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Detection and Classification of Cougars in Michigan Using Low Copy DNA Sources

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ABSTRACT.—Sporadic reports of cougars (*Puma concolor*) have occurred in Michigan since its official classification as extirpated in the 1930s. We collected 297 scats from 12 areas in Michigan with heavy sighting reports of cougars. Ten scats produced DNA profiles consistent with cougars. One scat was identified as having a North American origin; the other nine scats produced no useable sequences. One pre-Columbian sample, from a Native American burial site; also matched the current North American genotype. Based on the distance between cougar scats, we conclude that there were at least eight cougars in Michigan during the 3 y of this study. The mtDNA sequences also suggest that at least some of the matrilines currently and historically found in Michigan are the same as those found in current and historical western populations.

INTRODUCTION

There has been a recent resurgance in evaluating the status of the state-listed endangered cougar (*Puma concolor*) in Michigan due to questions regarding taxonomy of the eastern cougar and recent sighting reports (Evers, 1994). Federal protection of cougars east of the Mississippi River is based on range maps that recognize 15 North American subspecies (Downing, 1981; Hall, 1981). Michigan's Upper Peninsula is within the former range of *Puma concolor shorgeri*, a subspecies separated on the basis of 3 19th Century specimens from Wisconsin, Minnesota and Kansas (Jackson, 1955). The Lower Peninsula is within the range of the eastern cougar (*P. concolor couguar*), classified by the U.S. Fish and Wildlife Service as extinct, although there is a recovery plan for this subspecies (Downing, 1981). Several scientists (Jones, 1964; Bowles, 1975; Lazell, 1981; Scott, 1996) have noted that the original separation into 15 subspecies was based on inadequate sample sizes and questionable differences in morphology. Furthermore, Culver *et al.* (2000) found only two different mtDNA sequences in all of North America, extending down through Costa Rica and Panama. These samples included six musuem samples from *P. concolor couguar*'s range questioning the validity of the 15 subspecies.

The cougar in Michigan has been considered extirpated since the 1930s (Baker, 1983) and few historical specimens exist of cougars known to have been from Michigan. We examined records at Michigan museums and gained access to 1 pre-settlement skull that might yield cougar DNA. The skull was from a Native American burial pit excavated (Foster and Hagge, 1975) in 1966 in Saginaw County in the Lower Peninsula (Fig. 1). Its position in the burial pit, which also included the remains of four children, suggested that the skull was

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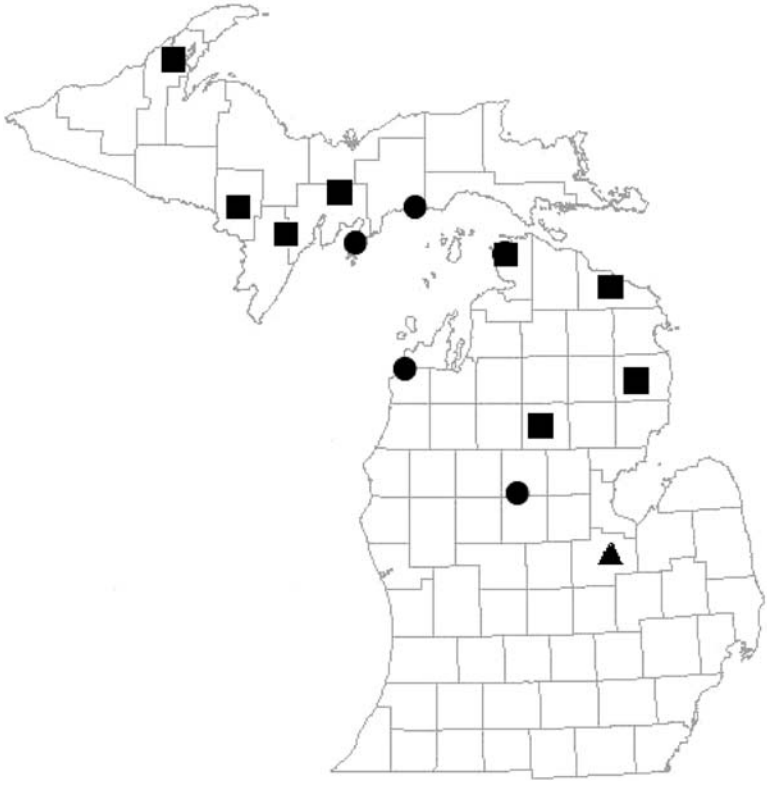

