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Selection of microsatellite loci for genetic monitoring of sloth bears

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Abstract: The sloth bear (*Melursus ursinus*) is a threatened species endemic to the Indian subcontinent. To date, no reliable method has been developed for identifying individuals or monitoring their populations. Here we describe a non-invasive genetic monitoring technique for individual identification of sloth bears. After testing 18 microsatellites developed for other carnivore species, including ursids, we optimized a panel of 7 highly polymorphic microsatellite loci that yielded a cumulative Probability of Identity between siblings value of 2.15E-03. We used this panel to identify 55 individual sloth bears from 190 fecal and 4 hair samples collected in tiger reserves in central India. This panel can be used for population genetic studies and population monitoring of sloth bears.

Key words: DNA, individual identification, *Melursus ursinus*, microsatellite, noninvasive sampling, sloth bear

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Sloth bears (*Melursus ursinus*) are endemic to the Indian subcontinent and are evolutionarily distinct from other bear species of the world (Servheen et al. 1999). It is a medium-sized bear (adult males 75–140 kg; females 55–95 kg) with a suite of adaptations for feeding on ants and termites. Sloth bears are listed as vulnerable on the International Union for Conservation of Nature red list of threatened species (Garshelis et al. 2008) and in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (http://www.cites.org). They are also protected under Schedule I of the Indian Wildlife Protection Act, 1972 (http://envfor.nic.in/legis/wildlife/wildlife1.html). Sloth bears are found in a variety of habitats, including wet or dry tropical forests, savannas, scrublands, and grasslands (Joshi et al. 1995, Sreekumar and Balakrishnan 2002, Akhtar et al. 2004, Ratnayeke et al. 2007). As a result of continued habitat destruction and degradation, sloth bear populations have declined or become fragmented. Habitat has been lost, degraded, and fragmented by over-harvesting of forest products (timber, fuel wood, fodder, fruits, and honey), establishment of monoculture plantations, and expansion of agricultural areas, human settlements, and roads (Santiapillai and Santiapillai 1990).

Non-invasive genetic sampling has been used to study most of the extant bear species (Paetkau and Strobeck 1998), but to our knowledge these methods have not been developed to monitor populations of the sloth bear. Here we describe a non-invasive genetic method for individual identification that can be used for population monitoring and genetic studies of sloth bears.

Methods

During November 2009–May 2010, we collected fecal and hair samples from wild sloth bears in four tiger reserves (TR) in Central India: Kanha, Satpura, Pench (in Madhya Pradesh), and Melghat (in Maharashtra). We sampled along forest trails and collected bear scats identified by their morphology (size, shape, and diameter) and the presence of ants, termites, or seeds. We stored these samples in 100% ethanol until DNA isolation in the lab.

We isolated genomic DNA using the QIAamp mini-stool kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol for isolation of DNA from stool for human DNA analysis. We carried out all pre-PCR procedures with aerosol barrier tips in a separate room to minimize cross-contamination.

To select a panel of microsatellite loci, we used 21 geographically isolated non-invasive samples (2 hair samples from known captive individuals, and 3 hair

