PROJECT REPORT

Determinación de sistemas de apareamiento en yacaré overo mediante marcadores moleculares. Aportes al uso sostenible.
(*Mating system determination on broad-snouted caiman using molecular markers. Contributions to the sustainable use)*

Genetic studies of wild populations have become an important issue for their conservation, management and sustainable use together with habitat conservation. *Caiman latirostris* (broad-snouted caiman) is an important species in wetlands of northeast Argentina, with commercial value at both national and international levels. Biological knowledge of its populations includes studies of the mating system in the wild.

This project has the goal of study reproductive characteristics and analyze the existence of multiple paternity behavior in *C. latirostris* wild populations. I have used primers developed for amplifications of microsatellite markers, invaluable tools in the study of variability and mating system.

Study sites and sample collection

A total of 8 families of *C. latirostris* were sampled from eight different localities of Santa Fe province, Argentina: Estancia El Lucero -29° 54' S 60° 50' O-, Estancia Piedras -29° 58' S 60° 44' O-, ciudad de Vera -29° 25'S 60° 12'O-, Lago artificial Granja La Esmeralda -31° 35'S 60° 41'O-, Reserva El Fisco -30°11'S, 61°0' O-, Estero Lote 114 -30° 42' S 60° 17' O-, Cañada Ombú -28°40'S 60° 4' O-, Flores -28°19'S 59° 57 O'-. Each family consisted of the nest-guarding *C. latirostris* female and the hatchlings of each nest.

Nest-guarding *C. latirostris* females were captured, measured and weighed. Blood samples of these females were obtained by puncture of internal jugular vein near the cervical vertebra (Tourn et al., 1993) and diluted (1:10) in lysis buffer (Longmire et al., 1988) for long term storage at room temperature according to White and Densmore (1992). Eggs from the nests were collected for artificial incubation at 31.5 °C, with 95% relative humidity, at "Proyecto Yacaré" facilities in Santa Fe City. Five days after hatching, between 9 and 16 hatchlings were randomly collected from each family. A 0.5 ml blood sample of was obtained from each hatchling using the same method as for adult females. DNA was isolated as described by Murray and Thompson (1980).

Samples obtained at this stage were analyzed together with four *C. latirostris* families previously sampled (Amavet et al., 2008).

Microsatellite genotyping

Four microsatellite primer pairs developed by Zucoloto et al. (2002) for *C. latirostr*is were used: *Cla*μ 2, *Cla*μ 6, *Cla*μ 9, *Cla*μ 10 (Table 1):

Primer	**5'-3' sequence**
*Cla*μ 2a	CCT TCA GGA CCC ACT TTC TT
*Cla*μ 2b	CGA ATC CCT CTT CCC AAA CT

*Cla*µ 6a	GAA ATA TGG GAC AGG GAG GA
*Cla*µ 6b	GGT TGG CTG CAT GTG TAT GT
*Cla*µ 9a	ACA GGG GAA AAG AAG AGC TG
*Cla*µ 9b	AAA ATC CCC CAC TCT TAC CC
*Cla*µ 10a	TGG TCT TCT CTT CGT GTC CT
*Cla*µ 10b	ATG AGC CCC TCT ATG TTC CT

Table 1: 5'-3' sequences of the used primers.

PCR conditions were those described by Zucoloto et al. (2002) with modifications as follows: amplifications were carried out in a final volume of 15 µl with 1X of buffer; 1.2 µl of each primer (0.1 nM/ µl); 1X of MgCl (except *Cla*µ 10: 0.75 µl); 0.6 µl dNTP mix (200 µM) for *Cla*µ 2 and *Cla*µ 6, and 1µl dNTP mix for *Cla*µ 9 and *Cla*µ 10; 0.06 µl *Taq* DNA polymerase (5 U/ µl) from PBL for *Cla*µ 2 and *Cla*µ 6, and 0.2 µl *Taq* DNA polymerase for *Cla*µ 9 and *Cla*µ 10; and 50 ng of genomic DNA. A negative control containing all reagents except genomic DNA was included in each set of reactions.

PCR reactions were performed in a MPI ® thermal cycler. Amplifications were conducted using annealing temperatures between 55 ºC to 65 ºC and different cycling programs (Table 2).

Microsatellite	Step 1	Step 2	Step 3
*Cl*aµ 2	30 cycles (1 min) 64° C		
*Cl*aµ 6	10 cycles (1 min) 65° C	10 cycles (1 min) 63° C	25 cycles (1 min) 60° C
*Cl*aµ 9	15 cycles (1 min) 64° C	20 cycles (1 min) 60° C	
*Cl*aµ 10	35 cycles (1.30 min) 66° C		

Table 2. Cycles and annealing temperatures number for DNA amplification using the different primers.