FIG. 1.—Location of search sites for cougar scats. Squares indicate locations where scats were found that showed a cougar DNA profile while the circles indicate sample locations were we did not find genetic evidence of cougars. The location of the pre-Columbian cougar skull is indicated with the triangle. Multiple scats were collected at each site

incorporated as part of a hide used as a robe or as a medicine bundle. This indicated to Foster and Hagge (1975) that it was an authentic artifact of a Michigan cougar rather than an imported object obtained through inter-tribal trade. Baker (1983) considered this skull the earliest cougar record in Michigan, possibly placed in the burial pit as early as pre-Columbian times. However, no datable artifacts were uncovered.

The advent of PCR has allowed researchers to better study rare, cryptic and elusive species, such as cougars, by using low copy DNA (lcDNA) sources such as scat or hairs to determine presence/absence of specific species (Höss *et al.*, 1992; Gerloff *et al.*, 1995; Morin *et al.*, 2001; Parson, 2001; Ernest *et al.*, 2002; Lucchni *et al.*, 2002; Palomares *et al.*, 2002). Since the intial uses of lcDNA (*e.g.,* Pääbo, 1990; Höss *et al.*, 1992) several pitfalls have been reported when trying to amplify lcDNA, especially when the molecular markers used are microsatellites. One of the most common problems associated with lcDNA is allelic dropout or the failure of an allele to amplify in an individual during a specific PCR reaction due to stochastic sampling of the targeted locus. An additional problem arises when the source of DNA, such as feces, contains PCR inhibitors which produce false alleles, or "misprinting." These two problems produce significant genotyping errors, with misprinting rates occuring in up to 11% of loci

sampled and allelic dropout found in up to 39% of the loci analyzed using lcDNA (Creel *et al.*, 2003). These errors are especially problematic when the molecular data are used to try and estimate census population sizes. Given the frequency of errors associated with lcDNA several methods have been developed to minimize the impact of the lcDNA errors, including such methods as running many PCR reactions per sample (Taberlet *et al.*, 1996), using maximum likelihood methods to determine genotype reliability (Miller *et al.*, 2002), quantifying the amount of amplifiable DNA present in a sample (Morin *et al.*, 2001) and allowing some some samples with non-identical genotypes to be counted as identical (Creel *et al.*, 2003). Typically, these problems are considered to be more common in nuclear genes than mitochondrial genes because of the lower frequency of nuclear genes in a cell relative to mitochondrial genes, although errors are associated with high copy DNA as well (Jeffery *et al.*, 2001).

Use of mtDNA reduces, but does not eliminate, the problems associated with analysis of lcDNA. The discover of numts, pieces of mtDNA translocated into the nuclear genome, has called into question the ability to use mtDNA as a tool for assesing evolutionary relationships (Thalmann *et al.*, 2004). Numts evolve independently of the mtDNA sequence from which they are derived (Actander, 1995) and can often assume multiple different sequences within the nuclear genome (Greenwood and Pääbo, 1999), confounding analyses of evolutionary relationships.

This study analyzed available samples from Michigan's pre-settlement and modern-day cougars to determine genetic relationships with other North American cougars. Accordingly, we sought genetic clues as to the status and origin of Michigan cougars. Our study avoids many of the problems associated with using lcDNA by using a modification of Foran *et al.*'s (1997) method of determine the species responsible for producing the scat based on the size of the fragment amplified and the presence of specific restriction sites rather than an individuals genotype or base pair sequence. No variation in size of the amplified cytochrome b/D-loop fragment or in the presence of the restriction sites has been found for these species (Foran *et al.*, 1997).

