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Short Communication

Cross-species microsatellite amplification in South American Caimans (*Caiman* spp and *Paleosuchus palpebrosus*)

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Abstract

Microsatellite DNA markers have been used to assess genetic diversity and to study ecological behavioral characteristics in animals. Although these markers are powerful tools, their development is labor intensive and costly. Thus, before new markers are developed it is important to prospect the use of markers from related species. In the present study we investigated the possibility of using microsatellite markers developed for *Alligator mississipiensis* and *Caiman latirostris* in South American crocodilians. Our results demonstrate the use of microsatellite markers for *Paleosuchus palpebrosus, Caiman crocodilus and Caiman yacare.*

Key words: SSR, STR, primers, crocodilians, Alligatorinae.

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Microsatellite DNA markers are simple sequence repeats (Tautz *et al.*, 1986) distributed along the genome (Litt and Luty, 1989) that have been used to assess genetic diversity and to study ecological behavioral characteristics such as mating system and dispersal pattern in reptiles and amphibians (Avise, 1994; Forstner and Forstner, 2002), including the timber rattlesnake *Crotalus horridus* (Villareal *et al.*, 1995), *Alligator mississipiensis* (Glenn *et al.*, 1996; Glenn *et al.*, 1998; Davis *et al.*, 2001a), and *Crocodylus spp.* (Dever *et al.*, 2001; FitzSimmons *et al.*, 2001; Verdade *et al.*, 2002).

Microsatellite markers are powerful research tools but their development is labor intensive and costly. Consequently, researchers have tried to use microsatellite markers developed for one species in another (Moore *et al.,* 1991). Microsatellite markers developed for *Alligator mississipiensis* have been successfully used in closely related Alligatorinae species (Glenn *et al.*, 1998); however, transference is more effective at the family or subfamily level (Glenn *et al.*, 1998; Zucoloto, 1998).

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All South American crocodilians (*Caiman spp.*, *Melanosuschus niger* and *Paleosuchus spp.*) belong to the Alligatorinae subfamily (King and Burke, 1989). To date the only Alligatorinae species with specific microsatellite markers currently developed are *Alligator mississipiensis* and *Caiman latirostris* (Glenn *et al.*, 1998; Zucoloto, 2002). Thus, transference of microsatellite markers to other Alligatorinae species could help conservation programs, genetic diversity studies as well as mating behavior and ecological studies

The present study tested the ability of microsatellite markers previously developed for *Alligator mississipiensis* (Glenn *et al.*, 1998) and *Caiman latirostris* (Zucoloto *et al.*, 2002) to amplify orthologous loci in the related South American Alligatorinae species *Caiman crocodilus*, *Caiman yacare* and *Paleosuchus palpebrosus*.

The blood samples used in this study were from the Brazilian crocodilians *P. palpebrosus, C. yacare and C. crocodiles.* Samples were obtained from crocodilians maintained at the Department of Zoology, São Paulo State University, Rio Claro, São Paulo (SP), Brazil (UNESP, Rio Claro, SP) and were stored at the Biotechnology laboratory, ESALQ, University of São Paulo, Piracicaba, SP, Brazil.

Blood was collected from three *C. crocodilus* specimens (Cc1, Cc2 and Cc3), three *C. yacare* specimens (Cy1, Cy2 and Cy3) and two *P. palpebrosus* specimens (Pp1 and Pp2) by puncturing the dorsal branch of the superior cava vein, which runs along the interior of the vertebral column of large reptiles (Olson, 1975). After collection, blood was mixed with lysis buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 0.5% SDS (w/v); 10 mM NaCl) (Hoelzel, 1992). The DNA from these samples was then purified by CTAB and chloroform extraction followed by isopropyl alcohol precipitation (Sambrook *et al.*, 1989).

