

Identification of Microsatellite DNA Markers for the Giant Anteater *Myrmecophaga tridactyla*

J. E. GARCIA, L. A. VILAS BOAS, M. V. F. LEMOS, E. G. DE MACEDO LEMOS, AND E. P. B. CONTEL

From the Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14049-900, Ribeirão Preto, SP, Brazil (Garcia and Contel); Departamento de Biologia Aplicada à Agropecuária, Faculdade de Ciências Agrárias e Veterinárias, Campus de Jaboticabal, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane, km 5, 14870-900, Jaboticabal, SP, Brazil (Lemos); Departamento de Biologia Geral, Universidade Estadual de Londrina, P.O. Box 6001, 86051-990, Londrina, PR, Brazil (Vilas Boas); and Departamento de Tecnologia, Faculdade de Ciências Agrárias e Veterinárias, Campus de Jaboticabal, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane, km 5, 14870-900, Jaboticabal, SP, Brazil (de Macedo Lemos). Current address: Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina, P.O. Box 6001, 86051-990, Londrina, PR, Brazil (Garcia) and Departamento de Biologia Geral, Instituto de Biologia, Universidade Federal da Bahia. Rua Barão de Geremoabo, 40170-290, Salvador, BA, Brazil (Vilas Boas).

Address correspondence to J. E. Garcia at the address above, or e-mail: jegarcia@uel.br.

The giant anteater (*Myrmecophaga tridactyla*) is found from Belize and Guatemala to Paraguay and Argentina. Its conservation status is considered vulnerable by IUCN. Here we report the isolation and characterization of six microsatellite loci. Positive loci for $(GT)_n$ were isolated using a magnetic bead hybridization selection protocol. The number of alleles per locus as well as the heterozygosity and PCR conditions are described. These loci will be useful for studying population structure, genetic diversity, and paternity in *M. tridactyla* wild populations.

Introduction

The giant anteater (*Myrmecophaga tridactyla*) is found from Belize and Guatemala to Paraguay and Argentina and is probably extinct in Uruguay (Messias-Costa et al. 2001).

Phylogenetic relationships within Xenarthra were established by using Bayesian approach on DNA sequences (Delsuc et al. 2003, 2004). The monophyly of the Xenarthra order, as well as its three groups (vermitongues, leaf-eaters, and cingulates), were strongly confirmed by these data. The estimated time of separation between the Xenarthra order and boreoeutherians was of 103 ± 5 Myr (million years), and the divergence between the pilosa group (vermitongues and sloths) and cingulate (armadillos) was estimated in 65 ± 5 Myr. The separation between the two genera of anteaters (*Myrmecophaga* and *Tamandua*) was estimated in 10 ± 2 Myr. In turn *Cyclopes* genus is separated from *Myrmecophaga/Tamandua* by 40 ± 4 Myr (Delsuc et al. 2004).

Giant anteaters are considered vulnerable by the IUCN (Edentate Specialist Group 1996) especially because of the

loss of numbers and habitat fragmentation. Based on observation of the continuous growth of factors that potentially contributed to the decline of the populations of *M. tridactyla*, genetic studies of the remaining populations, specifically through the utilization of polymorphic markers (e.g., microsatellites, SNPs, etc.), will contribute to a better understanding in population dynamics such as migration, genetic structure, similarities between isolated populations, and the effects of habitat fragmentation. This knowledge, associated with other biological data, will be used in a specific action plan for the conservation of the remaining populations of this species (Garcia 2003).

Microsatellite markers have become valuable tools for investigating a wide range of evolutionary questions (Beaumont and Bruford 2000). The main areas in which they have been used are related to studies of population history and phylogeography, for detecting population bottlenecks and inbreeding, social structure, gene flow, and genetic structure of endangered populations (Pastor et al. 2004; Randi et al. 2004).

Our goal was to report the first isolation and characterization of a panel of $(GT)_n$ microsatellite markers that can be used in population and individual assessments in giant anteaters.

Material and Methods

Two unrelated animals were used to obtain genomic DNA isolated from the pooled white blood cell pellets. The DNA was digested with *SAU3AI* and fragments were separated on

Table 1. Primers and characteristics of the 6 polymorphic microsatellite loci obtained from 15 free-living *Myrmecophaga tridactyla*

