Multiple paternity in the Black Caiman (*Melanosuchus niger*) population in the Anavilhanas National Park, Brazilian Amazonia

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Abstract. The formation of dominance hierarchies in which the female mates with a large dominant male is common among crocodilians. However, there is the possibility of polyandry, in which females mate with multiple partners during a single breeding season and generate offspring with multiple paternity. In the present study, eight pairs of heterologous primers developed for *Alligator mississippiensis* and *Caiman latirostris* were used to determine whether multiple paternity exists in the Black Caiman, *Melanosuchus niger*. For such, we analyzed 34 Black Caiman offspring from the Anavilhanas Archipelago in the Negro River (state of Amazonas, Brazil). The specimens came from six groups, each containing five or six hatchlings. Paternity exclusion and genetic identity indices were calculated to test the robustness of the microsatellite loci. Simple allele counts and maximum likelihood estimation of family clusters were used to determine the likelihood of occurrence of multiple paternity. Among the eight loci tested, five were effective at determining paternity, with paternity exclusion values close to 1.0 ($QC = 0.92$) and genetic identity values close to zero ($IC < 0.01$). Using the simple allele count, six cases of multiple paternity were detected and confirmed in three hatchling groups by four different microsatellite loci. However, maximum likelihood analysis indicated multiple paternity in all the groups analyzed, with five family clusters identified in one hatchling group alone. Considering that this species is listed according to IUCN as Lower Risk/Conservation Dependent, our results have direct conservation implications. Multiple paternity increases effective population size by maintaining genetic variation, and thus could be an important mechanism to maintain genetic diversity in isolated local populations.

Keywords: Alligatoridae, *Melanosuchus niger*, microsatellites, paternity.

The Black Caiman (*Melanosuchus niger*) is the largest alligatorid species and figures among the largest carnivores of the continental Americas (Ross, 1998). Its historical and actual distributions encompass parts of Bolivia, Brazil, Colombia, Ecuador, Peru, Guyana and French Guyana (Ross, 1998). As in all living crocodilian species, *M. niger* males grow to larger sizes than females and there are historic records of individuals of six meters total length – TL (Medem, 1983). The species is common in the Brazilian Amazonia, but most of the larger individuals are around four meters in TL (Medem, 1983), and probably are survivors from the intensive hunting for their skins that in the 1970s reduced many populations throughout the Amazonian biome (Plotkin et al., 1983; Rebêlo and Magnusson, 1983; Da Silveira and Thorbjarnarson, 1999).

Male territoriality and dominance-mating behavior is typical of all crocodilians (Pough et al., 2003) and is expected for *M. niger*, in which the largest females reach up to 2.8 m TL, or only about half the maximum TL of males (Da Silveira, 2002). Territorial control by larger crocodilians could reflect competition for food, high quality nesting sites and potential partners. Access to these resources could directly affect

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both sexes and limit access to potential mating partners (Pough et al., 2003).

In general, we would expect that male crocodilians would try to secure matings with as many females as possible, which in turn should prefer to mate with large dominant males, but long term studies indicate that mating behavior in crocodilians is not so simple (Garrick and Lang, 1977). Some females mate only with a single male during many consecutive breeding seasons but others can mate with up to three males during the same breeding season, as was recently demonstrated for *Alligator mississippiensis* (Lance et al., 2009).

Extra-pair paternity (polyandry) could be advantageous to the female because it increases the genetic variance of her offspring, and provides opportunity for sperm competition which in turn could result in the selection of the best spermatozoids and therefore result in increase in the health and fitness of the offspring (Parker, 1990; Madsen et al., 1992).

High-resolution genetic markers, such as microsatellites, are used to obtain detailed information on mating systems (Litt and Luty, 1989). Microsatellites are tandem repeat sequences in the genomes of eukaryotes (Goldstein and Schlötterer, 1999), generally in noncoding regions, and are the most polymorphic class of molecular markers available to date. These markers are widely employed in conservation programs, and in analysis of mating systems of natural populations (Jones and Ardren, 2003). In kinship analyses, microsatellites have advantages over other markers in that they have Mendelian inheritance, are co-dominant, and exhibit a high degree of genetic diversity (Hedrick and Parker, 1998). Often microsatellite markers developed for one species can be used in closely related species.

