellite loci – *FCA043*, *FCA057*, *FCA082*, *FCA090*, *FCA096*, and *FCA166* to obtain genotypes and distinguish individuals from the mountain lion samples collected in Kofa NWR. We found three of these microsatellite loci (*FCA082*, *FCA096* and *FCA166*) to be low in Polymorphism Information Content (PIC) for mountain lion samples from Kofa NWR. This result is not surprising since we tested the loci on individuals within a small geographic scale (limited to Kofa NWR) possibly related to each other. Moreover, Culver et al. (2000) indicate little microsatellite variability in North American pumas.

We are confident about our estimate on the minimum number of individual genotypes based on only 3 microsatellite loci because of the high PIC of the 3 loci and a low P (id) between two individuals when all 3 loci are used in combination. Based on these loci, it is possible that every 2.65 individual mountain lion samples may possess an

identical genotype when drawn at random from a population size of 1,000 individuals – a number much larger than the number of individual free-ranging mountain lions that could possibly exist in southwestern Arizona.

For wildlife agencies plagued by a lack of robust information on mountain lion populations, genetic techniques provide an effective, noninvasive and potentially less expensive way to sample populations over large areas. Our analysis of mountain lion scats provided data on the numbers and sex of mountain lions not obtainable through limited (because of personnel/budget constraints) collaring efforts or camera surveys. We foresee these techniques will become increasingly useful to wildlife managers as management agencies are called upon to obtain baseline information in support of management actions.

**ACKNOWLEDGEMENTS**

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# CHAPTER 3: UNIVERSAL PRIMER PCR ASSAYS TO IDENTIFY PREY REMAINS IN FECES OF LARGE TERRESTRIAL CARNIVORES

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## Universal primer PCR assays to identify prey remains in feces of large terrestrial carnivores

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## ABSTRACT

**The use of Universal Primer PCR Assays (UPPAs) has been very effective in DNA-based diet studies of marine predators, but the application of UPPAs to study large terrestrial carnivore diets has been limited. We used two UPPAs to document prey species from fecal specimens of American pumas (*Puma concolor*) in southwestern Arizona. We successfully identified to the species level, 38 of 83 fecal specimens and 64 of 64 prey remains (connective tissue and bone fragments) recovered from 32 of 34 puma fecal specimens. We determined that a majority (62 %) of prey was mule deer (*Odocoileus hemionus*). We present our methodology to demonstrate the efficacy of UPPAs in identifying both predator and prey species from fecal remains.**

**UPPAs can be used reliably and extensively to analyze diets of terrestrial carnivores.**

*Keywords*: fecal DNA, predator and prey identification, universal primers, cytochrome *b*, puma, southwestern Arizona

## INTRODUCTION

Molecular methods have been successfully used to identify prey in predator feces (Jarman *et al.* 2002, 2004; Deagle 2006; Matejusová *et al.* 2007; Deagle *et al.* 2009; Dunshea 2009), and most studies using these methods have examined the diets of invertebrate and vertebrate marine predators (Blankenship & Yayanos 2005; Deagle *et al.* 2005; Kvitrud *et al.* 2005; Parsons *et al.* 2005; Casper *et al.* 2007). Similar methods have been used to study diets of several terrestrial species (Agustí *et al.* 2003; Nyström *et al.* 2006; Pons 2006; Brenda *et al.* 2007; Deagle *et al.* 2007), however, such studies are uncommon. A few diet studies used genetic analyses to identify fecal specimens of large terrestrial carnivores, at the species level (Farrell *et al.* 2000; Krausman *et al.* 2006) and individual level (Fedriani & Kohn 2001), but did not perform genetic analyses on prey remains inside the feces.

For DNA-based diet analyses, Universal Primer PCR Assays (UPPAs) are considered superior to group-specific and species-specific PCR primer assays because use of universal primers does not require prior knowledge of species in diets of predators (reviewed by Valentini *et al.* 2008). UPPAs are more advantageous than microsatellite

genotyping and restriction fragment length polymorphism (RFLP) techniques designed for species identification because UPPAs return species identities on species of interest and non-target species. Although UPPAs have been proposed for extensive use in identifying predator diets, precautions must be taken to monitor contamination by predator DNA (Blankenship & Yayanos 2005; Dunshea 2009).

We used two UPPAs targeting two non-overlapping regions of the mammalian mitochondrial cytochrome *b* (*cytb*) gene to first identify and distinguish feces of puma (*Puma concolor*) from feces of sympatric carnivores, coyote (*Canis latrans*) and bobcat (*Lynx rufus*) in southwestern Arizona. We subsequently identified prey species from connective tissue and bone remains recovered from puma feces. Here, we present our findings and support the use of UPPAs on fecal DNA to identify sympatric terrestrial carnivores and to document prey species in their diet.

## MATERIALS AND METHODS

### *Sample collection*

From June 2006 to November 2008, we collected 83 fecal samples suspected to be of puma in origin based on size, morphology and contents (Chame 2003) from the Kofa National Wildlife Refuge, Arizona, USA. We stored each sample in a paper bag and then into a sealable plastic bag. If fecal was moist, we air dried it in an area devoid of sunlight or moisture and added silica desiccant beads in an approximate 4 (silica):1 (feces) ratio by weight. We transported samples to a freezer within 5 days of collection and then to

the Conservation Genetics Laboratory (University of Arizona, Tucson, AZ, USA) for long-term storage at -20 $^{o}$C.