METHODS

In the springs of 2001, 2002 and 2003, we conducted field surveys in six Upper Peninsula and six Lower Peninsula sites in Michigan (Fig. 1). We selected study areas ranging from 1 km^2–30 km^2 with long histories of cougar sightings (Rusz, 1991). Most of our study sites were in areas of mixed forest within 0.5 km of one or more swamps (>5 km^2) dominated by nothern white cedar (*Thuja occidentalis*), tamarack (*Larix laricina*), balsam fir (*Abies balsamea*), black spruce (*Picea mariana*) or speckled alder (*Alnus incana*). Four study areas in the Upper Peninsula's Delta, Mackinac and Schoolcraft counties, and two study areas in the Lower Peninsula's Benzie/Leelenau and Emmet counties, included stretches of Lake Michigan sand dunes and beaches (Fig. 1). All of the study areas were centered in remote lands with low human populations (<5 per km^2 in the areas surveyed) and few improved roads. However, the Benzie/Leelenau study area in the Lower Peninsual included the Sleeping Bear Dunes National Lakeshore, which attracts >1 million visitors annually.

Field crews generally consisted of two or three biologists who walked dune ridges, beaches, logging roads or trails in or near large swamps used as winter yarding areas by >100 white-tailed deer (*Odocoileus virginianus*). Scats consistent with cougar droppings in size and shape were collected, most often within about 30 d of spring snow melt. This was usually March 10–April 10 in the Lower Peninsula and April 20–May 20 in the Upper Peninsula. Scats were wrapped in tissue, placed in plastic bags, and cooled or frozen prior to transport to the laboratory where they were stored at –20 C.

The 297 scat samples were extracted in quadruplicate from different regions of each scat using Qiagen Stool Kits (Qiagen Inc. Valencia, California) and each extraction was amplified twice. This produced 8 PCR reactions from each sample, exceeding the seven independent amplifications suggested for non-invasive samples (Taberlet *et al.*, 1996). All of the extractions were performed in a fume hood only used for the scats and which was kept completely closed when not in use. Samples were removed from each scat with a unique razor blade, which was then discarded in a lidded sharps container approximately 15 feet away from the work area. Between working with individual samples all surfaces were wiped down with with DNA Displace (Fisher Scientific, Pittsburgh, Pennsylvania), allowed to dry, wiped down with molecular grade water and allowed to dry again. Each extraction was then amplified at a 500–1000 bp region containing portions of the cytochrome b and D-loop regions from the mtDNA (Kocher *et al.*, 1989; Foran *et al.*, 1997) using a modified reverse primer (5′TACACCAGTCTTGTAAACC 3′; D. Foran, pers. comm.). DNA was not quantified as it would not be possible to partition the total amount of DNA between bacterial, fungal, parasite and the animal producing the scat. Tissue samples from three known bobcats, cougars, wolves, and coyotes were extracted using Qiagen Tissue Kits (Qiagen Inc. Valencia, California) to serve as standards for comparison (Fig. 2).

PCR was performed on each extraction in a 20 μL cocktail containing 5 μl of extracted DNA, 250 μM dNTPs, 0.5 μM fluorescently labeled dCTP (ChromaTide Alexafluor 488-7, Molecular Probes, Eugene, OR), 0.16 μM of each primer, 1× HotMaster Taq buffer (Brinkman Instruments, Inc., Westbury, New York) and 1.5 units of HotMaster Taq polymerase (Brinkman Instruments, Inc., Westbury, New York). Every PCR reaction was run with a negative control to test for contamination. Amplification was conducted on an Eppendorf MasterGradient Thermocycler (Brinkman Instruments, Inc., Westbury, New York) and consisted of an initial denaturation step of 2 min at 94 C followed by 3 cycles of 20 s at 94 C, 20 s at 54 C and 15 s at 72 C. This was followed by 35 cycles of 15 s at 94 C, 15 s at 54 C and 15 s at 72 C followed by a terminal extension step of 4 min at 72 C for the cytochrome b/D-loop. Each PCR set of scat samples was jointly run with DNA from known bobcats, cougars, coyotes and wolves with a total of 75 ng of template DNA.

Following amplification, we visualized all samples on an ABI 310 Genetic Analyzer using GeneScan Analysis 3.1.2 (Applied Biosystems Foster City, California) software with collection time set at 60 min. TAMRA 2500 (Applied Biosystems Foster City, California) was run as an internal size stand with all samples. Scat samples were identified as canid or felid based on multiple PCR products in the felid samples and the smaller size of the fragments amplified in canids (Foran *et al.*, 1997). All samples that amplified and produced a felid banding pattern were further processed by separate restriction analysis using Rsa1 in conjunction with restriction digest of the amplified DNA from the known animals. Restriction digests followed conditions included with the restriction enzymes (New England Biolabs, Beverly, Massachusetts) and allowed to digest for 3 h. We analyzed the restriction fragments by running the samples on an ABI 310 Genetic Analyzer using GeneScan Analysis 3.1.2 software with collection time set at 60 min.

