

## PRIMER NOTE

# Dinucleotide microsatellite primers designed for a critically endangered primate, the black lion tamarin (*Leontopithecus chrysopygus*)

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## Abstract

**Black lion tamarin (BLT) monkeys (*Leontopithecus chrysopygus*) have suffered a severe reduction in their natural range and are consequently critically endangered. Because allozyme data showed very low levels of variation, it was not clear if these monkeys had much genetic diversity. We designed microsatellite primers for BLTs, and from them we identified nine polymorphic loci, seven of which were tested on golden lion tamarins (GLTs) (*Leontopithecus rosalia*). All of the seven polymorphic loci and two other monomorphic BLT loci were polymorphic in GLTs. The microsatellite markers identified here are directly applicable to ongoing lion tamarin population and conservation genetics studies.**

*Keywords:* genetics, *Leontopithecus*, microsatellites, New World monkey, polymorphism

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The black lion tamarins (BLTs), *Leontopithecus chrysopygus*, is a critically endangered Neotropical primates (Hilton-Taylor 2000). As a direct result of habitat destruction, its population size has dwindled to approximately 1000 individuals spread primarily among nine locations in the state of São Paulo, Brazil.

We present a set of species-specific microsatellite primers for BLTs that are useful for the conservation genetics research that is currently underway. In addition, we tested the utility of these primers in golden lion tamarins (GLTs), *Leontopithecus rosalia*, another critically endangered callitrichine primate.

Microsatellite loci were identified using standard library construction and screening protocols. BLT genomic DNA was digested with restriction enzymes *AluI*, *RsaI*, and *HaeIII*.

Fragments ranging in size from 250 to 350 base pairs were inserted into pGEM 5Zf(+) plasmid (Promega). The plasmid was chemically transformed into high efficiency JM 109 competent cells (Promega) using the TA cloning kit (Invitrogen). Over 1700 transformation-positive colonies (detected by blue/white screening) were hybridized onto nylon membranes and exposed to synthetic oligonucleotides of (AC)<sub>n</sub>/(TG)<sub>n</sub> and (AG)<sub>n</sub>/(TC)<sub>n</sub> repeats (Pharmacia) end-labelled with <sup>32</sup>P. One hundred of the microsatellite-positive clones were then cycle-sequenced via standard dye-terminator methods (Applied Biosystems) using pUC/M13 primers specific to the pGEM vector (Promega) and the fragments were separated on an ABI377 automated sequencer (Applied Biosystems). Thirty-eight loci that showed a sufficient amount of sequence flanking the repeat regions were chosen for primer design with OLIGO (version 5.1, National Bioscience) and MACVECTOR (version 6.53, Oxford Molecular Group) software. Of the 38

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**Table 1** Characterization of BLT primers and assessment of variation in BLTs and GLTs

