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Microsatellite DNA Library for *Caiman latirostris*

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ABSTRACT New genetic markers were characterized for the broad-snouted caiman (*Caiman latirostris*) by constructing libraries enriched for microsatellite DNA. Construction and characterization of these libraries are described in the present study. One microsatellite marker was developed from a $(ACC\text{-}TGG)_n$ enriched microsatellite DNA library, and 12 microsatellite markers were developed from a $(AC\text{-}TG)_n$ enriched microsatellite DNA library. These markers were tested in wild-caught animals, and these tests resulted in ten new polymorphic microsatellites for *C. latirostris*. *J. Exp. Zool. (Mol. Dev. Evol.) 294:346–351, 2002.* © 2002 Wiley-Liss, Inc.

The broad-snouted caiman (*Caiman latirostris*) is a palustrine, medium-sized South American crocodylian. The geographic distribution of the species spreads over Northern Uruguay, Northeastern Argentina, Southeastern Bolivia, Paraguay, and South to Northeast Brazil, including the Parana and São Fransisco river basins and a great number of small, hydrographic basins of the Brazilian Atlantic shore. In latitude, the São Paulo state in Brazil comprises the central part of the range of this species' distribution. This means that populations of the broad-snouted caiman in São Paulo may represent a genetic link between the southern and the northern populations of the species.

Landscape anthropogenic changes have been altering habitat use and dispersal patterns of the species, causing either local extinction or colonization of new available habitats such as cattle pounds and small reservoirs (Verdade and Lavorenti, '90; Verdade, '98).

In an effort to understand possible metapopulation structure of the broad-snouted caiman, we initially conducted studies to evaluate the microgeographic variation, both in terms of cranial morphometrics (Verdade, '97a) and microsatellite DNA genetic analyses of the species in small wetlands of the Tietê river system in central-eastern São Paulo (Zucoloto, '98). Significant variation for both methods was found for sites separated even by distances of a few kilometers. However, these genetic analyses were limited by the lack of DNA markers specific for *C. latirostris*; only four of the microsatellite loci developed by Glenn et al. ('98) for *Alligator mississipiensis* could be scored in the species we were studying.

Herein, we describe the development of new microsatellite DNA markers specific for the broad-snouted caiman (*C. latirostris*). This is an important first step that should help to establish conservation strategies and contribute to an understanding of the structure of wild, remnant populations for this species. Regarding the former, captive colonies could be more efficiently managed by establishing individual pedigrees that would help keep inbreeding coefficient (and inbreeding depression) as low as possible. With respect to the latter, genetic studies of the behavioral ecology of remnant populations will allow assessment of mating systems and dispersal patterns of wild individuals, helping researchers to understand how the remnant populations use the landscape.

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MATERIALS AND METHODS

DNA extraction, Sau3A I restriction, and size selection

Genomic DNA of *C. latirostris* was isolated from freshly collected blood by standard Proteinase K digestion, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (Sambrook et al., '89). Total DNA was digested with *Sau*3A I, to a final volume of 50 μl as follows: 5 μl of 10 X *Sau*3A I buffer, 0.2 U/μl of *Sau*3A I, and 0.1 μg/μl of genomic DNA at 37°C for two hours. Fragments between 400 bp and 800 bp were selected and gel extracted with sephaglass (Amersham-Pharmacia, Piscataway, NJ).

Sau3A I adapters

The *Sau*3A I digested and size-selected DNA was linked to *Sau*3A I adapters using *T4 DNA ligase* according to the following conditions in a final volume of 120 μl: 200 μM adapters, 5–10 μg of DNA, 1 X *T4 DNA ligase* buffer, and 0.05 U/μl of *T4 DNA ligase*, followed by incubation for 16 hours at 12°C.

Enrichment procedure and cloning

An enrichment procedure adapted from previously developed protocols (Taramino and Tingey, '96; Rafalski et al., '96; White and Powell, '97) was used to select the DNA fragments containing putative microsatellite regions. Briefly, biotin-labeled oligonucleotide probes $(ACC)_8$, $(AC)_{12}$ were hybridized to the DNA at 55°C and then purified using streptavidin-labeled paramagnetic beads.