*Predator identification*

We scraped the surface of each fecal sample and collected between 0.1 mg – 0.3 mg of fecal matter for DNA extraction. To minimize potential contamination by prey DNA, we scraped only the surfaces of fecal samples (based on the appearance and texture) that were likely to possess sloughed epithelial cells from the predator's intestine. We used QIAamp DNA Stool MiniKits (Qiagen, Valencia, CA, USA) and followed the manufacturer's protocol for DNA extraction and purification from the scraped fecal matter. To maximize DNA concentration in the final eluate, we incorporated the following changes: (1) We incubated lysed samples at 70 °C for 30 min instead of 10 min as recommended in the manufacturer's protocol, and (2) We repeated the final elution of DNA step twice (using 75 µL of the elution buffer each time) with a final mixing of the eluate, yielding ~150 µL of DNA.

We amplified a 472 bp region of mitochondrial *cytb* gene using universal PCR primers – *mcb398* and *mcb869* designed specifically for identification of mammals (Verma & Singh 2003). We also amplified a 474 bp region of *cytb* gene using another primer pair – *Cytb-1* and *Cytb-2* (Janczewski *et al.* 1995). We performed each PCR amplification in a 20 µL reaction volume with the following final concentration: 1 X PCR Buffer (Qiagen), 1 mM $MgCl_2$ (Qiagen), 0.2 mM dNTPs (Qiagen), 0.05 % BSA (Sigma-Aldrich, St. Louis, MO, USA), 0.5 U of *Taq* DNA Polymerase (Qiagen), 0.5 µM

each of forward and reverse primers , and 3 μL of template DNA. PCR conditions for both PCR analyses consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 51 °C for 1 min, extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. We performed all PCRs in *Mastercycler* PCR machines (Eppendorf, Westbury, NY). All PCR products were cleaned with *ExoSAP-IT* (USB Corporation, Cleveland, OH, USA) and sequenced on a 3730 Automated DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Arizona Genetics Core (http://uagc.arl.arizona.edu/).

We used BLAST to identify species of origin of our query DNA sequences. We identified species by selecting the first hits for each query sequence with an E-value cut-off of 0.0 and maximum identity ≥ 95 %. Fecal samples identified as puma from their DNA sequences were subsequently examined for prey contents – mainly bone or connective tissue fragments from primary mammalian prey.

*Prey remains identification*

We dissected all positive puma fecal samples and obtained a collection of prey remains (connective tissue or bone fragments) from each fecal sample. To possibly identify two different primary prey species from each fecal sample, we selected two prey remains that differed significantly in their morphology (mainly size and shape). We cleaned the surface of each bone sample with 20% sodium-hypochlorite solution (commercial grade bleach) to remove fecal material and other potential surface contaminants. We then washed each bone sample in double distilled, UV-irradiated water. To prevent DNA

damage, we did not subject connective tissue samples to 20% sodium-hypochlorite solution, but washed them with double distilled, UV-irradiated water. We dried bone and tissue samples at 56 °C in a sterilized incubator.

To extract DNA from bone samples and simultaneously test DNA extraction success from two methods, we divided bone samples into two groups for pulverization. For the first group, we wrapped each bone fragment in sterile weighing paper and in heavy-duty aluminum foil. Using a sterilized metal mortar and pestle, we pulverized samples by hand to a fine powder. For the second group, we pulverized samples with a *SPEX SamplePrep* Freezer/Mill 6770 cryogenic grinder (Spex CertiPrep, Metuchen, NJ, USA) following the manufacturer's instructions. We decalcified bone samples by adding 1.8 ml of EDTA, 0.5 M (pH 8.0), molecular grade (Promega, Madison, WI, USA) to 0.2-0.3 g of bone powder in a 2 ml vial. We incubated each vial on a rocking-platform for 12 hrs in a refrigerator at 4 °C three times and centrifuged samples to pellet bone powder and changed EDTA every 12 hrs. We complemented the decalcification with a wash step that involved centrifuging samples at high-speed (approx. 13,000 g) and re-suspension of bone powder in 1.8 ml of PCR grade water (Sigma-Aldrich). We used QIAamp Tissue & Blood Kits (Qiagen) and followed the manufacturer's protocol to extract DNA from bone and tissue samples. To maximize dissolution of samples, we doubled quantities of buffers *ATL*, *Proteinase K* and *AL* (provided in the kit) and ethanol. We performed PCR amplifications, DNA sequencing, and species identification for each sample as previously described for the predator fecal sample identification. We assembled a list and frequency of occurrence of each prey species identified in the sample set (see Figure 1).

*Contamination Precautions*

To prevent contamination, we performed DNA extractions and post-PCR analysis in separate laboratories. During sample preparation and DNA extraction, we wiped all surfaces and equipment with 20% sodium-hypochlorite solution. To maintain sample integrity, we placed equipment that directly contacted samples (trays, spatulas, forceps, grinding vials, mortar and pestle) in a 20 % sodium-hypochlorite bath for at least 10 min and used the *DNA-OFF* decontaminating solution (Takara Bio Inc.) to wipe all equipment between each use. We controlled for contamination by including DNA extraction controls (blanks) and PCR controls (negatives) and substituting samples with PCR grade water. We used an additional control sample in each prey remain DNA extraction batch where we rinsed the decontaminated mortar, pestle and grinding vials with EDTA and used 1.8 ml of rinsate as the control sample. We used *TipOne* sterile filter tips (USA Scientific, Ocala, FL, USA) for pipetting during manipulations. We wore protective face-masks while handling fecal samples and prey remains. We used sterile latex gloves for handling during all stages of the work.

**RESULTS**

Of the 83 fecal samples collected, we identified 34 puma, 3 coyote, and 1 bobcat. The remaining 45 fecal samples failed to yield PCR-amplifiable DNA.