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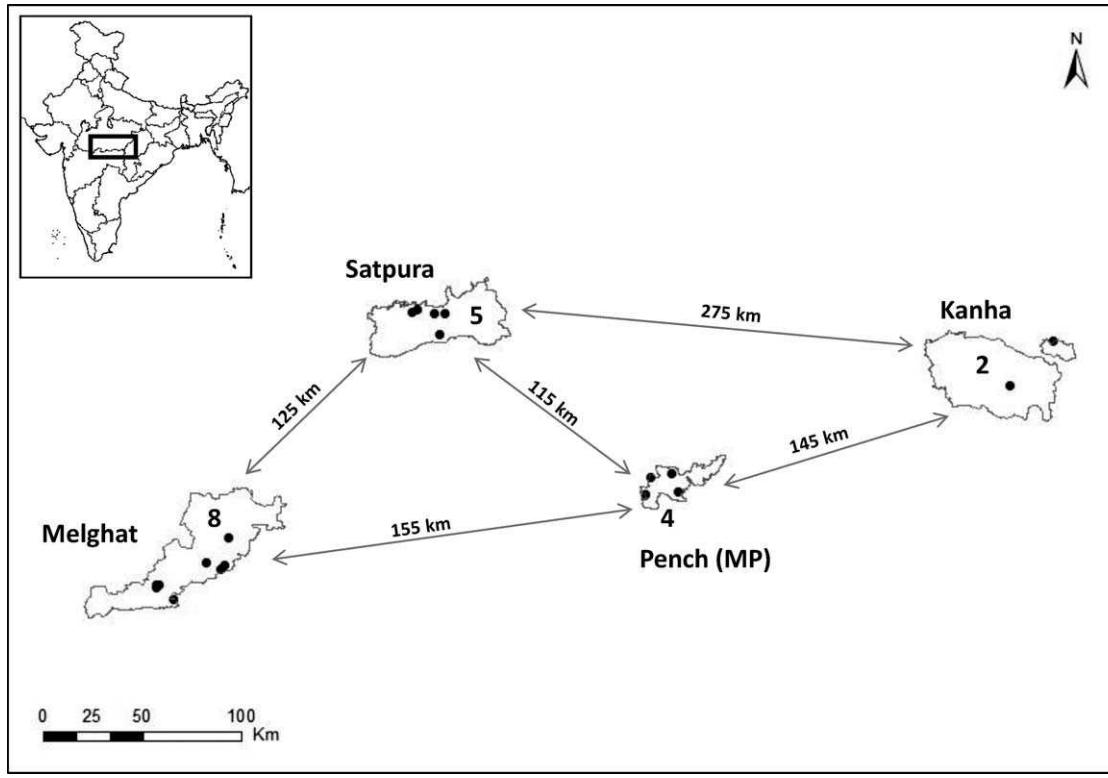

Fig. 1. The Satpura-Maikal landscape with its location in India (inset). Black filled circles represent locations of 19 sloth bear samples in each tiger reserve (indicated by grey polygons) that were used to screen 13 microsatellite loci. The number of samples used from each tiger reserve is shown inside the tiger reserve polygon. The arrows show distances between tiger reserves through the forest corridors. Two reference samples were obtained from captive sloth bears.

and 16 scat samples from geographically isolated sloth bear positive samples; Fig. 1). We used these sloth-bear-positive samples to screen 18 microsatellites developed on American black bear (*Ursus americanus*; Paetkau and Strobeck 1994, Paetkau et al. 1995, 1998), brown bear (*U. arctos*; Taberlet et al. 1997, Bellemain and Taberlet 2004), polar bear (*U. maritimus*; Poissant and Davis 2011), and domestic dogs (Ostrander et al. 1993, Cronin et al. 2009).

Because these primers were developed on other bear species and dogs, we screened them on known sloth bear samples using gradient PCR from 48°C to 60°C to optimize the annealing temperature. We used a multiplex pre-amplification method to increase the success rate of amplification of each locus and the quality of the amplified DNA fragments (Poissant and Davis 2011). We conducted pre-amplification PCR in 25 µL reactions containing 0.5 U Ampli Taq Gold DNA Polymerase (Applied Biosystems, Carlsbad, California, USA), 0.01 µM each fluorescently-labeled forward primer, 0.01 µM each unlabeled reverse primer, 2 mM $MgCl_2$, 1X Gold buffer, 0.1 mM of each dNTP, 2 µL 10X BSA, and 4–5 µL of the DNA extract. We performed a second round of PCR amplification for each locus as described above except the final reaction volume was 10 µL, and we added 0.2 µM of both forward and reverse primer and 1.5 µL of pre-amplified product as the DNA template. PCR conditions were: initial denaturation step (95°C for 10 min), 45 cycles of denaturation (95°C for 30 sec), annealing temperature (Ta, for 30 sec) and extension (72°C for 1 min), and a final extension step (72°C for 7 min).

Conditions for PCR pre-amplification were the same except the Ta (60°C for brown bear primers and 50°C for the remaining primers) and 25 cycles of amplification. We ran all PCR products on an ABI 3730xl sequencer, using GeneScan™ *–500 LIZ* (Applied Biosystems) size standard. We scored alleles using GeneMapper-4.1 (Applied Biosystems).

Table 1. Characteristics of 13 di-nucleotide microsatellites screened on 21 individual sloth bears from Satpura-Maikal landscape in India.

