Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA

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Abstract

Twelve microsatellite loci were characterized in California mountain lions (*Puma concolor*) and sufficient polymorphism was found to uniquely genotype 62 animals sampled at necropsy. Microsatellite genotypes obtained using mountain lion faecal DNA matched those from muscle for all of 15 individuals examined. DNA from potential prey species and animals whose faeces could be misidentified as mountain lion faeces were reliably distinguished from mountain lions using this microsatellite panel. In a field application of this technique, 32 faecal samples were collected from hiking trails in the Yosemite Valley region where seven mountain lions previously had been captured, sampled, and released. Twelve samples yielded characteristic mountain lion genotypes, three displayed bobcat-type genotypes, and 17 did not amplify. The genotype of one of the 12 mountain lion faecal samples was identical to one of the mountain lions that previously had been captured. Three of the 12 faecal samples yielded identical genotypes, and eight new genotypes were detected in the remaining samples. This analysis provided a minimum estimate of 16 mountain lions (seven identified by capture and nine identified by faecal DNA) living in or travelling through Yosemite Valley from March 1997 to August 1998. Match probabilities (probabilities that identical DNA genotypes would be drawn at random a second time from the population) indicated that the samples with identical genotypes probably came from the same mountain lion. Our results demonstrate that faecal DNA analysis is an effective method for detecting and identifying individual mountain lions.

Keywords: bobcat, cougar, faecal DNA, *Lynx rufus*, microsatellites, mountain lion, *Puma concolor*, scat analysis

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Introduction

Mountain lion (*Puma concolor*) sightings were considered an unusual event in California before the 1990s. After 1994, when two people were killed by mountain lions in the state, sightings and reports of potential threatening behaviours towards humans, pets, and livestock dramatically increased (Torres *et al.* 1996). Some speculate that lion numbers have risen, and that an increase in human

densities and loss of lion habitat due to housing development and agriculture have led to escalating interactions between lions and people. However, no empirically derived state-wide estimate for mountain lion population size in California is available. Population sizes have been estimated using data taken from mountain lions killed for attacking livestock, pets, or people (Mansfield & Torres 1994; Torres *et al.* 1996), telemetry studies (Beier 1993; Pierce *et al.* 1999), and tracking surveys (Smallwood & Fitzhugh 1995; but see Grigione *et al.* 1999). However, large home ranges and great mobility make mountain lions extremely difficult to detect and count. We propose that

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additional data for animal census and monitoring may be provided by faecal DNA analysis.

The analysis of faecal DNA from an obligate carnivore presents the challenge of discerning the DNA of the carnivore from that of its prey. To address this problem and develop methods to discriminate, monitor, and count individual mountain lions in the Yosemite Valley region of California, we tested and applied polymerase chain reaction (PCR) amplification of microsatellites to obtain genetic information from samples of blood, buccal swabs (cheek cells and saliva), faeces, hair, and muscle. Our experiments addressed the following questions: (1) Can mountain lion DNA be differentiated from its prey? (2) Can mountain lion DNA be differentiated from that of bobcats and other species with similar faeces? (3) Does

faecal DNA provide a reliable microsatellite genotype of a mountain lion? (4) Can individual mountain lions be differentiated using a panel of 12 microsatellites? With these questions addressed, we used DNA analysis to identify and estimate the minimum number of mountain lions near Yosemite Valley by collecting faecal samples from the field.

