Isolation and characterization of polymorphic tri- and tetra-nucleotide microsatellite loci for the south China tiger *Panthera tigris amoyensis*

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Abstract
Eleven tri- and tetra-nucleotide microsatellite loci from the south China tiger, *Panthera tigris amoyensis*, were isolated, and characterized in this paper. Among these microsatellite loci, one locus was monomorphic; among the remaining 10 loci, the number of observed alleles for each locus in 57 individual tigers ranged from four to nine, the expected and observed heterozygosity ranged from 0.366 to 0.805 and 0.400 to 0.837, respectively, and the mean polymorphic information content (PIC) was 0.609. Moreover, two loci (F41 and FCA391) were compared between the domestic cat and the south China tiger and differences in their flanking regions were found (5.9% for F41 and 4.8% for FCA391). These 10 polymorphic tri- and tetra-nucleotide microsatellite markers would be very useful in the evaluation of tiger population structure and the genetic relationships among the individual tigers.

Keywords: Microsatellite, *Panthera tigris amoyensis*, south China tiger, tetra-nucleotide, tri-nucleotide

Introduction
The south China tiger (*Panthera tigris amoyensis*) is a critically endangered subspecies in China: only about 60 individuals remain in zoos and no evidence of wild south China tigers has been found in the last 10 years (Zhang et al. 2006). In order to prevent its extinction, recovery efforts are focusing on a future supportive breeding programme, with the intent of using genetic markers to avoid inbreeding and outbreeding depression. Microsatellites are considered by many to be the most suitable genetic marker in genetic identification,
parentage, kinship, population variability assessment, and so on (Baron et al. 1992; Goldstein and Pollock 1997).

We have reported 12 polymorphic dinucleotide microsatellite loci in this tiger subspecies (Zhang et al. 2006). However, individual alleles of dinucleotide microsatellite loci often appear as stutter or shadow bands that refer to multiple peaks per allele, making it difficult to distinguish between homozygous and heterozygous states where alleles differ by a single repeat unit. In general, stutter increases as the repeat unit length decreases. For example, arrays with four base pair (bp) repeats tend to stutter less than arrays with three bp repeats, which stutter less than arrays with two bp repeats (Edwards et al. 1991). Therefore, the isolation of tri- and tetra-nucleotide microsatellite loci from the south China tiger might provide more information for the conservation of the tiger.

Two genomic libraries for (GAA)n and (GATA)n repeats were constructed using enrichment methods and the microsatellite-containing clones were screened by PCR (Zou et al. 2005; Xia et al. 2006; Zhang et al. 2006). Putatively recombinant clones of (GAA)n served as the template for amplification (35 cycles with 92°C denaturation, 54°C annealing, and 72°C extension) using (GAA)8 and Oligo A (5’-GGCCAGAGACCCCAAGCTTCG-3’) as primers; (GATA)n served as template for amplification (35 cycles with 92°C denaturation, 48°C annealing, and 72°C extension) using a (GATA)6 primer in combination with T7 promoter (5’-TAATACGACTCTATAGGGC-3’) forward and SP6 promoter (5’-ATTTAGGTGACACTATAGAATAC-3’) reverse primers. In the (GAA)n library, out of 672 recombinant clones screened, only seven (1.0%) positive clones were found and insured by sequencing; in the (GATA)n library, out of 384 recombinant clones screened, 101 (26.3%) positive clones were found, 35 positive clones were sequenced, and each contained microsatellite inserts.

Primer pairs were designed for seven sequences containing the (GAA)n insert and six sequences containing the (GATA)n insert. In these 13 sequences, four (GAA)n loci showed non-specific amplification and others yielded consistent product. At the same time, two microsatellite loci (F41 and FCA391) were selected from the domestic cat (Menotti-Raymond et al. 1999) and their motif repeats were obtained from one homozygous south China tiger allele. The F41 locus exhibited compound, uninterrupted, repeated structures and the FCA391 locus exhibited compound, interrupted, repeat structures, which were consentient with the homologous domestic cat microsatellite locus (Menotti-Raymond et al. 1999).