The *Ami*μ8, *Ami*μ11, *Ami*μ13 and *Ami*μ20 markers developed for *Alligator mississipiensis* (Glenn *et al.*, 1998) and successfully used in *Caiman latirostris* (Zucoloto, 1998) and the *Cla*μ2, *Cla*μ3, *Cla*μ5, *Cla*μ6, *Cla*μ7, *Cla*μ8, *Cla*μ9, *Cla*μ10 e *Cla*μ12 markers (Table 1) developed for *C. latirostris* (Zucoloto *et al.*, 2002) were tested. The PCR conditions were: 60 mM Tris-HCl and 25 mM Ammonium sulfate and different concentrations of Mg^{2+} and pH (Table 1), 0.2 mM each dNTP, 0.4 μM each primer pair, 1U *Taq DNA polymerase* and 100 ng DNA in a 25 μl reaction. After 3 min at 94 °C, 30 or 35 cycles (depending on the individual microsatellite) were performed for 1 min at 94 °C, 1 min at the annealing temperature specific for each locus (Table 1), 2 min at 72 °C, and a final extension step of 10 min at 72 °C.

The PCR products were loaded onto 2% agarose gel containing a positive control consisting of the amplification product of the locus analyzed in individuals of *C. latirostris* under the conditions described in Zucoloto (2002), a negative PCR control, and a ϕx *Hae* III DNA size marker to estimate the size of the amplified products. Positive amplifications were loaded in a Megabace 1000 DNA sequencer for genotyping. Allele sizes were obtained using the Genotyper software (GE Healthcare).

Markers developed for *A. mississipiensis* (*Ami*μ8, *Ami*μ11*, Ami*μ13 *and Ami*μ20) presented amplification products and polymorphism for all species tested with the exception of the *Ami*μ8 marker that showed no amplification for *P. palpebrosus.* The *Cla*μ2, *Cla*μ3, *Cla*μ5, *Cla*μ6, *Cla*μ7, *Cla*μ8, *Cla*μ9, *Cla*μ10 and *Cla*μ12 markers developed for *C. latirostris* presented amplification products but the *Cla*μ3 and *Cla*μ12 markers showed nonspecific amplification products for *C. latirostris* (*Cla*μ3) and *Palpebrosus* (*Cla*μ12). Several loci were monomorphic in at least one species, while the *Cla*μ12 marker was monomorphic in all the species investigated, although it would be premature to assume that these loci are truly monomorphic for the species investigated because only a small number of specimens were used in our study. An exception is the *Cla*μ12

Table 1 - Primers and amplification conditions.

Locus	5'-3' sequence	Buffer	Annealing temperature (°C)	Cycles
*Ami*μ8	F:CCTGGCCTAGATGTAACCTTC R: AGGAGGAGTGTGTTATTTCTG	(1.5 mM MgCl2, pH 8.5)	55	30
*Ami*μ11	F:AAGAGATGTGGGTGCTGCTG R:TCTCTGGGTCCTGGTAAAGTGT	(1.5 mM MgCl2, pH 8.5)	64	35
*Ami*μ13	F:CCATCCCCACCATGCCAAAGTC R: GTCCTGCTGCTGCCTGTCACTC	(1.5 mM MgCl2, pH 8.5)	64	35
*Ami*μ20	F:TTTTTCTTCTTTCTCCATTCTA R:GATCCAGGAAGCTTAAATACAT	(2 mM MgCl2, pH 9.0)	58	30
*Cla*μ2	F:CCTTCAGGACCCACTTTCTT R: CGAATCCCTCTTCCCAAACT	(1.5 mM MgCl2, pH 8.5)	58	30
*Cla*μ3	F:TGACTTCCAGCTATGGGTGA R: GTTCAAACCAGCAGTGACCA	(2.5 mM MgCl2, pH 8.5)	54	35
*Cla*μ5	F:GCGTAGACAGATGCATGGAA R:CAGTCTGAAGCTAGGGCAAA	(2 mM MgCl2, pH 9.0)	55	30
*Cla*μ6	F:GAAATATGGGACAGGGAGGA R: GGTTGGCTGCATGTGTATGT	(2 mM MgCl2, pH 9.5)	58	30
*Cla*μ7	F:CGGGGTCTTGGTGTTGACTA R: CGGGACCAGGAGCTGTATAA	(2 mM MgCl2, pH 9.0)	58	30
*Cla*μ8	F: CAGCCACTGAAGGAATTGAC R: CACATACCTGACCCAGCTTATC	(2 mM MgCl2, pH 9.0)	55	30
*Cla*μ9	F:ACAGGGGAAAAGAAGAGCTG R: AAAATCCCCCACTCTTACCC	(1.5 mM MgCl2, pH 8.5)	60	35
*Cla*μ10	F:TGGTCTTCTCTTCGTGTCCT R:ATGAGCCCCTCTATGTTCCT	(1.5 mM MgCl2, pH 8.5)	60	35
*Cla*μ12	F:AAAAAGCCTCGACTGGCTGT R: CACAGGGAAAGGTTTCTGGA	(1.5 mM MgCl2, pH 8.5)	55	30