Locus name	GenBank accession no.	Repeat motif	Primers sequences	Product size (pb)	Alleles	Ho/He	T _a (°C)
MtriUSP 04	AF465633	(gt) ₉	5'gggtcagatatacctaattggg3' 5'tgtcttctttactcagtgctcc3'	158	4	0.62/0.64	61
MtriUSP 07	AF465636	(gt) ₄₂	5'aggaggataagattaggcag3' 5'tgtgtcctgtgaagtaatgg3'	274	2	0.35/0.37	61
MtriUSP 11	AF465634	(gt) ₁₅	5'tgtctctgtttagggcttctc3' 5'tcaccctcattggagcttc3'	174	5	0.78/0.81	59
MtriUSP 13	AF465632	(gt) ₁₄	5'ctgctcaggtaacattcc3' 5'tggtaaagaatgaggtc3'	223	6	0.58/0.60	57
MtriUSP 17	AF465637	(gt) ₂₀	5'cccagaatgaacttacttg3' 5'actggcaactgtgttg3'	191	4	0.54/0.56	55
MtriUSP 20	AF465635	(gt) ₁₄	5'cttctctatctccctg3' 5'ctatatgcttgccttgg3'	157	5	0.79/0.82	55

2% LMP agarose gels. Those in the size range from 200 to 1,000 bp were eluted using the phenol chloroform protocol (Sambrook et al. 1989). Fragments recovered were ligated into synthetic adapters, denatured, and hybridized against biotinylated (AC)₁₀ oligonucleotides. Oligofragment hybrids were selectively separated from the remaining DNA using streptavidin-coated paramagnetic beads (Streptavidin Magna-Sphere Paramagnetic Particles, Promega). The DNA fragments containing simple sequence repeats (SSRs) were amplified by polymerase chain reaction (PCR) using adapter-specific primer (Refseth et al. 1997). PCR products were ligated into pUC18 (Sure Clone Ligation Kit, Amersham) following the manufacturer's instructions and cloned into *Escherichia coli* DH5 α competent cells (Invitrogen, CA). Three hundred eighty-four recombinant clones were sequenced using bigdye-terminator fluorescent (Applied Biosystems, CA) and products were sequenced on an ABI 377 sequencer (Applied Biosystems, CA).

Primers complementary to sequences flanking the microsatellites motifs were designed using the software Gene Runner. PCR amplifications were carried out using a 10 μ l reaction volume using PTC-100 thermocycler (MJ-Research) with \sim 50 ng of genomic DNA samples as templates. Final amplification consisted of 10 pmol of each primer, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM MgCl₂, 200 μ M each dNTP and 0.5 U *Taq* DNA polymerase (Biotools, Spain). The thermal profile for PCR amplification was 94°C for 2 min, followed by 35 cycles of 30 s at 92°C, a specific primer annealing temperature of 1 min (see Table 1), 2 min at 74°C, ending with an extension of 10 min at 74°C. PCR products were analyzed in 20% denaturing polyacrylamide gel electrophoresis and silver stained (Sanguinetti et al. 1994). Designed primers were tested by PCR in a captive maintained true family (male, female, and two cubs from the Sorocaba Zoo, Brazil) to verify the Mendelian inheritance.

Polymorphism levels for each primer were evaluated by PCR amplification in 15 samples, 7 collected from animals captured at Emas National Park (Goiás State, 18° 18' S/ 52° 54' W) and 8 from road-killed specimens collected at highways in the states of São Paulo and Mato Grosso do Sul (Brazil). DNA collected from the road-killed and captured animals was extracted from muscle biopsies (Medrano et al. 1990). Heterozygosity for each locus was calculated using

TFPGA software (Miller 1997); linkage disequilibrium and Hardy-Weinberg equilibrium were verified using Genepop (Raymond and Rousset 1995). Cross-species amplification of all primers was tested in two other species of Xenarthra: *Tamandua tetradactyla* and *Euphractus sexcinctus*.

Results

Repetitive motifs were found at 75 (19.5%) from the 384 sequenced clones. Twenty-one sequences showed features that were compatible with primers' design. Six out of the 21 primers showed polymorphism, with the number of alleles varying from 2 to 6 (Table 1). All loci were in linkage disequilibrium ($p \geq .95\%$) and did not fit Hardy-Weinberg equilibrium. Observed heterozygosity varied from 0.35 to 0.79 (mean of 0.61).

Cross-amplification with *Tamandua* and *Euphractus* DNA showed products with expected size only at MriUSP04, MriUSP07, and MriUSP11 loci. However, sequencing of the fragments demonstrates that the repetitive motifs were substituted by nonrepetitive inespecific sequences.

Discussion

In the order Xenarthra there are few studies with microsatellites, and only one species, the nine-banded armadillo (*Dasypus novencinctus*), was analyzed using these markers. The polyembryony of this species was confirmed and the spacial distribution of brothers was traced in North America using microsatellites (Prodöhl et al. 1996). The same markers were used to verify the paternity and maternity in individuals of a local Florida population (Prodöhl et al. 1998). As far as the species *M. tridactyla* is concerned, this is the first report on specific microsatellite markers for the giant anteater.