Locus	Primer sequence (5'-3')	Repeat motif of clone	BLT/ GLT $T_a$	PCR product size range BLT/GLT	BLT/ GLT $N_a$	GenBank Accession nos
Leon2	CTGCTTCTTGTTCCACTTCTTCTC GTTTGGGTGGTTGCCAAG	(CA) <sub>18</sub> (CG)(CA) <sub>3</sub>	55/55	206–208/206–212	2/2	AY706915
Leon3c20	CTGTATGTGATCGCTTTTACCTG AAGGCAATCTAACTAATCAACACTC	(GT) <sub>22</sub>	60/60	294–296/296–300	2/3	AY706916
Leon15c85	CTGATCCTTGAAGCAGCATTTG GGTTAAAGGGGTTCTTCTGTG	(GA) <sub>17</sub>	60/60	270–274/279–281	2/2	AY706920
Leon21c75	CAGTTGAGGGAACAGGAATTA CACTGCACTGACAGAGCAAG	(GT) <sub>19</sub> (NA) <sub>1</sub> (GT) <sub>5</sub>	60/62	278–282/274–282	3/4	AY706922
Leon26c10	TTCATCTCAATGACACGAAAC CATCGAGTGTCTCTGCTGT	(TG) <sub>17</sub> (AG) <sub>15</sub> (GT) <sub>3</sub> (CT)(GT)	50/55	250/250–262	1/2	AY706924
Leon27c13	AAGCGCAGATTTATTTGATAGG TGCAGGTAAATGATGGTAATG	(CA) <sub>11</sub>	60/60	195/190–196	1/3	AY706925
Leon28c15	TCATGTGTGGGAAGTTGAG GTTCCCTAACATTCCTTAACA	(GT) <sub>21</sub>	60/–	NS/250–258	NS/2	AY706926
Leon30c73	GGACCTGATTGAAGCAGTC TTCCTTGAGAACTAATGGAG	(TC) <sub>25</sub> (AA)(TC)(TG) <sub>16</sub>	60/60	255–269/260–264	6/3	AY706927
Leon31c97	TGGTCCAGAGAAATGATGTC GTAATTCCTTGGATTTATGCC	(GA) <sub>2</sub> (CA) <sub>2</sub> (GA) <sub>19</sub> (TT)(GA)(CA) <sub>4</sub>	55/55	311–323/311–323	4/5	AY706928
Leon11c72	AGGATTACAGGTGCCAC TTGCATATTTGTGTTCAACTTC	(GT) <sub>21</sub>	60/–	296–304/–	2/–	AY706921
Leon35c42	GTGAAAGGTTTCAGAAATATC TGCAGTTGTCCACACTTTA	(CT) <sub>16</sub> (CA) <sub>9</sub> (T)(AC) <sub>3</sub>	58/–	201–205/–	3/–	AY706929
Leon4c32	TACCTAATGCACGCAGG CCTCTGTTATCTCCCC	(CA) <sub>21</sub>	60/–	305/–	1/–	AY706917
Leon5c23	CGAGGTATAGCCAGACAGATGC CACCTGCCTACTCTGCCATAGG	(CA) <sub>16</sub>	60/–	294/–	1/–	AY706918
Leon10c46	CATGGCATTAAACAGGTTTC CATGAGTATCTGCTGTGTCG	(CA) <sub>18</sub>	60/–	228/–	1/–	AY706919
Leon22c02	AAACCTGCTAATAGTGGTGAG TCAATAGCAAAGACTGAACT	(CA) <sub>17</sub> (CG)(CA) <sub>4</sub>	55/–	202/–	1/–	AY706923

$T_a$ , annealing temperature;  $N_a$ , number of alleles; NS, Marker was not scored due to stutter excess.

primer pairs, 28 amplified DNA, and 21 of these were further evaluated. Fifteen of the latter 21 primer sets were optimized and used to screen 14 BLT individuals from four remnant populations. Nine of the BLT-specific primers were re-optimized for GLTs and used to screen five individuals from the largest remnant population of this species in the Poço dos Antes reserve in Rio de Janeiro, Brazil.

Genomic DNA for all BLT and GLT samples were extracted from blood using the QIAGEN blood kits. Polymerase chain reactions (PCRs) for all loci in both species consisted of 0.16  $\mu$ M per primer (forward and reverse), 0.1 mM dNTPs, 2.25 mM MgCl<sub>2</sub>, 10 mM Tris-HCL, 50 mM KCl, and approximately 1 ng of DNA in a 10  $\mu$ L reaction. *Taqstart* (CloneTech) was used with 1 U of *Taq* polymerase (Promega) at a 1 : 1 unit ratio. PCR cycling conditions began with a 95 °C denaturation period for 1 min and continued with 42 cycles of the following steps: 95 °C for 45 s, 55–63 °C for 45 s (Table 1), and 72 °C for 45 s. The cycles ended with a 72 °C hold for 15 min.

PCR products were separated on an ABI 377 automated DNA sequencer (Applied Biosystems). Gels were prerun for 1 h, according to the recommendation of Fernando *et al.* (2001) to avoid electrophoresis artefacts. PCR products were sized with respect to a TAMRA-350 or TAMRA-500 internal standard (Applied Biosystems) using GENESCAN software (version 3.1). Alleles were scored by eye from the electropherograms using the method outlined by Ziegler *et al.* (1992) for discrimination of stutter and true bands. Observed and expected heterozygosities were calculated with GENALEX (Peakall & Smouse 2001; <http://www.anu.edu.au/BoZo/GenALEX/>). Tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed using ARLEQUIN 2.001 (Schneider *et al.* 2000, <http://lgb.unige.ch/arlequin>).

