

Phylogeographic and Conservation Genetic Analysis of the Black Caiman (*Melanosuchus niger*)

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ABSTRACT We assessed the spatial distribution of the genetic variability of *Melanosuchus niger* from 11 localities in South America using 1,027 base pairs of the mitochondrial cytochrome *b* gene. Screening 132 animals, we found 41 haplotypes, high values of genetic diversity, low values of nucleotide diversity and significant deviations from neutral expectation of allelic frequencies in some localities. Mantel test and nested-clade analysis indicated that isolation-by-distance was an important population dynamic for the species as a whole. Wright's fixation indexes analyses showed that hydrogeographically separated populations from French Guiana together with Amapá state population in Brazil are genetically differentiated from all other populations that are found in the Amazon drainage basin. These indexes also disclosed that the population from Ecuador is genetically differentiated in relation to the populations from Brazil, Peru and French Guiana. Within the Amazon Basin little differentiation exists, and genetic and geographic distances are not correlated. Demographic data as well as population genetic data suggest that *M. niger* is recovering in some protected regions. However, part of this apparent recovery may be owing to the movement of animals into protected regions. *J. Exp. Zool.* 309A:600–613, 2008. © 2008 Wiley-Liss, Inc.

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The six crocodylian species that occur in Brazil belong to the family Alligatoridae (King and Burke, '81; Magnusson, '85) and are divided among three genera: *Caiman*, *Paleosuchus* and *Melanosuchus*. The last genus is monotypic, containing *Melanosuchus niger*, the black caiman, which is the largest alligatorid predator of continental America, reaching to over 5 m in length (Medem, '83). The black caiman is a habitat specialist found in the Amazonian várzea floodplain (Herron, '94). It is also found in the Rupununi Swamp of the Essequibo River watershed, and in a number of drainages of Guiana Shield draining directly into the Atlantic Ocean (Ross, '98).

Crocodylians have traditionally been subject to various forms of legal and illegal exploitation, and more recently to managed exploitation. Economically important crocodylian products include eggs and meat that provide an important protein

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source for Amazonian communities, but these products are also sold clandestinely at national and international markets (Da Silveira and Thorbjarnarson, '99), leather and teeth that are used by the garment and shoe industries and for manufacture of artisan products and fat that is used by the riverine communities in traditional medicines against rheumatism (Santino Mendes, personal communication). Furthermore, similarly to the Amazon river dolphin *Inia geoffrensis*, the black caiman is also used as bait for the carrion-feeding vulture catfish (*Calophysus macropterus*), which are then illegally exported to Colombia and sold as fillets of desirable catfish species (Da Silveira, 2002; Da Silveira and Viana, 2003; da Silva, 2004; WWF_Colombia, 2005). Crocodylians clearly play an important part in the Amazonian economy, although many of the current uses of crocodylians are still illegal and appear to be unsustainable.

Although historically the harvest of crocodylian products was a subsistence activity, a large-scale commercialization began in the 1930s (Smith, '80; Medem, '83; Plotkin et al., '83). Of all the species in South America, the black caiman was the most heavily exploited and consequently the most affected species (Plotkin et al., '83). The preference for black caiman hides over other species was owing to large size and little subcutaneous ossification resulting in high-quality, commercially valuable leather. The hunt intensified in the beginning of the 1950s (Fittkau, '70) and in this form continued until the late 1970s (Smith, '80; Rebêlo and Magnusson, '83). As a result, the black caiman became patchily distributed, the total census population size may have decreased to less than 1/10th the population size estimated in early 20th century, and this population reduction contributed to the demographic fragmentation (Ross, '98). Concomitantly the black caiman also experienced an accelerated rate of habitat loss owing to the settlement of the Amazonian várzea that contains the vast majority of human settlements of the Amazon Basin, and the conversion of these areas to agricultural and pastoral economies (Goulding et al., 2003). The várzea constitutes approximately 3% of the total area of the Amazon Basin making it the most highly threatened Amazonian ecosystem (Goulding et al., 2000). Because *M. niger* is a habitat specialist with a slow growth (Rebêlo and Magnusson, '83; Herron, '94; Thorbjarnarson, '96), and it competes ecologically with the spectacled caiman *Caiman crocodylus*, a much more opportunistic and fast-growing

species (Herron, '94), this may account for the apparent or real low densities and patchy distribution. However, it should be noted that historical and current distribution of the black caiman remains poorly understood, and that in areas that have been well studied, the black caiman appears to be quite commonly observed (Uruena, '90; Da Silveira and Thorbjarnarson, '99; Da Silveira, 2002) and populations in protected areas such as the Mami-rauá Reserve appear to be demographically recovering (Da Silveira et al., '97; Da Silveira and Thorbjarnarson, '99).

Demographic changes induced by anthropogenic pressures can have significant and inevitably negative consequences for natural populations especially if those populations remain at low census numbers (Frankham et al., 2002; Martínez-Cruz et al., 2004). Population genetic studies of crocodylians where nearly all species were heavily impacted by the 20th century skin trade have shown little genetic evidence of population reduction. In a study of the American alligator (*Alligator mississippiensis*), Glenn et al. (2002) found only four mtDNA cytochrome *b* haplotypes in 25 animals sampled from the complete distributional range of this North American species. The authors attributed the low haplotype diversity in the American alligator to a Pleistocene—a prehistoric—population bottleneck, but reasoned that historical demographic bottleneck was unlikely to result in the low genetic diversity observed. In a study of *Crocodylus moreletii*, Dever et al. (2002) did not find reduction in heterozygosity or genetic diversity at nine microsatellite loci despite near extinction of this species in Belize during 1940s–1960s. A microsatellite analysis of several black caiman populations found significant genetic bottlenecks, but attributed them to population history than to recent threats (de Thoisy et al., 2006).