Microsatellite-enriched DNA was PCR-amplified using the short sequence *Sau*3A I adapter as primer and cloned into pUC18/*Sma* I plasmid using a SureClone kit (Amersham-Pharmacia). Transformed DH5α *Escherichia coli* competent cells were screened with $(ACC)_8$ and $(AC)_{12}$ labeled probes (ECL 3′ oligolabeling kit, Amersham-Pharmacia), respectively, according to manufacturer instructions, and positive clones were sequenced using the Big Dye terminator Kit under ABI prism 377 (PE Applied Biosystems, Foster City, CA).

Primer design and microsatellite nomenclature

Primer pairs were designed to flank microsatellite observing the following criteria: (1) a lack of tandem repeats and a distance of 10–60 base pairs in both directions from any microsatellite; (2) a size of 20–24 bases with 50–55% of G and C; (3) an annealing temperature between 45°C and 65°C with a maximum 4°C difference between the two primers of any single pair; (4) no primer dimer formation, either with itself or any other member of the pair; and (if possible) (5) 5′ and 3′ terminal bases that were either G or C.

The new microsatellite markers were named according to the scientific name of the organism, using the first letter of the genus in capitals and the two first letters of the species name not capped, followed by the Greek letter μ identifying the oligo as a microsatellite primer, plus the number representing the crescent numerical order. Thus, as an example, the first new microsatellite marker was named as *Cla*μ1.

DNA extraction for genotyping, PCR conditions, and product size measurement

Two to three individuals from wild populations of *C. latirostris* from the State of São Paulo were analyzed per microsatellite marker. Immobilization of animals was mechanical, without anesthetics or muscle relaxants (Verdade, '97b). Blood was collected by puncturing the dorsal branch of the superior cava vein, which runs along the interior of the vertebral column of large reptiles (Olson, '75). After collection, blood was stored in lyses buffer: 100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS (w/v) as in Hoelzel ('92). DNA from these samples was purified by CTAB and chloroform extraction followed by isopropyl alcohol precipitation.

Microsatellite loci were amplified under the following conditions: 94°C for 3 min followed by 30 cycles of 94°C for 45 sec, the appropriate annealing temperature (Table 1) for 1 min, and amplification at 72°C for 1 min and 15 sec. The samples were amplified in a 25 μl final volume of 1 X PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM of each primer, 0.02 U/μl Taq DNA polymerase, and 100 ng of DNA. Products were stored at 4°C until ready to be analyzed and scored.

The alleles for marker *Cla*μ1 (whose direct primers were 5′FAM labeled) were observed in ALF DNA sequencer (Amersham-Pharmacia). The alleles of the other new markers were detected in 3% agarose gel under ultra-violet light and their sizes were measured by the use of the program Kodak 1D Digital Science v. 3.0.2: Scientific Imaging System (Eastman Kodak Company, New Haven, CT), a system for analyses of scientific

images, Φx *Hae* III fragments as size standards. The observed size range for each locus in Table 1 is defined as the variation from minor to major bands appearing at genotypes of individuals sampled for each marker.

RESULTS AND DISCUSSION

Two enriched microsatellite libraries containing 384 clones each were constructed, one for probe $(ACC)_8$ and another for $(AC)_{12}$. The hybridization selection of $(ACC\text{-}TGG)_n$ library indicated 29 clones with repeat regions recognized by probe $(ACC)_8$, which means 7.55% of total library. These clones yielded one new marker (3.45% of selected clones), *Cla*μ1. From the $(AC\text{-}TG)_n$ library, 51 clones were selected (13.28% of total library). The 51 selected clones generated 12 new microsatellite markers (23.53%), nine redundant sequences (17.65%), ten bad sequences with confirmed microsatellites (19.61%), one sequence in which microsatellite position in the clone disabled primers design (1.96%), 14 sequences without microsatellites (27.45%), and five bad sequences where the presence of microsatellites was not determined (9.80%). In a screening with two to three animals per microsatellite locus, ten out of 13 total microsatellites (76.92%) showed polymorphism. Markers *Cla*μ11 and *Cla*μ13 did not amplify any specific product with PCR conditions applied on the current work and require additional optimization. An agarose gel illustrating characteristic polymorphism is presented in Fig. 1 for locus markers *Cla*μ4 to *Cla*μ13.