Although microsatellite makers have not been developed specifically for *M. niger*, a study by de Thoisy et al. (2006) characterized seven populations of *M. niger* widespread throughout the Amazon Basin using markers originally developed for *Caiman latirostris* (Zu-

coloto et al., 2002) and *A. mississippiensis* (Glenn et al., 1998; Davis et al., 2002). Of the original seven *Caiman latirostris* and 28 *A. mississippiensis* markers, eight markers were successfully amplified and were polymorphic in *M. niger*, thereby allowing their use as heterologous primers. These eight primers were used in the present study to obtain data on the occurrence of multiple paternity.

Multiple paternity appears to be a frequently encountered pattern in reptiles including lizards (e.g., Laloi et al., 2004; Fitze et al., 2005; Eizaguirre et al., 2007), snakes (e.g., McCracken et al., 1999; Garner and Larsen, 2005; Voris, 2008), turtles and tortoises (e.g., FitzSimmons, 1998; Valenzuela, 2000; Johnston et al., 2006; Pearse et al., 2006; Fantin et al., 2008) and crocodilians such as *Alligator mississippiensis* (Davis et al., 2001), *Caiman latirostris* (Verdade et al., 2002; Amavet et al., 2008), *Caiman crocodilus crocodilus* (Oliveira, 2010), *Caiman crocodilus yacare* (Godshalk, 2002), *Crocodylus moreletii* (McVay et al., 2008) and *Alligator sinensis* (Hu and Wu, 2010). A molecular analysis of the mating system of *Melanosuchus niger* has yet to be studied, however. Our aim in the present study was to genetically analyze the mating pattern of *M. niger* in an archipelago of the Negro River located in the Anavilhanas National Park, Central Amazonia, using the eight heterologous microsatellite loci used by de Thoisy et al. (2006).

Muscle-tissue samples from caudal scales were obtained during a long term caiman mark-recapture program undertaken in the Anavilhanas Archipelago located in the lower Negro River, state of Amazonas, Brazil (Da Silveira et al., 1997). The Anavilhanas Archipelago encompass 100 000 ha, it is the second largest freshwater Archipelago in the world with more than 100 islands, and represents almost 30% of the total area of the Anavilhanas National Park, which lies between 02°00′ and 03°02′S, and 60°27′ and 61°07′W.

Seventeen sub adults of *M. niger* were sampled to test the robustness of the genetic markers and 34 hatchlings from six groups were sampled for the paternity test. The Anavilhanas Archipelago is in a black water system that supports a small population of *M. niger* characterized by naturally very low abundance (<10/km of shoreline during dry season). Most of the non-hatchling caiman surveyed in the Anavilhanas Archipelago were the spectacled caiman (*Caiman*

crocodilus) and only 3.5% were *M. niger* (Da Silveira et al., 1997). Nests of *M. niger* in Anavilhanas are very rare and only one nest of this species was found during a decade-long study in the area. Annually 1000 km of shoreline in 1992, 1995, 1996, 1997 and 1998 were surveyed, and during each survey no more than nine groups of hatchlings were found (Da Silveira, 2002). During the surveys, hatchlings from six different groups, presumably representing six different nests were sampled. Samples from five to seven hatchlings per group were collected. Crocodilian hatchlings can move between hatchling groups. However, this is very unlikely in the case of *M. niger* in the Anavilhanas, where there are few nesting females, and hatchling groups were generally kilometers from each other.