We obtained morphologically dissimilar prey remains (tissue or bone, or both) from 32 of 34 puma fecal samples. No tissue or bone fragments were recovered from 2 puma fecal samples. Although plant and insect remains were found in these 2 fecal

specimens, we did not consider these as primary prey items and did not include these in our data set. DNA from all prey remains (64 total) from puma feces yielded PCR products with both UPPAs.

All predator and prey PCR products yielded DNA sequences that matched reference mammalian species' haplotypes deposited in GenBank. All matches returned with an E-value of 0.0 and a maximum identity of 95 % - 99 %. We identified 5 different mammalian prey species for pumas in this study area – mule deer (*Odocoileus hemionus*), American badger (*Taxidea taxus*), bighorn sheep (*Ovis canadensis*), domestic sheep (*Ovis aries*), and gray fox (*Urocyon cinereoargenteus*). We also determined that a majority (62 %) of puma prey in this study area is mule deer (Figure 3).

In 29 of the 32 sets of prey remains analyzed from puma feces, both prey remains in the same fecal sample corresponded to the same prey species. In the other two sets, one set comprised bighorn sheep and American badger, and the other set comprised bighorn sheep and mule deer.

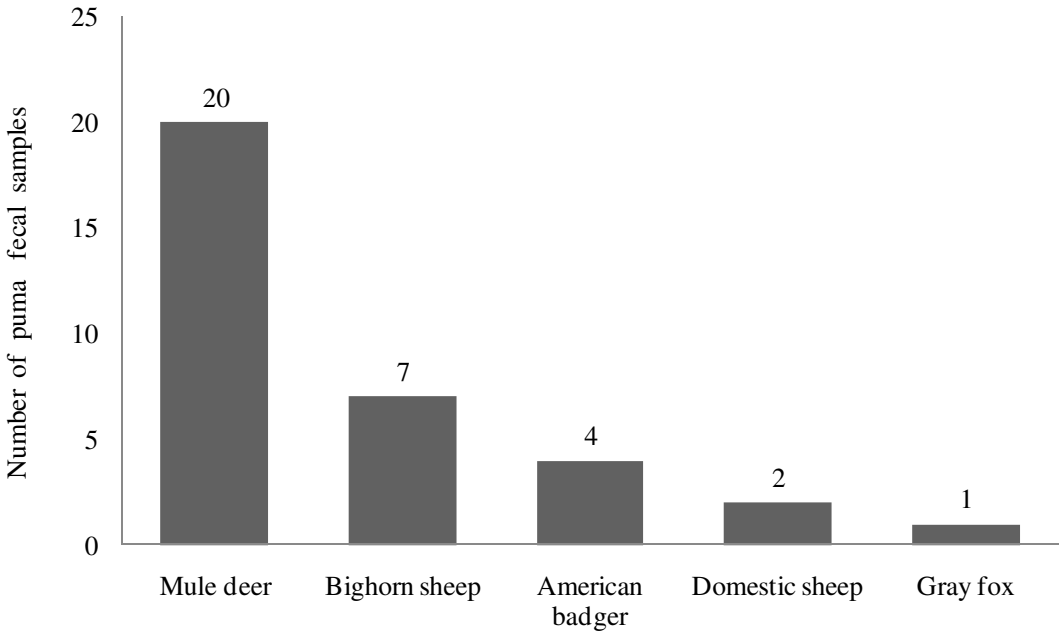


**Figure 3** Frequency of occurrence of prey species in puma fecal samples, Kofa NWR, Arizona.

*Contamination*

We did not observe any amplified PCR products in the controls (DNA extraction blanks and PCR negatives). In the case of one puma fecal sample in which the prey remains were identified as gray fox, we initially identified one of the two prey remain fragments to be puma in origin. Upon further genotyping the DNA samples from both predator and prey, using microsatellite DNA loci, we found that both DNA samples belonged to the same individual puma (Naidu *et al.*, unpublished data). We re-investigated the fecal sample for another prey remnant and repeated the analysis. This other prey remnant was identified as gray fox.

## DISCUSSION

We report 100 % success in the identification of prey species from tissue and bone remains found inside feces of pumas, which suggests that this method can yield similar results for other terrestrial large carnivores. We found no difference in success resulting from the two different bone fragment pulverization methods (mortar and pestle versus Freezer/Mill) suggesting that either method could be used in the future depending on accessibility of equipment. The puma diet results (62 % mule deer) correspond with puma diet studies conducted in southwestern United States that present mule deer as being the major food source for pumas (Rosas-Rosas *et al.* 2003).

The limited success in identifying predator species from feces (83 scats collected, 38 identified to species level) is potentially due to DNA degradation caused by exposure of the scats to microbial activity and natural UV light (Lindahl 1993) prior to collection. In contrast, the high success in identification of prey remains (tissue and bone fragments) is possibly due to the preservation of DNA in bones (Götherström *et al.* 2002) complemented with the protection offered by scat material to the prey remains against direct exposure to moisture and sunlight.

We based our choice of universal primers (*mcb398*, *mcb869* and *Cytb-1, Cytb-2*) on their use previously in studies involving species identification (e.g. Verma *et al.* 2003; Bhagavatula & Singh 2006). In 2007, these primer pairs were used in identifying field collected fecal specimens belonging to tiger (*Panthera tigris*), leopard (*Panthera pardus*) and dhole (*Cuon alpinus*) in south-central India (Bhagavatula *et al.*, unpublished data). We also based our choice on two other important reasons: (1) the abundance of

mammalian *cytb* sequences deposited in GenBank for referencing, and (2) *cytb* provides a high inter-specific resolution for mammalian species identification (Shanan *et al.* 2009).