A single premolar was removed from a cougar skull found in a Native American burial mound (Michigan State University Museum Catalog Number 13178) and sectioned by the Michigan DNR. The saw was thoroughly cleaned with DNA Displace and rinsed with molecular grade water (Fisher Scientific, Pittsburgh, Pennsylvania). Prior to sectioning of the tooth swab samples were taken from the blade of the saw and the water reservoir. During sectioning of the tooth the water reservoir was filled with molecular grade water to prevent contamination. The tooth was cut just below the jaw line, cutting off the two roots associated with the tooth. Visual examination under a 1000× magnification showed that there were

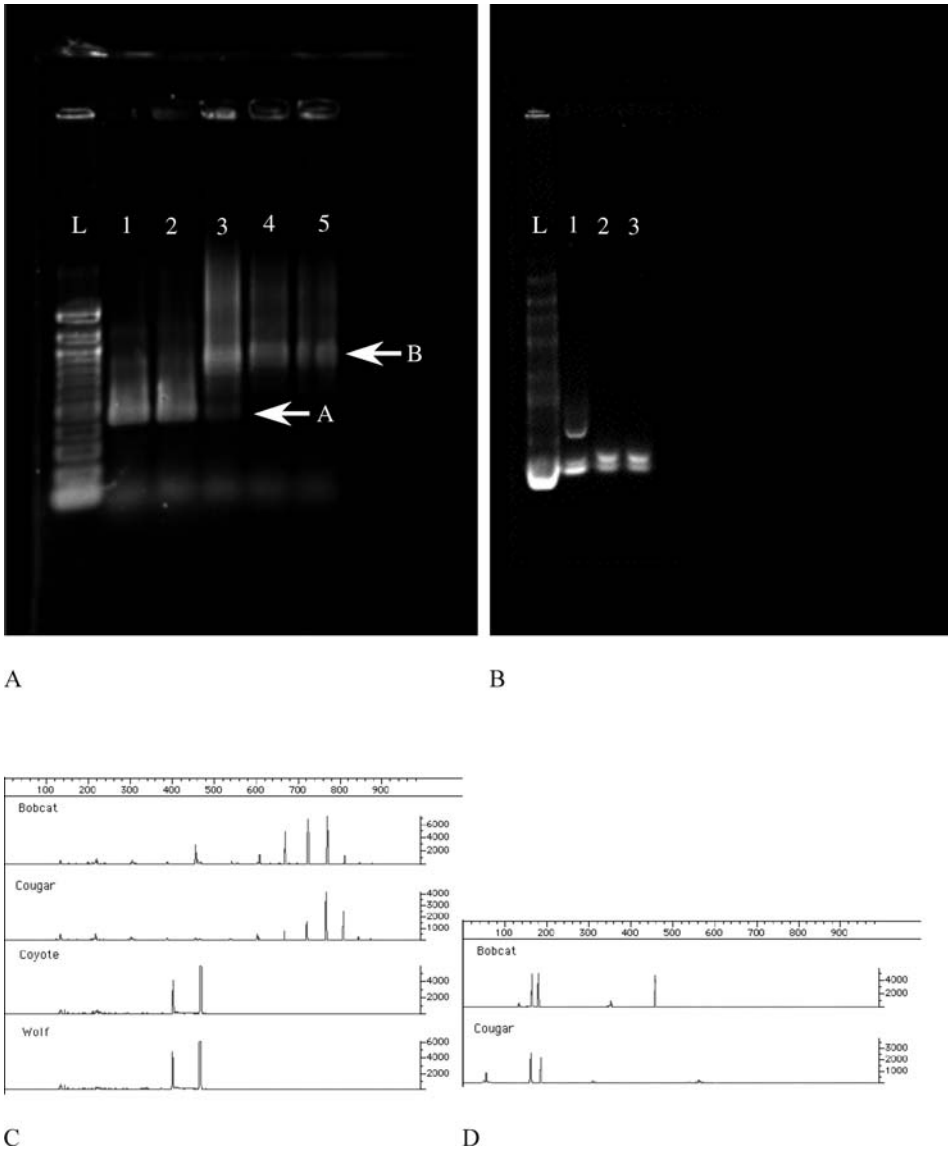

FIG. 2.—A. Results of D-loop amplification for coyote (lane 1), wolf (lane 2), bobcat (lane 3), cougar (lane 4), a scat sample (lane 5) and a 100 bp ladder (lane L). The canids (1 and 2) show amplification of a 500 bp fragment (arrow A) while the felids (3 and 4) show amplification of an 800 bp fragment (arrow B). Note the presence of a faint second band at 500 bp (arrow A) for the bobcat sample (3). B. Results of Rsa1 digestion, for bobcat (lane 1), cougar (lane 2) and unknown scat sample from 2A (lane 3) C. Genescan results of D-loop amplification for bobcat, cougar, coyote and wolf. D. Genescan results of Rsa1 digestion for bobcat and cougar

still cells in the pulp cavity. DNA was extracted using Qiagen Tissue Kits following Qiagen's published protocols for tissues (Qiagen, 2000). We amplified the extracted DNA at three mtDNA loci, 16s rRNA, NADH-5 and ATPase-8 (Johnson *et al.*, 1998; Johnson and O'Brien, 1997), which Culver *et al.* (2000) used to investigate the evolutionary relationship of North and South American cougars. Our PCR cocktail and thermal program were based on Culver *et al.*'s (2000) methods and was run for a total of 40 cycles of 30 s at 94 C, 30 s at 50 C and 60 s at 72 C following an initial denaturation of 94 C for 2 min. A terminal elongation period occurred for 4 min at 72 C. The PCR products were sequenced in both directions using Prism Big Dye Termination kits (Applied Biosystems Foster City, California) following the included instructions and sequenced on an ABI 310 automated sequencer and compared to sequences published by Culver *et al.* (2000).

