Notes

Genetic Analysis of Scats Reveals Minimum Number and Sex of Recently Documented Mountain Lions

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Abstract

Recent records of mountain lions *Puma concolor* and concurrent declines in desert bighorn sheep *Ovis canadensis mexicana* on Kofa National Wildlife Refuge in Arizona, United States, have prompted investigations to estimate the number of mountain lions occurring there. We performed noninvasive genetic analyses and identified species, individuals, and sex from scat samples collected from the Kofa and Castle Dome Mountains. From 105 scats collected, we identified a minimum of 11 individual mountain lions. These individuals consisted of six males, two females and three of unknown sex. Three of the 11 mountain lions were identified multiple times over the study period. These estimates supplement previously recorded information on mountain lions in an area where they were historically considered only transient. We demonstrate that noninvasive genetic techniques, especially when used in conjunction with camera-trap and radiocollaring methods, can provide additional and reliable information to wildlife managers, particularly on secretive species like the mountain lion.

Keywords: *Puma concolor*; Arizona; noninvasive genetics; identification; species; individual; sex

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Introduction

Mountain lions *Puma concolor* were previously thought to be transient in southwestern Arizona, United States (Shaw et al. 1988; Germaine et al. 2000). Additionally, there was uncertainty over whether a distinct subspecies, the Yuma puma *P. c. brownii*, occurred in that region (McIvor et al. 1995). Culver et al. (2000) subsumed subspecies designations for mountain lions in North America (north of Nicaragua), from 15 subspecies to only 1—*P. c. couguar*, based on genetic analysis. In the Kofa National Wildlife Refuge (Kofa NWR), until recently, the only verifiable record of a mountain lion was in 1944 (Halloran 1946). However, between 2003 and 2008, direct sightings, detection of tracks, and a camera-trap survey revealed the presence of at least five individual mountain lions occupying the Kofa and Castle Dome Mountains in the Kofa NWR (Smythe 2008). This documentation, along with the state and federal wildlife management concern over a declining population of desert bighorn sheep *Ovis canadensis mexicana* (U.S. Fish and Wildlife Service 2009), resulted in the implementation of an adaptive, site-

specific predator-management plan directed at removing any mountain lion responsible for more than one bighorn sheep kill during any 6-mo period (Arizona Game and Fish Department [AGFD] 2007). Recent analysis of movement data from four Global Positioning System (GPS)–radio-collared mountain lions also indicated that the Kofa NWR is providing habitat to mountain lions with large home ranges that included movements between five different mountain complexes in and around the Kofa Predation Management Area (U.S. Fish and Wildlife Service and AGFD, unpublished data; AGFD 2010). These preliminary findings, along with the concern for the impact of predation by mountain lions on declining desert bighorn sheep numbers, prompted further investigation into the distribution, abundance, and ecology of mountain lions occurring on the Kofa NWR. Mountain lions, unlike other felids such as tigers *Panthera tigris* and jaguars *Panthera onca*, possess few natural markings that vary from individual to individual, thus requiring a high level of expertise to distinguish individuals through photographs from camera-traps (Kelly et al. 2008). In addition, radiocollaring of mountain lions is labor-intensive and expensive. Here we investigate the use of noninvasive genetic analysis to obtain additional information on the minimum number and sex of mountain lions occurring on the Kofa NWR.

Methods

Study site

The Kofa NWR is located in Yuma and La Paz Counties of Arizona, spans 2,693 km^2 of the Sonoran Desert, and encompasses two major block-faulted mountain ranges (the Kofa and Castle Dome Mountains), as well as portions of the Little Horn, Tank, and New Water Mountains. The predominantly shallow and stony soils of these mountain ranges are separated by Lower Colorado River Valley vegetation communities in deep soils on alluvial fans and valley floors (USDI 1996). Average rainfall on the Kofa NWR is 17 cm, and temperatures can range from an average of 5 to 48°C (Western Regional Climate Center, Kofa Mine Station, http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?az4702, accessed 27 December 2010).

