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Mating system and population analysis of the broad-snouted caiman (*Caiman latirostris*) using microsatellite markers

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Abstract. The knowledge about reproductive strategies of a species contributes to their conservation. Multiple paternity is a reproductive behavior increasing effective population size, which could increase genetic diversity particularly in populations submitted to bottlenecks events. In Argentina, wild populations of *Caiman latirostris* are subject of a management plan devoted to their preservation and sustainable utilization based on its commercial interest. This program started in response to the evident numeric reduction of the populations, as a consequence of hunting pressure and habitat modification; it had a remarkable success in population recovery allowing the commercial use of *C. latirostris*. Data on reproductive behavior of *C. latirostris* are limited because mating occurs in the water and the information about their genetic diversity is scarce too. Our specific aims were to study the mating system and population genetic structure applying microsatellite markers in twelve *C. latirostris* families. The obtained results showed highly significant difference among populations and a lack of correspondence between geographical distance and genetic differentiation suggesting that populations of *C. latirostris* represent unstable metapopulations. In the paternity analysis was detected more than one father in two nests, which could be explained by capacity of storage sperm, proposed in females of a related species. The behavior of multipaternity could contribute to maintain viable populations of *C. latirostris*, since the maintenance of genetic variability within populations could help increase their capacity to respond to selective pressure. Further studies employing genetic and behavioral framework are needed to better understand the reproductive biology of *C. latirostris*.

Keywords: *Caiman latirostris*, molecular markers, multiple paternity, population genetics, sustainable use.

Introduction

The Broad-snouted caiman (*Caiman latirostris*) is one of two crocodilian species cited for Argentina. It has a wide geographic distribution that embraces diverse aquatic environments of the Paraná River basin, which usually are shallow water and vegetated marshes (Larriera, 1992). As it is an opportunistic carnivorous consumer in the top of trophic nets, it could be considered as a flag species in local ecosystems, whose viability guarantees the whole system conservation.

In Argentina, the wild populations of *C. latirostris* from Santa Fe province are subject of a management and monitoring plan carried out within the frame of a special conservation program (Proyecto Yacaré-Gob. de Santa Fe/MUPCN) devoted to the preservation and the sustainable utilization of this species based on its commercial interest. This management plan started in 1990 in response to the evident numeric reduction of the populations as a consequence of a strong hunting pressure on one hand and the habitat modification on the other. The use of ranching system (harvest of wild eggs for captivity rearing) had a remarkable success in terms of population recovery (Larriera et al., 2008). As a consequence of this, since 1997, *C. latirostris* in Argentina is listed in the Appendix II of the CITES (Convention on Inter-

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national Trade in Endangered Species of Wild Fauna and Flora). This legal frame allows the commercial use of *C. latirostris* under strict control (Larriera, 1998). Due to the high quality of their leather and meat it turns into a species of zootechnical interest, representing a regionally important economic activity that acts as a direct incentive for habitat conservation based on the community involvement.

Molecular analyses of genetic variability in a wide variety of species including especially crocodilians, have provided valuable data about reproductive mechanisms, gene flow, population effective size, geographic distribution and genetic variability measurements (Forstner and Forstner, 2002), all of them representing fundamental information for the selection of appropriate management strategies.

Initially, allozyme analyses showed low heterozygosity in *Alligator mississippiensis* wild populations (Gartside et al., 1976; Menzies et al., 1979; Adams et al., 1980; Lawson et al., 1989). These authors suggested that such a low variability might be due to important bottlenecks (genetic drift) underwent for several decades, plus to particular characteristics of the species such as big size and long life span (Gartside et al., 1976; Lawson et al., 1989). These analyses were later applied on other crocodilian species (Jurgens et al., 1994; Flint et al., 2000), and indicated that they show few allozyme polymorphic loci.

In relation to DNA studies, analyses on mitochondrial genes were conducted. Phylogenetic analyses were performed by Densmore and White (1991), and more recently by Hrbek et al. (2008), who studied crocodilian phylogenetic relationships including the genus *Caiman*; also, Weaver et al. (2008) conducted phylogenetic studies on *Crocodylus rhombifer.* In relation to variability analysis performed using mitochondrial genes, Glenn et al. (2002) found low variation among populations of *A. mississippiensis*; by contrast, Ray et al. (2000) and Farias et al. (2004) estimated high variability on *Osteolaemus tetraspis tetraspis*, and good differentiation among populations of *Melanosuchus niger* and *Caiman crocodylus*.