RESULTS

We were able to amplify 12 of the 297 scats (4%). Ten scats were identified as cougar, 1 as bobcat and 1 as canid based on the size of the PCR products and restriction digest patterns (Fig. 2). The canids produced a single band following amplification of the cytochrome b/D-loop, while the felids produced multiple bands, products of an 80-bp insertion in the mtDNA (Lopez *et al.*, 1996; Foran *et al.*, 1997). The remaining 285 scats produced no visible products following cytochrome b/D-loop amplification. Following the restriction digest with Rsa1, we found two fragments between 150–180 bp and a third fragment around 445 bp in the bobcat and only the two fragments between 150–180 bp in the cougar (Fig. 2).

We were able to obtain 184 bp of sequence from the 16S gene, 197 bp of sequence from the NADH-5 gene and 163 bp of sequence from the ATPase-8 gene from the tooth sample taken from the pre-settlement skull. The scat sample found in Delta Count in the fall of 2001 produced 364 bp of sequence from the 16S gene, 288 bp of sequence from the NADH-5 gene and 181 bp of sequence from the ATPase-8 gene. Neither the tooth, nor the Delta Count Scat sample showed any deviations from the M haplotype of Culver *et al.* (2000). The tooth sample differed from the all South American genotypes by at least 1 nucleotide substitution in the 16S sequence, 1 substitution at the ATPase-8 sequence and 2 substitutions in the NADH-5 gene, all of which were found in the M haplotype of Culver *et al.* (2000). The longer sequences we obtained from the scat sample allowed us to compare additional sequence data with Culver *et al.*'s (2000) data and all differences found in the M haplotype were found in our scat sample.

DISCUSSION

Our data show that there are no genetic differences in the sequence of a historical cougar sample from Michigan or from a contemporary Michigan cougar compared to cougars currently resident elsewhere in North America. There is considerable debate regarding an eastern subspecies of cougar in North America (Downing, 1981; Scott, 1996; Culver *et al.*, 2000). Our data are among the first to indicate that at least some of the cougars found in the lower peninsula of Michigan were genetically the same as is currently found, and was historically found, throughout North America.