When amplifying prey DNA, the use of blocking primers (Vestheim & Jarman 2008; Deagle *et al.* 2009; Dunshea 2009) that inhibit the amplification of predator DNA may not be necessary if precautions are taken to minimize surface contamination (i.e., fecal material possibly containing predator DNA) on prey remains. The use of blocking primers likely allows for the quantity of template DNA from prey remains to be much larger than any potential contaminant DNAs and have a higher probability of being amplified during PCR. However, in our analysis, this was not necessary. In the case that species identification from prey remains matches with that of the predator species, we recommend genotyping both prey and predator DNA samples to identify if both samples are from the same individual (predator).

With the use traditional methods such as morphological identification, it is often cumbersome and time consuming to get to species-level identity on unknown biological specimens. Genetic analyses and the use of UPPAs offer a high level of accuracy by providing information to the species and subspecies levels. We conclude that UPPAs can be very effective in reliably distinguishing between sympatric large carnivore feces and identifying their diet. We recommend their use by researchers and wildlife managers to complement diet studies of terrestrial large carnivores.

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# CHAPTER 4: NOVEL PRIMERS FOR COMPLETE MITOCHONDRIAL CYTOCHROME *B* GENE SEQUENCING IN MAMMALS

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## Novel primers for complete mitochondrial cytochrome *b* gene sequencing in mammals

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## ABSTRACT

**We developed a primer set for complete cytochrome *b* (*cytb*) gene sequencing in a large number of mammalian species for use in database development and user verification of sequence data in species identification, barcoding and phylogenetic analyses. An *in-silico* PCR test with this primer set provided positive results in species representing 27 orders of mammals. We also tested this primer set through PCR on DNA from 19 species representing 7 orders of mammals and submitted complete *cytb* gene sequences of 10 species to GenBank. We recommend the use of such primer sets to obtain sequences from known specimens and to validate reference sequences deposited in online databases.**

*Keywords*: Mammalian cytochrome *b*, primers, DNA sequence, database, species identification

Mitochondrial DNA sequences from a myriad of species have become widely available and are used increasingly for multiple applications such as species identification, phylogenetic analyses and barcoding (reviewed in Valentini *et al.* 2008). DNA sequences from the cytochrome *b* (*cytb*) gene and the cytochrome c oxidase subunit 1 (CO1) gene have become popular recently for identifying species, especially in mammals, and have yielded reliable species identification in wildlife DNA forensics (reviewed in Ogden *et al.* 2009). Also, many studies sequence DNA from unknown, degraded, trace or ancient biological material for the purpose of species identification and phylogenetic analyses (Beja-Pereira *et al.* 2009). Although many mammalian species are well represented in online reference databases for DNA sequences such as GenBank, more often than not, only partial DNA sequences from the *cytb* gene are deposited. These partial *cytb* sequences are derived from several smaller regions of the gene, either due to convenience or technical limitations (e.g. degraded DNA from non-invasive or ancient specimens). For a particular species, it is likely that a region queried may not overlap or match completely with partial sequence(s) present in the database for that species. This may lead to incorrect conclusions on species identification. To avoid misidentifications on species, deposition of complete gene or coding sequences into online databases can be particularly useful as reference sequences, as they provide full coverage in local alignments with query sequences amplified from partial regions of genes.

We developed a single PCR primer set to enable sequencing of the complete *cytb* gene (approximately 1140bp) in mammals. A recent sequence analysis by Shanan *et al.*

(2009) has shown that, in mammals, the *cytb* gene has higher inter-species variation than the CO1 gene and that the use of *cytb* gene sequences can be more informative for mammalian species identification. If the *cytb* gene will be used as a standardized gene region for mammalian species barcoding and identification in the future, this primer set can contribute to DNA sequence database initiatives such as the iBOL (http://www.ibolproject.org/), BOLD (http://www.boldsystems.org/) and the CBOL (http://barcoding.si.edu/).

*Primer design*

We downloaded a ~1740 bp region (DNA sequence) of the mitochondrial genome of species representing 6 mammalian orders from GenBank (Table 1). These DNA sequences included the complete *cytb* gene coding sequence (cds). Flanking sequences spanned ~300 bp on either side of the *cytb* cds. We aligned these sequences using BioEdit software v7.0.9.0 (Hall 1999) and identified conserved regions in the 300 bp flanking sequences upstream and downstream of the *cytb* cds. We designed a forward primer *MTCB-F* (5' – CCHCCATAAATAGGNGAAGG – 3') and a reverse primer *MTCB-R* (5' – WAGAAYTTCAGCTTTGGG – 3') located at positions 14588-14607 and 15988-16006, respectively, according to Anderson *et al.* (1981). The forward primer is located at the NADH dehydrogenase subunit 6 gene, whereas the reverse primer is located at the transfer RNA-Pro of the mammalian mitochondrial genome. We placed degenerate bases in primers corresponding to bases that were not conserved throughout the alignment.

*Primer testing and sequencing*

To test the functionality and scope of this primer set, we used AMPLIFY 3 software v3.1 (http://engels.genetics.wisc.edu/amplify/) to run an *in-silico* PCR on mammalian mitochondrial genome (reference) sequences representing 27 mammalian orders. These sequences were different from the reference sequences used in primer design. We obtained amplification in all target species. The amplified (target) fragment size ranged between 1415 and 1442 bp (Table 1). We also observed amplification of non-target fragments in some species indicating that gel purification of the target fragment may be required before sequencing.