Materials and methods

Sampling procedures

In co-operation with the California Department of Fish and Game and the United States Geological Survey (USGS), tissue samples were obtained from 62 northern California mountain lions (Fig. 1, Table 1). Tissue samples
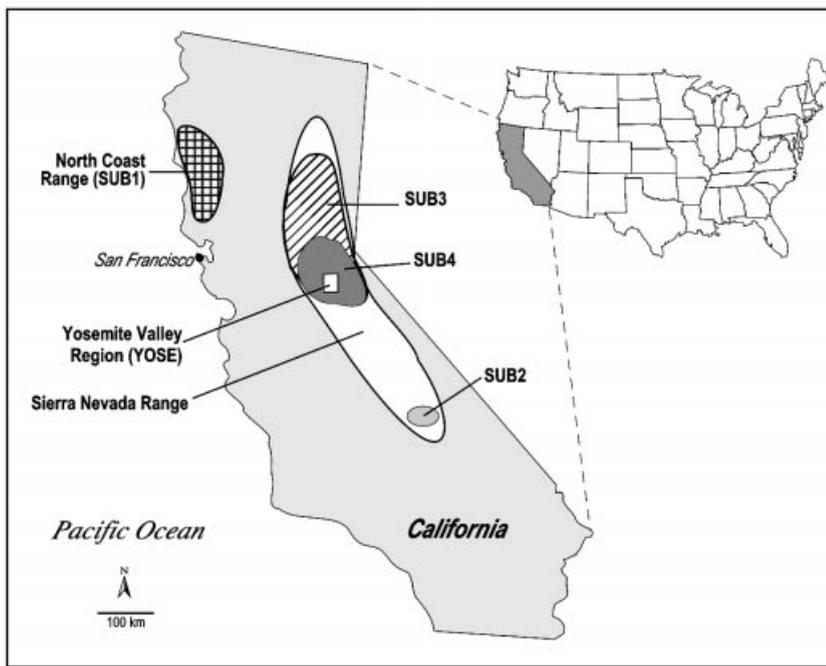
Fig. 1 Map of California showing mountain lion sampling regions. The following abbreviations are used: SUB1, tissue and faecal samples ($n = 7$) collected at necropsy from mountain lions sampled in the North Coast Range; SUB2, tissue and faecal samples ($n = 2$) collected at necropsy from mountain lions sampled > 200 km from Yosemite Valley in the Sierra Nevada Range; SUB3, tissue ($n = 36$) and faecal samples ($n = 3$) collected at necropsy from mountain lions sampled 50–200 km from Yosemite Valley in the Sierra Nevada Range; SUB4, tissue ($n = 17$) and faecal samples ($n = 3$) collected at necropsy from mountain lions sampled within 50 km of Yosemite Valley in the Sierra Nevada Range; YOSE, faecal samples ($n = 32$) field collected in a 1185 sq. km area located in Yosemite National Park and Stanislaus National Forest (Yosemite Valley region).

Table 1 Collection information for 62 mountain lion tissue samples and 32 field-collected faecal samples

Sample code	Location	Sample analysis groups (n)	Collection method (n)
SUB1	> 200 km from Yosemite Valley in North Coast Range	Faecal validation (7)	Necropsy (7)
SUB2	> 200 km from Yosemite Valley in Sierra Nevada Range	Faecal validation (2)	Necropsy (2)
SUB3	50–200 km from Yosemite Valley in Sierra Nevada Range	50–200 km subset (35*)	Necropsy (36)
		Faecal validation (3)	
SUB4	Within 50 km of Yosemite Valley in Sierra Nevada Range	0–50 km subset (17)	Necropsy (10)
		Faecal validation (3)	Capture (6)
			Capture and necropsy (1)
Grouped subset	Within 200 km of Yosemite Valley in Sierra Nevada Range	0–200 km subset (52)	Necropsy (45)
		SUB3 (35) + SUB4 (17)	Capture (6)
			Capture and necropsy (1)
YOSE	Yosemite Valley region	Faecal DNA analysis (32)	Field collected (32)

*One of the SUB3 animals was excluded from allele frequency calculations because that mountain lion was suspected of being a sib of another lion in the subset.

included blood, buccal swabs, hair, and muscle collected directly from mountain lions either by necropsy or by capture. Of the 62 mountain lions, seven were sampled by capture from March 1997 to August 1998 in a 1185 sq. km area located in Yosemite National Park and Stanislaus National Forest (Yosemite Valley region, Fig. 1, Table 1). Faecal samples were also collected for validation of faecal DNA techniques from 15 of the 62 animals. Additional samples were collected from animals whose faeces might be misidentified as mountain lion and from potential prey animals whose DNA might be present in mountain lion faeces: bobcats (*Lynx rufus*, muscle or blood collected on to blotting paper and stored at room temperature, $n = 20$, from Sierra Nevada, $n = 11$ and Coast Ranges, $n = 9$), dogs (*Canis familiaris*, blood, $n = 4$), coyotes (*C. latrans*, muscle, $n = 5$), mule deer (*Odocoileus hemionus*, blood, $n = 5$), bighorn sheep (*Ovis canadensis*, blood, $n = 2$), domestic sheep (*O. aries*, blood, $n = 2$), cattle (*Bos taurus*, blood, $n = 3$), horse (*Equus caballus*, blood, $n = 1$), and human (*Homo sapiens*, buccal swab, $n = 1$). In the Yosemite Valley region, USGS staff collected 32 faecal samples thought to be from mountain lions (YOSE, Fig. 1, Table 1). Faecal samples, ranging in estimated age from under 12 h to over 2 weeks, were collected opportunistically from predetermined transects and while searching for lions. All samples were stored at –20 °C upon collection, until DNA was extracted, except as noted above for bobcat samples.

