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Genetic diversity of microsatellite loci in *Leopardus pardalis, Leopardus wiedii* and *Leopardus tigrinus*

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ABSTRACT. The microsatellite loci FCA045, FCA077, FCA008, and FCA096 are highly variable molecular markers which were used to determine the genetic diversity in 148 captive *Leopardus* sp. The PCR-amplified products of microsatellite loci were characterized in ABI Prism 310 Genetic Analyzer. Allele numbers, heterozygosity, polymorphism information content, exclusive allele number, and shared alleles were calculated. Sixty-five alleles were found and their sizes ranged from 116 to 216 bp in four microsatellite loci. The heterozygosity ranged from 0.36 to 0.81 in *Leopardus pardalis*, 0.57 to 0.67 in *L. tigrinus* and 0.80 to 0.92 in *L. wiedii*. The polymorphism information content was from 0.80 to 0.88 in *L. pardalis*, 0.76 to 0.88 in *L. tigrinus* and 0.77 to 0.90 in *L. wiedii*. The margay (*L. wiedii*) showed the highest index of polymorphism among the three species in this study. These results imply that

microsatellite DNA markers can help in the study of the genetic diversity of *Leopardus* specimens.

Key words: Heterozygosity, *Leopardus pardalis*, *Leopardus tigrinus*, *Leopardus wiedii*, Microsatellite

INTRODUCTION

The ocelot (*Leopardus pardalis*), margay (*L. wiedii*) and tigrina (*L. tigrinus*) are Neotropical felids whose survival assured in Conservation Units or captivity. These species are listed as vulnerable or endangered according to the International Union for the Conservation of Nature (IUCN) and U.S. Fish and Wildlife Service listing, respectively (O'Brien et al., 1996). The ocelot, margay and tigrina are threatened by habitat loss and fragmentation, hunting and human persecution (Eizirik et al., 1998). Crossing programs are planned to avoid inbreeding. These strategies are indispensable to brighten the outlook for the remaining population. Reproduction in captivity is an important tool to safeguard the genetic diversity of felids (Foose, 1983; Avise, 1994; Oliveira, 1994).

DNA analysis shows us genetic variability and allows us to refine the genealogies of savage and captive populations (Mace et al., 1996). Microsatellites, due to their abundance in the genome and their high mutation rates, are molecular markers used to detect levels of genetic variability and to plan future breeding strategies. An important characteristic of microsatellites is that primers developed for a specific species can be used for other species of related taxons (Menotti-Raymond and O'Brien, 1993, 1995; Shankaranarayanan et al., 1997). Ten highly polymorphic microsatellite loci of dinucleotide repetitions (dC.dA)n/(dG.dT)n developed from the domestic cat genome showed amplified products of the same size in lions, cheetahs, pumas, leopard cat, and geoffroy cat (Menotti-Raymond and O'Brien, 1995). A broad range of heterozygosity was observed among the species for a single locus and among loci within a single feline species. This characteristic demonstrates that a microsatellite is an important informative marker (Menotti-Raymond and O'Brien, 1993, 1995).

The aim of the preasent research was to determine the potential usefulness of microsatellite markers of *Felis catus* to verify inter- and intra-group genetic level diversity of captive species of *L. pardalis*, *L. tigrinus* and *L. wiedii*.

MATERIAL AND METHODS

The feline samples were collected by workers from Associação Mata Ciliar (Jundiaí, SP, Brazil) and the animals were housed in different zoos in Brazil (Table 1). The domestic cat (*Felis catus*) was used as control and it was supplied by Menotti-Raymond from the Laboratory of Genomic Diversity, Frederick, MD, USA.

Genomic DNA was isolated from peripheral blood leukocytes from specimens of *L. tigrinus* (N = 46), *L. wiedii (*N = 25) and *L. pardalis* (N = 77). Proteinase K digestion and treatment with phenol-chloroform was used for DNA extraction (Sambrook et al., 1989).

