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Twenty polymorphic microsatellite markers in the Asiatic lion (*Panthera leo persica*)

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Abstract

The Asiatic lion (*Panthera leo persica*) is driven to a single habitat in Gir forests in India for its survival. In order to devise adequate conservation and management strategies for this critically endangered species, it is important to characterize its genetic diversity and understand its population structure. Here we report twenty microsatellite loci, in addition to seven reported earlier, from the genome of a pure Asiatic lion. The microsatellite loci described here will provide potentially useful markers for the assessment of genetic variability in the only existing wild population of the Asiatic lions and other big cat species.

The Asiatic lion (*Panthera leo persica*), which was once widespread throughout Southwest Asia, is today, restricted to a single location in the wild, the Gir forest in Gujarat state of India. A major problem with Asiatic lions is the isolation of the population leading to inbreeding and reduction of genetic variation. In order to seriously save this species from extinction, information about its population genetic structure and the state of isolation is essential. Microsatellites or simple sequence repeats (SSR) have become the markers of choice for study of population genetics and its application to the conservation biology (Sunnucks 2000). We have previously reported development and characterization of seven polymorphic microsatellite loci in this critically endangered species (Singh et al. 2002). The present study involves the development of additional 20 polymorphic microsatellite markers for this species, derived from the same genomic library that gave rise to the first seven markers.

Genomic DNA was isolated from blood sample drawn from an adult Asiatic lion housed at Nehru Zoological Park, Hyderabad, India, using standard phenol–chloroform method (Sambrook et al. 1989). Genomic DNA was digested with restriction enzyme *Sau*3AI. DNA fragments were excised and purified from a size range of 500–1500 bp. A size-selected library was constructed by ligating the DNA fragments into dephosphorylated pBluescript-KS plasmid (Stratagene, USA) digested with *Bam*H1. Plasmids were transformed into electrocompetent *E. coli* DH10B cells by electroporation [*E. coli* pulser, BioRad, USA]. Approximately 10,000 recombinant colonies were screened using γ^{32}P-[dATP] radiolabelled dinucleotide $(CA)_{15}$, $(GA)_{15}$, $(GC)_{15}$ and $(AT)_{15}$; trinucleotide $(GCC)_{10}$, $(CTG)_{10}$, and $(CAA)_{10}$; and tetranucleotide $(GATA)_8$ and $(GACA)_8$ repeats as probes.

In addition to the 30 clones identified earlier (Singh et al. 2002), 60 clones more with good microsatellite repeats were identified from a partial

Table 1. Twenty polymorphic microsatellite loci isolated and characterized from *Panthera leo persica*

Loci	Repeat motif	Primer sequence (5′ – 3′)[§]	Ta (°C)	Size range (bp)	Asiatic lions ($n = 22$)				Hybrid lions ($n = 15$)				Accession number
					K	H_O	H_E	PIC	K	H_O	H_E	PIC	
Ple18	$(TTTTA)_5$	F: AGCCAGCTCTCTCTTTGACAC R: GATCTTGCGGTTTGTGAG	52	325–366	4	0.53	0.51	0.42	2	0.05*	0.32	0.21	AY244426
Ple21	$(CA)_{19}$	F: TCTCTGTGCCTCCGTTTCTT R: GATGTGGGGCTTGAACTCAT	59	222–232	6	0.95	0.68	0.61	6	0.92	0.79	0.72	AY244428
Ple22	$(CA)_{17}$	F: ATCATGACCTGAGCCGAAGT R: CCACCCTTCGCTCTATGTGT	57	149–153	3	0.53	0.50	0.42	5	0.92	0.75	0.69	AY244429
Ple34	$(CA)_{17}$	F: ACCACACACATATCCGCATC R: CCGATCCTTGAAGATTTTGC	55	118–124	5	0.57	0.61	0.53	3	0.50	0.51	0.40	AY244430
Ple37	$(CA)_{18}$	F: CCTTTCCTCTCGTGAACTGC R: CACCAAGTGACTGAAGCCAG	56	180–182	2	0.84*	0.50	0.36	4	0.85*	0.76	0.68	AY095493
Ple38	$(T_3A)(T_5A_4)_2$ $(T_3A)(GA)_{12}$	F: TGTCTGCCTTCAAAACTGC R: AACTCCACATCAGGCTCCAT	56	187–193	4	0.89	0.55	0.44	2	0.92*	0.51	0.37	AY244431
Ple40	$(CAAAA)_5$	F: CTATATCCTGCCCTGCCTA R: GCATCTAACCGGAAAGCAC	56	194–199	3	0.78	0.65	0.50	4	0.92	0.67	0.60	AY244432
Ple44	$(GA)_{13}$	F: GCTCTTTGCTGGCAGCTC R: AGGATTCTCGCAGAGTCGTG	62	176–182	4	1.00	0.71	0.64	5	1.00	0.79	0.71	AY244433
Ple52	$(AAAAC)_6$ $(GA)_{10}$	F: CTTCATACCTACGAGATGTCT R: CCTGGACCTGTTTCCGAT	52	443–450	2	0.05*	0.55	0.36	5	0.21	0.75	0.64	AY244434
Ple53	$(CA)_{23}$	F: GGATGTGAACTGGTGCAAAG R: CGAGTGGTACTGCTGAGTCTG	59	117–129	6	0.94	0.77	0.72	6	1.00	0.84	0.79	AY244435
Ple54	$(CA)_{25}$	F: TCCTGGAGACTGGAAGGAGG R: CCCAGGATATGGCTTGTGG	59	191–201	3	1.00*	0.56	0.44	4	1.00*	0.61	0.50	AY095495
Ple56	$(CA)_{23}$	F: GCACGCAGACGTATAACTCC R: GGTAGCTTCCATCGGAGAAA	60	168–180	5	0.89	0.67	0.59	7	0.92	0.84	0.78	AY244436
Ple58	$(AT)_{24}$	F: TGCCACTGATGAGTCAAGTA R: GTCCAAGATTCAGTGATCCA	58	221–227	4	0.63	0.62	0.53	5	0.71	0.67	0.57	AY244437
Ple62	$(AAAT)_5$	F: CCCTCTCCCTGGTCACAC R: GCCAACTGAGTTTGAGTCCC	59	155–169	6	0.68*	0.77	0.69	5	0.85	0.69	0.60	AY244438
Ple65	$(GA)_{12}$	F: GGAGCGAAACACGAAAACAG R: CAGGAGCCTCATGCAGAGAT	59	110–120	5	0.84	0.75	0.69	6	1.00	0.79	0.73	AY244439
Ple86	$(GA)_{27}$	F: GAGCCTGCTTCGGATTCTG	60	171–187	6	0.78	0.72	0.65	6	0.78	0.67	0.60	AY244440