Another molecular technique applied to genetic variability analysis on crocodiles, is RAPD technique (Random Amplified Polymorphic DNA). There are previous works using this method on genus *Alligator* (Dessauer, unpublished data; Wu et al., 2002), and *Crocodylus acutus* (Porras Murillo et al., 2008). In Argentina we conducted population genetic studies using RAPD markers and morphometric traits on *C. latirostris* and we found relatively high estimates of polymorphism and heterozygosity (Amavet et al., 2007, 2009).

A technique applied more recently to evaluate genetic structure and variability is microsatellite analysis. These specific molecular markers are short tandem arrays of simple DNA sequences (Tautz and Schlötterer, 1994) also known as SSR (simple sequence repeats). They are frequently chosen because they are highly polymorphic, allow to detect heterozygous genotypes and they are distributed throughout the whole genome (Bruford and Wayne, 1993); and have been shown to be important tools in ecological and genetic research in the analysis of population variability, as well as kinship between individuals. Applying these markers variability estimates on crocodilians were higher than values obtained using methods previously mentioned (Glenn et al., 1998; Davis et al., 2000, 2002; Fitzsimmons et al., 2000, 2002; Dever et al., 2002; de Thoisy et al., 2006). Several authors have also used microsatellite amplification for genealogical and mating system studies on crocodilians (Davis et al., 2001; Isberg et al., 2004; McVay et al., 2008; Weaver et al., 2008; de Oliveira et al., 2010; Hu and Wu, 2010).

The information about genetic diversity on *C. latirostris* is scarce. Verdade et al. (2002), using four microsatellite markers originally developed for *Alligator mississippiensis*, found microgeographic variation in Brazilian *C. latirostris* populations. Later, using *C. latirostris* specific microsatellites (developed by Zucoloto et al., 2002; Zucoloto, 2003), Villela (2004) recorded

high values of genetic diversity and heterozygosity and, more recently, Villela et al. (2008) found a consistent relationship between geographic distance and genetic differentiation along the species latitudinal range in Brazil. The same primers developed for *C. latirostris* were also successfully applied to reveal SSRs in *Paleosuchus palpebrosus*, *Caiman crocodylus* and *C. yacare* (Zucoloto et al., 2006).

An important contribution to the conservation of a species is knowledge of its breeding strategies. Multiple paternity (i.e. the existence of more than a father siring one nest) has been shown theoretically to increase effective population size (Sugg and Chesser, 1994), thus potentially increasing the overall genetic diversity of a population, particularly those populations that have recently undergone a genetic bottleneck (McVay et al., 2008). This mating strategy has been reported in different vertebrate groups: fishes (Weir et al., 2010), birds (Leisler and Wink, 2000; Dunn et al., 2009), mammals (Kitchen et al., 2006; Gottelli et al., 2007), amphibians (Adams et al., 2005), turtles (Jensen et al., 2006), lizards (Berry, 2006), and snakes (Madsen et al., 2005).

Data on reproductive behavior of *C. latirostris* are limited because mating occurs in the water between groups of males and females (which are not easily distinguishable) and unambiguous observations are not often possible (Lang, 1989). Thus, microsatellite markers are useful in helping to understand mating patterns of *C. latirostris* due to the possibility of its use as fingerprints. Zucoloto et al. (2009) applied SSR analysis to assess parentage among individuals from a captive colony of *C. latirostris.* In relation to mating system analyses in the wild, preliminary results obtained in our laboratory using SSRs in paternity analysis found evidence of multipaternity in one of four families analyzed (Amavet et al., 2008). Although these results were based on a small sampling, they were consistent with findings on different crocodilian species (Davis et al., 2001; McVay et al., 2008; Hu and Wu, 2010).