Table 1. Zoo location (in Brazil) and specimen registration number in Associação Mata Ciliar of *Leopardus pardalis*, *L. tigrinus* and *L. wiedii*.

Zoo location (city/town - state) and registration number of feline		
Leopardus pardalis	*Leopardus tigrinus*	*Leopardus wiedii*
Alfenas - MG; 243	Belo Horizonte - MG; 039	Jundiaí - SP; 01, 08, 013, 014, 047, 1440, 1494
Belo Horizonte - MG; 076, 241	Ipatinga - MG; 040	São Bernardo do Campo - SP; 05
Ipatinga - MG; 040, 087, 092, 093, 094	Vargínia - MG; 041	Sorocaba - SP; 07
Pouso Alegre - MG; 097, 144	Uberaba - MG; 050	Curitiba - PR; 040, 041, 042, 043, 044
Uberaba - MG; 019, 272, Janine (no number)	Goiânia - GO; 046	Maringá - PR; 80, 81, 88, 86
Uberlândia - MG; 022,150, 151, 152, 153, 256, 257, 275	Brasília - DF; 042, 043, 044	Cascavel - PR; 1162, 1347
Vargínia - MG; 040, 042, 098, 161, 209, 237, 238, 239, 262	Bauru - SP; 021, 024	Brusque - SC; 30
Goiânia - GO; 049, 050, 048	Campinas - SP; 08, 09, 010	Camboriú - SC; 032, 033
Brasília - DF; 044, 072, 242, 273, Tica (no number)	Jundiaí - SP; 04, 05, 038, 049, 079, 083	Joinville - SC; 025, 026
Bauru - SP; 09	São B. do Campo - SP; 03, 06, 07	
Campinas - SP; 015, 016	São Carlos - SP; tigrina (no number)	
Jundiaí - SP; 061, 233, 252, 261	São José do Rio Preto - SP; 018	
Mogi Guaçu - SP; 122, 181	Sorocaba - SP; 015,162	
Pedreira - SP; 141	Cascavel - PR; 081, 082, 087, 088	
Piracicaba - SP; 023, 024	Curitiba - PR; 084, 085, 086, 092, 093	
São Bernardo do Campo - SP; 232	Maringá - PR; 034, 090, 091	
São Carlos - SP; Tica 1, Tica 2, Tica 3, Tica 4 (no number)	Brusque - SC; 078	
São J. do Rio Preto - SP; 011, 081, 082, 083, 084, 100, 227, Ceguinha (no number)	Camboriú - SC; 1431, 1454	
Sorocaba - SP; 4748, 035, 1478	Joinville - SC; 057	
Cascavel - PR; 1647, 260	Pomerode - SC; 058, 059, 077, 260	
Curitiba - PR; 54, 254, 278, Jack (no number)		
Maringá - PR; 003, 020		
Camboriú - SC; 249, AGP (no number)		
Pomerode - SC; 250		

DF = Distrito Federal; GO = Goiás; MG = Minas Gerais; PR = Paraná; SC = Santa Catarina; SP = São Paulo.