Historical and ecological processes will also impact phylogeographic patterns. We tested whether sampling localities from coastal Atlantic drainages that are hydrologically unconnected to the Amazon Basin are differentiated from those localities sampled in the Amazon Basin. We also tested for the existence of panmixia within the Amazon Basin. Based on four sampling localities, Farias et al. (2004) suggested differentiation of black caiman populations from the Atlantic coastal drainages and those of the Amazon Basin, and lack of differentiation within the Amazon Basin except for the contrast between animals originating from black water and white water drainages. This pattern was observed in a microsatellite analysis

of the black caiman (de Thoisy et al., 2006) and also in *C. crocodilus* (Vasconcelos et al., 2006).

Given our still limited knowledge of the ecological genetics for the black caiman, the goals of this study were to: (1) detect patterns of genetic diversity; (2) evaluate possible genetic differentiation of Amazon Basin from non-Amazon Basin populations and ecological-based differentiation within the Amazon Basin (Farias et al., 2004; de Thoisy et al., 2006) and (3) test if populations are in a genetic equilibrium considering the over-exploitation of the recent past (Fittkau, '70; Smith, '80; Medem, '83; Plotkin et al., '83; Rebêlo and Magnusson, '83). In this way we hope to provide information that might be used for the formulation of better conservation and management strategies of *Melanosuchus* in Amazônia.

MATERIALS AND METHODS

Field protocol

Material for molecular analyses was difficult to collect owing to several factors including obtaining appropriate field and laboratory data collection permits, logistic difficulties associated with accessing remote areas of suitable habitat, low densities and extreme shyness of these animals resulting from years of hunting as well as the inherent danger of handling such large and dangerous predators. Nevertheless, we sampled 132 adults from 11 localities throughout the distributional range of this species. Eight localities were within the Amazon Basin, with the remaining three localities represented by Atlantic coastal drainages descending from the Guiana Shield; these three localities are hydrologically isolated from the Amazon Basin (Fig. 1). This sampling design allowed us to test hypotheses not only of intra-Amazon Basin differentiation, but also of differentiation between animals inhabiting the Amazon Basin to those outside the Amazon Basin. The majority of the collections were nocturnal. Each sample was obtained during routine marking of specimens for mark-recapture studies, and consisted of one or more scutes removed from the tail. Scutes were preserved in 95% ethanol, and kept at ambient temperature until delivered to the laboratory.

Laboratory protocol

Total genomic DNA was extracted from tail scutes using standard phenol-chloroform method: samples were digested with a proteinase K/sodium dodecyl sulfate solution, followed by phenol-

chloroform extraction, the addition of 5 M NaCl and a final 70% ethanol precipitation of DNA product (Sambrook et al., '89).

A mitochondrial fragment consisting of near-complete cytochrome *b* gene, tRNA threonine and partial tRNA proline genes was amplified with the polymerase chain reaction (PCR) primers L14254 (5'-ATGACCCACCAACTACGAAAAT-3') published by Glenn et al. (2002) and H15990 (5'-TTAGAAYGTCGGCTTTGG-3') published by Farias et al. (2004). All PCR reactions were carried out in a final volume of 25 μ L and contained 11.7 μ L of ddH₂O, 3 μ L of 25 mM MgCl₂, 2.5 μ L of 10 mM dNTPs, 2.5 μ L of 10 \times PCR buffer (100 mM Tris-HCl, 500 mM KCl), 2 μ L of each 2 μ M primer, 0.3 μ L of 5 U/ μ L Taq DNA polymerase and 1 μ L of DNA (between 50 and 100 ng).

Cycling conditions were as follows: denaturation at 92°C for 35 s, annealing at 55°C for 35 s and extension at 72°C for 90 s repeated for 35 cycles. PCR products were evaluated on a 1% agarose gel and purified on Qiagen spin-columns (Valencia, CA). Purified PCR product was used in cycle sequencing PCR reactions. Approximately 30 ng of the PCR product was used as a template for each cycle sequencing reaction containing 3–4 μ L of PCR products, 2 μ L of 2 μ M primer, 2 μ L of 5 \times sequencing buffer (400 mM Tris-HCl pH 9.0, 10 mM MgCl₂) and 2 μ L of the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (GE Healthcare). Cycle sequencing primers used were L14254 and L14732 (5'-TCGTGCCATGAATTTGAG-3') from Glenn et al. (2002). Cycle sequencing reactions were precipitated with ethanol according to manufacturer's recommendations. Precipitated product was resuspended in 6 μ L of deionized formamide (Sigma), injected and resolved on the MegaBACE 1000 DNA Analysis Systems (GE Healthcare, São Paulo, Brazil).

Data analysis

Sequence data were verified by blasting them to GenBank, and by direct comparison with published cytochrome *b* sequences of *A. mississippiensis* (AF318548–AF318557 published by Glenn et al., 2002), *C. crocodilus* (AY462456–AY462487 published by Farias et al., 2004) and *M. niger* (AY462488–AY462533 published by Farias et al., 2004). Sequence data were edited, and combined in the program BioEdit 6.0.7 (Hall, '99). Our final data set was an alignment of 1,027 base pairs without indels, representing 132 individuals from