	$(ATTTT)_5$	R: CCCAGTCTTCCATTTCTCCA											
Ple251	$(GA)_{11}$	F: AGCTCTGGAAGGTCCTCATTC	60	153–161	4	0.84	0.60	0.52	3	0.71	0.62	0.53	AY244442
		R: CCCACTCATGCGTACACG											
Ple487	$(CAT)_6$	F: CTCTCCCAAGAGCTCCACAG	62	179–181	3	0.78	0.62	0.54	3	1.00*	0.58	0.46	AY244443
		R: ACCAGCTTCAGAAAGCCTCA											
Ple495	$(GTG)_7$	F: GATCGCTCGTTTCCATGATT	58	182–186	3	0.26	0.28	0.22	5	0.57	0.56	0.50	AY095494
		R: TGAGGGCTGGATTTACCAAG											
Ple523	$(GA)_{16}$	F: CTCATTTTGGGGTCAGAG	58	171–175	3	0.57	0.53	0.45	3	0.57	0.65	0.50	AY244444
		R: GAGCCTGGAGCCTCCTTC											

[§]All forward primers were fluroscence labelled, T_a is the annealing temperature, n is the number of samples, K is the number of alleles per locus, H_O and H_E are observed and expected heterozygosity, respectively and PIC is polymorphism information content. * depicts significant departure from Hardy–Weinberg equilibrium.

genomic library of Asiatic lion. Out of these, 50 repeat sequences were selected based on their properties and length of the repeats. Primers were subsequently designed on the basis of the sequences flanking the repeat region using the program PRIMER 3.0 (Rozen and Skaletsky 2000). Twenty primer set, out of these 50, produced bright resolvable PCR products and were selected for further characterization (Table 1). PCR amplification was carried out in DNA engine (M J Research, USA) in 20 μl of reaction mixture containing 50 ng of genomic DNA, 1×PCR buffer, 100 μM dNTPs, 1.5 mM $MgCl_2$, 5 pmoles of each primer, and 0.5 units of amplitaq gold (Applied Biosystems, USA). The reaction conditions were: initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 30 sec; annealing at 52–62 °C for 20 sec; and extension at 72 °C for 1 min (30 cycles) with final extension at 72 °C for 7 min. These 20 primer sets, which gave reproducible amplification, were fluorescent labeled. Fluorescent-tagged amplification products were size fractionated, visualized and allele size was determined as described earlier (Singh et al. 2002).

Here we have employed these novel microsatellite markers derived from a pure Asiatic lion to study genetic variability among a captive population of 22 Asiatic lions endemic to Gir forests as well as 15 lions which were hybrid between the Asiatic and the African lion (Table 1). Genetic variation in the population was analyzed using software ARLEQUIN version 2.0 (Schneider et al. 2000). Tests for deviation from Hardy–Weinberg and linkage disequilibrium was done using exact tests based on a Markov chain algorithm and Fisher's exact test in program GENEPOP version 1.2 (Raymond and Rousset 1995), respectively. All the loci amplify well and are generally polymorphic. In Asiatic lions, the number of alleles per locus ranged from 2 to 6 (average = 4.05) per locus with a mean observed heterozygosity (H_O) of 0.71; a mean expected heterozygosity (H_E) of 0.60 and a mean polymorphism information content (PIC) value of 0.52. Four of the loci, Ple 37, 52, 54 and 62 showed significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$), which may be a consequence of the small number of samples genotyped. In hybrid lions, the number of alleles per locus ranged from 2 to 7 (average = 4.45) per locus with a mean observed heterozygosity (H_O) of 0.77, a mean expected heterozygosity (H_E) of 0.66

and a mean polymorphism information content (PIC) value of 0.58. Five of the loci, Ple 18, 37, 38, 54 and 487 showed significant departure from Hardy–Weinberg equilibrium ($P < 0.001$). Exact test for linkage disequilibrium yielded no significant value for all the loci.

The results suggest that the level of heterozygosities between pure Asiatic and hybrid lions was comparable. The additional microsatellite loci described here showed good amplification with DNA extracted from non-invasively obtained scat and hair samples also. These microsatellite markers provide potentially useful tools for the assessment of genetic variability within and across wild populations of the Asiatic lions and other endangered big cat species like tiger and leopard, to understand their population structure, phylogeography and species relationships.

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