We tested the primer set on DNA from 19 species representing 7 orders of mammals (Table 1). The DNA sample set included two bobcat (*Lynx rufus*) DNA samples – Bobcat-1 (isolated from bobcat feces) and Bobcat-2 isolated from bobcat tissue. The DNA from all the other species in the sample set was obtained from field-collected tissue or blood samples where species identification was done with the specimen in hand. DNA was extracted using the QIAamp Tissue & Blood Kit (Qiagen) following the manufacturer's protocol. To test the specificity of the primer set, we also included 2 non-mammalian species – burrowing owl (*Athene cunicularia*) and freshwater mussel (*Anodonta californiensis*). We quantified DNA concentration using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). We used 0.1 – 100 ng/µL of DNA as template for PCR. We performed amplifications in a 20 µL reaction volume with the following final concentration: 1X PCR Buffer (Qiagen), 1 mM $MgCl_2$ (Qiagen), 0.2 mM dNTPs (Qiagen), 0.05 % BSA (Sigma-Aldrich, St. Louis, MO,

USA), 0.5 U of *Taq* DNA Polymerase (Qiagen), 0.5 µM each of forward and reverse primers. PCR cycling conditions for both reactions consisted of initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. We used *Mastercycler* PCR machines (Eppendorf, Westbury, NY) for all PCR amplifications. We obtained amplicons from 18 of 19 mammalian species. DNA from the Baja-California pocket mouse (*Chaetodipus rudinoris*) and one bobcat sample (Bobcat-1) failed to amplify (Figure 1). Bobcat sample likely failed to amplify because of the degraded condition of DNA in feces. We electrophoresed PCR products on a 1 % agarose gel. In cases where non-target amplifications were obtained along with amplification of the target region (~1420 bp), we gel-purified the target fragment for sequencing. In all other cases, we prepared PCR products directly for sequencing with the *ExoSAP-IT* PCR Clean-up kit (USB Corporation, Cleveland, OH, USA) using manufacturer's recommendations. PCR products were sequenced in both the forward and reverse directions using primers *MTCB-F* and *MTCB-R* on a 3730xl Automated DNA Analyzer (Applied Biosystems) at the University of Arizona Genetics Core (http://uagc.arl.arizona.edu/). We repeated amplification and sequencing on all samples twice to check for base calling errors, frame shifts, insertions and deletions.

*Sequence analysis and deposition*

We assembled and edited the sequences from each species using Sequencher software v4.9 (Gene Codes Corporation). We trimmed the flanking sequences and created

consensus sequences of the *cytb* cds (~1140 bp). We submitted 10 of the 18 mammalian species' consensus *cytb* gene sequences to GenBank (GenBank accession GU175434–GU175443).

**Table 1** Sequences and DNA samples used in primer design and testing.

| Order | Species | GenBank accession | Amplify 3 (in-silico) test result | |
|---|---|---|---|---|
| | | | Target fragment size (bp) | Non-target fragment size (bp) |
| *Sequences used in primer design* | | | | |
| Didelphimorphia | Opossum (*Didelphis virginiana*) | Z29573.1 | | |
| Primates | Human (*Homo sapiens*) | J01415.2 | | |
| Carnivora | Cheetah (*Acinonyx jubatus*) | AF344830.1 | | |
| Proboscidea | Elephant (*Loxodonta africana*) | AJ224821.1 | | |
| Cetartiodactyla | Deer (*Cervus elaphus*) | AB245427.2 | | |
| Rodentia | Mouse (*Mus musculus*) | DQ874614.2 | | |
| *Sequences used in primer testing with Amplify 3 software v3.1* | | | | |
| Monotremata | Platypus (*Ornithorhynchus anatinus*) | X83427.1 | 1416 | 370, 1689 |
| Didelphimorphia | Opossum (*Didelphis virginiana*) | Z29573.1 | 1436 | - |
| Paucituberculata | Shrew opossum (*Rhyncholestes raphanurus*) | AJ508399.1 | 1432 | 97 |
| Dasyuromorphia | Quoll (*Dasyurus hallucatus*) | AY795973.1 | 1430 | 59, 252, 367, 884, 975 |
| Peramelemorphia | Bandicoot (*Isoodon macrourus*) | AF358864.1 | 1428 | 719 |
| Diprotodontia | Wombat (*Vombatus ursinus*) | AJ304826.1 | 1427 | - |
| Notoryctemorphia | Marsupial mole (*Notoryctes typhlops*) | AJ639874.1 | 1429 | 534, 1098, 1173 |
| Afrosoricida | Hedgehog tenrec (*Echinops telfairi*) | AJ400734.2 | 1433 | - |
| Erinaceomorpha | Hedgehog (*Erinaceus europaeus*) | X88898.2 | 1421 | - |
| Soricomorpha | Shrew (*Crocidura russula*) | AY769264.1 | 1422 | 364, 373, 1465 |
| Macroscelidea | Elephant shrew (*Macroscelides proboscideus*) | AJ421452.1 | 1417 | 60 |
| Scandentia | Treeshrew (*Tupaia belangeri*) | AF217811.1 | 1415 | 761 |
| Dermoptera | Colugo (*Cynocephalus variegatus*) | AJ428849.1 | 1420 | - |
| Chiroptera | Fruit-eating bat (*Artibeus jamaicensis*) | AF061340.1 | 1419 | 60, 92, 635, 973 |
| Primates | Lemur (*Eulemur macaco*) | AB371088.1 | 1419 | 1504 |
| Carnivora | Mongoose (*Herpestes javanicus*) | AY873843.1 | 1421 | - |
| Hyracoidea | Hyrax (*Procavia capensis*) | AB096865.1 | 1442 | 1305 |
| Proboscidea | Elephant (*Elephas maximus*) | DQ316068.1 | 1419 | 186, 309, 897 |
| Sirenia | Dugong (*Dugong dugon*) | AJ421723.1 | 1420 | - |
| Perissodactyla | Horse (*Equus caballus*) | X79547.1 | 1427 | 703 |
| Cetartiodactyla | Llama (*Lama glama*) | AP003426.1 | 1421 | - |
| Xenarthra | Armadillo (*Dasypus novemcinctus*) | Y11832.1 | 1425 | 363, 633 |
| Pholidota | Pangolin (*Manis tetradactyla*) | AJ421454.1 | 1419 | - |
| Tubulidentata | Aardvark (*Orycteropus afer*) | Y18475.1 | 1426 | 718, 764 |
| Cetacea | Dolphin (*Grampus griseus*) | EU557095.1 | 1422 | 93, 877, 1075 |
| Rodentia | Dormouse (*Myoxus glis*) | AJ001562.1 | 1421 | 248, 633, 871, 971 |
| Lagomorpha | Pika (*Ochotona collaris*) | AF348080.1 | 1415 | 654, 716, 873, 1056, 1214 |
| *DNA samples amplified (shown in agarose gel image - Figure 1)* | | | | |
| Carnivora | Jaguar (*Panthera onca*) | GU175435 | | |
| Rodentia | Mt. Graham red squirrel (*Tamiasciurus hudsonicus grahamensis*) | GU175443 | | |
| Cetartiodactyla | Desert bighorn sheep (*Ovis canadensis mexicana*) | - | | |
| Carnivora | Bobcat-1 (*Lynx rufus*) | - | | |
| Cetacea | Burmeister's porpoise (*Phocoena spinipinnis*) | - | | |
| Carnivora | Striped skunk (*Mephitis mephitis*) | GU175440 | | |
| Rodentia | Dulzura kangaroo rat (*Dipodomys simulans peninsularis*) | GU175437 | | |
| Chiroptera | Southern long-nosed bat (*Leptonycteris curasoae*) | GU175441 | | |
| Chiroptera | Spotted bat (*Euderma maculatum*) | - | | |
| Rodentia | Domestic rat (*Rattus norvegicus*) | - | | |
| Rodentia | Laboratory mouse (*Mus musculus*) | - | | |
| Carnivora | Black bear (*Ursus americanus*) | - | | |
| Rodentia | Baja pocket mouse (*Chaetodipus rudinoris*) | - | | |
| Lagomorpha | Black-tailed jackrabbit (*Lepus californicus*) | - | | |
| Primates | Spider monkey (*Ateles geoffroyi*) | - | | |
| *DNA samples amplified (not shown in agarose gel image)* | | | | |
| Cetartiodactyla | Sonoran pronghorn (*Antilocapra americana sonoriensis*) | GU175434 | | |
| Carnivora | Bobcat-2 (*Lynx rufus*) | GU175436 | | |
| Rodentia | Merriam's kangaroo rat (*Dipodomys merriami melanurus*) | GU175438 | | |
| Carnivora | Raccoon (*Procyon lotor*) | GU175439 | | |
| Carnivora | American puma (*Puma concolor couguar*) | GU175442 | | |