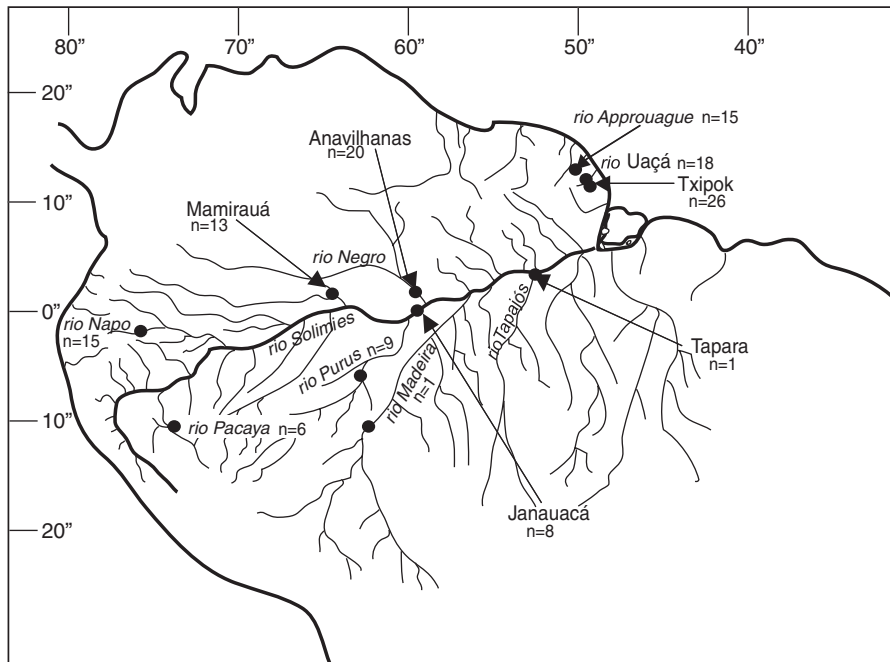


Fig. 1. Geographic distribution of the black caiman populations analyzed and the respective sample numbers per locality. The hatched area indicates the distribution of species *M. niger*. The following coordinates correspond to the central points of the collection localities: Anavilhanas Archipelago (2°32'S and 60°15'W); Purus River (4°43'S and 62°21'W); Janauacá Lake (3°26'S and 60°17'W); Uaçá River (3°45'N and 51°36'W); Txi-pok Lake (3°40'N and 51°17'S); Tapará Community (0°77'S and 97°65'W); Mamirauá Lake (2°59'S and 64°53'W); Madeira River (9°22'S and 6°57'W); Pacaya-Samíria Reserve (4°19'S and 76°55'W); Approuague River (4°40'N and 52°10'W) and Napo River (0°38'S and 76°9'W).

11 collection localities (Fig. 1). Sequence data were further verified by translation into putative amino acids; no stop codons or other anomalies were observed.

Among the various methodologies used to address historical questions of spatial distribution of genetic diversity stands out the nested-clade phylogeographic analysis (NCPA; Templeton, 2004). This analysis reduces the original haplotype network into a hierarchically nested series of haplotype networks using the principles of haplotype grouping described in Templeton et al. ('87) and Templeton and Sing ('93). With each haplotype and nested haplotype is an associated phenotype, and in the case of the NCPA the phenotype is the geographic locality of the haplotype or nested haplotype. The hierarchically nested networks are then tested for significant differences in the means of haplotype geographic localities represented as the clade distance (D_c), the nested-clade distance (D_n) and the interior-tip distances (I- T_c and I- T_n). Significance of differences in mean are tested using 10,000 random permutations and evaluated against the χ^2 distribution—implementation in the program GeoDis 2.0 (Posada et al., 2000). Results of the NCPA

were interpreted using the November 11, 2005, version of the inference key available at <http://darwin.uvigo.es/>. Geographic distances between localities were calculated along river courses, a geographic distance measure most appropriate for aquatic animals (see Fetzner and Crandall, 2003).

We also estimated levels of genetic diversity. Assuming that genetic diversity is a probability that two sequences chosen randomly from a population are different (Li, '97), this measure of polymorphism is then equivalent to the expected heterozygosity of a codominant marker. Additional estimators included the number of segregating sites between sequences (S), Nei's ('87) nucleotide diversity (π), Nei's ('87) gene diversity (\hat{H}) and Watterson's ('75) theta (θ), all calculated in the programs Arlequin 3.1 (Excoffier et al., 2005) and DnaSP 4.0 (Rozas et al., 2003).

To test for mutation-drift equilibrium in the mitochondrial sequence data, we used Tajima's ('89) D statistic and Fu's ('97) F_s statistic. Although both statistics were designed to test for deviations from selective neutrality, in cases where selection can be discounted, mutation-drift disequilibria may be interpreted from a demographic perspective. For example, in a situation of population expansion, the

number of segregating sites will increase significantly compared with average pair-wise sequence divergence, resulting in a significantly negative value of D (Tajima, '89). Similarly, under this scenario one will observe a large excess of rare alleles resulting in significantly negative F_s (Fu, '97). Methodology for estimating both statistics and their significance is implemented in the program Arlequin 3.1 (Excoffier et al., 2005).

The analysis of molecular variance (AMOVA; Excoffier et al., '92) also implemented in the program Arlequin 3.1 (Excoffier et al., '92) was used to test if molecular variation is nonrandomly distributed among user-defined groups. In this study we used AMOVA to test two hypotheses: (1) that samples from the Amazon Basin do not have a significantly different genetic composition from samples originating in the non-Amazonian Atlantic Ocean drainage systems and (2) that sampling localities from the Amazon Basin are not genetically differentiated from each other. Both test the null hypothesis of panmixia, however, at different hierarchical levels. Inferences from AMOVA were confirmed by an analogue of Fisher's test of exact population differentiation (Raymond and Rousset, '95).