DNA techniques

The Chelex® protocol of Walsh *et al*. (1991), the salting out procedure of Miller *et al*. (1988), or the QIAamp® Blood and Tissue Kit (Qiagen) were used to extract DNA in duplicate from blood, buccal swabs, hair, and muscle. A useful protocol for extracting DNA from faecal samples was developed following a modification of the techniques of Sambrook *et al*. (1989). Approximately 50–100 mg of faecal material was measured into 1.5 mL microcentrifuge tubes in four to six extraction replicates. Positive (faecal samples from lions with known microsatellite genotypes) and negative (only extraction reagents and sterile water) controls were included with each extraction run. The lysis buffer contained 200 mM NaCl, 100 mM Tris–HCl pH 8.0, 2.0% sodium dodecylsulphate (SDS), 50 mM ethylene diamine tetraacetic acid (EDTA), 0.5% Triton X-100. Lysis buffer (500 µL) and 20 mg/mL proteinase K (12.5 µL) were added and tubes incubated overnight with rotation at 50 °C. The samples were treated with 1.25 µL of RNase (10 mg/mL) and incubated at 50 °C for another 30 min. Phenol (500 µL) pH 8.0 was added to the tubes, which were then vortexed, and centrifuged at 13 000*g* for 10 min. The top aqueous layer was pipetted to a clean tube and the bottom layer with faecal solids was discarded. Chloroform:phenol 1:1 (500 µL) was added to the tubes, which were vortexed and centrifuged. The top layer was transferred to a clean tube. This procedure was repeated with chloroform:isoamyl 24:1 (500 µL). DNA was precipitated by adding 900 µL of 95% ETOH and stored at –20 °C for 20 min to overnight. The tubes were centrifuged (13 000*g*) for 10 min to pellet the DNA. After decanting the ethanol, the pellets were air dried for 30–45 min in a fume hood. DNA was resuspended in 500 µL of 300 mM sodium acetate (pH 5.0), with tubes kept on ice and vigorously mixed every 10 min until the pellets were dissolved. The ethanol precipitation and air dry steps were repeated. The DNA pellets were dissolved in 70 µL of sterile water and resuspended overnight at 4 °C. Each sample was purified by elution through sephacryl columns (MicroSpin S-400 HR®, Pharmacia Co.), then stored at either 5 °C or –20 °C until PCR was run.

Twelve domestic cat microsatellite primers (Menotti-Raymond & O'Brien 1995; Menotti-Raymond *et al*. 1997, 1999) denoted Fca 8, Fca 23, Fca 26, Fca 35, Fca 43, Fca 77, Fca 78, Fca 90, Fca 96, Fca 126, and Fca 132 were used for PCR amplification. The primers were fluorescently labelled with dyes HEX, TAMRA, or 6-FAM (Applied Biosystems Inc.). Fifteen microlitre reactions contained 150 µg/mL of bovine serum albumin (BSA), 0.2 mM of each of four dNTP, 1 × PCR buffer (Promega), 2.5 mM MgCl$_2$, 3 µL of faecal DNA or 1 µL of blood, buccal swab, hair or muscle DNA, and 1.2 pmol (Fca 8, Fca 23, Fca 35, Fca 43, Fca 45, Fca 77, Fca 90, Fca 96, Fca 126, and Fca 26) and 3.6 pmol (Fca 78 and Fca 132) each of forward and reverse primers. Microsatellite primer sets were run either individually or in multiplexed groups. One unit of *Taq* DNA polymerase (Promega) was added to each reaction after hot start of 95 °C for 1 min. PCR thermocycling was performed at 94 °C for 15 s, 53 °C for 30 s, 72 °C for 45 s, for 45–52 cycles (faecal DNA) or 32–35 cycles (blood, buccal swab, hair, and muscle DNA) followed by a final extension step at 72 °C for 15 min. Positive (muscle samples from lions with known microsatellite genotype) and negative (only sterile water and PCR reagents) controls were included in PCR runs. Each allele was confirmed by at least two, and usually three or more, independent PCR reactions.

The PCR products were electrophoresed on 6% acrylamide 7 M urea gels using an Applied Biosystems 373 DNA sequencer, with a fluorescent-labelled base pair size standard (ROX-350, Applied Biosystems) in each lane. Image analysis and fragment size determination were carried out using GENESCAN 672 Analysis and Genotyper software programs (Applied Biosystems). Genotypes were then classified as heterozygotes if two DNA fragments were observed and homozygotes if only a single fragment was observed.

Data analysis

We tested three subsets (SUB3, SUB4, and grouped subset; Table 1, Fig. 1) of tissue samples from mountain lions in the north-central Sierra Nevada Range collected within 200 km of Yosemite Valley for Hardy–Weinberg equilibrium and genotypic disequilibrium using the GENEPOP software program version 3.1d (Raymond & Rousset 1995), with alpha levels set at 0.05. SUB3 ($n = 35$) was the subset of samples collected 50–200 km from Yosemite Valley, SUB4 ($n = 17$) was the subset of samples collected within 50 km of Yosemite Valley, and the grouped subset was the sum of SUB3 and SUB4 (Fig. 1, Table 1). We estimated F_{ST} between the SUB3 and SUB4 by a weighted analysis of variance (GENEPOP; Weir & Cockerham 1984).