Each locus was evaluated for polymorphism and heterozygosity in a total of 57 south China tigers, including 50 living captive tigers from 11 Chinese zoos and seven museum specimens (in which, four were from wild and three were from captive tigers that died at least 20 years ago). DNA was extracted from the seven museum specimens following the method of Fang and Wan (2002). PCR amplification conditions consisted of 15 pmol unlabeled reverse primer, 15 pmol fluorescently labelled forward primer (Table I), 200 µM each dNTP, 3.5 µl 1 µg µl⁻¹ BSA, about 50 ng DNA sample and 0.5 U of AmpliTaq Gold® DNA polymerase (ABI) and a primer-specific volume of 25 mM MgCl2 (Table I) in 10 µl total volume. The PCR was performed on a GeneAmp® 9700 thermal cycler (ABI) with the following conditions: 10 min at 95°C followed by 20 cycles of 30 s at 94°C, 45 s at stepwise lowering of the annealing temperature from 58°C to 49°C and 55 s at 72°C; 15 cycles of 30 s at 90°C, 45 s at 53°C, and 55 s at 72°C. The final extension time was 30 min at 72°C. The amplified products were separated on a Long-Ranger denaturing acrylamide gel (Cambrex BioScience) on an ABI 373 DNA sequencer (ABI). Fragment lengths (Table I)
Table I. Locus name, primer sequence, repeat motif, MgCl2, allele size range, number of alleles observed (\(A\)), observed heterozygosity (\(H_O\)), expected heterozygosity (\(H_E\)) and HWE \(P\) values for 11 microsatellite loci in south China tigers.

| Locus primer | Sequence (5’–3’)\(^a\) | Repeat | \(\text{MgCl}_2\) (\(\mu\)l) | Size (bp) | \(A\) | \(H_O\) | \(H_E\) | \(P\) values |
|--------------|------------------------|--------|-----------------|--------|------|-------|-------|------------|
| Ptia1        | F: TET-TTTAATTCCTCTCTGCCAA R: CTCTGTTGCTGACAGCTCA | (CTT)\(_{14}\) | 1.0 | 212–233 | 4 | 0.419 | 0.366 | 1.0000 |
| Ptia5        | F: TET-GACTGAGCCACCCCAAGAG R: AAGGAACCTCCCTCAAACCAT | (GAA)\(_{17}\) | 0.6 | 275–287 | 5 | 0.545 | 0.612 | 0.0163 |
| Ptia8        | F: TET-CAAGAGTCAGACGCCTCAA R: GCTAATTCATTTTGACATC | (CTT)\(_{3}\)CTCTTT(CTT)\(_{3}\)CCT(CTT)\(_{7}\) | 0.6 | 264–279 | 5 | 0.723 | 0.665 | 0.3290 |
| Ptia9        | F: TET-AGCAGGAGCTACGCACCAG R: ACTCAAGGGACTCAGAAA | (CTT)\(_{14}\) | 0.8 | 193–226 | 8 | 0.740 | 0.746 | 0.0786 |
| Ptia16       | F: TET-TGAGACCAGCTTGCCCTAC R: ATCCCAAAGGTTCTCTCCAG | (GAA)\(_{4}\)GGA(GAA)\(_{9}\) | 0.6 | 165–180 | 5 | 0.612 | 0.705 | 0.0033 |
| Ptia17       | F: FAM-CTTGGCCCACCTCTACCTTT | (TTC)\(_{20}\) | 0.6 | 168–212 | 8 | 0.596 | 0.652 | 0.4516 |
| Ptia18       | F: FAM-GATCCCTCTCCTCTCTACTTTGCTCT | (GAA)\(_{17}\)(GGA)\(_{4}\) | 0.6 | 380–413 | 8 | 0.630 | 0.753 | 0.3409 |
| Ptia15       | F: FAM-AAACCTGTCCCAAAGGCTC | (TATC)\(_{10}\)AGGCT(GA)\(_{15}\) | 1.0 | 140–176 | 9 | 0.837 | 0.762 | 0.3633 |
| Ptia19       | F: FAM-GGATCATGGGATAAGCCCTT | (GATA)\(_{8}\) | 0.8 | 289 | 1 | 0.000 | 0.000 | 0.000 |
| F41          | F: TET-GTCTGAGCTCTTCAAATAGGA R: GTACCTGAGTGGCTTGTTGA | (A)\(_{11}\)(AAGG)\(_{2}\)(AAAG)\(_{17}\) | 0.6 | 134–176 | 7 | 0.400 | 0.494 | 0.0801 |
| FC-A391      | F: FAM-GGCCCTAAACTCTCCTTGAGA R: TTTAGGTAGCCCATTTCATCA | (ATGG)\(_{3}\)ATT(GATG)\(_{2}\)G(GATA)\(_{12}\) | 0.6 | 200–224 | 7 | 0.796 | 0.805 | 0.0538 |