Sample collection and DNA extraction

Between December 2006 and August 2009, we collected 105 scat samples suspected to be from mountain lions (based on scat morphology from the Kofa and Castle Dome mountains in the Kofa NWR). We collected scats opportunistically during mountain lion capture efforts and while searching for mountain lion signs at known predation sites based on GPS radiocollar data. We also performed directed searches for scats by following washes and ridgelines used by radiocollared mountain lions within the study area. We combined scat-sample collection times into seasonal periods: December–March, April–July, and August–November. To prevent degradation of DNA after collection, we stored samples in paper bags placed inside of sealable plastic bags containing desiccant silica in an approximate ratio of 4(silica):1(scat) by weight. We transported samples to the Conservation Genetics Laboratory at the School of Natural Resources and the Environment, University of Arizona (Tucson, Arizona) for cold-storage at −20°C. In the laboratory, we scraped the surface of each sample to obtain sloughed epithelial cells from the predator's large intestine. We used 0.1–0.3 mg of the scrapings for DNA extraction. We used QIAamp DNA Stool Mini Kits (Qiagen, Valencia, CA), and followed the manufacturer's protocol for DNA extraction and purification, with a few modifications. To maximize DNA concentration in the final extract (or eluate), we incorporated the following modifications: 1) lysed samples were incubated at 70°C for 30 min instead of 10 min as recommended in the manufacturer's protocol; and 2) final elution of DNA was performed twice (using 75 μL of the elution buffer each time) with a final mixing of the eluate, yielding approximately 150 μL of DNA in solution.

Species identification

We used Polymerase Chain Reaction (PCR) amplification of partial mitochondrial cytochrome b (cytb) gene, followed by DNA sequencing for species identification. We used forward and reverse PCR primers, mcb398 and mcb869, designed for mammalian species identification (Verma and Singh 2003) to amplify a cytb gene region sized 472 base-pairs (bp) from the scat DNA samples. We subjected scat DNA samples that did not amplify with mcb398 and mcb869 to a second set of PCR primers, Cytb-1 and Cytb-2 (Janczewski et al. 1995), designed for the same gene but targeting a different region sized 476 bp. We performed PCR amplifications in a 20-μL reaction volume with the following final concentration: 1× PCR Buffer (Qiagen), 1 mM $MgCl_2$ (Qiagen), 0.2 mM dNTPs (Qiagen), 0.05% BSA (Sigma-Aldrich, St. Louis, MO), 0.5 U of *Taq* DNA Polymerase (Qiagen), 0.5 μM each of forward and reverse primers, and 4 μL of template (sample) DNA. Conditions for both PCRs consisted of initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 51°C for 1 min, and extension at 72°C for 2 min. The final extension step was set at 72°C for 10 min. We used Mastercycler PCR machines (Eppendorf, Westbury, NY) for all PCRs. We prepared PCR products for sequencing with the ExoSAP-IT PCR Clean-up kit (USB Corporation, Cleveland, OH) using manufacturer's recommendations. We submitted cleaned PCR products to the University of Arizona Genetics Core (http://uagc.arl.arizona.edu/) for DNA sequencing on a 3730 Automated DNA Analyzer (Applied Biosystems, Foster City, CA). All primers, mcb398, mcb869, Cytb-1, and Cytb-2, were used in DNA sequencing.

We used Sequencher v4.1 (Gene Codes Corporation, Ann Arbor, MI) to assemble and edit DNA sequences. We then used BLAST (Altschul et al. 1990) to query DNA sequences and identify species of origin based on best matches obtained with reference sequences in GenBank (*Supplemental Material*, Text S1; http://dx.doi.org/10.3996/042010-JFWM-008.S1). For increased confidence in our species identification results, 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.

Individual identification

We used a nuclear microsatellite genotyping assay to identify individuals within our 105 scat DNA samples and from 4 additional tissue samples from radiocollared animals (collected by AGFD). In this genotyping assay, we used 12 microsatellite loci—*FCA043*, *FCA057*, *FCA082*, *FCA090*, *FCA096*, *FCA166*, chosen from the domestic cat genetic linkage map (Menotti-Raymond et al. 1999), and *PcoB105*, *PcoA2*, *PcoD8*, *PcoD301*, *PcoD329*, *PcoB010w*, chosen from Kurushima et al. (2006). We chose these microsatellite loci based on their use in geographically proximal mountain lion populations (e.g., Culver et al. 2000; Sinclair et al. 2001; Ernest et al. 2003; McRae et al. 2005; Kurushima et al. 2006), and their performance for scat DNA-PCR applications. We amplified the microsatellites via PCR using conditions described by Menotti-Raymond et al. (1999) for all FCA-loci, and Kurushima et al. (2006) for all Pco-loci. 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 (allele) size data using Genotyper v3.7 (Applied Biosystems). We genotyped each sample three times to check for allelic dropout and false alleles—issues commonly encountered during noninvasive genetic analyses (Waits and Paetkau 2005). We excluded samples that resulted in inconsistent allele sizes or missing size data after the three genotyping attempts. We identified individuals that exhibited consistent allele sizes for unique multilocus genotypes resulting from all three genotyping attempts.