Successful amplification was confirmed by separation on 2 % agarose gels in TBE 0.5 X buffer (0.89 M Tris, 0.89 M boric acid and 0.11 M EDTA, pH 8.3). Gels were stained with Gel Green® (Biotium) and analyzed on Dark Reader® transilluminator. PCR products were analyzed by electrophoresis on 10% polyacrylamide gels running at 2200 V and 75W, and stained with $AgNO_3$ solution (Bassam et al., 1991). Molecular weights in base pairs (bp) were estimated using a 100 bp ladder (PB-L). We also used CTT Allelic Ladder Mix, FFv Allelic Ladder Mix and STR III Allelic Ladder Mix (Gene Print Silver STR III System) from Promega, as an additional method for estimating molecular weight.

Genetic analysis

Allele counts and genotype were used to test for the presence or absence of more than two parents in each nest by single locus Minimum Method. This method assigns multiple paternity within a clutch, assuming that all alleles not accounted for by the maternal genotype were contributed by "fathers" (Myers and Zamudio, 2004). We assumed multiple paternity when the maternal genotype could be reconstructed and three or more additional alleles were present in the clutch. Data analyses were carried out using Cervus 3.0 (Marshall et al., 1998) and Gerud 2.0 (Jones, 2005). Cervus 3.0 is a program that examines genetic data from co-dominant genetic markers and performs parentage analyses. Parentage tests (paternity) were performed using family groups (one female with at least 9 hatchlings). Gerud 2.0 reconstructs parental genotypes from half-sib progeny arrays with known or unknown parents. Using each family genotype group and allelic frequencies, we performed a determination of compatible mothers and then tested all possible paternal genotypes to search for the minimum combination of fathers can best explain the data set. We calculated relative probabilities based on allele frequencies and Mendelian segregation.

Results

All females and hatchlings were successfully genotyped using the four microsatellite markers: *Cla*μ 2, *Cla*μ 6, *Cla*μ 9 and *Cla*μ 10. All four microsatellite loci were polymorphic with allele size varying from 135 bp (*Cla*μ 9) to 262 bp (*Cla*μ 10) (Table 3).

*Cla*μ 2		*Cla*μ 6		*Cla*μ 9		*Cla*μ 10	
Allele size (bp)	Freq	Allele size (bp)	Freq	Allele size (bp)	Freq	Allele size (bp)	Freq
204	0.189	153	0.092	135	0.003	210	0.201
206	0.167	159	0.183	139	0.201	214	0.061
210	0.113	161	0.167	164	0.235	216	0.061
214	0.403	163	0.386	166	0.124	218	0.223
218	0.022	165	0.056	168	0.438	220	0.373
240	0.107	179	0.118			248	0.045
						262	0.038

Table 3. Allele sizes and frequencies for the four microsatellite loci analyzed.

All families were successfully assigned to the candidate mother. Applying the Minimum method we found evidence of multiple paternity in two of the twelve analyzed families. Using Cervus 3.0 for the first parentage analysis we considered only offspring and candidate mother genotypes (the female guarding the nest), and we found three inconsistent genotypes among known mother-offspring pairs, all of which occurred in locus *Cla*μ 2 of family 8, which can be attributed to the possibility of the presence of eggs from different females in the nest or misinterpretation of the obtained bands.

Utilizing Gerud 2.0 we found that in eleven of the twelve analyzed families the candidate mother (the nest-guarding female) was determinate to be the most likely mother for all offspring. We determined that in family 8 there appeared to be more than one compatible mother, which corresponded to the inconsistency found using Cervus 3.0. In

two families the clutch had an assignment minimum of two fathers (families 3 and 5), verifying evidence of multiple paternity, in agreement with our previous results (Amavet et al., 2008). In the rest of the families, the clutch had an assignment minimum of one father.

Discussion

According to paternity analyses, more than one father was detected in two *C. latirostris* nests. The Minimum Method is not able to detect multiple paternity by males with similar genotypes (Myers and Zamudio, 2004) therefore, the number of fathers detected represents a minimum estimate, and, so the occurrence of multiple paternity might be a relatively frequent condition. The obtained results using two different programs were consistent, despite the differences in the statistical approaches applied (see Jones and Ardren, 2003).