Wright's inbreeding coefficients (F) were used to characterize genetic differentiation between populations. We used the method of Cockerham and Weir ('93) to estimate F_{ST} . Statistical significance of F_{ST} values was estimated using bootstrapping implemented in the program Arlequin 3.1 (Excoffier et al., '05), and adjusted using the method of sequential Bonferroni for multiple comparison (Rice, '89). We tested the hypothesis of isolation-by-distance using the Mantel ('67) test, estimating the significance of correlation between a matrix of F_{ST} values and a matrix of geographic distances calculated along river lengths and along the Atlantic coast when comparisons between localities of the Amazon Basin with those outside the basin were being made. In all instances, geographic distances were calculated along river courses, a geographic distance measure most appropriate for aquatic animals (see Fetzner and Crandall, 2003). The Mantel test is also implemented in the program Arlequin 3.1 (Excoffier et al., 2005).

RESULTS

In the aligned matrix 1,027 nucleotides collected for 132 individuals from 11 collecting localities, we observed 41 haplotypes differentiated by 52 polymorphic sites (Table 1). Haplotypes are deposited in GenBank under numbers EU271969–

EU272009. Haplotypes H1 and H2 were most frequent and widely distributed, occurring in approximately 60% of the sampled individuals (Table 2). These two haplotypes differ from each other by a transition (C ↔ T) at nucleotide position 614. Neither one of these two haplotypes were observed in animals from the Napo River, Ecuador. Translation of the 1,027 base pair haplotypes in the program BioEdit (Hall, '99) resulted in 340 amino acids and no unexpected stop codons or other aberrations. The characteristic mtDNA anti-G bias (Zhang and Hewitt, '96) was observed in all sequences, leading us to conclude that we have not amplified and sequenced nuclear pseudogenes.

The nested-clade structure shown in Figure 2 illustrates the terminal nodes that differ by just one base substitution, and are contained in one-step clades numbered 1-1 through 1-16. The nested clades 1-3 and 1-6 that included the haplotypes H1 and H2—the likely oldest haplotypes in this analysis—were used in NCPA clades with both geographic and genetic variation. Thus they were the only nested clades that could be statistically analyzed. In the second nested-clade level, the null hypothesis of no association between geographic and genetic differentiation could not be rejected for all the clades. Significant values (permutational $\chi^2 = 74.0000$, $P < 0.0001$) were observed in clade 2-3; however, the sampling scheme did not allow us to test among alternate hypotheses of historical differentiation between the sample from Ecuador (clade 1-4) and remaining samples from the Amazon Basin (clade 1-3). The nested-clade levels 3-1 and 3-2 are not statistically significant; thus the hypothesis of panmixia at this nesting level cannot be rejected. In the final nested level (4-1), which is composed of clades 3-1 and 3-2, significant association of geographic and genetic distance exists (permutational $\chi^2 = 53.9282$, $P < 0.0001$); however, owing to the lack of an internal haplotype, no conclusions about the process can be drawn. This final contrast is between all the animals from the Amazon Basin and those from the Guianas region, i.e. coastal drainages that flow directly into the Atlantic Ocean without first emptying into the Amazon Basin.

To confirm and expand on the results obtained from the NCPA, we performed a posteriori analysis of population genetic structure. Using AMOVA (Excoffier et al., '92), we tested three hypotheses: (1) the first hypothesis tested differentiation among localities; (2) the second hypotheses tested differentiation between the samples

TABLE 2. Distribution of the 41 haplotypes found in the cytochrome *b* fragment (1,027bp) of 132 individuals of *M. niger*

Haplotypes	Brazil								Peru	French Guiana	Ecuador	Total
	Anavilhanas Archipelago	Purus River	Janaucá Lake	Uaçá River	Txipok Lake	Tapará Community	Mamirauá Lake	Madeira River	Pacaya-Samíria	Approuague River	Napo River	
H1	10	4	4	2	6	-	7	1	3	3	-	40
H2	1	2	2	9	10	1	4	-	-	10	-	39
H3	-	-	-	-	-	-	-	-	-	-	11	11
H4	4	-	-	-	-	-	-	-	-	-	-	4
H5	1	-	-	-	-	-	-	-	-	-	-	1
H6	-	-	-	-	-	-	-	-	-	1	-	1
H7	-	-	-	-	-	-	-	-	1	-	-	1
H8	-	-	-	-	-	-	-	-	1	-	-	1
H9	-	-	-	-	1	-	-	-	-	-	-	1
H10	-	-	-	1	-	-	-	-	-	-	-	1
H11	-	-	1	-	-	-	-	-	-	-	-	1
H12	-	1	-	-	-	-	-	-	-	-	-	1
H13	-	-	-	-	-	-	-	-	-	-	1	1
H14	-	-	-	-	-	-	-	-	-	-	1	1
H15	-	-	-	-	-	-	-	-	-	-	1	1
H16	-	-	-	-	-	-	-	-	-	-	1	1
H17	-	-	-	-	1	-	-	-	-	-	-	1
H18	-	-	-	-	-	-	1	-	-	-	-	1
H19	-	-	-	-	1	-	-	-	-	-	-	1
H20	-	-	-	1	-	-	-	-	-	-	-	1
H21	-	-	-	1	-	-	-	-	-	-	-	1
H22	-	-	-	1	-	-	-	-	-	-	-	1
H23	-	-	-	-	-	-	-	-	-	1	-	1
H24	-	-	-	1	-	-	-	-	-	-	-	1
H25	-	-	-	1	-	-	-	-	-	-	-	1
H26	-	-	-	1	-	-	-	-	-	-	-	1
H27	-	-	-	-	1	-	-	-	-	-	-	1
H28	-	-	-	-	1	-	-	-	-	-	-	1
H29	-	-	-	-	1	-	-	-	-	-	-	1
H30	-	-	-	-	1	-	-	-	-	-	-	1
H31	-	-	-	-	1	-	-	-	-	-	-	1
H32	-	-	-	-	1	-	-	-	-	-	-	1
H33	-	-	-	-	1	-	-	-	-	-	-	1
H34	1	-	-	-	-	-	-	-	-	-	-	1
H35	1	-	-	-	-	-	-	-	-	-	-	1
H36	-	2	-	-	-	-	-	-	-	-	-	2
H37	-	-	1	-	-	-	-	-	-	-	-	1
H38	-	-	-	-	-	-	-	-	1	-	-	1
H39	-	-	-	-	-	-	1	-	-	-	-	1
H40	1	-	-	-	-	-	-	-	-	-	-	1
H41	1	-	-	-	-	-	-	-	-	-	-	1
Total	20	9	8	18	26	1	13	1	6	15	15	132