In order to contribute to a better knowledge of broad-snouted caiman reproductive ecology and population dynamics in relation to conservation issues, we investigated twelve families of *C. latirostris* from Santa Fe province, Argentina, using microsatellite markers. Specific aims were: a) to study the mating system; b) to use the allelic diversity to analyze population genetic structure.

Materials and methods

Study sites and sample collection

A total of 12 families (150 specimens) of *C. latirostris* were sampled between 2005 and 2008 from eight different localities of Santa Fe province, Argentina (table 1, fig. 1).

Table 1. Localities, geographical coordinates, and acronyms used in fig. 1 for each family analyzed of *C. latirostris*.

Families	Localities	Geographical coordinates	Acronym
1	Los Amores	28°06′S-59°59′W	LA
2	La Selva-Ruta 30	28°10′S-59°22′W	LS
3	La Selva-Ruta 30	28°10′S-59°22′W	LS
4	Flores-Ruta 3	28°19′S-59°57′W	FL
5	Estancia El Lucero	29°54′S-60°50′W	EL
6	Estancia El Lucero	29°54′S-60°50′W	EL
7	Estancia El Lucero	29°54′S-60°50′W	EL
8	Estancia Piedras	29°58′S-60°44′W	EP
9	Estancia Piedras	29°58′S-60°44′W	EP
10	Reserva El Fisco	30°11′S-61°00′W	EF
11	Estero Lote 114	30°42′S-60°17′W	LO
12	Lago artificial Granja La Esmeralda	31°35′S-60°41′W	GL

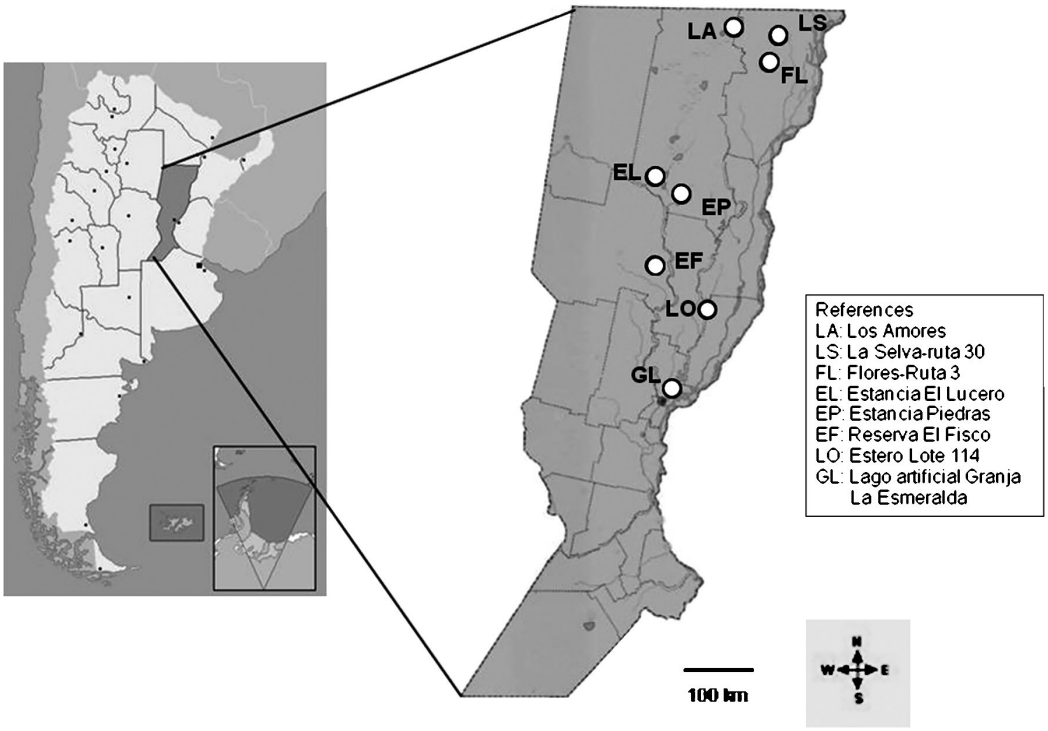

Figure 1. Geographic location of sampling sites of the families analyzed of *Caiman latirostris*.