Information about all markers, including repeat motif, expected size, observed sizes, PCR conditions, and other characteristics, is presented in Table 1. Each microsatellite marker is classified

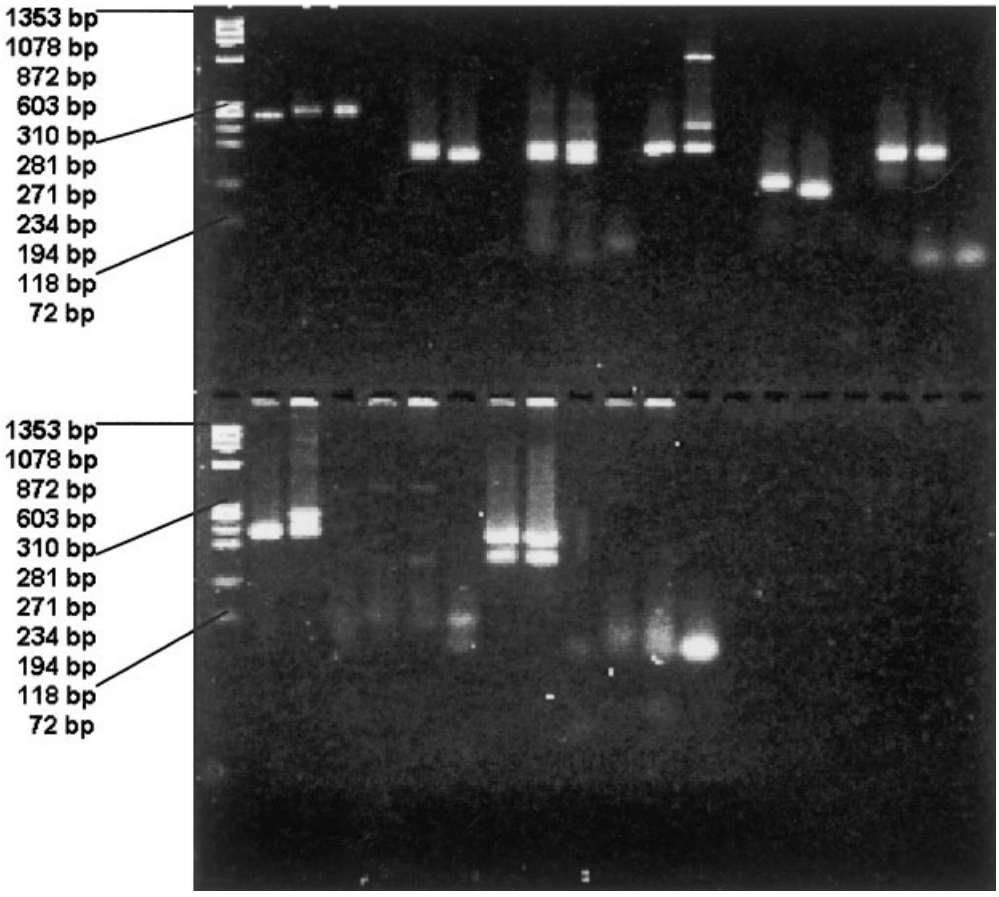

Fig. 1. Inspection of polymorphism in markers *Cla*μ4 to *Cla*μ13 at 3% agarose gel, Φx *Hae* III DNA ladder in lane 1, the samples of markers were distributed in other lanes being the last lane of each marker is the negative control, the distribution was: *Cla*μ4 from lanes 2–5, *Cla*μ5 from lanes 6– 8, *Cla*μ6 from lanes 9–11, *Cla*μ7 from lanes 12–14, *Cla*μ8 from lanes 15– 17, *Cla*μ9 from lanes 18–20, Φx *Hae* III DNA ladder in lane 21, *Cla*μ10 from lanes 22–24, *Cla*μ11 from lanes 25–27, *Cla*μ12 from lanes 28–30, *Cla*μ13 from lanes 31–34.