\(^a\)F, forward; R, reverse.
were assigned using GeneScan software (ABI) and a GeneScan-500 [Tamra] size standard.

FCA391 had non-overlapping size ranges and F41 had overlapping size ranges between the tiger and the cat (Menotti-Raymond et al. 1999). However, F41 and FCA391 loci differences in the flanking regions immediately 5′ and 3′ of the repeat between the cat and the tiger were 5.9% and 4.8%, respectively. These differences would suggest that interspecific comparisons between cat and tiger with the two microsatellite loci should be made with care because a number of genetic distance measures describing evolution at microsatellite loci rely on the assumption that differences between alleles are due entirely to changes in the number of repeat units (Goldstein et al. 1995).

From the PCR results of the museum specimen, it was found that microsatellite loci F41, FCA391, Ptia9, Ptia15, Ptia16, and Ptia17 tended to produce results more readily than the remaining loci, since most museum specimen DNA generally congregated around 300 bp or so and loci with a median product size of less than 250 bp generally tend to produce results more readily. These results showed fragment size had a much stronger influence on the success and repeatability of microsatellite amplification from a museum specimen, which was consistent with Sefc et al. (2003). Moreover, allele 224 in FCA391, 208 in Ptia9, and 172 in Ptia15 were present only in wild museum specimens and not represented in any of the living captive tigers.

Three alleles (285, 289, and 291) of loci Ptia19 were found from the 57 tigers and every individual was heterozygous in loci Ptia19. The three alleles were sequenced and it was found that only one allele (289) included a microsatellite repeat motif, which indicates that Ptia19 was monomorphic.

The program CERVUS 2.0 (Marshall et al. 1998) was used to determine heterozygosity estimates for all loci (Table I). All of the examined loci (except Ptia19) showed a distinct allelic variation ranging from four to nine in the tigers examined, the expected and observed heterozygosity ranged from 0.366 to 0.805 and 0.400 to 0.837, respectively, and the mean polymorphic information content (PIC) was 0.609. Tests for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were performed using GENEPOP 3.4 (Raymond and Rousset 1995). Because some museum specimens were only successful in the amplification of certain loci and did not have offspring among living captive tigers, HWE tests were only performed on the 50 living captive tigers and revealed that one locus (Ptia5) deviated from HWE at \( P<0.05 \). One locus (Ptia16) significantly deviated from HWE at \( P<0.01 \) (Table I). The data from parents and offspring were consistent in the 11 microsatellite loci through pedigree and no evidence for allelic drop was found. Moreover, the 50 living captive tigers were the offspring of six founders (determined through pedigree) and there is no evidence for different captive populations recently exchanging individuals, thus effectively resulting in out-breeding. So, the departure from HWE may be due to the presence of inbreeding. Following Bonferroni correction, highly significant LD tests were not shown for any pair of loci.

These 10 polymorphic tri- and tetra-nucleotide microsatellite markers would be very useful in the evaluation of tiger population structure and the genetic relationships among the tiger individuals. This is not only because they were high polymorphic, but also they were readily scored and separated using simple polyacrylamide and silver-staining.

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