from localities that are within the Amazon Basin and those that are outside of it; and (3) the third hypothesis tested differentiation within the Amazon Basin. Testing the first hypothesis we observe that sampling localities are differentiated ($F_{ST} = 0.3000$, $P < 0.0001$); however, the contrast between the localities that are within the Amazon Basin and those that are outside of it is not significant ($F_{CT} = 0.1596$, $P = 0.098$). The great majority of genetic variance is concentrated within groups (70.71%). Results of the test of the third

hypothesis indicate structuring within the Amazon Basin ($F_{ST} = 0.2726$, $P < 0.0001$). Differences are observed even when the most divergent locality, the Cuyabeno Faunal Reserve on the Napo River, is removed from the analysis ($F_{ST} = 0.2690$, $P < 0.0001$). An exact test of global population differentiation (Raymond and Rousset, '95) also supported the hypothesis of differentiation among localities ($P < 0.0001$); however, it fails to reject nondifferentiation within the Amazon Basin.

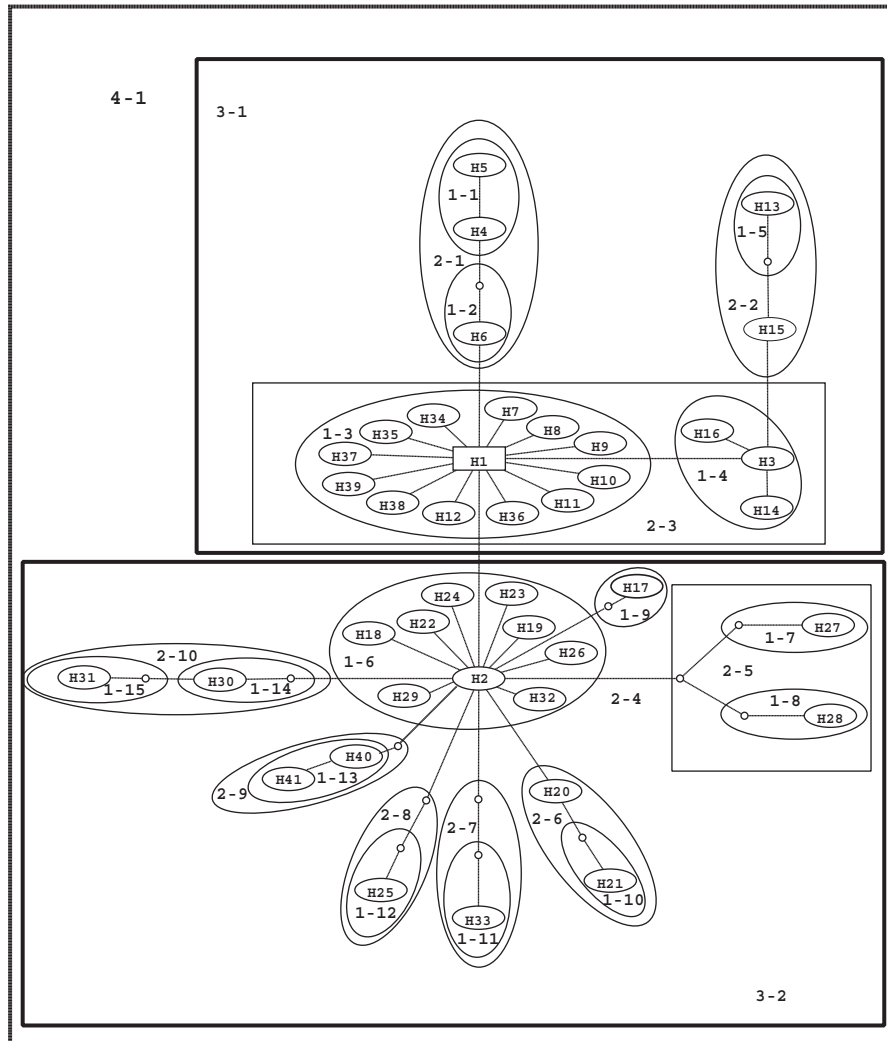


Fig. 2. A statistical parsimony network among the 41 mtDNA haplotypes detected in *M. niger*. Each line in the network represents a mutational change. The small circles represent inferred but not detected haplotypes, and * indicates significant association between genetic divergence and geographic distance detected by NCPA. NCPA, nested-clade phylogeographic analysis.

A significant correlation between geographic and genetic distances could be observed ($r = 0.3133$, $P = 0.014$). The differentiation among sampled localities observed in AMOVA may therefore be compatible with the model of isolation-by-distance at large geographic distances. However, after the removal of the Atlantic coast localities, there no longer is significant correlation between genetic and geographic distances ($r = 0.1245$, $P = 0.323$).