Our method was a conservative procedure when attempting to determine the species of origin from scats. We only assumed species identity if the sizes of the amplified DNA and the products of the restriction digest matched samples from known species, reducing the impact of independently evolving numts. Two possibilities exist for numts to produce false readings. First, a felid numt could be a reduced version of the mtDNA region in which we were interested, producing a canid pattern. However, if this were the only band produced we

would conclude that the animal was a canid, not the questionable cougar. Alternatively, a numt could be amplified in addition to the repeated felid mtDNA regions, producing a banding pattern that did not agree with any of the controls used and would be assumed to be neither canid nor felid. It is unlikely that numts from a canid would falsely produce a felid pattern because the numt would have to be larger than the mtDNA sequence and inserted multiple times.

It would also be possible for a numt to alter the banding pattern produced from the restriction digest. However, this would also make it unlikely that we would falsely conclude that a sample came from a cougar. Even with variation found in numts (Greenwood and Pääbo, 1999), it is unlikely that we would see mutations producing restriction sites such that similar sized fragments were produced. Given this, and not needing to worry about allelic dropout or misprinting since we were not working with microsatellites, we are confident that our results accurately represent the species which produced the 12 scats we were able to identify.

With the exception of two samples from the same sites in Roscommon and Dickinson Counties, the shortest distance between locations of cougar scats was 56 km (Dickinson and Menominee Counties), with all other samples being separated by >90 km (Fig. 1). While juvenile cougars often disperse >60 km (Scott, 1996) and adult home ranges vary from 41 km^2–461 km^2 (Lindzey, 1984), it is unlikely that we could have found scat dropped by the same cougar (dispersing or resident) at any two locations given our limited sampling. Our restriction enzyme analysis suggests that there were at least 8 cougars in Michigan, over the three years of this study, with at least one of them of North American origin. There are several methods for more accurately estimating population size using molecular data, such as genotype mark-recapture (*e.g.,* Creel *et al.*, 2003). Sampling genotypes from hair pulls over a given region allows the production of density estimates with confidence intervals. Individual capture events are recorded by how often novel genotypes are found relative to previously sampled ones using traditional mark-recapture estimators (*e.g.,* Otis *et al.*, 1978; Seber, 1982). However, these methods still face the problems associated with lcDNA sources and thus need to be adjusted for factors such as allelic dropout and misprinting (Creel *et al.*, 2003). The genotypic mark-recapture methods however are costly and time consuming which put them beyond the scope of this project.

The age of our scat samples is the most likely explanation for our inability to amplify the ctochrome b/D-loop region we used in our species determination; we did not use a systematic searching of a given area to obtain the freshest scats. Our sampling method was a haphazard sampling of areas and we collected all scat samples regardless of age. This collection technique resulted in our collecting many scats that had been exposed to the environment for unknown, but often, long periods of time. Thus, the majority of our scat samples had likely been exposed to several freeze-thaw cycles and extensive amounts of precipitation and UV radiation, all of which degrade DNA (Kohn *et al.*, 1995; Foran *et al.*, 1997). A more systematic and repeated searching of the same location over shorter periods of time would likely to produce more scats with amplifiable DNA.

One of the most contentious debates regarding cougars in Michigan is if the current animals are released pets, natural recolonization (Heist *et al.*, 2001), or a relict population and if there is a breeding population. Our methods were unable to determine the population of origin for the cougars in Michigan. Other techniques, such as microsatellite assignment based methods, would allow more accurate determination of where the Michigan cougars originated. However, this would require an extensive database of microsatellite genotypes from multiple locations throughout the cougar's range as well as a solid database for current or historical Michigan cougars. Similarly, it is not possible to distinguish between

released cougars that originated in North America and natural recolonization or residual populations of cougars without more extensive data. If the Michigan cougar population has the same mtDNA lineage of the rest of the North American cougars, it would only be possible to determine if an animal was a released pet if it had a non-North American genotype. Pets raised from females originating from North American female breeding stock would all show the M-genotype, as would natural recolonizers and relict populations. Evidence of breeding status would require microsatellite genotypes and finding scat from both a mother and her offspring, as well as allele frequencies for the population, none of which were available for this study. While non-invasive DNA sampling makes it easier to find evidence of cryptic, rare or elusive species, it typically requires major expenditures of time and money to produce accurate estimates of population size and breeding status.