Sex identification

To identify sex for all individuals, we used a felid sex-identification PCR assay designed by Pilgrim et al. (2005) for noninvasive genetic samples, using the conditions as originally described. This assay distinguishes females from males based on Y-chromosome deletions in the amelogenin region. We used PCR primers targeting the amelogenin locus (as recommended by K.L. Pilgrim, Rocky Mountain Research Station, personal communication) and electrophoresed the PCR products on a 2% agarose gel to obtain a single band, sized 214 bp for females, versus two bands, sized 214 bp and 194 bp for males. We used tissue DNA samples from the four known male mountain lions and one additional female mountain lion (radiocollared by AGFD near Sabino Canyon, Tucson, Arizona) as positive controls of known sex.

Contamination precautions

We used sterile latex gloves to collect scat samples, and stored them in fresh bags. We performed DNA extractions in a dedicated ancient-DNA laboratory, located in a separate building where no contemporary animal DNA work (including animal PCR amplification of any kind) is conducted. 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 (Takara Bio, Madison, WI) decontaminating solution 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) during all pipetting manipulations. We wore protective facemasks while scraping scat samples and wore sterile latex gloves during all stages of laboratory work.

Results

We collected 105 scat samples and obtained sufficient DNA sequence data to identify 68 (65%) of the samples to the species level: 53 mountain lion, 12 bobcat *Lynx rufus*, and 3 coyote *Canis latrans* scats. The remaining 37 scats failed to yield PCR products or yielded poor-quality DNA sequences and, therefore, were not identified to species level.

All 12 microsatellite loci amplified consistently in the four radiocollared mountain lion tissue samples (known males), but only 3 microsatellite loci—*FCA057*, *FCA090*, and *FCA043* amplified consistently in 21 of 53 (40%) mountain lion scat samples. Microsatellite loci *FCA057*, *FCA090*, and *FCA043* were polymorphic in all samples and displayed four, six, and six alleles, respectively, with a combined probability of identity (*P*[ID]) value, of 2.65×10^{-3} (calculated using Genalex v6.1; Peakall and Smouse 2005). Among the 25 genotypes from tissue and scat samples, we identified 11 unique genotypes (individuals; Figure 1). Genotypes from 14 scat samples matched with three of the four radiocollared male tissue samples from the Kofa NWR. In addition to the four male tissue samples, we identified two individual female and two individual male mountain lions from scat samples alone (*Supplemental Material*, Table S1; http://dx.doi.org/10.3996/042010-JFWM-008.S2). We were unable to identify the sex of three individuals, whose genotypes did not match with any of the tissue samples, because DNA from their scat samples failed to amplify with the sexing PCR assay.

We identified 8 of the 11 individuals to be from samples collected in 2007, 5 from 2008, and 3 from 2009 (*Supplemental Material*, Table S2; http://dx.doi.org/10.3996/042010-JFWM-008.S3). On the basis of matching genotypes, we likely genetically captured two individuals in all 3 y, one individual in 2007 and 2009, and all other individuals in only 1 of the 3 y. One male, identified only from tissue DNA genotype, did not match with any of the scat DNA genotypes (Figure 2).

Discussion

We documented presence of mountain lions in the 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, occurred on the Kofa NWR between December 2006 and August 2009 (the scat collection period). These estimates supplement GPS–tracking and camera-trap data on mountain lions occurring on the Kofa NWR between 2006 and 2008, as presented by Smythe (2008).

Although it is quite possible that the individuals genetically captured over two or more consecutive years are residing on the Kofa NWR, we cannot conclusively