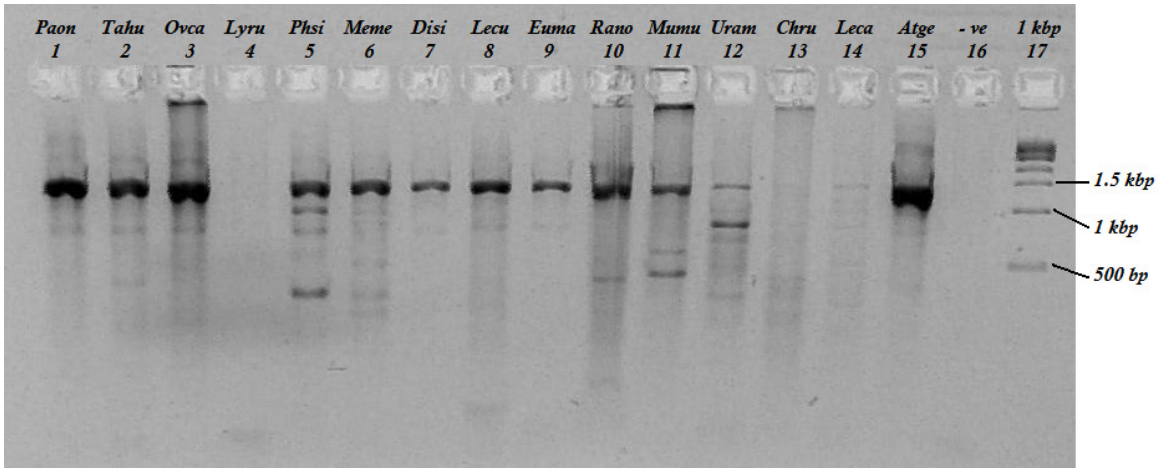


**Figure 4** Gel image showing amplification of some of the DNA samples used in primer testing. Lane 1 – 17: *Panthera onca, Tamiasciurus hudsonicus grahamensis, Ovis canadensis mexicana, Lynx rufus* (Bobcat-1 DNA sample), *Phocoena spinipinnis, Mephitis mephitis, Dipodomys simulans peninsularis, Leptonycteris curasoae, Euderma maculatum, Rattus norvegicus, Mus musculus, Ursus americanus, Chaetodipus rudinoris, Lepus californicus, Ateles geoffroyi,* PCR negative control, 1 kbp DNA ladder (Invitrogen).