Each family consisted of the nest-guarding *C. latirostris* female and the hatchlings of each nest.

Twelve 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).

Microsatellite genotyping

Four microsatellite primer pairs developed by Zucoloto et al. (2002) for *C. latirostris* were used: *Cla*µ 2, *Cla*µ 6, *Cla*µ 9, and *Cla*µ 10 (table 2).

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 1 μl dNTP mix for *Cla*µ 9 and *Cla*µ 10; 0.06 μl *Taq* DNA polymerase (5 U/μl) from PB-L 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.

Table 2. 5′-3′ sequences of the used primers.

Primer	5′-3′ sequence
*Cla*µ 2a	CCTTCAGGACCCACTTTCTT
*Cla*µ 2b	CGAATCCCTCTTCCCAAACT
*Cla*µ 6a	GAAATATGGGACAGGGAGGA
*Cla*µ 6b	GGTTGGCTGCATGTGTATGT
*Cla*µ 9a	ACAGGGGAAAAGAAGAGCTG
*Cla*µ 9b	AAAATCCCCCACTCTTACCC
*Cla*µ 10a	TGGTCTTCTCTTCGTGTCCT
*Cla*µ 10b	ATGAGCCCCTCTATGTTCCT

PCR reactions were performed in a MPI® thermal cycler. Amplifications were conducted using annealing temperatures between 55°C to 65°C and different cycling programs (table 3). 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 75 W, and stained with $AgNO_3$ solution (Bassam et al., 1991). Molecular weights in base pairs

Table 3. Cycles and annealing temperatures number for DNA amplification using the different primers.

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

Table 4. Allele sizes and frequencies for the four microsatellite loci analyzed.

*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

(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.

For population genetic analyses we used R_{ST} statistics (Goodman, 1997). This coefficient is analogue to F_{ST} (Wright, 1969), but it is more appropriate under the stepwise mutation model assumed for microsatellite evolution. Global and pairwise R_{ST} were estimated using the software RST CALC (Goodman, 1997). In this analysis we assumed each family as one population. The matrix of pairwise R_{ST} estimates was compared with the corresponding matrix of geographic distances by means of Mantel test using the package *ade4* (Dray and Dufour, 2007) of R software ver. 2.13 (R Development Core Team, 2011).

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 4).

Estimated statistics using Cervus 3.0 showed variability values moderate to high depending of the locus considered. The same software was also used to test null alleles and the locus *Cla*μ 2 showed the highest frequency (table 5). R_{ST} value averaged over all loci was highly significant ($R_{ST} = 0.445$, $P < 0.01$ with 2000 permutations). Pairwise R_{ST} estimates are not correlated with geographical distances according to Mantel test ($r = 0.16$, $P = 0.14$, based on 1000 replicates), indicating that population structure does not fit the expectation for the equilibrium under the isolation by distance model.

Table 5. Variability statistics for each locus: Observed Heterozygosity (*Ho*), Expected Heterozygosity (*He*), Polymorphic Information Content (*PIC*), number of alleles (*A*), null allele frequency estimate (*F Null*).

Statistics	*Cla*μ 2	*Cla*μ 6	*Cla*μ 9	*Cla*μ 10
Ho	0.113	0.601	0.593	0.459
He	0.752	0.767	0.700	0.763
PIC	0.716	0.734	0.648	0.726
A	6	6	5	7
F Null	+0.7381	+0.1291	+0.0706	+0.2593

In the parentage analysis, 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 (families 3 and 5) which where sampled in different localities (table 1, fig. 1). 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 (table 6). This fact can be attributed to the possibility of the presence of eggs from different females in the nest.

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

Genotypes were successfully detected and assigned using the silver-stained polyacrylamide gel protocol.

Table 6. Genotypes for family 8 at locus *Cla*μ 2. Asterisk (*) marks inconsistent genotypes among the candidate mother and offspring pairs.

