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Isolation and characterization of new microsatellite markers for application in population genetic studies of *Caiman latirostris* and related species

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Abstract. Wild populations of *Caiman latirostris* are subject to sustainable use programs in Argentina, becoming a species with important impact in the regional economy, based in their skin and meat. Genetic studies are fundamental to acquire information on important parameters for conservation and management, which may be obtained from analysis of molecular markers. Some microsatellites have been previously isolated in this species, but due to some difficulties in using them, we obtained new ones using Next Generation Sequencing approach. This study reports eight new microsatellites for *C. latirostris* and tests their utility in a related species, *Caiman yacare*, with successful application in population genetics and mating systems studies. In addition, we shared data about a novel and fast bioinformatics tool to find microsatellites and to design their corresponding primers.

Keywords: conservation genetics, crocodilians, SSRs, sustainable use.

The Broad-snouted caiman (*Caiman latirostris*) is one of two crocodilian species cited for Argentina. It has a wide geographic distribution including diverse aquatic environments of the Paraná River basin, which usually are shallow water and vegetated marshes (Larriera, 1992). As it is an opportunistic carnivore that occupies the top of trophic nets, plus the fact that for the local inhabitants it is considered a charismatic species with a strong presence in the regional culture, could play the role of a flag species in local ecosystems, whose viability guarantees the whole system conservation.

Caiman latirostris populations decreased in number due to a strong hunting pressure and habitat loss during the 70s and 80s, and that fact motivated the creation in 1990 of a management program based on a "ranching system" to increase wild populations' density. The "Proyecto Yacaré" (Gob. de Santa Fe/MUPCN) started in 1990 harvesting wild eggs for captive rearing. Since then, this program has been very successful; and taking into account the population numerical increase verified by systematic monitoring, in 1997 *C. latirostris* in Argentina is listed in the Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Larriera, Imhof and Siroski, 2008). This legal frame allows the commercial use of *C. latirostris* products under strict controls (Larriera, 1998). Due to the high quality of its skin and meat it turns into a species of great interest, representing a regionally important economic activity that acts as a direct incentive for habitat conservation, through the local community involvement. Although the com-

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mercial success of sustainable use, there is little information about population genetic structure of *C. latirostris* throughout its vast distributional area, and most of these data were reported by our group (Amavet et al., 2007, 2009).

Genetic studies using molecular markers are fundamental to acquire information on population structure, patterns of dispersal and gene flow, hybridization, among other parameters important for conservation and management. In a species with multipaternity evidence as *C. latirostris* (Amavet et al., 2008, 2012), microsatellite markers are also the best choice for mating system characterization.

Some microsatellite markers have been isolated previously for *C. latirostris* (Zucoloto, Verdade and Coutinho, 2002) but our experience showed that most of them are not easy to use. These markers are difficult to amplified and were optimized using buffers of specific composition (different Mg^{2+} concentrations and pH), from PCR Optimizer Kit – Invitrogen® (Zucoloto et al., 2006), increasing costs and making it difficult the methodology. We tested these microsatellites in previous works (Amavet et al., 2008, 2012) but of the 13 published markers only 6 could be optimized. It is known that to obtain robust data it is better to analyze many markers as possible (ten at least) and Next Generation Sequencing (NGS) approaches allows us to develop new markers fast and cost effective way. We reported here eight new microsatellite markers for *C. latirostris*, suitable to be applied successfully in population genetics and mating systems studies, which cross-amplified in a related species: *Caiman yacare* (Hrbek et al., 2008). In addition, we shared the data of a novel and fast bioinformatics tools to find microsatellites and to design their corresponding primers.

Microsatellite loci were isolated by partial sequencing of the genome employing NGS methods. Total genomic DNA was isolated from 20 mg of skeletal muscle tissue of *C. latirostris* obtained in Santa Fe province, using a commercial kit (AccuPrep® Genomic DNA Extraction Kit from Bioneer) following the manufacturer's instructions. The extract was resuspended in buffer TE to obtain a concentration of 500 ng/μl.