The probability that two individuals in a population could have the same microsatellite genotype (match probability) was computed using the likelihood ratio equations detailed below (Balding & Nichols 1994; National Research Council 1996; Evett & Weir 1998):

$$\Pr\left(G_X = A_iA_i \,\middle|\, G_Y = A_iA_i\right) = \frac{[2\theta+(1-\theta)p_i][3\theta+(1-\theta)p_i]}{(1+\theta)(1+2\theta)} \quad (1)$$

$$\Pr\left(G_X = A_iA_j \,\middle|\, G_Y = A_iA_j\right) = \frac{2[\theta+(1-\theta)p_i][\theta+(1-\theta)p_j]}{(1+\theta)(1+2\theta)} \quad (2)$$

where eqn (1) is the conditional probability ('match probability, θ method') that the genotype of individual X is A_iA_i given that the genotype of individual Y is A_iA_i. Here, θ is the probability that two alleles drawn randomly from a population are identical by descent and p_i is the population frequency of allele A_i. We assumed that *de novo* mutations to pre-existing allelic states did not occur over the timescale of interest (one or few generations). Eqn (2) is the analogous conditional probability for the case where individuals X and Y are heterozygous

for alleles A_i and A_j. The parameter θ takes population structure into account and is equivalent to F_{ST} (Weir & Cockerham 1984). Probabilities for each locus were multiplied, assuming independence of loci, as supported by the linkage map of microsatellite loci in the domestic cat (Menotti-Raymond *et al.* 1999) except in the following cases, where two pairs of loci were located less than 50 centiMorgans (cM) apart on the linkage map. Microsatellite loci Fca 78 and Fca 35 were 38 cM apart on chromosome D2 and Fca 26 and Fca 132 were 22 cM apart on chromosome D3. For each of these pairs of loci, the locus with higher conditional probability was excluded from the calculations. To assess the sensitivity of the θ method to changes in θ, we calculated the match probabilities using an arbitrarily low $\theta = 0.001$ and an arbitrarily high $\theta = 0.25$.

Two other estimates of match probability, the product rule (using p^2 for homozygotes and $2pq$ for heterozygotes) and $2p$ rule (using $2p$ for homozygotes and $2pq$ for heterozygotes) were calculated, as they have been used in human forensic DNA analyses (National Research Council 1996; Evett & Weir 1998).

Results

Polymorphism was observed at 10 of the 12 microsatellite loci (Table 2), and each of 62 mountain lions in this study exhibited a unique genotype. Amplification of microsatellites was not observed from nonfelid potential prey species (dog, coyote, horse, cattle, bighorn sheep, domestic sheep, mule deer, and human). Amplification of bobcat DNA yielded microsatellite genotypes that were unique and distinguishable from mountain lion genotypes (Fig. 2). No amplification of Fca 35 was detected for the 20 bobcats, while all mountain lions exhibited alleles at this locus. Three loci (Fca 8, Fca 45, and Fca 77) exhibited different alleles with nonoverlapping size ranges for bobcats and mountain lions. Bobcats and lions had

Locus	Allele size in base pairs (frequency)					
Fca 8	152 (0.73)	164 (0.27)				
Fca 23	142 (1.00)					
Fca 26	140 (0.64)	142 (0.11)	144 (0.24)	152 (0.01)		
Fca 35	123 (0.48)	135 (0.52)				
Fca 43	124 (0.21)	134 (0.49)	136 (0.30)			
Fca 45	127 (1.00)					
Fca 77	129 (0.40)	133 (0.60)				
Fca 78	186 (0.25)	188 (0.71)	190 (0.04)			
Fca 90	105 (0.46)	107 (0.16)	113 (0.15)	117 (0.04)	119 (0.18)	
Fca 96	191 (0.26)	201 (0.62)	205 (0.04)	209 (0.08)		
Fca 126	131 (0.18)	137 (0.50)	139 (0.22)	143 (0.10)		
Fca 132	162 (0.36)	174 (0.52)	178 (0.06)	180 (0.01)	182 (0.03)	186 (0.03)