Locus	Ta	Size range	Amplification success (%)	Number of alleles	*Ho*	*He*	P_{ID}[a]	$P_{ID(sibs)}$[b]	$P_{ID(cum)}$[c]	$P_{ID(sibs)cum}$[d]	ADO[e]	FA[f]
CXX203[g]	50	122–146	95	10	0.81	0.82	3.25E-02	3.54E-01	3.25E-02	3.54E-01	0.029	0
G10B[g]	50	133–143	100	4	0.43	0.36	3.75E-01	6.75E-01	1.22E-02	2.39E-01	0.028	0
G10L[g]	50	120–126	100	4	0.81	0.6	2.14E-01	5.07E-01	2.61E-03	1.21E-01	0.044	0
G10J[g]	50	93–111	95	10	0.62	0.78	3.95E-02	3.77E-01	1.03E-04	4.56E-02	0.038	0
G1A[g]	50	177–193	90	7	0.52	0.68	1.08E-01	4.48E-01	1.11E-05	2.04E-02	0.045	0.05
UarMu26[g]	50	119–173	100	4	0.52	0.51	2.39E-01	5.67E-01	2.66E-06	1.16E-02	0.045	0.025
Umar2[g]	52	185–227	100	7	0.62	0.6	1.49E-01	5.00E-01	3.96E-07	5.79E-03	0.019	0
G10C	50	100–106	90	4	0.14	0.15	6.93E-01	8.57E-01	2.74E-07	4.96E-03	0.167	0.014
G10O	50	201–205	76	3	0.57	0.54	2.82E-01	5.57E-01	7.74E-08	2.76E-03	0.104	0.028
G1D	50	166–174	81	4	0.57	0.53	2.60E-01	5.59E-01	2.01E-08	1.55E-03	0.021	0.028
UarMu23	60	238–242	24	2	0.05	0.42	3.99E-01	6.46E-01	8.02E-09	9.99E-04	0.25	0.025
Umar4	52	114–116	90	2	0.05	0.05	8.88E-01	9.50E-01	7.12E-09	9.49E-04	0.25	0
Umar10	52	216–220	76	3	0.19	0.53	2.46E-01	5.55E-01	1.76E-09	5.27E-04	0.125	0.015

[a]Probability of identity.
[b]Probability of identity among siblings.
[c]Cumulative probability of identity.
[d]Cumulative probability of identity among siblings.
[e]Allelic drop out.
[f]Proportion of false alleles.
[g]Loci selected for final panel.

We included negative controls in all PCR reactions to detect any contamination. To minimize genotyping errors, we followed a modified multitube approach (Taberlet et al. 1996) and repeated each PCR 4 times for each sample and for each microsatellite locus.

We used CERVUS-3.0.3 (Kalinowski et al. 2007) for individual identification. We used GIMLET-1.3.3 (Valière 2002) to obtain measures of genetic variation such as mean number of alleles per locus, observed (H_o) and expected (H_e) heterozygosities, and P_{ID} (probability of identity) and $P_{ID(sibs)}$ value (Waits et al. 2001). We used MICRO-CHECKER-2.2.3 (van Oosterhout et al. 2004) to detect loci containing errors due to scoring or stuttering and large allele dropout. We estimated PCR success and genotyping error rates (false alleles [FA], and allelic drop out [ADO]) using GIMLET. We conducted tests for deviations from Hardy Weinberg equilibrium (HWE) and tests for linkage disequilibrium (LD) in GENEPOP-3.4 (Raymond and Rousset 1995, Rousset 2008) with a Bonferroni correction (Rice 1989).

Results and discussion

Wecollected 190 fecal samples and 4 hair samples from wild sloth bears during field sampling. In the trial dataset, we were able to successfully generate genotyping data for 13 microsatellite loci, while 5 loci (UarMU10, UarMU50, UarMU51, G10P, and G10X) failed to amplify. We could reliably distinguish each of the 21 individuals using these markers.

We found no evidence of stuttering errors and large allele dropout in any of the 13 microsatellite loci. Amplification success across loci ranged from 24–100%. All loci were polymorphic with a mean of 4.9 alleles (range: 2–10) and mean H_o of 0.45 (Table 1). Ten of 13 loci (except G1A, G10O, and Umar10) were found to be in HWE and were not in LD in GENEPOP.