Individuals	Genotypes
Mother	210
	210
Offspring 1	210
	210
Offspring 2	206
	210
Offspring 3	214*
	214*
Offspring 4	206
	210
Offspring 5	210
	214
Offspring 6	206*
	206*
Offspring 7	206
	210
Offspring 8	206*
	206*
Offspring 9	206
	210
Offspring 10	210
	214

Genetic variability averaged values ($Ho = 0.4415$ and $He = 0.7455$) obtained using microsatellite markers are higher than the results of our previous work ($He = 0.165$) using RAPD markers (Amavet et al., 2009), a result consistent with the usually very high variability associated to microsatellite markers (Queller et al., 1993; Ellegren, 2004).

In the analysis of population structure, R_{ST} value averaged over all loci was 0.445. This structure index is higher than F_{ST} value (0.27) obtained in our previous work (Amavet et al., 2009). The difference in results can be attributed to several causes: (1) in the previous work four populations were taken as units, each represented by 10 non related individuals, whereas in the present study, we used as units groups of related individuals (families represented by the mother and 9-16 hatchlings); (2) the number of individuals analyzed here is much higher than

in the previous one; (3) the geographical range sampled here is also wider; (4) the markers differ in their ability to detect variation at different levels as a consequence of dominance, number of alleles, and number of loci analyzed in each case. However, both studies demonstrate a highly significant difference among populations and a lack of correspondence between geographical distance and genetic differentiation, suggesting that populations of *C. latirostris* do not represent a structured stable system but unstable metapopulations.

Results from previous and present analyses are also consistent showing a higher differentiation among populations for molecular markers (in terms of F_{ST} or R_{ST}) than that observed for quantitative traits (measured as Q_{ST} values). As postulated in Amavet et al. (2009) the lower values of Q_{ST} in comparison to F_{ST} suggests a higher contribution of neutral than adaptive loci to the genetic differentiation among populations. The high R_{ST} observed in the present study is consistent with the previous result based on RAPD markers. Quantitative traits are probably more related with fitness and the differentiation among populations remained relatively low. The high variability within populations and the significant differentiation among populations for neutral loci show that, in spite of past events of overexploitation of *C. latirostris* resulting in a reduction in population size, the genetic variability remains relatively high.

We found three inconsistent genotypes among the candidate mother and offspring pairs at locus *Cla*μ 2 in family 8. This can be attributed to the possibility of the presence of eggs from different females in the nest, in agreement to observations of Larriera et al. (2002) in this species. In addition, Zucoloto et al. (2009) found in a study conducted in six *C. latirostris* clutches that two females who were assigned by genetic analysis as mothers each one to one nest were not the same as those indicated by maternal behavior. The authors explained the display of maternal behavior by nonmothers as a behavioral malfunction caused by the captive environment or species social adaptation. In our study, the results seem to show that the cause of genetic disparity mother-offspring is the oviposition of different females within the same nest.

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). It should be noted that the finding of high frequencies of null alleles at some loci may indicate that our estimate of multiple paternity may be underestimated because some of these null alleles may be an extra paternal allele.

Multiple paternity detected here in *C. latirostris* is similar to cases reported by Davis et al. (2001) in *Alligator mississippiensis*, McVay et al. (2008) in *Crocodylus moreletii*, Hu and Wu (2010) in *Alligator sinensis*, and Muniz et al. (2011) in *Melanosuchus niger*. 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.

Loss of genetic diversity has been associated with an increase in vulnerability to population depletion and also a higher extinction risk (Lande and Shannon, 1996; Frankham, 2005). This is applicable to other crocodilian species, because of population reductions due to overexploitation in the 1950s and 1960s (Thorbjarnarson, 1999), and the concomitant decreases in genetic variability that typically follow such reductions. The use of multiple paternity as a mating strategy contributes to greater genetic variability as a population is recovering, since we assume there is also an increase in effective population size (Sugg and Chesser, 1994). Maintaining sufficient genetic variability within wild populations helps to increase the ability of populations to respond to selective pressures and adapt to changing environmental conditions (Frankham, 2005). It is clear that understanding mating behavior is important in maintaining viable populations of exploited taxa like *C. latirostris*. This knowledge will in turn help indicate the proportion of males and females needed for reintroduction into the wild in order to maximize effective population size. Further studies on mating system employing both a genetic and behavioral framework are now needed in order to better understand the reproductive biology of *C. latirostris*.

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