Our suggestion that at least eight animals were in Michigan over the 3 y of this study would seem to cast doubt on the suggestion that all the animals were released pets. However, in 2000, Michigan passed the Large Carnivore Act, prohibiting the ownership of large carnivores such as cougars. Individuals who owned animals prior to the passage of the act were allowed to keep their animals although they were subjected to expensive and intrusive regulations. Thus, it is possible that more individuals dumped their animals to avoid having to upgrade the facilities of their cougars to meet the new requirements. Since most pet cougars have their front claws removed it is unlikely they would still be surviving today if they had been released during the last several years. While we were able to show that cougars are present in the Michigan further work is needed. An additional study, which collects scats in a more uniform and timely fashion, would produce higher quality DNA and allow the use of microsatellite DNA to produce more detailed conclusions regarding the status of the cougar in Michigan.

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LITERATURE CITED

ARCTANDER, P. 1995. Comparison of a mitochondrial gene and a corresponding pseudogene. *Proceedings of the Royal Society of London Series B,* **262**:13–19.

BAKER, R. H. 1983. Michigan mammals. Michigan State University Press, East Lansing. 536–544 p.

BOWLES, J. B. 1975. Distribution and biogeography of mammals of Iowa. Texas Tech University Museum Special Publications, Lubbock. 184 p.

CREEL, S., G. SPONG, J. L. SANDS, J. ROTELLA, J. ZEIGEL, L. JOE, K. M. MURPHY AND D. SMITH 2003. Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Moelcular Ecology,* **12**:2003–2007.

CULVER, M., W. E. JOHNSON, J. PECON-SLATTERY AND S. J. O'BRIEN. 2000. Geonomic ancestry of the American puma (*Puma concolor*). *Journal of Heredity,* **91**:186–197.

DOWNING, R. L. 1981. Eastern cougar—recovery plan. U.S. Fish and Wildlife Service, Atlanta, Georgia. 17 p.

ERNEST, H. B., E. S. RUBIN AND W. M. BOYCE. 2002. Fecal DNA analysis and risk assesment of mountain lion predation of bighorn sheep. *Journal of Wildlife Management,* **66**:75–85.

EVERS, D. C. (ed.). 1994. Endangered and threatened wildlife of Michigan. University of Michigan Press, Ann Arbor. 59–63 p.

FORAN, D. R., K. R. CROOKS AND S. C. MINTA. 1997. Species identification from scat: an unambiguous genetic method. *Wildlife Society Bulletin,* **25**:835–839.

FOSTER, D. W. AND D. R. HAGGE. 1975. A unique secondary burial of four children found in Taymouth Township, Saginaw County, Michigan. *Michigan Archaeologist,* **21**:63–70.

GERLOFF, U., C. SCHLOTTERER, K. RASSMANN, I. RAMBOLD, G. HOHMANN, B. FRUTH AND D. TAUTZ. 1995. Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living Bonobos (*Pan paniscus*). *Molecular Ecology,* **4**:515–518.

GREENWOOD, A. D. AND S. PÄÄBO. 1999. Nuclear insertions sequences of mitochondrial DNA predominate in hair but not in blood of elephanst. *Molecular Ecology,* **8**:133–137.

HALL, E. R. 1981. The mammals of North America, 2nd ed. J. Wiley & Sons, New York. 2 vols. 1081 + 90 p.

HEIST, E. J., J. R. BOWLES AND A. WOOLF. 2001. Record of a North American cougar (*Puma concolor*) from Southern Illinois. *Transactions of the Illinois State Academy of Science,* **94**:227–229.

HÖSS, M., M. KOHN, S. PÄÄBO, F. KNAUER AND W. SCHRÖDER. 1992. Excrement analysis by PCR. *Nature,* **359**:199.

JACKSON, H. H. T. 1955. The Wisconsin puma. *Proceedings of the Biological Society of Washington,* **68**:149–150.

JEFFERY, K. J., L. F. KELLER, P. ARCES AND M. W. BRUFORD. 2001. The development of microsatellite loic in the song sparrow, *Melospiza melodia* (Aves) and genotyping errors associated with good qualtiy DNA. *Molecular Ecology Notes,* **1**:11–13.

