Di-, tri- and tetranucleotide microsatellite loci for the giant panda, *Ailuropoda melanoleuca*

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**Abstract**

We describe 10 polymorphic microsatellite loci for the giant panda, *Ailuropoda melanoleuca*. Microsatellite sequences were isolated from three partial genomic libraries of giant panda DNA that were enriched for (i) (GT), (ii) (GAA) & (CAA), and (iii) (GATA) repeat sequences. The markers were tested for polymorphism in up to 82 pandas. Number of alleles at each locus varied between four and 11, and the observed and expected heterozygosities varied between 0.267 and 0.732, and between 0.601 and 0.799, respectively.

**Keywords**: *Ailuropoda melanoleuca*, giant panda, microsatellite, ursidae, VNTR

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Giant pandas, *Ailuropoda melanoleuca* (*Carnivora, Ursidae*) have a highly specialized biology and are indigenous to China. They are under threat from extinction as a result of the effects of habitat loss. As part of an attempt to augment the giant panda population, the Chengdu Research Base of Giant Panda Breeding (CRB) is one of several institutions that undertakes an *ex situ* breeding program. Fertilization in captive giant pandas is ensured by artificial insemination with semen from several males during natural copulation, and therefore the paternity of the cubs is not known. While there is some debate about the specific effects of reduced genetic variability, there is evidence that inbreeding is detrimental to many species (e.g. Saccheri et al. 1998). Maintaining diversity has therefore been identified as crucial to successfully managing both the *in situ* and *ex situ* giant panda populations. Although some dinucleotide microsatellites have already been constructed in this species (Lu et al. 2001), we wished to (i) expand the existing panel of markers, and (ii) take advantage of reduced stuttering of tri- and tetranucleotide microsatellites to provide unambiguous genotypes. Using an enrichment procedure based on a protocol described by Gardner et al. (1999), we developed supplementary dinucleotide — (CA)$_n$ — loci as well as libraries of tri- and tetranucleotide microsatellites.

Genomic DNA was extracted (Sambrook et al. 1989) from blood obtained from giant pandas during routine veterinary procedures at the CRB. Ten micrograms of DNA (from a pool of five pandas) was digested with *Sau*3AI (Boehringer Mannheim) and ligated to phosphorlated linkers (S61 5′-GGCCAGAGACCCCAAGCTTCG-3′ annealed to S62 5′-PO4-GATCCGAAGCTTGGGGTCTCTGGCC-3′; Refseth et al. 1997). DNA fragments between 500 bp and 1000 bp were excised from a 2% NuSieve GTG (FMC Bioproducts) gel and purified using a QIAquick gel extraction kit (QIAGEN). For enrichment, we used 1 mg of M2-80 streptavidin-coated magnetic beads (Dynal) incubated with 200 pmol of 3′-biotin-labelled oligonucleotides (MWG Biotech) [either (i) (GT)$_{12}$, (ii) (GAA)$_8$/(CAA)$_8$, or (iii) (GATA)$_5$]. After a series of differential stringency washes, the enriched DNA was recovered, and amplified by polymerase chain reaction (PCR). The DNA fragments were then purified using a QIAquick PCR purification kit (QIAGEN), ligated into pGEM-T (Promega) and transformed into JM109 *Escherichia coli* competent cells (Promega); recombinant clones were identified using black/white screening on S-gal agar plates (Sigma). Plasmids containing a microsatellite insert were identified by two or more amplified products after PCR (see Gardner et al. 1999; but...
with 50 pmol SP1 and 25 pmol (GT)$_{12}$ oligonucleotide) or after hybridization with (GAA)$_6$ (CAA)$_6$ or (GATA)$_5$ γ32P-ATP end-labelled probes (see Sambrook et al. 1989). Positive clones were cycle sequenced using Big Dye chemistry (PE Applied Biosystems) and electrophoresis on an ABI PRISM 377 following standard protocols. Full details of the enrichment and library screening protocols are provided by Bloor et al. (2001). Oligonucleotide primers flanking the tandem repeat regions were designed using PRIMER 2 (S.J. Kemp, unpublished data).

Loci were tested for polymorphisms on up to 82 individuals. Eighteen of them were collected from four different in situ populations (6 from Qinling Mountain, 3 from Minshan Mountain, 6 from Qiong Lai Mountain and 3 from Liangshan Mountain), and 64 of them were captive-born from Chengdu Research Base of Giant Panda Breeding (49), Beijing Zoo (8), the Breeding Centre of Wolong nature reserve (4), and the Breeding Centre of Longquantai nature reserve (3). The captive population are composed of F$_1$ (11), F$_2$ (3), F$_3$ (2) and the overlap generation (48, most of them are offspring of F$_2$). PCR amplification was undertaken in a 10-μL final volume using ReddyMix PCR Mix (ABgene) on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems Inc.). PCR conditions were: (i) 3 min at 95 °C, followed by 6 cycles of (ii) 95 °C for 30 s, 30 s at $T_a$ (the annealing temperature for each locus, Table 1) and 45 s at 72 °C, and then (iii) 26 cycles of 30 s at 92 °C, 30 s at $T_a$ and 55 s at 72 °C, and finally (iv) 72 °C for 10 min. Each reaction contained 1.5–3.0 mm MgCl$_2$ (Table 1), 75 mm Tris-Cl, 20 mm (NH$_4$)$_2$SO$_4$, 0.01% (v/v) Tween 20, 0.2 mm each dNTP, 10–20 ng template DNA, 10 pmol each forward and reverse primer (Table 1) and 0.25 U of Taq polymerase (ABgene). PCR products were separated by capillary electrophoresis through a denaturing acrylamide gel matrix on an ABI 310 automated sequencer (Applied Biosystems Inc.). Alleles were sized using GeneScan-350 (TAMRA) size standard within the GENOTYPER analysis software version 3.7 (Applied Biosystems Inc.).

The program CERVUS 2.0 (Marshall et al. 1998) was used to determine heterozygosity estimates for all loci. Ten (4 dinucleotide, 3 trinucleotide, 3 tetranucleotide) out of 44 examined loci showed a distinct allelic variation ranging from four to 11 (the successful amplification ranging from 14 to 71 individuals in the pandas examined). Observed and expected heterozygosities varying between 0.267 and 0.732, and between 0.601 and 0.799, respectively, and mean polymorphic information content (PIC) was 0.627. This is approximately consistent with the results of separately analysis of the ex-situ and in-situ data, except the in-situ population has higher expected heterozygosities (Table 1).
Tests for linkage disequilibrium (LD) and deviations from Hard–Weinberg equilibrium (HWE) were performed using GENEPOP 3.3 (Raymond & Rousset 1995). When all data are considered, one marker (Panda-21) showed significant deviations from HWE. When taken separately, ex-situ data, Panda-12 and Panda-21 vary from HWE. And for in-situ data, three markers (Panda-06, Panda-21 and Panda-41) showed departure from HWE. Following Bonferroni correction, LD tests revealed that two pairs of these loci (Panda-05 & Panda-06; Panda-05 & Panda-29) showed highly significant LD at $P < 0.0001$.

There were lacks of amplifications for some markers and individuals. These markers showed not only high PIC but also high null allele frequency, it might explain that these microsatellite sequences have high mutation rate in giant pandas.

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References