BLTs are polymorphic for nine of the 15 (60%) species-specific primers examined (Table 1). One primer (Leon28c15) had a large amount of stutter and was not scored in BLTs, but was informative in GLTs. Another primer, Leon11c72,

**Table 2** Heterozygosities for polymorphic loci

Locus	Sample size		$H_O$		$H_E$	
	BLT-CP	BLT-P	BLT-CP	BLT-P	BLT-CP	BLT-P
Leon2	5		0.4		0.48	
	8		0.5		0.469	
Leon3c20	5		0.6		0.42	
	5		0.2		0.18	
Leon15c85	7		0.286		0.541	
	5		0.4		0.54	
Leon21c75	5		0.2		0.18	
	8		0.125		0.117	
Leon26c10	5		0.8		0.48	
	5		0		0	
Leon27c13	9		0.333		0.426	
	4		0.75		0.469	
Leon28c15	—		—		—	
	5		—		—	
Leon30c73	5		0.4		0.320	
	—		—		—	
Leon31c97	5		0.6		0.54	
	—		—		—	
Leon11c72	5		0.6		0.50	
	2		1.0		0.5	
Leon35c42	7		0.143		0.133	
	4		0.75		0.656	
Leon31c97	5		1.0		0.580	
	8		0.250		0.219	
Leon11c72	4		1.0		0.813	
	3		0.333		0.278	
Leon35c42	9		0.00		0.00	
	—		—		—	
Leon35c42	4		0		0.375	
	7		0.286		0.245	
			—		—	

Heterozygosities, observed ( $H_O$ ) and expected ( $H_E$ ), for two BLT regions, central São Paulo (CP) and Pontal (P), and the main population of GLTs, are provided. Heterozygosity for Leon28c10 in BLTs was not calculated because of the excessive stuttering that affected scoring.

consistently produced two amplicons, one at 286–290 bp and the other at 296–304. The scored locus, which was 296–304, was chosen because its size-range matched the size of the targeted cloned microsatellite sequence and is in HWE for the only forest-fragment population in which it showed variation ( $n = 3$ , Table 2, and for the same population,  $n = 7$ , data not shown). Finally, one locus, Leon30c73, showed three or more alleles in three of the 14 individuals. This is likely a result of fetal chimerism. Lion tamarins, like other callitrichine primates, have dizygotic twins, which often, but do not always, share blood vessels of the placenta (placental anastomosis), and thus precursor haematopoietic cells, during early embryonic development (cf. Dixson *et al.* 1992;

Haig 1999; Signer *et al.* 2000). Consequently, tissue derived from precursor haematopoietic cells (such as blood) contains a mix of cells that are both constitutive and from a twin. Given the sensitivity of PCR to pick up all the alleles in a chimeric pool of cells, it may be that some homozygotes appear to have 'heterozygous' genotypes, but in reality reflect the admixture of fraternal homozygote twins. However, it should be noted that chimerism does not have an effect on our assessment of polymorphism.

All of the loci were in HWE for BLTs and GLTs and heterozygosities are reported in Table 2. No loci showed significant LD in the BLT central São Paulo population as well as in GLTs with the exception of one locus pair in GLTs, Leon3c20 and Leon30c73 ( $P = 0.0159$ ). There were several loci in the BLT Morro do Diabo population which were significantly associated with each other and are as follows: Leon2 with Leon15c85, Leon21c75, and Leon31c97; Leon3c20 with Leon35c42; Leon21c75 with Leon15c85; Leon31c97 with Leon15c85.

With two to six alleles at nine polymorphic loci and an average diversity of  $0.295 \pm 0.189$  ( $\pm$  SD), BLTs show much more variation from prior allozyme data than expected (Forman *et al.* 1986: 47 loci,  $P = 3\%$ ,  $H = 0.003$ , Valladares-Padua 1987: 25 loci,  $P = 0$ ). Seven of the nine BLT polymorphic loci were also variable in GLTs, as were two other loci that were monomorphic in BLTs (Table 1). In all, GLTs exhibited more alleles than BLTs at five loci (Table 1). Because the sample sizes for this initial survey are small, a statistical comparison was not performed between GLTs and BLTs, but the average diversity of GLT ( $H_E = 0.526 \pm 0.141$  ( $\pm$  SD) (compare to  $0.542 \pm 0.099$  calculated from Grativol *et al.* 2001), vs. BLT ( $H_E = 0.295 \pm 0.189$  ( $\pm$  SD)), suggests a trend of greater microsatellite variability in GLTs than BLTs. These preliminary results are interesting because GLTs are suspected to have undergone a more severe population bottleneck in the recent past with possibly as few as 300–400 surviving individuals vs. the *c.* 1000 surviving BLTs (see Coimbra-Filho & Mittermeier 1973; Dietz *et al.* 1986; Valladares-Padua & Cullen 1994). The microsatellite primers described here significantly add to the primers available for lion tamarins (Grativol *et al.* 2001; Di Fiore & Fleisher 2004), and are currently being used in research informing management decisions for the lion tamarins.

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