Table 2 Microsatellite allele sizes and frequencies for 52 mountain lions sampled within 200 km of Yosemite Valley, Sierra Nevada Range, California (grouped subset; Table 1)
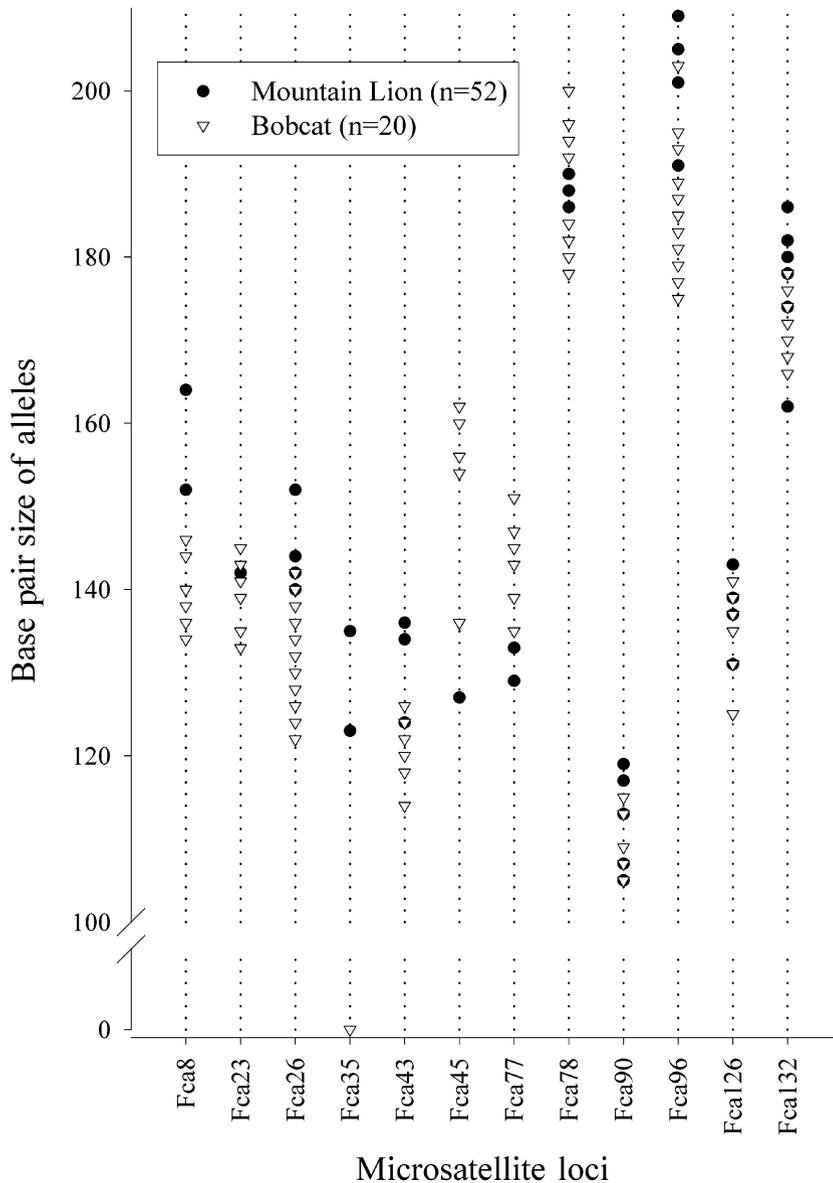
Fig. 2 Allele base pair sizes for 12 micro-satellite loci examined in mountain lions (grouped subset, $n = 52$) and bobcats ($n = 20$).

different alleles at two loci (Fca 78 and Fca 96), although allele size ranges overlapped. In addition, Fca 23 had different alleles for the two species. However, three alleles (142 bp for lions and 141 bp and 143 bp for bobcats) differed by only 1 bp in size. Although the differences at Fca 23 were repeatable in our laboratory, we deemed the locus less important as a species discriminator, as two of the bobcat alleles were close in size to the mountain lion allele. Fca 23 and Fca 45 were monomorphic in mountain lions and polymorphic in bobcats. At Fca 45, mountain lions exhibited only a 127 bp allele, while bobcats had 136, 154, 156, 160, and 162 bp alleles. Of the 103 unique alleles observed in mountain lions and bobcats, only 11 were common between the two species. For 10 loci, bobcats displayed a higher number of alleles per locus than mountain lions. Across all loci, the bobcats exhibited 77 alleles, while the mountain lions showed 37.

Greater than 75% of faecal DNA extracts contained amplifiable DNA. The microsatellite alleles obtained from mountain lion faecal samples matched those of muscle samples from the same individuals ($n = 15$) for each of 12 loci examined. Allele dropout (lack of amplification of an allele) occurred in about 8% of faecal PCR runs and in < 1% of blood/muscle PCR runs. Therefore, we ran at least four PCR replicates for each faecal DNA sample, and at least two replicates of each blood, buccal, hair, or muscle sample, to minimize the misidentification of heterozygotes as homozygotes.