In pair-wise F_{ST} comparisons of differentiation among individual localities, all instances that involved the Napo River Cuyabeno Faunal Reserve locality of Ecuador, the Approuague River of French Guiana and the Uaçá River and Txipok Lake from the Atlantic drainages of Brazil were significant. Significant differences were not ob-

served within the Amazon Basin or among the Atlantic coastal drainages (Table 3).

For population genetic analyses, we removed the Madeira and Tapará Communities both of which had only one individual. In the remaining localities we observed high values of genetic diversity (\hat{H}) in comparison with nucleotide diversity (π). When all localities were combined, both Tajima's ('89) D and Fu's ('97) F_s tests showed significant negative deviation; $D = -2.527$ ($P < 0.0001$) and $F_s = -27.622$ ($P < 0.0001$), respectively. When sampling localities were analyzed separately, only in the Txipok Lake was selective neutrality rejected by Tajima's D test. Selective neutrality was also rejected in Txipok Lake and Uaçá River, the two localities of Brazil that drain directly into

TABLE 3. Matrix of pair-wise F_{ST} values using distance method with the respective P values (below diagonal) and effective number of migrants (N_m) between the pairs of populations with the respective geographic distances (above diagonal)

Populations	Anavilhanas Archipelago	Purus River	Janauacá Lake	Uaçá River	Txipok Lake	Mamirauá Lake	Pacaya-Samiría	Approuague River	Napo River
Anavilhanas Archipelago	-	6.9613 (255 km)	9.9846 (105 km)	1.4898 (1,800 km)	2.1418 (1,850 km)	4.7495 (605 km)	9.0285 (1,655 km)	1.7849 (1,970 km)	0.8233 (1,835 km)
Purus River	0.0670 ($P = 0.0900$)	-	∞ (250 km)	1.8000 (720 km)	4.0344 (1,750 km)	27.8114 (450 km)	9.0000 (1,450 km)	1.5340 (2050 km)	0.5062 (1,680 km)
Janauacá Lake	0.0476 ($P = 0.1891$)	-0.0215 ($P = 0.5765$)	-	2.2248 (1,700 km)	5.9356 (1,750 km)	∞ (590 km)	12.8372 (1,670 km)	1.9140 (1,750 km)	0.5036 (820 km)
Uaçá River	0.2512* ($P < 0.0001$)	0.2173* ($P < 0.0001$)	0.1834* ($P < 0.0001$)	-	79.4831 (50 km)	3.3861 (2,290 km)	1.0276 (3,340 km)	∞ (170 km)	0.3912 (3,520 km)
Txipok Lake	0.1892* ($P < 0.0001$)	0.1102* ($P < 0.0001$)	0.0776 ($P = 0.0270$)	0.0062 ($P = 0.2522$)	-	10.9352 (2,340 km)	2.0517 (3,390 km)	∞ (220 km)	0.5963 (3,550 km)
Mamirauá Lake	0.0952 ($P = 0.0450$)	0.0176 ($P = 0.0307$)	-0.0307 ($P = 0.2342$)	0.1286 ($P = 0.0090$)	0.0437 ($P = 0.0991$)	-	3.1934 (1,050 km)	4.2193 (2,450 km)	0.4433 (1,230 km)
Pacaya-Samiría	0.0524 ($P = 0.1711$)	0.0526 ($P = 0.2072$)	0.0374 ($P = 0.5225$)	0.3273* ($P < 0.0001$)	0.1959* ($P = 0.0991$)	0.1353 ($P = 0.0720$)	-	0.7110 (3,070 km)	0.53187 (350 km)
Approuague River	0.2188* ($P < 0.0001$)	0.2458 ($P = 0.0360$)	0.2071 ($P = 0.0270$)	-0.0032 ($P = 0.5225$)	-0.0211 ($P = 0.8828$)	0.1059 ($P = 0.0991$)	0.4128* ($P < 0.0001$)	-	0.2871 (3,680 km)
Napo River	0.3748* ($P < 0.0001$)	0.4918* ($P < 0.0001$)	0.4911** ($P < 0.0001$)	0.5570* ($P < 0.0001$)	0.4560* ($P < 0.0001$)	0.5354* ($P < 0.0001$)	0.4845* ($P < 0.0001$)	0.6323* ($P < 0.0001$)	-

*Shows the significance level after the Bonferroni correction ($P < 0.0014$).

TABLE 4. Indexes of genetic diversity and test of populational equilibria in black caiman populations

Population	N	hp	S	θ	π	\hat{H}	Tajima's D (P value)	Fu's F_s (P value)
Anavilhanas Archipelago	20	4	10	2.818696 ± 1.264173	0.002173 ± 0.001396	0.7316 ± 0.0940	-0.72839 (0.2380)	-1.81775 (0.1350)
Purus River	9	1	3	1.103811 ± 0.738453	0.000974 ± 0.000817	0.7778 ± 0.1100	-0.35929 (0.3860)	-1.03877 (0.1180)
Janauacá Lake	8	2	3	1.157025 ± 0.781078	0.000904 ± 0.000788	0.7500 ± 0.1391	-0.81245 (0.2620)	-1.38724 (0.046)
Uaçá River	18	7	10	2.907355 ± 1.321162	0.001451 ± 0.001027	0.7582 ± 0.1056	-1.75532 (0.0340)	-5.05416* (< 0.0001)
Txipok Lake	26	16	20	5.241148 ± 1.991315	0.002028 ± 0.001305	0.8154 ± 0.0627	-2.16030* (0.0020)	-5.92452* (0.0020)
Mamirauá Lake	13	2	3	0.966741 ± 0.631371	0.000799 ± 0.000686	0.6538 ± 0.1060	-0.47825 (0.3010)	-0.94022 (0.1210)
Pacaya-Samiría	6	3	3	1.313869 ± 0.909766	0.000974 ± 0.000871	0.8000 ± 0.1721	-1.23311 (0.0960)	-1.81298 (0.0120)
Approuague River	15	2	3	0.922633 ± 0.597805	0.000668 ± 0.000602	0.5429 ± 0.1327	-0.76384 (0.2780)	-1.16127 (0.0850)
Napo River	15	4	5	1.537722 ± 0.847536	0.000760 ± 0.000656	0.4762 ± 0.155	-1.66013 (0.0240)	-2.16724 (0.0140)
Amazon Basin	71	15	24	4.966027 ± 1.603120	0.001646 ± 0.001081	0.8479 ± 0.0444	-2.04341* (0.002)	-18.02110* (< 0.0001)
All samples	130	39	53	9.741036 ± 2.582490	0.001769 ± 0.001135	0.9116 ± 0.0236	-2.52310* (< 0.0001)	-27.60205* (< 0.0001)