Ratios of light absorbance at 260/280 nm and at 260/230 nm were determined to be 1.80 and 0.63, respectively, using a Gene Quant II (Pharmacia, Biotech) spectrophotometer. Production of enriched DNA libraries was performed following protocols of Rapid Library Preparation Method of Roche® 454 (http://454.com/my454/) which were conducted by Institute of Agrobiotechnology of Rosario (INDEAR, CONICET-BIOCERES, www.indear.com) staff. The pyrosequencing of libraries (1/16 titanium plate) was performed according to the provisions of the Sequencing Method Manual Titanium Roche® 454 series sequencer, also by INDEAR staff.

From the partial sequences of genome, contigs were constructed by INDEAR and then, microsatellite were searched and their corresponding primers were designed using *FullSSR* software (http://sourceforge.net/projects/fullssr/?source=directory), a software created by graduate students of Bioinformatics to perform both tasks together. The obtained results were then corroborated using MISA® (Microsatellite Identification Tool) and Primer3® 1.1.4 (Rozen and Skaletsky, 2000), both traditional programs for microsatellite discovery and design of primers, respectively. A total of 24 concatenated sequences of between 111 and 2048 bp length, containing microsatellite motifs with free flanking regions were found employing these software programs.

All primers pairs were analyzed with the DNASTAR software and Oligo Analyzer 3.1 (IDT)®, studying their hairpin properties, self-dimer or hetero-dimer possibilities and PCR conditions.

Analysis of polymorphism and genetic diversity were conducted with samples obtained from 20 *C. latirostris* collected from 4 sampling sites of Santa Fe province (Argentina) and 1 site of Corrientes province; and 11 *C. yacare* specimens collected from 2 sampling sites of Corrientes province.

Genomic DNA was extracted from blood as described by Amavet et al. (2012). For amplification of these new eight specific microsatellites of *C. latirostris* the following conditions were used: PCR were carried out in a final volume of 15 μl with 1× standard buffer, 1 mM each of the fluorescent label forward and unlabelled reverse primer, 2 mM MgCl, 20 mM dNTP mix, 0.5 U *Taq* DNA polymerase (Invitrogen® for *Cl* 811, *Cl* 58 and *Cl* 315 and PB-L® for other markers) and 50 ng of DNA. A negative control containing all reagents except DNA was included in each set of PCR reactions performed.

Amplifications were performed in a thermocycler with gradient Bioer Life Express® of 96 wells. All programs include 30 cycles with denaturation step at 94°C for 1 min, 1 min annealing at optimum temperature for each marker (see table 1) and extension at 72°C from 1.15 min at each iterative cycle. Besides, each amplification program used an initial denaturation of 94°C for 4 min and a final extension at 72°C for 30 min. The genotypes of each marker samples were obtained by genotyping service request to the Unidad de Genómica-Instituto de Biotecnología-CICVyA – CNIA-INTA (Argentina) and Macrogen Inc. (Korea).

PCR products were scored for length using LIZ500 as the internal lane-size standard. Fragment lengths were assigned

Table 1. Designation, primer sequences, repeat motif, annealing temperatures (T_a), product size range, and GenBank accession numbers of the new specific markers. Diversity parameters for *C. latirostris* and *C. yacare*: allele number (N_a), observed heterozigosity (H_o), and expected heterozigosity (H_e). X: denotes the absence of amplification.

Locus	Primer sequence (5′-3′)	Repeat motif	T_a (°C)	Size range (pb)	GenBank accession no.	*Caiman latirostris* ($n = 20$)			*Caiman yacare* ($n = 11$)		
						N_a	H_o	H_e	N_a	H_o	H_e
Cl 058	F:CTCTCGGCAGAAGCTACTGG R:AATGAATGCGCGTGTCTGTG	$(AC)_6$	60	370-400	KP849485	3	0.40000	0.61053	X	X	X
Cl 100	F:ACGACTTGGAGGTCTTGGTG R:GGAAGGTGCAGGAGACTCAC	$(GCT)_6$	56	460-520	KP849486	8	0.55556	0.75163	2	0.33333	0.33333
Cl 236	F:TGAACCAGATGCCAGTGGAC R:TCCAACTGATCGCTGTCTCTG	$(GA)_6$	58	380-420	KP849487	2	0.00000	0.50526	2	0.00000	0.53333
Cl 279	F:AAGAGCATGATGTGTGCCAA R:CCTGCACTGATCTCCTGGAA	$(GT)_7$	58.5	170-230	KP849488	2	0.05882	0.45098	8	1.00000	0.95556
Cl 309	F:TCTAAGATTCCAGCCTTATTCTCTG R:ACAATCACAGGAAGAGCAGCA	$(TC)_6$	64.2	170-210	KP849489	6	0.31250	0.63306	2	0.50000	0.50000
Cl 315	F:GCCTTCCAGCAACAATCTGC R:GTGACTGAGGTACTGGTAGGC	$(AT)_6$	57	330-370	KP849490	5	0.28571	0.75824	3	0.50000	0.83333
Cl 567	F:CCTTACAGAGTGCGCCTGTT R:GTATGGTCTCTGCCACACGT	$(GCT)_8$	64	190-230	KP849491	5	0.88889	0.77124	3	1.0000	0.83333
Cl 811	F:AGCAGAGAGGATGGGGACT R:CCCTTCCTTATCTTAAATTCCAG	$(GA)_{10}$	66	60-100	KP849492	9	0.66667	0.83678	6	0.80000	0.8444