As already pointed out in the Introduction, *M. tridactyla* is considered vulnerable by the IUCN especially because of habitat fragmentation, mainly due to the agricultural expansion in its native habitats. In Brazil, road accidents seem to be an important source for population decline, but there is no reference about the true impact of the highway accidents on the natural population status of this species. Road accidents, combined with the progressive loss of habitat, can contribute to a rapid decline of this species

(Garcia 2003). The microsatellites described here will be valuable tools for investigating a wide range of evolutionary and biological questions regarding the giant anteater.

Although the similarity in the size of amplified fragments, the lack of the repetitive regions in *Tamandua* and *Euphractus* is possibly associated with the evolutionary divergence time estimated for these species. Considering that the *Tamandua* is the genus most closely related to *Myrmecophaga* within the Xenarthra order, the markers described are not suitable for studies in other species of the order.

Acknowledgments

This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP, proc. no. 98/11707-6 and Fundação de Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto (FAEPA). We are grateful to Dr. José Mauricio B. Duarte for his important suggestions and to João Airton Boer, Michele Mantovani Gonçalves, Angela Talarico, Eliane Cristina da Cunha Alves and Irlan Leite de Abreu for their technical assistance. We also thank the “Parque Zoológico Municipal Quinzinho de Barros—Sorocaba/SP” for the samples and Mariângela Pereira Pinho for the anesthetic assistance.

References

Beaumont MA and Bruford M, 2000. Microsatellites in conservation genetics. In: Goldstein DB and Schlötterer C, eds., *Microsatellites, evolution and applications*. Oxford University Press, New York, pp 165–182.

Delsuc F, Stanhope MJ, and Douzery EJ, 2003. Molecular systematics of armadillos (Xenarthra, Dasypodidae): contribution of maximum likelihood and Bayesian analyses of mitochondrial and nuclear genes. *Mol Phylogenet Evol* 28(2):261–275.

Delsuc F, Vizcaíno SF, and Douzery JP, 2004. Influence of tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. *BMC Evol Biol* 4(1):11.

Edentate Specialist Group 1996. *Myrmecophaga tridactyla*. In: IUCN, 2003 IUCN red list of threatened species. Available online at www.redlist.org (accessed October 18, 2004).

Garcia JE, 2003. Genetic studies in living Xenarthra. *Sencken Biol* 83(1):19–26.

Medrano JF, Aasen E, and Sharrow L, 1990. DNA extraction from nucleated red blood cells. *Biotechniques* 8(1):43.

Messias-Costa A, Beresca AM, Cassaro K, Diniz LSM, and Esbérard C, 2001. Order Xenarthra (Edentata) (Sloths, Armadillos, Anteaters). In: Fowler ME, ed., *Biology, medicine, and surgery of South American wild animals*. Iowa University Press, Ames, pp 238–255.

Miller MP, 1997. Tools for population genetic analysis, version 1.3. Department of Biological Sciences, Northern Arizona University, Flagstaff.

Pastor T, Garza JC, Allen P, Amos W, and Aguilar AJ, 2004. Low genetic variability in the highly endangered mediterranean monk seal. *J Hered* 95(4):291–300.

Prodöhl PA, Loughry WJ, McDonough CM, Nelson WS, and Avise JC, 1996. Molecular documentation of polyembryony and the micro-spatial dispersion of clonal siblings in the nine-banded armadillo, *Dasypus novemcinctus*. *Proc Royal Soc London B* 263:1643–1649.

Prodöhl PA, Loughry WJ, McDonough CM, Nelson WS, Thompson EA, and Avise JC, 1998. Genetic maternity and paternity in a local population of armadillos assessed by microsatellite DNA markers and field data. *Am Nat* 151:7–19.

Randi E, Alves PC, Carranza J, Milosevic-Zlatanovic S, Sfougaris A, and Mucci N, 2004. Phylogeography of roe deer (*Capreolus capreolus*) populations: the effects of historical genetic subdivisions and recent nonequilibrium dynamics. *Mol Ecol* 13(10):3071–3083.

Raymond M and Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J Hered* 86:248–249.

Refseth UH, Fangan BM, and Jakobsen KS, 1997. Hybridization capture of microsatellites directly from genomic DNA. *Electrophoresis* 18: 1519–1523.

Sambrook J, Fritsch EF, and Maniatis T, 1989. *Molecular cloning: a laboratory manual*, 2d ed. Cold Spring Harbor Laboratory Press, New York.

Sanguinetti C, Dias-Neto E, and Simpson AJG, 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* 17(5):209–214.

Received January 12, 2004

Accepted March 25, 2005

Corresponding Editor: Robert Fleischer