We then selected a panel of 7 microsatellites based on amplification success, allele numbers, H_o level, and low genotyping error rates. We amplified all 194 samples for 3 loci (Umar2, CXX203, and G10L) to select high-quality samples for further analysis. Only those samples that amplified for 2 of these 3 loci were used for amplifying remaining loci. Only 89 samples out of 194 passed this test and were amplified for other loci. Of these, 58 samples that amplified for more than 5 loci were retained for further analysis. We identified 55 individual sloth bears using the selected panel with $P_{ID(cum)}$ of 2.61E-08 and $P_{ID(sibs)(cum)}$ of 2.15E-03 (Table 2). There were 6 recapture events of 5 individuals in 2 tiger reserves. In Melghat, 2 individuals were recaptured once, while in Satpura 2 individuals were recaptured once, and one was recaptured twice.

Table 2. Genetic variability of 55 individual sloth bears at 7 microsatellite loci from a study in Satpura-Maikal landscape in India.

Locus	Size range	Success	No. of alleles	H_o	H_e	P_{ID}[a]	$P_{ID(SIBS)}$[b]	$P_{ID(cum)}$[c]	$P_{ID(SIBS)cum}$[d]	ADO[e]	FA[f]
CXX203	122–146	0.87	12	0.65	0.86	2.76E-02	3.29E-01	2.76E-02	3.29E-01	0.037	0.018
G10B	133–143	0.58	4	0.27	0.47	2.92E-01	5.91E-01	8.06E-03	1.94E-01	0.067	0
G10J	89–113	0.78	12	0.58	0.88	1.81E-02	3.14E-01	1.46E-04	6.10E-02	0.031	0
G10L	114–130	0.96	7	0.89	0.68	1.44E-01	4.49E-01	2.10E-05	2.74E-02	0.02	0
G1A	173–193	0.78	11	0.38	0.72	8.70E-02	4.16E-01	1.83E-06	1.14E-02	0.063	0
UarMu26	111–173	0.82	7	0.38	0.69	1.37E-01	4.41E-01	2.50E-07	5.03E-03	0.079	0
Umar2	185–227	0.85	9	0.58	0.71	1.05E-01	4.26E-01	2.61E-08	2.15E-03	0.063	0

[a]Probability of identity.
[b]Probability of identity among siblings.
[c]Cumulative probability of identity.
[d]Cumulative probability of identity among siblings.
[e]Allelic drop out.
[f]Proportion of false alleles.
[g]Loci selected for final panel.

Of the samples that passed the quality test, amplification success was 81%. Sloth bears showed moderate genetic variation with a mean of 8.8 alleles (range: 4–12) and mean H_o of 0.53. Four of 7 loci (G1A, G10J, G10L, and UarMU26) deviated from HWE when samples from all TRs were analyzed as a single population. When samples were grouped by their sampling locality, only 2 loci in Melghat (G1A, and UarMU26) deviated from HWE, while all loci from remaining 3 TRs were in HWE. No loci pair out of 84 pairwise comparisons was in significant LD after Bonferroni corrections.

These microsatellite loci were polymorphic with low P_{ID} values for reliable identification of individuals. The low rates of PCR success can be attributed to sample age, degraded DNA in non-invasive samples, and presence of PCR inhibitors due to the plant based diet of sloth bears. Limiting sampling to fresh fecal samples and use of hair-snares to obtain fresh hairs would certainly improve PCR success. Non-invasively collected samples have shown promising results in unraveling important questions about population interactions at landscape level and species' biology in Central Indian landscape including tigers (*Panthera tigris tigris*; Sharma et al. 2013a, b), leopards (*Panthera pardus fusca*; Dutta et al. 2012, 2013a, b), and jungle cats (*Felis chaus*; Sharma et al. 2013c). Similarly, several important questions about sloth bear biology can be answered by using non-invasive DNA based molecular ecology studies. One limitation of our study was the unavailability of paired samples of blood and tissue and fecal samples. We recommend future studies use paired samples and genotype multiple first-order relatives for estimating error rates and to increase potential use of non-invasive samples.

Molecular genetic tools combined with noninvasive genetic sampling have been increasingly used to study cryptic carnivore species, where obtaining tissue or blood samples is often very difficult. We have demonstrated that noninvasive genetic sampling and its use in monitoring sloth bears is possible by amplifying a suite of polymorphic microsatellite loci. These loci can also be used to answer questions about population genetics, relatedness, and forensic investigations of sloth bears across their geographic range.

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