N , number of individuals sampled; hp, number of unique haplotypes observed; S , number of segregating (polymorphic) sites; θ , Watterson's theta; π , Nei's nucleotide diversity; \hat{H} , Nei's gene diversity. *Significant values after Bonferroni correction ($P < 0.0056$).

the Atlantic Ocean. Other localities are in mutation-drift equilibrium (Table 4).

DISCUSSION

Genetic diversity and population genetic estimates

In this study, elevated indexes of genetic diversity ($\hat{H} = 0.9116 \pm 0.0236$; $\hat{H} = 10.8479 \pm 0.0444$ within the Amazon Basin) were found for the black caiman. These values were greater than those previously observed by Farias et al. (2004) who analyzed four localities ($\hat{H} = 0.715 \pm 0.049$). The observed genetic diversity in this study was also greater than that observed for *C. crocodilus* ($\hat{H} = 0.733 \pm 0.042$) sampled from largely overlapping localities (Vasconcelos et al., 2006). The North American alligator studied by Glenn et al. (2002) has low genetic diversity ($\hat{H} = 0.313 \pm 0.332$), as does Morelet's crocodile from Central America ($\hat{H} = 0.502 \pm 0.048$) reported in Ray et al. (2004). In general, indexes of nucleotide diversity π (Nei, '87) were low in all studies of crocodylians including this study. Elevated values of \hat{H} together with low values of π , as observed in the black caiman, have been suggested to characterize populations that are undergoing demographic expansion after a period of low effective population size (Grant and Bowen, '98). The significantly negative values of Tajima's *D* and Fu *F*'s test statistics can also be interpreted as a signature of a demographic expansion in situations where selective nonneutrality can be disproved.

In spite of clear and drastic reduction in numbers of the black caiman, the mitochondrial DNA data do not detect a signature of a significant population reduction. On the contrary, in the case of the Uaçá River and Txipok Lake, the data suggest a population expansion. There are at least three explanations for this pattern: (i) The current demographic population reduction may be masqueraded by a previous demographic expansion that left a strong signature on the mitochondrial data (e.g. pacific crabs studied by Lavery et al., '96); (ii) the large-scale exploitation of the last 100 years is relatively short with respect to the black caiman's generation time to be detected in the mitochondrial DNA data; (iii) because of large historical census sizes and the black caiman's longevity, the black caiman still has relatively large census sizes even if only it represents 10% of the estimated numbers in the earlier part of the last century, and so reduction in genetic diversity owing to genetic drift will be difficult to detect

over relatively short ecological time scales. The observed sign of a population expansion in the Uaçá River and Txipok Lake may represent a true demographic event. In these regions of the Amapá state, the indigenous population almost exclusively hunts *C. crocodilus* whose survey numbers are very depressed, whereas the black caiman is abundant and its census numbers are expanding.

Similarly, the relatively large values of θ correspond to a genetically diverse species, and are indicative of a large inbreeding effective population size; in our case an estimate on the order of 10^5 effective female individuals—estimated from $\theta = 4N_e\mu$ —in the Amazon Basin, and twice that number when individuals from outside the Amazon Basin are included. These estimates assume a mitochondrial DNA mutation rate of 10^{-8} per site per generation. Even these rough inbreeding effective population size estimates do not appear to reflect the current demographic status of the black caiman. The estimate more likely reflects a long-term or historical effective population size (Garrigan et al., 2002), which is relevant in historical but not ecological time owing to lag in the response time of genetic diversity coming to an equilibrium with the black caiman's current survey size. This phenomenon has been observed in a number of species. For instance, recent population isolation neither resulted in significantly lower genetic diversities in the American alligator (Ryberg et al., 2002) nor in the Australasian skink (Sumner et al., 2004). Similar genetic patterns have also been observed in the Atlantic cod, a species that has had a long history of intense commercial exploitation, and in the early 1990s its fishery stocks collapsed, yet inbreeding effective population sizes inferred from DNA data remain high, and show no significant decrease over time (Ruzzante et al., 2001; Poulsen et al., 2006). Therefore, the lack of observed signal of population decline and relatively large effective population sizes in the mitochondrial DNA markers should not be necessarily considered as a lack of a possible historical overexploitation or an indication of current abundance.