All samples were stored in tubes containing 95% alcohol. DNA isolation was performed according to the proteinase K/phenol-chloroform protocol (Sambrook et al., 1989). The genomic DNA was used as template for the PCR-based amplification of microsatellite regions using the heterologous primers used for the characterization of *M. niger* populations by de Thoisy et al. (2006). PCR conditions were based on protocols reported by Zucoloto et al. (2002) for *Caiman latirostris* and Glenn et al. (1998) and Davis et al. (2002) for *A. mississippiensis*. In all PCR amplifications, we included a second round of 15 cycles with an annealing temperature at 53°C used for the incorporation of a 6-FAM labeled M13 (-21) primer (Schuelke, 2000). In the PCR reactions, we used the primers Claμ 6 and Claμ 8, developed for *Caiman latirostris* Zucoloto et al. (2002), and the primers Amiμ 8, Amiμ11, Amiμ13, Amiμ 14, Amiμ 16 and Amiμ 20 developed for *A. mississippiensis* (Glenn et al., 1998). All forward primers had an M13 (-21) tail added to their 5′ end. The PCR product was combined with a ROX size standard (DeWoody et al., 2004), and resolved on the automatic sequencer ABI 3130xl (Applied Biosystems). Fragments were binned, and sizes were estimated in the software GeneMapper (Applied Biosystems).

To estimate the efficiency of the markers in detecting multiple paternity, the probability of genetic identity for each locus (I) was calculated, and a combined probability of genetic identity – IC (Paetkau et al., 1995), and the probability of paternity exclusion for each locus (Q), estimated by the joint probability of paternity exclusion method – QC (Weir, 1996).

We used two methods to estimate multiple paternity. First, we used a simple method of counting the number of observed alleles, and comparing it to the expected number of alleles in each nest (Myers and Zamudio, 2004). The maximum number of expected alleles in each nest under the assumption of monogamy is four, unless one observes homozygous progeny (FitzSimmons, 1998; Valenzuela, 2000). Each

homozygous genotype indicates that alleles are shared between parents, and therefore the number of expected alleles decreases to three with one homozygous genotype, and to two with two homozygous genotypes. The allelic count method is conservative and accurate (Myers and Zamudio, 2004). Second, we carried out analyses using the program Colony v 2.0 (Wang, 2004). Colony uses Bayesian Inference to estimate the number of full sib family clusters in each nest (Jones and Wang, 2009). Inference of full sib families is possible even with no a priori knowledge of parental genotypes, as this program uses information of allelic sharing at multiple loci (multi-locus genotypes) among individuals in each nest.

Of the eight microsatellites used, three (Claμ 8, Amiμ 13 and Amiμ 14) had an unclear pattern and were therefore removed from analyses. Additionally, locus Amiμ 8 in nest 4 was not used since only three of the six individuals in this nest could be genotyped, indicating the presence of a null allele in this group.

Of the five microsatellite loci analyzed, the locus Claμ 6 was the most polymorphic with seven alleles, and the locus Amiμ 11 had the smallest number of alleles (table 1). The five microsatellite loci had a high power to discriminate paternity when analyzed together since the probability of joint paternity exclusion (QC) was very close to one, and the joint probability of genetic identity (IC) was close to zero (table 1).

Table 1. Number of different alleles detected for each locus for each microsatellite. Q = index of paternity exclusion for each locus; QC = combined index of paternity exclusion; I = probability of genetic identity; and IC = combined index of probability of genetic identity.

Locus	Alleles	Paternity exclusion (Q)	Genetic identity (I)
Claμ 6	7	0.62	<0.01
Amiμ 8	6	0.10	0.30
Amiμ 11	2	0.19	0.40
Amiμ 16	6	0.53	0.11
Amiμ 20	5	0.40	0.19
All		$QC = 0.92$	$IC < 0.01$

Table 2. Simple allelic counting method: Columns Claμ 6 to Amiμ 20 shows the maximum expected number of alleles/actual number of alleles observed; * = indicates a case of multiple paternity. Colony: inferred number of full sib families, and associated likelihoods. Smaller or larger number of full sib families than the reported number in each nest is associated with smaller likelihood for the specific nest.