marker, which showed no polymorphism in *C. latirostris* even when more than 90 individuals were tested (Zucoloto *et al.*, 2002). An interesting observation was that we found that although *Cla*μ3 gave poor amplification results in *C. latirostris* it worked well in *C. crocodilus*, *C. yacare* and *P. palpebrosus*.

Despite some exceptions, allele sizes for *C. crocodilus* and *C. yacare* were in agreement with the size range observed for *C. latirostris* by Zucoloto *et al.* (2002) (Table 2). The *Ami*μ8 marker showed no PCR amplification product for *P. palpebrosus* and allele sizes for *C. crocodilus* and *C. yacare* were out of the range of those observed for *C. latirostris* (Table 2). Amplification for *P. palpebrosus* diverged from that observed for the other species, as can be observed in Table 2 for the *Ami*μ13, *Ami*μ20, *Cla*μ3, *Cla*μ5, *Cla*μ6, *Cla*μ7, *Cla*μ8 and *Cla*μ10 markers.

The efficiency of heterologous amplification observed in this study was 100% among the caimans and 84.6% between *C. latirostris* and *P. palpebrosus* (Table 2). These results were to be expected considering the evolutionary distance between the species (Primmer *et al*, 1996).

This study supplied the first set of data showing heterologous amplification of microsatellites for *C. crocodilus, C. yacare and P. palpebrosus.* Future studies with larger sample sizes are necessary to establish if the markers show Mendelian segregation and determine polymorphism information content (PIC) in the '*caiman* complex'. Once these markers are fully characterized they may be able to contribute to the evaluation of genetic diversity, conservation efforts and the elucidation of possible genetic flow between *C. crocodilus crocodilus* and *C. crocodilus yacare*.

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Table 2 - Sample genotypes with alleles size in base pairs.

Locus	Allele range in *Caiman latirostris**	*Caiman crocodilus*			*Caiman yacare*			*Paleosuchus palpebrosus*	
		Cc1	Cc2	Cc3	Cy1	Cy2	Cy3	Pp1	Pp2
*Ami*μ8	115-117	101/101	101/101	101/101	101/113	101/101	101/113	NP	NP
*Ami*μ11	223-249	229/229	229/237	223/229	229/237	229/237	229/229	229/239	223/237
*Ami*μ13	228-272	272/272	252/252	252/252	252/252	248/272	272/276	232/234	232/234
*Ami*μ20	106-164	142/156	142/156	170/170	160/160	164/164	164/164	156/156	124/126
*Cla*μ2	195-241	171/171	171/171	171/171	171/171	173/173	173/173	173/173	173/173
*Cla*μ3	NS	391/391	331/391	339/339	391/391	333/333	333/333	387/387	387/387
*Cla*μ5	161-199	223/235	195/199	161/201	219/243	237/243	235/249	167/167	167/167
*Cla*μ6	155-227	221/221	247/247	247/247	235/247	247/247	247/247	223/227	223/225
*Cla*μ7	181-277	183/183	187/187	183/213	181/183	183/183	163/163	163/163	155/159
*Cla*μ8	101-235	095/095	097/109	109/109	095/095	095/095	095/095	099/099	099/099
*Cla*μ9	161-179	157/163	163/165	157/163	161/165	163/165	161/165	161/161	161/161
*Cla*μ10	216-258	208/212	214/216	214/216	208/212	208/212	208/212	216/222	216/216
*Cla*μ12	207	207/207	207/207	207/207	207/207	207/207	207/207	NS	NS

*Data from Zucoloto (2002), with at least 90 specimens.
NP = No PCR product; NS = Non specific bands.

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