We used four dinucleotide primers (FCA 008, FCA045, FCA077, and FCA096) that were developed by Menotti-Raymond and O'Brien (1995). Multiplex PCR amplifications were performed in a final volume of 10 µL containing 10 ng feline genomic DNA, 2 pmol forward and reverse primers (Invitrogen Life Technologies), 200 mM of the four deoxyribonucleoside 5'-triophosphates (dATP, dCTP, dGTP, and dTTP) (Invitrogen Life Technologies), 25 mM $MgCl_2$ (Applied Biosystems), 2 units *AmpliTaq* GOLD (5 U/µL) (Applied Biosystems), 1.5 µL PCR buffer (containing 200 mM Tris-HCl and 500 mM KCl) (Applied Biosystems) and completed with ultrapure water (Invitrogen Life Technologies). The forward sequences were fluorescently labeled using tetramethyl-rhodamine, hexachloro-6-carboxyfluorescein and 6-carboxyfluorecein (Table 2). Amplification was performed using PTC-100 Thermocycler (MJ-Research). The amplification parameters were: one cycle of 94°C for 10 min followed by 10 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, 10 cycles of 89°C for 15 s, 55°C for 30 s, 72°C for 30 s, one cycle of 72°C for 30 min, and the samples were cooled to 4°C. All PCR fragments were submitted to automated DNA sequencing. Pooled product (1 µL) was mixed with 12 µL deionized formamide and 0.5 µL DNA standard ROX 500 molecular weight marker (Applied Biosystems). The samples were denatured in a PTC-100 Thermocycler (MJ-Research) at 95°C for 5 min, snap-cooled on ice and placed in an auto-sample tray in ABI PRISM 310 Genetic Analyzer (Applied Biosystems) for automatic injection. The samples were run for 25 min at constant temperature of 60°C in polymer GEL POP4 (Performance Optimized Polymer 4, Applied Biosystems). The electrophoresis data were analyzed using ABI PRISM GENESCAN 500 2.0 and GENOTYPER 2.0 DNA software (Applied Biosystems).

It was possible to determine heterozygote or homozygote genotypes and the size of alleles for loci of each animal through the electrophoretograms. CERVUS program model 2.0 (Marshall et al., 1998) was used to obtain results on expected and observed heterozygosity, polymorphism information content (PIC), number of alleles for loci, and Hardy-Weinberg equilibrium. The exclusive alleles of each specimen, the shared alleles among species investigated and the total number of alleles observed for each microsatellite locus were characterized (Table 2).

RESULTS AND DISCUSSION

Menotti-Raymond and O'Brien (1995) demonstrated that ten pairs of cat microsatellite primers amplified predicted size products in the lion, cheetah, puma, Asian leopard cat, and geoffroy cat. Eizirik et al. (2001) used 29 *Felis catus* microsatellite markers to investigate genetic diversity in *Panthera onca* specimens. Our results showed that the four primers (FCA008, FCA045, FCA077, and FCA096) can amplify products in *L. pardalis, L. tigrinus* and *L. wiedii*, suggesting their evolutionary conservation across Felidae.

Chakraborty and Kimmel (2000) observed that dinucleotide microsatellite alleles showed the highest mutation rate. The high allele number average for loci observed in Table 2 can be related to the fact that the microsatellites were multiallelic and dinucleotide repetitions. Microsatellites determine the possibility of repetitions in different species, which are able to change quickly among limited states. The number of repetitions would provide comprehension of the peculiar ability of a species to adapt to environmental changes. We observed a difference in allele size variation and high allele number for loci among the three species (Table 2). The four microsatellite primers produced an allele average of 12.5 for *L. pardalis*, 11.5 for *L. tigrinus*

Table 2. Characterization of feline microsatellite loci, repetition numbers (CA; CT; GT), primer sequences (5'-3'), number of alleles, number of exclusive alleles, shared alleles, and PCR product size (bp) from *Leopardus pardalis* (Lpa), *L. tigrinus* (Lti) and *L. wiedii* (Lwi).

Microsatellite loci (locus accession No.)	Repetition	Primer sequence	Number of alleles			Number of exclusive alleles			Shared alleles	PCR product size range (bp)		
			Lpa	Lti	Lwi	Lpa	Lti	Lwi		Lpa	Lti	Lwi
FCA008 (AF130476)	(CA) 26	*F[1] actgtaaatttctgagctggcc R ccatacccagaacagtctgtca	12	9	10	4	2	1	6	122-160	120-140	116-142
FCA045 (AF130489)	(CA) 15	*F[4] tgaagaaaagaatcaggctgtg R cacgaacacagagatgctcatac	12	9	9	4	2	-	6	131-155	137-155	143-161
FCA077 (AF130506)	(GT) 20	*F[3] gcacctataactaccagtgtga R ccaaaatttatttccccagagat	12	12	13	2	2	1	8	125-151	127-151	117-149
FCA096 (AF130519)	(CA) 20; (CT) 18 (CA) 3	*F[2] cacgccaaactctatgctga R gtgttcttggacggcacattg	14	16	12	2	1	3	5	180-216	176-216	176-200
Average			12.5	11.5	11							

*Fluorescent dye (Forward primer): F[1] - tetramethylrhodamine, F[2] and F[3] - 6-carboxyfluorecein, F[4] - hexachloro-6-carboxyfluorescein.