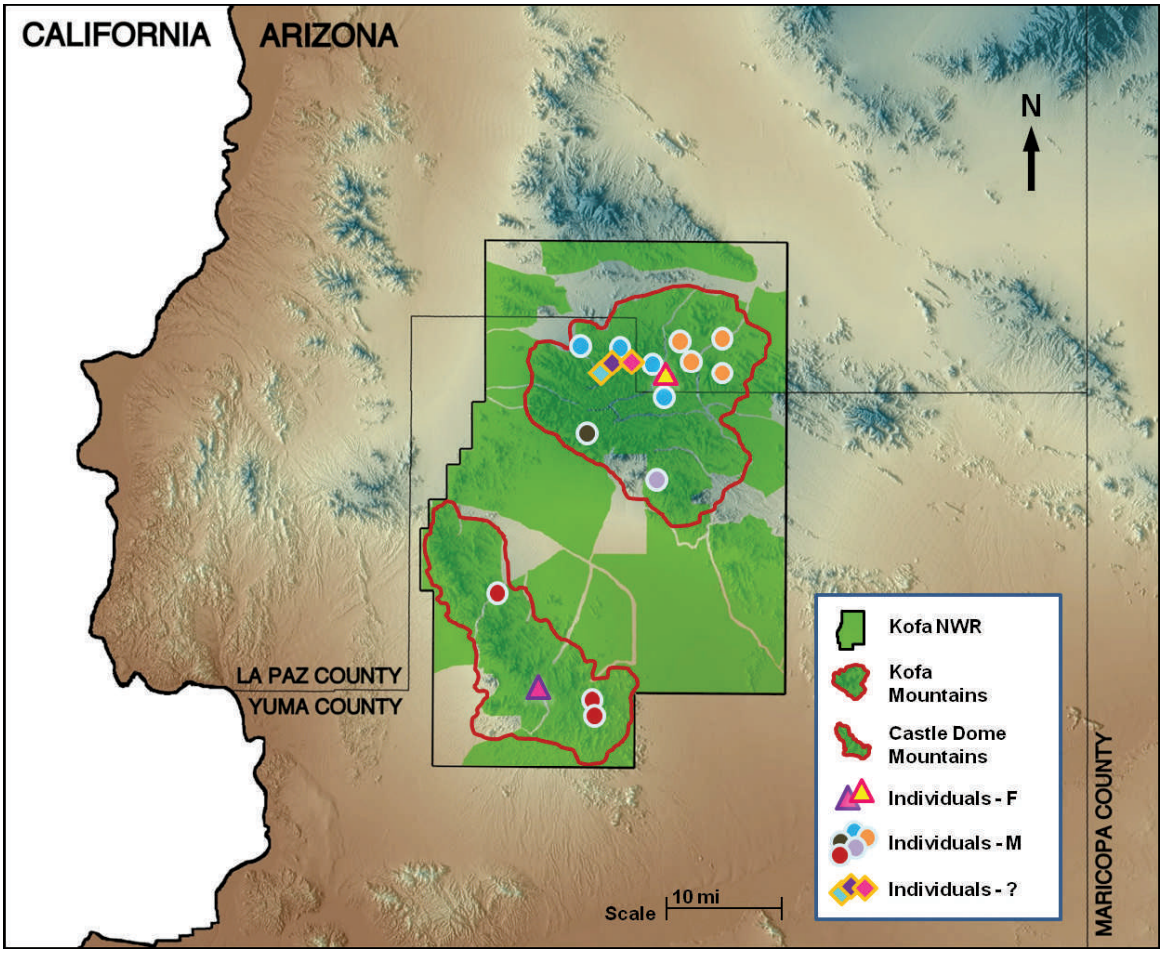

Figure 1. Scat sample locations for 2 individual females (F), 5 individual males (M), and 3 individuals of unknown sex (?) mountain lions *Puma concolor* during 2007 to 2009 on the Kofa National Wildlife Refuge (NWR), Arizona, United States. Multiple dots of same color indicate collections from the same individual.

confirm residency or transient usage of the Kofa NWR by these individuals due to two major reasons: 1) an insufficient sample size, and 2) potential for genotypes (particularly of closely related individuals) to mismatch at the other microsatellite loci not assayed here, resulting in an under-counting of individuals. Hence, our current genotype data must only be inferred to convey the minimum number of individuals identified on the basis of a differing genotype. Future studies using a similar approach, wherein sample collection is based on a randomized or stratified random sampling design, followed by mark–recapture analyses on such baseline multilocus genotype data, may further elucidate absolute abundance, relatedness between individuals, and the extent of individual residency or transiency on the Kofa NWR.

We are confident about our estimate on genetic recaptures of genotypes based on only three microsatellite loci because of the high polymorphism information content of the loci and a low *P*(ID) between two individual genotypes (when all three loci are used in combination). Based on the information from 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. This *P*(ID) value is within the theoretical guidelines outlined in Waits et al. (2001).