JOHNSON, W., M. CULVER, J. A. IRIARTE, E. EIZRIK, K. L. SEYMOUR AND S. J. O'BRIEN. 1989. Tracking the evolution of the elusive Andean mountain cat (*Oreailurue jacobita*) from mitochondrial DNA. *Journal of Heredity,* **89**:227–232.

——— AND S. J. O'BRIEN. 1997. Phylogenetic reconstruction fo the Felidae using 16 S rRNA and NADH-5 mitochondrial genes. *Journal of Molecular Evolution,* **44**:S98–S116.

JONES, J. K., JR. 1964. Distribution and taxonomy of mammals of Nebraska. *University of Kansas Publication of the Museum of Natural History,* **16.** 356 p.

KOCHER, T., W. THOMAS, A. MEYER, S. EDWARDS, S. PÄABO, F. VILLABLANCA AND A. WILSON. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of the Sciences,* **86**:6196–6200.

KOHN, M., F. KNAUER, A. STOFELLA, W. SCHRODER AND S. PÄÄBO. 1995. Conservation genetics of the European brown bear—a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology,* **4**:95–103.

LAZELL, J. D., JR. 1981. Diagnosis and identification of the races of *Felis concolor* in eastern North America. U.S. Fish and Wildlife Service, Washington. 18 p.

LINDZNEY, F. 1984. Mountain lion, p. 656–659. *In:* M. Novak, J. A. Baker, M. E. Obbard and B. Malloch (eds.). Wild furbearer management and conservation in North America. Ontario Ministry of Natural Resources, Toronto.

LOPEZ, J. V., S. CEVARIO AND S. J. O'BRIEN. 1996. Complete nucleotide sequences of the domectic cat (*Felis catus*) mitochrondrila genome and a transposed mtDNA tandem repeat (Numt) in the nuclear genome. *Genomics,* **33**:229–246.

LUCCHINI, V., E. FABBRI, F. MARUCCO, S. RICCI, L. BOITANI AND E. RANDI. 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Moleculare Ecology,* **11**:857–868.

MILLER, C. R., P. JOYCE AND L. P. WAITS. 2002. Assessing allelic dropout and genotype reliability using maximum likelihood. *Genetics,* **160**:357–366.

MORIN, P. A., K. E. CHAMBERS, C. BOESCH AND L. VIGILANT. 2001. Quantitative polymerase chain reation analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology,* **10**:1835–1844.

OTIS, D. L., K. P. BURNHAMD, G. C. WHITE AND D. R. ANDERSON. 1978. Statistical inference from capture data on closed animal populations. *Wildlife Monographs,* **62**:1–135.

PÄÄBO, S. 1990. Amplifying ancient DNA, p. 159–166. *In:* M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (eds.). PCR protocols eds. Academic Press, San Diego.

PALOMARES, F., J. A. GODOY, A. PIRIZ, S. J. O'BRIEN AND W. E. JOHNSON. 2002. Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology,* **11**:2171–2182.

PARSONS, K. M. 2001. Reliable microsatellite genotyping of dolphin DNA from faeces. *Molecular Ecology Notes,* **1**:341–344.

QIAGEN. 2000. DNeasy stool kit handbook. Qiagen. Valencia, California, USA.

RUSZ, P. J. 1991. The cougar in Michigan: sightings and related information. Technical Publication, Bengal Wildlife Center, Bath. 64 p.

SCOTT, F. W. 1996. Updated status report on eastern populations of the cougar or puma, Puma concolor couguar, in Canada. Acadia University Department of Biology, Wolfville. 28 p.

SEBER, G. A. F. 1982. Estimation of animal abundance. Charles Griffin, London. 229 p.

TABERLET, P., S. GRIFFIN, B. GOOSSENS, S. QUESTIAU, V. MANCEAU, N. ESCARAVAGE, L. WAITS AND J. BOUVET. 1996. Reliable genotyping of samplples with very low DNA quantiteis using PCR. *Nucleic Acid Research,* **24**:3189–3194.

THALMANN, O., J. HEBLER, H. N. POINAR, S. PÄÄBO AND L. VIGILANT. 2004. Unreliable mtDNA data due to muclear insertions: a cautionary tale from analysis from humans and other great apes. *Molecular Ecology,* **13**:321–335.

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