to allelic classes with Peak Scanner® 1.0 Software (Applied Biosystems).

Genetic diversity among individuals was quantified by the number of alleles per locus, allele size range, observed heterozygosity (H_o) and expected heterozygosity (H_e) for each species using Arlequin 3.5 (Excoffier, Laval and Schneider, 2005). We use MICRO-CHECKER software (van Oosterhout et al., 2004) to detect null alleles.

A total of 131 microsatellites were found in 815 *C. latirostris* contigs built from 25 000 reads. The software avoided repeated SSRs (simple sequences repeats), and those very short, imperfect or compound. There are previous studies using NGS in amphibians (Drechsler et al., 2013; Wang et al., 2013) and reptiles (Castoe et al., 2010; Geser et al., 2013) in which the authors express the advantages of NGS, such as reducing cost and time through more efficient microsatellites obtaining. Our results are similar to those reported by other authors working in reptiles regarding the number of microsatellites designed (Geser et al., 2013) employing NGS approach.

Microsatellites obtained were analyzed to select those whose flanking regions could be optimal for designing suitable primers. From the 131 microsatellites sequences, 16 microsatellites were selected and a primer pair was designed for each. Eight of these markers successfully amplified DNA samples of *C. latirostris* and seven markers in *C. yacare* samples demonstrating its potential to cross-species amplification, agreeing with others authors working in crocodiles (Miles et al., 2009b; Bashyal et al., 2014).

All of them proved to be polymorphic and have a product of analyzable size, ranging between 74 bp and 520 bp. The general characteristics of these new markers and population diversity parameters are shown in table 1.

The eight novel microsatellite loci for *C. latirostris* and *C. yacare* are suitable markers for population genetics studies. The number of alleles per locus ranged from 2 to 9. H_o levels ranged from 0.000 to 1.0000 and H_e levels ranged from 0.33333 to 0.95556. Null alleles were not detected. Among the 131 microsatellites sequences found, only the 6% were proven suitable for population genetics analysis. Drechsler et al. (2013) stated that normally NGS approaches result in tens of thousands of sequence reads, which are expected to lead to a large amount of suitable microsatellite loci. However, the correlation between the initial number of sequence reads obtained and the number of usable polymorphic microsatellite loci may be low as the number of potentially amplifiable loci is negatively influenced by many factors including sequence read quality, motif length, and the presence, quality, and necessary length of the primer design region, in addition to the amplification success and confirmed polymorphism of loci across the studied species.

These data also reflect and reinforce the need of update, search and design new markers for this scarcely studied species. While there is currently a project of sequencing the genomes of three species of crocodiles (St John et al., 2012), microsatellite markers remain useful tools for population genetics and phylogeography studies (Frankham, 2010; Dawson et al., 2013) which is the focus of our research group.

Other researchers working in crocodiles have developed microsatellites by means of traditional methods using enriched genomic libraries (Miles et al., 2009a; Oliveira et al., 2010) but that methodology implies a lot of time and costs.

The employments of these new microsatellites expands the pool of available specific markers for the *Caiman* genus and, by this way, could extend the robustness of complete genetics population studies and mating system analyses. According to our results, these eight novel markers can be used as new molecular genetics tools in *C. latirostris* and *C. yacare*, and probably also in other related species. Besides, these new microsatellites constitute an important contribution to the genetic characterization of these crocodilian species particularly useful to assist in the adjustment of management and sustainable use programs to which their populations are subject.

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