Multiple paternity detected here in *C. latirostris* is similar to cases reported by Davis et al. (2001) in *Alligator mississippiensis*, Mc Vay et al. (2008) in *Crocodylus moreletii*, Hu and Wu (2010) in *A. sinensis*. It was suggested that the existence of multiple paternity in crocodilians could be result of multiple copulations in a single mating period, or due to the existence of sperm storage in females between one mating period and the next. The capacity of storage sperm has been proposed by Davenport in 1995 to *Paleosuchus palpebrosus* (belonging to the family Alligatoridae as *C. latirostris*), and more recently was found by Gist et al. (2008) in *A. mississippiensis* females, which may be able to accumulate sperm in their oviducts glands for at least one year. This mechanism could be a cause of detection of multipaternity in this species. There are no records in relation to this aspect of the reproductive biology in *C. latirostris*, but the finding of multiple paternity in this study could be explained by similar mechanisms to those described in the related species.

In order to have greater confidence in the occurrence of multiple paternity, it would be prudent to sample more microsatellite loci, so to reduce the probability that two or more males with similar multilocus genotype cannot be recognized as a function of the offspring genotypes.

Literature cited:

-Amavet, P.; Rosso, E.; Markariani, R.; Piña, C.I. 2008. Microsatellite DNA markers applied to detection of multiple paternity in Caiman latirostris in Santa Fe, Argentina. Journal of Experimental Zoology Part A, Ecology Genetics and Physiology 309A: 637-642.

-Bassam, B. J.; Caetano- Anolles, G.; Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry 196: 80-83.

-Davenport, M. 1995. Evidence of possible sperm storage in the Caiman, *Paleosuchus palpebrosus.* Herpetological Review 26: 14-15.

-Davis, L.M.; Glenn, T.C.; Elsey, R.M.; Dessauer, H.C.; Sawyer, R.H. 2001. Multiple paternity and mating patterns in the American alligator, *Alligator mississippiensis*. Molecular Ecology 10:1011-1024.

-Gist, D.H.; Bagwill, A.; Lance, V.; Sever, D.M.; Elsey, R.M. 2008. Sperm storage in the oviduct of the American alligator. Journal of Experimental Zoology 309A: 581-587.

-Hu, Y. ; Wu, X.B. 2010. Multiple paternity in Chinese alligator (*Alligator sinensis*) clutches during a reproductive season at Xuanzhou Nature Reserve. Amphibia- Reptilia 31: 419-424.

-Jones, A.G. 2005. GERUD 2.0: A computer program for the reconstruction of parental genotypes from half-sib progeny arrays with known or unknown parents. Molecular Ecology Notes 5: 708-711.

-Jones, G.; Ardren, W.R. 2003. Methods of parentage analysis in natural populations. Molecular Ecology 12: 2511-2523.

-Longmire, J.L.; Lewis, A.K.; Brown, N.C.; Buckingham, J.M.; Clark, L.M.; Jones, M.D.; Meincke, L.J.; Meyne, J.; Ratliff, R.L.; Ray, F.A.; Wagner, R.P.; Moyzis, R.K.1988. Isolation and molecular characterization of a highly polymorphic centromeric tandem repeat in the family Falconidae. Genomics 2:14-24.

-Marshall, T.C.; Slate, J.; Kruuk, L.; Pemberton, J.M. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7: 639-655.

-McVay, J.D.; Rodriguez, D.; Rainwater, T.R.; Dever, J.A.; Platt, S.G.; McMurry, S.T.; Forstner, M.R.J.; Densmore, L.D. 2008. Evidence of multiple paternity in Morelet's Crocodile (*Crocodylus moreletii*) in Belize, CA, inferred from microsatellite markers. Journal of Experimental Zoology 309A: 643-648.

-Murray, M.G.; Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Research 8: 4321-4325

-Myers, E.M.; Zamudio, K.R. 2004. Multiple paternity in an aggregate breeding amphibian: the effect of reproductive skew on estimates of male reproductive success. Molecular Ecology 13:1951-1963.

-Tourn, S.; Imhof, A.; Costa, A.L.; von Finck, M.C.; Larriera, A. 1993. Colecta de sangre y procesamiento de muestras en *Caiman latirostris* (Informe de avance). Memorias del IV Workshop sobre Conservación y Manejo del yacaré overo (*Caiman latirostris*). Santa Fe. Argentina: 25-30.

-White, P.S., Densmore, L.D. 1992. Mitochondrial DNA isolation. In: Molecular Genetic Analysis of Populations. A Practical Approach. The Practical Approach Series, p. 29-57. Hoelzel, A.R., Ed., Oxford, Oxford University Press.

-Zucoloto, R.B.; Verdade, L.M.; Coutinho, L.L. 2002.Microsatellite DNA library for *Caiman latirostris*.Journal of Experimental Zoology (Molecular Development and Evolution) 294: 346–351.