TABLE 1. Primer parameters and observed values for Caiman latirostris

Name	Length	Tm	gc%	Primer sequence 5′ to 3′	Expected size	Tandem repeat Classification as Weber ('90)	Observed allele size range	Annealing (°C)	Cycles	Polymorphism
*Cla*μ1a	20	59.82	50.00	CAT AAA CCT TGG GGC TGG TA	276	$(AC)_{46}$	232–288	60	30	Yes
*Cla*μ1b	20	60.26	50.00	ACA CCA TGC TGA AAG AAG CC		Perfect				
*Cla*μ2a	20	58.23	50.00	CCT TCA GGA CCC ACT TTC TT	205	$(GA)_{16}$, $(GT)_{10}$	283–293	55	30	Yes
*Cla*μ2b	20	60.43	50.00	CGA ATC CCT CTT CCC AAA CT		Compound				
*Cla*μ3a	20	59.24	50.00	TGA CTT CCA GCT ATG GGT GA	377	$(TG)_{18}$, $(TGTA)_6$	373–441	55	30	Yes
*Cla*μ3b	20	59.73	50.00	GTT CAA ACC AGC AGT GAC CA		Compound				
*Cla*μ4a	18	53.23	50.00	CCA TGA GT GCT TGA ACA G	269	$(AC)_{15}$	279–293	55	30	Yes
*Cla*μ4b	20	53.07	50.00	CAG TCT CTA CCC AAG ATG TG		Perfect				
*Cla*μ5a	20	59.83	50.00	GCG TAG ACA GAT GCA TGG AA	203	$(AC)_{16}$, $(AC)_{22}$	221–229	55	30	Yes
*Cla*μ5b	20	58.26	50.00	CAG TCT GAA GCT AGG GCA AA		Imperfect				
*Cla*μ6a	20	58.79	50.00	GAA ATA TGG GAC AGG GAG GA	229	$(AC)_{17}$	219–231	55	30	Yes
*Cla*μ6b	20	59.45	50.00	GGT TGG CTG CAT GTG TAT GT		Perfect				
*Cla*μ7a	20	60.94	55.00	CGG GGT CTT GGT GTT GAC TA	201	$(TG)_{12}$, $(TG)_6$	229–273	55	30	Yes
*Cla*μ7b	20	60.09	55.00	CGG GAC CAG GAG CTG TAT AA		Imperfect				
*Cla*μ8a	20	58.29	50.00	CAG CCA CTG AAG GAA TTG AC	123	$(AC)_{26}$	183–195	55	30	Yes
*Cla*μ8b	22	58.60	50.00	CAC ATA CCT GAC CCA GCT TAT C		Perfect				
*Cla*μ9a	20	58.52	50.00	ACA GGG GAA AAG AAG AGC TG	163	$(TG)_{11}$, $(CG)_5$	225	55	30	No
*Cla*μ9b	20	59.15	50.00	AAA ATC CCC CAC TCT TAC CC		Compound				
*Cla*μ10a	20	57.42	50.00	TGG TCT TCT CTT CGT GTC CT	220	$(CA)_{12}$, $(CT)_{19}$	266–292	55	30	Yes
*Cla*μ10b	20	57.67	50.00	ATG AGC CCC TCT ATG TTC CT		Compound				
*Cla*μ11a	20	59.57	50.00	GGC TCG TAT GTT GTG TGG AA	341	$(CA)_9$, $(TG)_7$, $(TG)_{19}$, $(TG)_4$		55	30	Undetermined
*Cla*μ11b	22	60.25	50.00	GCC AGA ATA GCA GGT TGA TAG C		Compound				
*Cla*μ12a	20	61.31	50.00	AAA AAG CCT CGA CTG GCT GT	205	$(TG)_5$, $(TG)_6$, $(TG)_3$	221–251	55	30	Yes
*Cla*μ12b	20	60.08	50.00	CAC AGG GAA AGG TTT CTG GA		Imperfect				
*Cla*μ13a	19	57.09	52.63	AGC CTA GAG CCG AAT TCA C	161	$(TG)_4$, $(TG)_9$, $(TA)_4$		55	30	Undetermined
*Cla*μ13b	24	57.37	50.00	CTG AGA GAG TAC TGA GTC ATC AGG		Compound				