Table 3. Observed heterozygosity (OH) and expected heterozygosity (EH), Hardy-Weinberg equilibrium (HW) and polymorphism information content (PIC) in *Leopardus pardalis*, *L. tigrinus* and *L. wiedii* for the four microsatellite loci.

Microsatellite loci	*Leopardus pardalis*				*Leopardus tigrinus*				*Leopardus wiedii*			
	Heterozygosity		HW	PIC	Heterozygosity		HW	PIC	Heterozygosity		HW	PIC
	OH	EH			OH	EH			OH	EH		
FCA045	0.60	0.89	NS	0.88	0.67	0.80	NS	0.88	0.75	0.81	NS	0.77
FCA008	0.81	0.80	NS	0.80	0.70	0.80	NS	0.80	0.85	0.86	NS	0.84
FCA077	0.80	0.87	NS	0.88	0.70	0.83	NS	0.77	0.88	0.92	NS	0.90
FCA096	0.36	0.82	S	0.82	0.57	0.90	S	0.76	0.67	0.80	NS	0.82
Average	0.64	0.84		0.83	0.66	0.83		0.80	0.81	0.82		0.85

(S - significant; NS - not significant).

and 11 for *L. wiedii*. The existence of exclusive alleles for each species was observed. The difference among alleles was probably due to extensive inter-specific biologic diversity resulting from the mutation of microsatellite markers.

It has been suggested that differences in the mutation rate for individual microsatellite loci are determined by the wide heterozygosity levels (Menotti-Raymond and O'Brien, 1995; Menotti-Raymond et al., 1999). The species that showed the highest heterozygosity average for the four loci was *L. wiedii* (0.81), and the lowest level (0.64) was observed in *L. pardalis* (Table 3). The difference between observed heterozygosity and expected heterozygosity was significant in *L. pardalis* for the FCA096 locus, showing that this population was not in Hardy-Weinberg equilibrium for this locus. The heterozygosity levels from medium to high polymorphism indicated that there is high genetic variability among the species studied (Table 3). Medium to high heterozygosity levels observed within the species studied demonstrate that they have not lost their heterozygosity, even while living in captivity.

The degree of polymorphism observed in a microsatellite sequence is usually correlated with the length of the repeat unit. Microsatellites with more than ten dinucleotide repeats tend to be highly informative (Weber, 1990). According to DeWoody (1995), PIC values are useful to determine the extent of polymorphism of the marker for each locus, and therefore PIC values were calculated. Each of the four markers has PIC values higher than 0.5, being highly informative for genetic studies of *Leopardus*. PIC determination obtained from CERVUS analysis, for the three species, indicated values higher than 0.5 for all loci (Table 3). The PIC average values indicated a high efficiency rate of primers in characterizing the genetic variability of these species. Moreno et al. (2006) found, in other felids specimens (*Herpailurus yagouaroundi*, *Puma concolor* and *Panthera onca*), PIC values between 0.41 and 0.92 for the same microsatellite loci. In this study, it was found PIC values between 0.76 and 0.90, which indicate that in *Leopardus* these microsatellite loci show a high level of polymorphism, and they, therefore, seem to be suited for genetic studies of these species.

In conclusion, among the species analyzed, *L. wiedii* has the highest genetic variability for these microsatellite markers. The research proved that the genetic variability of these felid species, even in captivity, still exists. Molecular analysis of genetic structure and integration of ecology, history and feline reproduction could provide a greater comprehension of the factors to be considered in the management of threatened species.

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