Nested-clade analysis and genetic structure

Although NCPA could not differentiate among several potential processes that could have resulted in the observed pattern of genetic structuring over the geographic landscape, it did reject the null hypothesis of panmixia confirming a previous

study of Farias et al. (2004). The populations of Amapá, Brazil—Uaçá River and Txipok Lake—and of the Approuague River, French Guiana, are differentiated from populations of the Amazon Basin, and genetic distances are correlated with geographic distances. Discounting the possibility that the differentiation is owing to insufficient sampling, the most likely explanation for the observed diversification is due to the fact that the Amapá and French Guiana populations occur in coastal Atlantic drainages, whereas all of the other populations occur in the Amazon Basin. Thus, these two areas are hydrologically separated, and their respective *M. niger* populations appear to be genetically differentiated. Genetic differentiation observed between localities from Atlantic drainages and those from the Amazon River Basin corroborates a hypothesis of vicariant differentiation previously proposed by Vasconcelos et al. (2006) for *C. crocodilus*. The salt water of the Atlantic Ocean is inferred to function as a physical barrier for the dispersal for this species in a similar way that salt water is a barrier to dispersal for other alligatorids owing to their inability to excrete excess salt (Taplin and Grigg, '89).

The results of Mantel test indicate that there is a significant correlation between genetic and geographic distances among sampled localities of the black caiman. This observation contrasts with other crocodylian studies, none of which have encountered a pattern of isolation-by-distance (e.g. Ray et al., 2004). A similar geographic sampling of the spectacled caiman also does not show significant correlation between geographic and genetic distance even across 3,070 km sampling range ($r = 0.4621$, $P = 0.058$; Vasconcelos et al., 2006). However, even for the black caiman, significant correlation between genetic and geographic distances is observed only at large geographic distances; the correlation does not exist among localities within the Amazon Basin. The pattern of lack of correlation between genetic and geographic distances within the Amazon Basin is also observed in other várzea vertebrates such as the fishes *Arapaima gigas* (Hrbek et al., 2005) and *Colossoma macropomum* (Santos et al., 2007), the manatee *Trichechus inunguis* (Cantanhede et al., 2005) and birds of the genus *Xiphorhynchus* (Aleixo, 2006), and may, therefore, represent a common pattern for várzea-associated organisms. The várzea is the floodplain of the Amazon River and many of its tributaries, and therefore it may act as a conduit for the exchange of individuals between different regions of the Amazon Basin.

In the present analysis we do not find significant differences associated with water type, although genetic differentiation within water type is smaller than between water types. Although nonsignificant in this study, this observation should be taken into consideration in management plans. Farias et al. (2004) found a weak differentiation between white and black water systems of Central Amazônia, and de Thoisy et al. (2006) observed significant differentiation among geographically proximal black and white waters in Brazil and French Guiana using highly variable microsatellite DNA markers. It is therefore probable that local adaptations exist. The existence of ecological differentiation based on water type has also been suggested by Da Silveira (2002). Together these genetic and ecological findings suggest that the observed black water/white water differentiation might be a real geographic structuring factor in Amazônia by reducing genetic exchange between limnologically differentiated systems. Limnological differences result in difference in prey availability, different prey species compositions and also differences in available nutrient levels.

Conservation benefits

A recent change in the World Conservation Union's classification of the black caiman as "vulnerable" and no longer as "endangered" and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) change from Appendix I to Appendix II listed species reflect, in spite of recent demographic recoveries, that its current demographic status still remains tentative and highly dependent on conservation efforts at the population level (Hilton-Taylor, 2000). The few recent field surveys indicate that the black caiman is locally extinct in many places throughout Amazônia, and occurs in strongly reduced densities in many others (Rebêlo and Lugli, 2001; de Thoisy, 2004); however, in some protected areas such as the Mamirauá Reserve the black caiman appears to be increasingly abundant (Da Silveira and Thorbjarnarson, '99; Da Silveira, 2002). From demographic studies it is clear that the number of individuals has increased since the extremely depressed levels of 1970s and early 1980s, although it is unlikely that population levels are anywhere near the levels observed before the beginning of the hide trade in 1930s (Bates, 1864). This increase in census sizes has recently prompted the Brazilian environmental agency IBAMA to approve a pilot-managed hunt of the black caiman

in the Mamirauá RDS, Amazonas, Brazil (source: official website of the Secretaria de Estado de Meio Ambiente e Desenvolvimento Sustentável/SDS—www.sds.am.gov.br/noticia.php?xcod = 922).

Within the Mamirauá Reserve it is interesting to note that the distribution of size classes appears not to be heavily skewed toward young animals, and that genetic indexes do not indicate disequilibrium. The apparent increase in number of animals in the Mamirauá Reserve may therefore be owing in part to immigration of individuals from surrounding unprotected areas. Another possibility is that a significant portion of the collected individuals were relatives, which would limit the power of Tajima's *D* and Fu's *F*s to detect a genetic disequilibrium. Our data show that populations of Uaçá River and Txipok Lake, both of which are located on the Uaçá indigenous lands, most likely are experiencing a demographic growth. This is supported by the observations that spectacled caiman (*C. crocodilus*) is heavily hunted as food sources, whereas the black caiman is not consumed or hunted. This probably allows demographic expansion of the black caiman into areas and the ecological niche previously occupied by the spectacled caiman.

Despite the fact that many of the genetic indexes observed in this study reflect the historical and not the current population, and that some of the observed demographic increases may be caused by immigration rather than mortality decrease, it seems that the black caiman is starting to recover in some areas of the Amazon Basin. As with many organisms of the Amazon region, the black caiman is an important component of the regional socio-economic and cultural landscape. Given recent demographic trends and the genetic structure of the species, there is some potential for the sustainable use and harvest of this species in regulated areas of its geographic distribution. However, further genetic studies that not only supply basic information on the spatial and temporal patterns of genetic variability and on reproductive strategies, but also monitor perturbations owing to harvesting of individuals, are needed to provide supporting data for the development and support a large-scale management program that preserves the genetic heritage of this species while it also respects local socioeconomic needs.

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