Nest	N° of hatchlings	Simple allelic counting method					Colony	
		Claμ 6	Amiμ 8	Amiμ 11	Amiμ 16	Amiμ 20	N° of full sib families	Log likelihood
N1	6	3/3	3/4*	3/2	4/3	4/5*	3	−3.997574
N2	5	4/3	3/2	3/2	3/3	3/3	2	−2.427482
N3	5	3/2	2/3*	3/2	3/2	3/2	4	−1.819115
N4	6	2/2	X	3/2	3/2	3/2	4	−1.373169
N5	5	3/2	2/2	4/2	3/2	4/3	2	−1.660341
N6	7	4/5*	3/3	3/2	2/4*	3/5*	6	−5.653093

For all analyses of multiple paternity using the simple allelic count method, we tested all the clutches for deviation from Hardy-Weinberg to avoid the underestimating the number of null alleles. None of the clutches showed a significant deviation from Hardy-Weinberg after Bonferroni correction for multiple comparisons (Rice, 1989).

Using simple counting of alleles methods, we detected six cases of multiple paternity (table 2). The maximum number of offspring alleles assuming a monogamous mating system where both parents are heterozygous and do not share any alleles is four offspring alleles. When parents share one or more alleles, observed as the presence of homozygous offspring, the number of expected alleles in the offspring is reduced to three (when only one homozygous offspring type is observed) and two (when two homozygote offspring types are observed). For example, in N1, the microsatellite marker Amiμ 20 had five alleles, and since no homozygote offspring was observed, the maximum expected number of offspring alleles was four. Therefore, the presence of a fifth allele necessitates the contribution of an additional parent to the offspring clutch. Similarly, in N6 the marker Amiμ 16 had four alleles, however, two types of homozygotes were observed in this nest, resulting in the maximum expectation of two offspring alleles. The presence of four alleles in this situation also indicates a case of multiple paternity.

Results of maximum-likelihood analysis of full sib families performed in the program Colony using five microsatellite loci indicated multiple paternity in all six groups analyzed. In each group, at least two full sib family clusters were inferred (table 2). In group 6, there was an indication of the contribution of more than two males since six full sib families were inferred.

We can affirm that five of the eight tested primers were variable and had sufficient discriminatory power to infer multiple paternity in all six groups of *M. niger* from the Anavilhanas Archipelago. The discovery of polyandrous behavior in *M. niger* has important implications with regard to conservation strategies for the species, as the understanding of mating systems is important for the maintenance of viable populations.

Polyandry offers genetic benefits by improving the chances that females will have access to "good genes", and pass on these genetic benefits to their offspring (Yasui, 1998). A female could also increase her fitness and that of her offspring by copulating with higher quality males, but if the female could determine the quality of the male a priori, she should avoid polyandry. Given low densities of *M. niger* in the Anavilhanas, the observed 100% incidence of polyandry speaks against the choosy-females hypothesis but rather in favor of a bet-hedging mechanism to maximize the chance of producing at least some high-quality offspring.

Incidences of multiple paternity increase effective population sizes with respect to census sizes, and such a population harbors increased

genetic variability and evolutionary potential (Chesser and Baker, 1996). The presence of multiple paternity in *M. niger* may, therefore, be important in maintaining genetic variability of this species. Observed heterozygosity in natural populations from Anavilhanas and Janauacá Lake were 0.70 and 0.67, respectively (de Thoisy et al., 2006). These values are high compared to heterozygosity in other crocodiles (Rodriguez et al., 2008; Hekkala et al., 2010; Milián-García et al., 2011). Therefore, multiple paternity can counter the effects of male territoriality and dominance, as male dominance and territoriality reduce effective population sizes and, consequently, genetic variability of the species. High levels of genetic variability increase a species' or population's evolutionary potential and adaptability to environmental variations. Knowledge of the mating system of an organism is, therefore, important for developing management and conservation strategies, especially when isolated populations are comprised of small numbers of individuals, because multiple paternity has direct consequences for effective population sizes and evolutionary potential (Sugg and Chesser, 1994; Chesser and Baker, 1996).

McVay et al. (2008) posed the following question: "Is polyandrous behavior a common ancestral strategy among crocodilians or did it arise independently in different taxa?" In order to answer this question, studies involving the paternity test are needed for many other crocodilian species that have yet to be investigated, but the present study contributes to answering this question. This study is an unequivocal demonstration of multiple paternity in *M. niger*. However, because of the, albeit remote, possibility of movement of some hatchlings among groups, further analyses are needed, with a greater number of nests from different locations, to determine the precise frequency of multiple paternity among and between nests in this species.

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