Applications of this primer set (*MTCB-F, MTCB-R*) include: (1) *cytb* gene sequence database development for mammalian species, (2) user verification of *cytb* sequence data before and after deposition into online databases (Harris 2003, Song *et al.* 2008), and (3) complete *cytb* gene sequencing via PCR using a single primer set for various phylogenetic and DNA barcoding analyses. Limitations to this primer set include: (1) restricted amplification success in some genera of mammals such as *Chaetodipus* (as tested, see Figure 1), and (2) their use on degraded, low-yield, and ancient DNA.

Harris (2003) questioned the reliability of sequences submitted to GenBank and this may well be the case when sequences submitted to GenBank are not linked with voucher specimens from a museum. This primer set can be used as one of the recommendations for 'double-checking' detailed in Harris (2003). We recommend the use of such primer sets to obtain sequences from known specimens (including field-collected voucher samples from well documented species) to verify sequences deposited in online databases up to the sub-species level. In light of the concerns expressed for data sharing among the scientific community (e.g., Noor *et al.* 2006), we strongly believe that this primer set and the design and use of other such primer sets will promote successful data sharing and minimize errors (such as incorrect bases, frame shifts, insertions and deletions) in DNA sequence submissions.

This study belongs to the Category I of wildlife forensic genetic research (reviewed in Ogden *et al.* 2009). Methods used in this study are consistent with protocols recommended for DNA barcoding by the CCDB (http://www.dnabarcoding.ca/).

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# CHAPTER 5: CONCLUSION

Genetic analysis of mountain lion scat has provided invaluable information on the numbers, sex, and diet of mountain lions that could not be obtained through collaring efforts or camera surveys (because of personnel/budget constraints). Concerns over mountain lion management have increased in the recent past in southwestern Arizona. Management decisions in the future will at least require information on how the Kofa mountain lions fit within the larger regional mountain lion population. Information on source populations, movements, and relatedness will be critical to adequately plan regional mountain lion management. We plan to obtain representative mountain lion DNA samples from the surrounding areas including mountain ranges in southern California and to the north and south of Kofa NWR; Plomosas, Harcuvar and Harquahalas, Black Mountains, Cabeza Prieta NWR, Gila Bend Mountains and Pinacate Biosphere Reserve (Mexico). In addition to the information gathered from this project, in the near future we plan to generate individual genotypes from all representative mountain lion DNA samples and ultimately analyze relatedness and movement patterns of mountain lions at a landscape level.

The lack of robust information on mountain lion populations plagues many agencies responsible for managing mountain lions and their prey. Genetic analysis of mountain lion scat provides a non-invasive, more feasible, and potentially less expensive method to sample mountain lion populations over a large area. This will become increasingly important as agencies are called upon to obtain baseline information on mountain lion populations to support management actions.

# APPENDIX A: GENOTYPES OF MOUNTAIN LIONS ON KOFA NWR

| Individual | Samples ↓ / Microsat. loci → | *FCA057* a | *FCA057* b | *FCA090* a | *FCA090* b | *FCA043* a | *FCA043* b | Sex |
|---|---|---|---|---|---|---|---|---|
| **1** | **KM01 (Tissue)** | 1 | 5 | 3 | 9 | 10 | 14 | **M** |
| **2** | **KM02 (Tissue)** | 4 | 5 | 8 | 9 | 9 | 10 | **M** |
| | **#23** (Scat) | 4 | 5 | 8 | 9 | 9 | 10 | **M** |
| | **#9M** (Scat) | 4 | 5 | 8 | 9 | 9 | 10 | **M** |
| **3** | **KM03 (Tissue)** | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#7** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#25** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#48** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#52** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#5M** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| | **#13M** (Scat) | 1 | 1 | 2 | 8 | 14 | 14 | **M** |
| **4** | **KM04 (Tissue)** | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| | **#22** (Scat) | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| | **#28** (Scat) | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| | **#11M** (Scat) | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| | **#12M** (Scat) | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| | **#4c** (Scat) | 1 | 5 | 2 | 8 | 10 | 14 | **M** |
| **5** | **#36** (Scat) | 1 | 1 | 8 | 9 | 14 | 14 | **M** |
| | **#37** (Scat) | 1 | 1 | 8 | 9 | 14 | 14 | **M** |
| **6** | **#7Ma** (Scat) | 1 | 5 | 8 | 9 | 10 | 14 | **M** |
| **7** | **#7Mb** (Scat) | 1 | 5 | 8 | 11 | 10 | 13 | **?*** |
| **8** | **#11** (Scat) | 1 | 5 | 2 | 2 | 10 | 10 | **?*** |
| **9** | **#1c** (Scat) | 1 | 5 | 2 | 8 | 9 | 14 | **F** |
| **10** | **#8** (Scat) | 1 | 5 | 2 | 8 | 12 | 14 | **F** |
| **11** | **#54** (Scat) | 5 | 5 | 10 | 10 | 11 | 11 | **?*** |