We amplified microsatellite DNA from 15 of 32 faecal samples (YOSE) collected from trails in the Yosemite

Table 3 Match probabilities for selected mountain lion tissue and faecal samples based on comparison with animals sampled within 200 km of Yosemite Valley (grouped subset; Table 1). Lion nos 94 and 41 had the lowest and highest match probabilities, respectively. Two unique genotypes with low match probabilities were obtained for lion no. 518 and faeces no. 520, and for faecal sample nos 467, 469, and 473. Five methods of calculating match probabilities are shown for comparison: product rule, θ method with θ = 0.001 (a very low θ), 0.03 (actual population value of θ), and 0.25 (a very high θ), and 2*p* rule

Match probabilities	Lion no. 41	Lion no. 94	Lion no. 518 and faeces no. 520	Faeces nos 467, 469, and 473
Product rule	1.5×10^{-4}	3.3×10^{-10}	4.1×10^{-6}	2.7×10^{-6}
θ method (θ = 0.001)	7.7×10^{-5}	2.6×10^{-11}	3.4×10^{-8}	1.8×10^{-7}
θ method (θ = 0.03)	2.6×10^{-4}	1.5×10^{-8}	8.8×10^{-6}	7.4×10^{-6}
θ method (θ = 0.25)	4.1×10^{-3}	5.0×10^{-5}	1.6×10^{-4}	4.5×10^{-4}
2*p* rule	3.1×10^{-1}	2.3×10^{-5}	1.8×10^{-4}	1.6×10^{-3}

Valley region. Samples yielding DNA spanned all ages (< 12 h to > 2 weeks). For 11 of these samples, data were obtained for 12 loci. All 12 loci exhibited alleles that were observed in the 52 samples collected within 200 km of the Yosemite Valley region (grouped subset), with the exception of sample no. 459 described below. Seven loci (Fca 8, Fca 23, Fca 35, Fca 45, Fca 77, Fca 78, and Fca 96) had alleles seen in mountain lions but not in bobcats, and five loci (Fca 26, Fca 43, Fca 90, Fca 126, and Fca 132) had alleles observed in both species. One sample (no. 459) yielded data for only nine loci. All nine loci had alleles observed in mountain lions, five loci (Fca 35, Fca 45, Fca 77, Fca 78, and Fca 96) had alleles not seen in bobcats, and four loci (Fca 26, Fca 43, Fca 90, and Fca 126) had alleles observed in both species. From these results we concluded that these 12 faecal samples were from mountain lions. The microsatellite genotype from one of these faecal samples (no. 520) was identical to one of the lions (no. 518) that had previously been captured, sampled, and released near Yosemite Valley (Table 3). Of the remaining 11 mountain lion faecal samples, three had the same microsatellite genotype (nos 467, 469, and 473), while the other eight were unique (Table 3). Two of the 15 faecal samples that amplified were identified as bobcat samples as they contained bobcat-like alleles (Fig. 2) at 11 loci and no amplification at Fca 35. A third putative bobcat faecal sample yielded amplified DNA for only four of the 12 microsatellite primers, but all alleles were bobcat-like. Genotypes for the three bobcat faecal samples were unique from each other indicating that they represented three individual bobcats. DNA was not amplified from the remaining 17 faecal samples, indicating that the faeces were of nonfelid origin, DNA was degraded, or faecal compounds inhibited detection.

We selected the grouped subset (*n* = 52) allele frequency data (Table 2) to calculate match probabilities for Yosemite Valley region faecal samples. The grouped subset had a larger sample size than SUB4 (*n* = 17), and did not depart significantly from Hardy–Weinberg equilibrium (Fisher's method). Genotypic disequilibrium tests showed significant *P*-values (0.008, 0.044, 0.045) at only three of 45 comparisons (2.25 would be expected by chance alone at alpha = 0.05). This suggests the absence of linkage disequilibrium, considering the number of comparisons that were made. F_{ST} (= θ) across all loci was estimated to be 0.03 between SUB3 and SUB4. The grouped subset contained five low-frequency alleles (Fca 26: 152 bp; Fca 90: 117 bp; Fca 96: 205 bp; Fca 132: 180 and 182 bp) that were not observed in SUB4 alone. One faecal sample (no. 459) contained the allele Fca 132: 172 bp not observed in the grouped subset data set, but was within the 162–186 bp size range observed in the grouped subset.