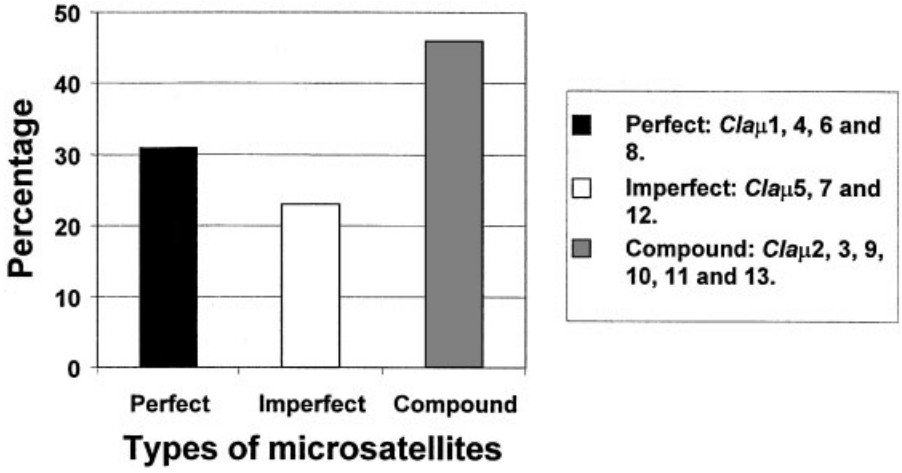

Fig. 2. Percentages of each of the classes of microsatellite markers using the system of Weber ('90) for the 13 loci characterized for *Caiman latirostris*.

as one of three types according to Weber ('90): (a) perfect sequences without any interruptions in the repeats; (b) imperfect sequences with one or more interruptions in the run of repeats; and (c) compound sequences with a run of CA or GT repeats, adjacent to a block of short tandem repeats of different sequence, usually another type of nucleotide repeat (GA, TC, or AT repeats, Fig. 2). The minor number of tandem repeats for the perfect, imperfect, and compound microsatellites were 15, 3, and 4, respectively, and the major number of tandem repeats were 46, 22, and 19 (Table 1).

The enrichment procedure that we employed was successful, and the number of microsatellites per library obtained in our study was comparable to those reported by Glenn et al. ('98) for *A. mississipiensis*, by FitzSimmons et al. (2001) for *Crocodylus porosus* and *C. johnstoni*, and by Brondani et al. ('98) for *Eucalyptus grandis* and *E. urophylla*.

None of the markers developed from the $(ACC\text{-}TGG)_n$ library presented tri-nucleotide repeats. The motif in *Cla*μ1 that came from this library was $(AC)_{46}$, and this may indicate that tri-nucleotide repeats are not common in Alligatoridae, as previously reported by Glenn et al. ('98). In future studies, we will further characterize the primers obtained by verifying segregation, heterozygosity, and PIC level.

The development of these new microsatellite markers significantly increases our capability to assess broad-snouted caiman genetic diversity. These new markers will improve exclusion power for maternity tests and the resolution of parentage identification among wild individuals in *C. latirostris* (Zucoloto, '98).

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