**?*** = Unidentified

# APPENDIX B: INDIVIDUAL MOUNTAIN LION SAMPLE LOCATIONS ON KOFA NWR AND THEIR DIET (PREY) COMPONENTS

| Individual | Samples | Sex | Date collected | GPS locations (coordinates) | Diet component |
|---|---|---|---|---|---|
| 1 | **KM01** (Tissue) | M | 2007 | Captured/Radio-collared | |
| 2 | **KM02** (Tissue) | M | 2007 | Captured/Radio-collared | |
| | **#23** (Scat) | M | Jun/17/2007 | 33° 01.294' N, 114° 00.632' W WGS84 | Bighorn sheep |
| | **#9M** (Scat) | M | Feb/25/2009 | 33° 02.291' N, 114° 00.739' W WGS84 | *(NYP)* |
| 3 | **KM03** (Tissue) | M | 2007 | Captured/Radio-collared | |
| | **#7** (Scat) | M | Dec/16/2006 | 33° 23.546' N, 113° 55.973' W NAD83 | Domestic sheep |
| | **#25** (Scat) | M | Jun/19/2007 | 33° 26.023' N, 113° 58.886' W WGS84 | Mule deer |
| | **#48** (Scat) | M | Apr/17/2008 | 33.29438° N, 113.92554° W WGS84 | Gray fox |
| | **#52** (Scat) | M | Apr/22/2008 | 33.34080° N, 114.06504° W WGS84 | Bighorn sheep |
| | **#5M** (Scat) | M | Oct/4/2008 | 11S 0776115, 3700541 UTM WGS84 | *(NYP)* |
| | **#13M** (Scat) | M | Mar/23/2009 | 33.41678° N, 113.94360° W NAD83 | *(NYP)* |
| 4 | **KM04** (Tissue) | M | 2009 | Captured/Radio-collared | |
| | **#22** (Scat) | M | May/24/2007 | 33.45379° N, 113.85067° W WGS84 | American badger |
| | **#28** (Scat) | M | Oct/7/2007 | 33° 27.859' N, 113° 51.007' W WGS84 | Ovis canadensis |
| | **#11M** (Scat) | M | Mar/23/2009 | (Not collected), Wilkerson Seep, Kofa Mts. | *(NYP)* |
| | **#12M** (Scat) | M | Mar/23/2009 | 33.41678° N, 113.94360° W NAD83 | *(NYP)* |
| | **#4c** (Scat) | M | Nov/22/2008 | (Not collected), 4-peaks Dam, Kofa Mts. | *(NYP)* |
| 5 | **#36** (Scat) | M | Dec/30/2007 | 33.33723° N, 114.06542° W WGS84 | Mule deer |
| | **#37** (Scat) | M | Dec/30/2007 | 33.34023° N, 114.06490° W WGS84 | Bighorn sheep |
| 6 | **#7Ma** (Scat) | M | Oct/4/2008 | 777956, 3698998 UTM | Mule deer |
| 7 | **#7Mb** (Scat) | ?* | Oct/4/2008 | 777956, 3698998 UTM | Mule deer |
| 8 | **#11** (Scat) | ?* | Oct/2/2007 | 33° 25.580' N, 113° 57.187' W NAD83 | Mule deer |
| 9 | **#1c** (Scat) | F | Oct/5/2008 | 33.04557 N, 114.08076 W WGS84 | *(NYP)* |
| 10 | **#8** (Scat) | F | Dec/17/2006 | 33° 24.376' N, 113° 55.914' W NAD83 | Domestic sheep |
| 11 | **#54** (Scat) | ?* | Oct/10/2007 | 33.42925° N, 113.97248° W WGS84 | American badger |

**?*** = Unidentified, *(NYP)* = Not yet processed

## APPENDIX C: IN-FIELD SCAT SAMPLE HANDLING, COLLECTION AND STORAGE PROTOCOL

### Requirements

1. Paper bags and sealable plastic bags
2. Silica desiccant gel or beads (commercial or laboratory grade)
3. Permanent marker pens
4. Hand gloves (plastic, latex or rubber)

### Procedure

1. Put on new gloves to handle scat sample (if no gloves: use leaves, pebbles or twigs found near scat site). Do not touch or handle scat sample with bare hands.
   *Parts of scat pile must be selected based on requirements for further analysis*. For example, only the parts of scat pile containing bones or hair (required for diet analysis) or only the surface of the scat possessing sloughed off large intestine epithelial cells (required for predator DNA analysis).*

2. Place the selected parts of scat pile in the paper bag and then place paper bag inside a sealable plastic bag containing silica desiccant. Ensure minimal damage to the scat sample by preventing transfer of other elements such as grass, sand, etc.
   *To prevent cross contamination, place only one uniquely identified scat sample in each paper bag. If multiple scat piles exist, handle separately and collect in separate bags.*

3. Using permanent ink, label both paper and plastic collection bags with details of scat sample – date, suspected species, GPS location, area, collector's name, etc.

4. If the scat sample is moist, add silica desiccant to the plastic bag containing the paper bag in an approximate 4(silica):1(scat) ratio by weight. Use silica desiccant accordingly. Silica desiccant is not required if the scat sample appears desiccated.
   *DNA in scat samples exposed to moisture and sunlight can be affected by microbial activity and UV light activity. Both these activities cause DNA degradation. Hence, ensure minimal exposure of scat sample to both elements (moisture and sunlight) after collection.*

5. Store sample bags in a cool, dry place. Transport samples to the laboratory for long-term storage at 4 $^{0}$C as soon as logistically possible for further analyses.

*Note: Collection must be done strictly based on requirement as removal of complete scat pile(s) from any location may affect or disturb animal markings, communication, behavior or movements.

## VITA

Ashwin Naidu has a Bachelor of Technology degree in Biotechnology from the Vellore Institute of Technology, 2007 and expects to receive a Master of Science in Natural Resources from the University of Arizona, December 2009. Dr. Melanie Culver of the University of Arizona is serving as Ashwin Naidu's thesis advisor. Ashwin is currently employed as a research assistant at the School of Natural Resources' Conservation Genetics Laboratory and has been with the laboratory for nearly 2 years. Prior to that, Ashwin worked 12 months as a project assistant at the Centre for Cellular and Molecular Biology, Hyderabad, India and as a volunteer for the Hyderabad Tiger Conservation Society, India. Ashwin has on-going interests in conservation genetics and wildlife DNA forensics, and has extensive experience with non-invasive genetic techniques and wildlife field research methods.