The match probability for lion no. 518 and faeces no. 520 was 8.8×10^{-6}, while the match probability for any two of the three faecal samples nos 467, 469, and 473 was 7.4×10^{-6} (Table 3). Because these match probabilities were low, we concluded that faeces no. 520 originated from lion no. 518, and that faeces nos 467, 469, and 473 were all from another single individual. Therefore, 10 individual mountain lions were identified by faecal DNA, and nine of the 10 were not previously detected by lion capture. Match probabilities using the product rule, θ method, and 2*p* rule differed by orders of magnitude, with the θ method intermediate to the other two (Table 3 and additional data not shown). Match probabilities calculated using the θ method, with θ = 0.001, 0.03, and 0.25, also differed by orders of magnitude, but the resulting match probabilities were still lower than the 2*p* rule (Table 3).

Discussion

In this study we showed that microsatellite primers developed from the domestic cat genome (Menotti-Raymond & O'Brien 1995; Menotti-Raymond *et al*. 1997, 1999) successfully amplified DNA fragments in mountain lions and bobcats (Table 2). In addition to species identification, PCR amplification of microsatellite loci allowed us to identify and differentiate individual mountain lions, unlike previous bile acid chromatography (Fernandez *et al*.

1997) and mitochondrial analyses (Foran *et al.* 1997). Faecal and muscle DNA yielded identical microsatellite genotypes, as shown by analysis of paired samples from 15 mountain lions. This study adds to the list of species for which faecal material has been validated as a source of DNA, including bears (Höss *et al.* 1992; Kohn *et al.* 1995), seals (Reed *et al.* 1997), and primates (Constable *et al.* 1995; Gerloff *et al.* 1995).

DNA obtained from seven field-captured animals and from faecal samples was used to identify and estimate the minimum number of mountain lions present near Yosemite Valley from March 1997 to August 1998. Twelve of 32 faecal samples were determined to be from mountain lions and three from bobcats. One of the 12 mountain lion faecal samples matched the microsatellite genotype from one of the captured lions (Table 3). Three samples yielded identical microsatellite genotypes and represented a single individual, while the other eight samples represented eight different mountain lions. Thus, we identified a minimum of 16 lions in the Yosemite Valley region (seven from capture, nine from faeces). The majority of lions were documented through faecal DNA rather than capture, a significant finding given the high cost (often hundreds of US dollars per animal) and safety risks to humans and animals associated with mountain lion captures (Hornocker 1970; McCown *et al.* 1990). We recognize that our estimate of 16 lions is a minimum and that better estimates might be obtained by collecting more faecal samples. We also suggest that it should be possible to bound estimates of animal numbers by using mark–recapture methods (Seber 1982; White & Garrott 1990) with faecal DNA data.

The sex of the mountain lions sampled by faecal DNA was unknown. In our laboratory, we performed trials to determine the sex of mountain lions using ZFX–ZFY (Aasen & Medrano 1990) and SRY (Griffiths & Tiwari 1993) regions amplified from faecal DNA. The results in these trials were equivocal. While these assays correctly assigned sex in all of 35 muscle samples, we observed fragments of sizes that were compatible with the male SRY fragment in faecal samples from three of four female mountain lions (data not shown). Felids have very short gastrointestinal transit times, with undigested prey tissues often present in faeces (Ernest, unpublished data). The observation of a male DNA fragment in samples was possibly due to coamplification of prey DNA present in faeces.

To determine the probability that faecal samples with the same microsatellite genotype came from one individual, we calculated match probabilities (Table 3). This calculation, drawn from the human forensic genetics literature, is the probability that the same genotype would be drawn at random a second time from the given population. Captured lion no. 518 and faecal sample no. 520 had identical microsatellite genotypes and a θ method match probability of 8.8×10^{-6} (one chance in 113 962). Another Yosemite Valley region lion was detected via three faecal samples (sample nos 467, 469, and 473) with a θ method match probability of 7.4×10^{-6} (one chance in 134 721). The low match probabilities are very strong evidence that lion no. 518 was sampled by faecal DNA at two locations along Bridalveil Creek, and that another lion, identified only through faecal DNA, was sampled near Mono Meadow, Badger Pass, and Cathedral Rock in Yosemite National Park.