We surmise that success in identifying, genotyping, and sexing mountain lion scats can be limited by DNA degradation caused by exposure of scats to environmental factors and high temperatures (Hájková et al. 2006) prior to collection, age of scats (Santini et al. 2007), and PCR inhibitors in DNA extracts from scats (Waits and Paetkau 2005). Our success rate (105 scats collected, 53 of 105 scat samples identified as mountain lion, 21 of 53 mountain lions genotyped and 8 of 11 individuals sexed) resembles previous noninvasive genetic research using scat DNA samples (reviewed by Taberlet et al. 1999; Waits and Paetkau 2005).

Noninvasive genetic techniques are becoming increasingly useful to help wildlife managers direct management actions. For wildlife agencies needing 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 provided data (in conjunction with ongoing camera-trap surveys and radiocollaring efforts) on the minimum number and sex of mountain lions that would have been difficult to obtain with other techniques. Wildlife

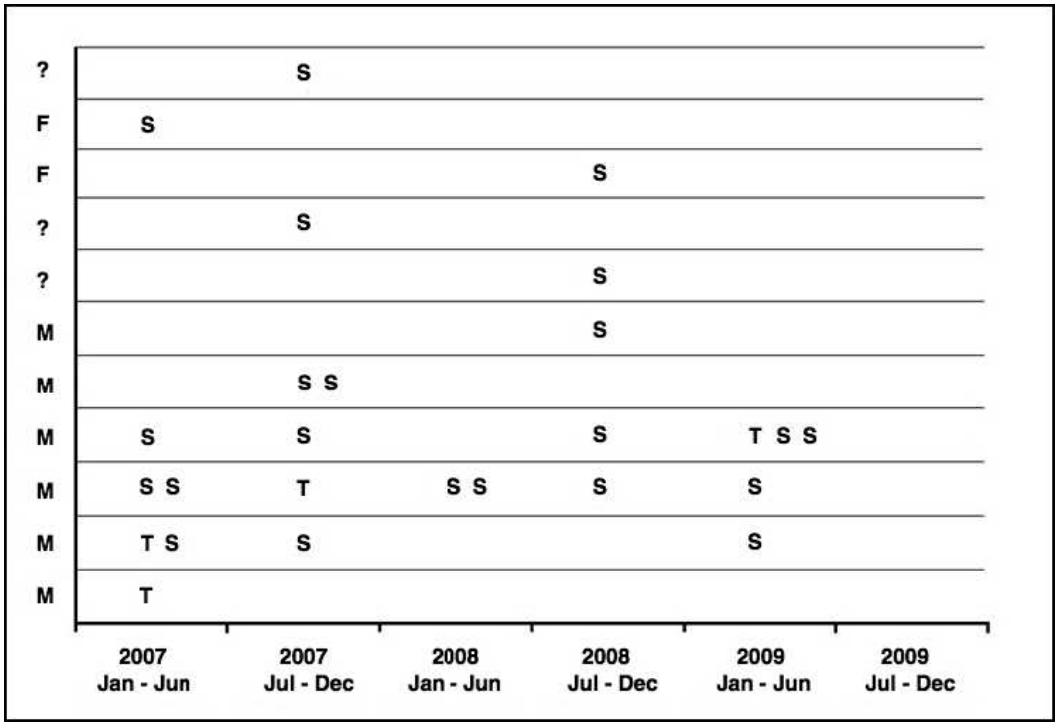

Figure 2. Genetic captures of individual mountain lions *Puma concolor* on the Kofa National Wildlife Refuge, Arizona, United States, during 2007 to 2009, listed by sex (M = male, F = female, ? = unknown sex). T = tissue samples from radiocollared mountain lions, S = scat samples.

managers may exploit such genetic techniques and analyses to monitor wild populations, particularly of secretive species like the mountain lion.

Supplemental Material

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Text S1. Reference sequences in GenBank used for species identification.

Found at DOI: http://dx.doi.org/10.3996/042010-JFWM-008.S1 (36 KB DOC).

Table S1. Genotypes of individual mountain lion *Puma concolor* samples and their allele sizes (bp) at each microsatellite locus. Allele size range for overall mountain lion genotypes sample set: FCA057 (171–179 bp), FCA090 (125–143 bp), FCA043 (147–158 bp).

Found at DOI: http://dx.doi.org/10.3996/042010-JFWM-008.S2 (409 KB DOC).

Table S2. Individual mountain lion *Puma concolor* sample collection dates and locations. AGFD, Arizona Game and Fish Department; Mts., Mountains.

Found at DOI: http://dx.doi.org/10.3996/042010-JFWM-008.S3 (198 KB DOC).

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