Given that the results of many wildlife studies are hindered by small sample sizes, allele frequency data may be available only from pooled data sets. If data sets with population substructure display Hardy–Weinberg and linkage equilibria (as was the case with our Sierra Nevada data), match probabilities may be calculated using the θ method (Balding & Nichols 1994; Evett & Weir 1998). For loci that may contain nonamplifying or null alleles (Pemberton *et al.* 1995), $2p$ or p may be substituted for p^2 in the product rule equation. However, this latter approach provides elevated probability estimates (National Research Council 1996; Weir 1996). We favoured the use of the θ method over the product rule because the θ method incorporates information on matching genotypes through conditional probability calculations, applies knowledge of population substructure, and allows for inbreeding and relatedness of individuals in the population. The National Research Council (1996) recommended that the θ method be adopted for analysis of DNA evidence from crime scenes and suspects. Our θ-value of 0.03 was calculated from a small set of samples and may not accurately represent the population genetic structure of mountain lions in the Sierra Nevada. Therefore, we assessed the sensitivity of the θ method to changes in θ with our data (Table 3). Even at an extremely high theoretical $\theta = 0.25$ (Wright 1978), match probabilities for the faecal samples were calculated to be 1.6×10^{-4} (lion no. 518 and faecal sample no. 520) and 4.5×10^{-4} (sample nos 467, 469, and 473). The $\theta = 0.25$ value was still sufficiently low to preclude the likelihood that more than one lion was represented in samples with the same genotype.

The exclusion of data from two informative loci based on the domestic cat–leopard cat linkage map raised match probabilities by about one order of magnitude (data not shown). Menotti-Raymond *et al.* (1999) noted that 'small inversions and other chromosomal rearrangements between domestic cat and leopard cat chromosomes may suppress recombination and result in a shorter map or some other ambiguity in marker order'. If this is true for mountain lion chromosomes, the apparently close-mapped domestic cat loci may have a sufficient recombination interval to warrant their inclusion in match probabilities. Also, North American mountain lions may have experienced a genetic bottleneck

as recently as 10 000 years ago (Culver 1999). Although linkage disequilibrium may persist at longer linkage distances in bottlenecked populations than in stable populations, population expansion after a founder event causes rapid decay of allelic disequilibrium (Slatkin 1994). In our study, linkage disequilibrium was not significant and for the purpose of population measures (e.g. match probabilities), these loci may be considered as independent (Evett & Weir 1998). Our exclusion of the data from loci less than 50 cM but greater than 10 cM in distance imparts a conservative (i.e. high) match probability estimate for lions in the Yosemite region.

Sample collection and analysis of faecal DNA warrant technical considerations that increase the time, effort, and cost of analyses when compared with tissue or blood. The lower quality and quantity of DNA in faeces may require less stringent PCR conditions (decreased annealing temperature, for example), and increased number of amplification cycles. This can increase the risk of amplifying an extraneous source of DNA or nonspecific sequences from target DNA. PCR inhibitors such as bile may impede amplification of DNA; however, the addition of BSA may improve amplification (Kohn & Wayne 1997). Multiplexing of primers may be problematic because limited copies of template DNA may be available or because amplification of DNA from other sources may interfere with the classification of alleles among multiple loci. Because we observed an 8% microsatellite allelic dropout in individual PCR reactions, we ran four replicates of DNA extracts and PCR reactions. DNA from faeces must be differentiated from closely related sympatric species, as demonstrated in this study with mountain lions and bobcats. For a bobcat to be misidentified as a mountain lion would require as yet undiscovered lion-like alleles at the six loci for which these two species do not appear to share alleles (Fca 8, Fca 35, Fca 45, Fca 77, Fca 78, and Fca 96; see Fig. 2). If we assume that the lion-like alleles were simply not observed in our original sample of 20 bobcats, then we may assume that these alleles occur at a frequency of less than 1/40. The total probability that an individual bobcat might exist with lion-like alleles at all six of these loci must be less than $(1/40)^{12}$ or $< 6 \times 10^{-20}$. DNA in mountain lion faeces may originate from the animal depositing the faeces, or other sources, such as hair ingested while grooming another mountain lion (such as a mother grooming a cub), and prey tissues. Deer are the primary prey of North American mountain lions (Young & Goldman 1946). Other prey may include closely related felid species such as bobcats and domestic cats (Young & Goldman 1946; Koehler & Hornocker 1985), and even other mountain lions through cannibalism (Anderson 1983; Young & Goldman 1946). Given the allele differences between mountain lions and bobcats observed in this study, a DNA admixture of two individuals (two lions or one lion + one bobcat) would be extremely likely to yield a composite genotype with three or four alleles (rather than the expected two) at one or more loci. Because we did not observe more than two alleles in any of the samples of this study, we concluded that we did not genotype more than one individual per faecal sample. Finally, field sampling difficulties may also arise because mountain lion faeces can be difficult to locate.

Faecal DNA analysis of mountain lions is a practical and useful technique for wildlife research, offering a method for locating animal travel routes and identifying individuals. In this study, mountain lion DNA in faeces was differentiated from bobcat and prey DNA, faecal DNA provided reliable microsatellite genotypes, and individual mountain lions were differentiated using a panel of 12 microsatellites. A promising area for future research is the development and application of faecal DNA methods to determine sex and estimate population sizes for mountain lions and other species.

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