



## Population genetic analysis of *Caiman crocodilus* (Linnaeus, 1758) from South America

William R. Vasconcelos<sup>1</sup>, Tomas Hrbek<sup>1,2</sup>, Ronis Da Silveira<sup>3</sup>, Benoit de Thoisy<sup>4</sup>, Boris Marioni<sup>5</sup> and Izeni P. Farias<sup>1</sup>

<sup>1</sup>Universidade Federal do Amazonas, Departamento de Ciências Biológicas, Laboratório de Evolução e Genética Animal, Mini Campus ICB, Manaus, AM, Brazil.

<sup>2</sup>University of Puerto Rico, Biology Department, San Juan, Puerto Rico.

<sup>3</sup>Universidade Federal do Amazonas, Departamento de Ciências Biológicas, Laboratório de Zoologia, Mini Campus ICB, Manaus, AM, Brazil.

<sup>4</sup>Association Kwata, Cayenne, French Guiana.

<sup>5</sup>Instituto Piagaçu-Purus, Manaus, AM, Brazil.

### Abstract

The genetic structure of *Caiman crocodilus* was investigated using a 1085 bp mtDNA fragment of the cytochrome *b* gene. Inferences were based on 125 individuals from nine localities in Peru, Brazil and French Guiana. With the exception of Mamirauá Lake, Anavilhanas Archipelago and the Taparú Community which show a signal of demographic expansion, the sampled localities are in a mutation-drift genetic equilibrium. Divergence between the Amazon basin and extra-Amazon basin localities is significant; however, inference from Nested Clade Analysis cannot distinguish between continuous range expansion, long distance colonization or past fragmentation; however, past fragmentation is unlikely due to low number of mutational steps separating these two regions. The divergence is probably maintained by the reduced ability of *C. crocodilus* to cross salt water barriers. Within the Amazon basin, continuous range expansion without isolation-by-distance is the most likely process causing genetic structuring. The observed genetic patterns are compatible with the ecology of *C. crocodilus*, and history of human exploitation. As commercial hunting depleted more valuable species, *C. crocodilus* expanded its range and ecological niche, prompting hunters to harvest it. Following a period of intense hunting, *C. crocodilus* is now experiencing recovery and a second population expansion especially in protected areas.

*Key words:* genetic structure, phylogeography, genetic diversity, demographic expansion, cytochrome *b*, *Caiman crocodilus*.

Received: April 13, 2005; Accepted: October 24, 2005.

### Introduction

The study of spatial and temporal distribution of intraspecific genetic variability is one of the principal foci of molecular ecology. They provide important data that shed light on evolutionary processes and spatio-temporal dynamics of often complex natural populations of the Neotropics. It is these evolutionary processes that allow species to adapt to dynamically changing environments that should be conserved (Smith *et al.*, 1997; Smith *et al.*, 2001). Therefore, molecular ecological studies can provide vital information for the conservation and management of biological diversity.

Send correspondence to Izeni Pires Farias. Universidade Federal do Amazonas, Departamento de Ciências Biológicas, Laboratório de Evolução e Genética Animal, Mini Campus ICB, Av. Gen. Rodrigo Octávio Jordão Ramos 3000, 69077-000 Manaus, AM, Brazil. E-mail: izeni\_farias@ufam.edu.br.

Brazil, and in particular Amazônia, is rich in biodiversity (Myers *et al.*, 2000). Of the seven alligatorid crocodylians (family Alligatoridae), five to six species occur in Brazil and four to five of those occur in Amazônia. The Brazilian species are classified in the genera *Caiman*, *Melanosuchus* and *Paleosuchus*. *Melanosuchus* is restricted to the Amazon, Essequibo and Oiapoque basins, while *Paleosuchus* is also found in the Orinoco basin and coastal drainages of The Guianas and the littoral of Brazil. *Caiman* has a much wider distribution, and is found from southern Mexico to northern Argentina, including all major South American drainages. *Caiman crocodilus* (the spectacled caiman) can reach 2.5 m of total body length. Females reach sexual maturity at three to four years of age (Staton and Dixon, 1977), the same age as *Alligator mississippiensis*, which is much less than the average age of

nine years at female sexual maturity found in other crocodylian species (Brisbin Jr., 1988).

The taxonomy of *Caiman* is not firmly established, but most recent taxonomic studies recognize the species *C. crocodilus*, *C. yacare* and *C. latirostris* (Busack and Pandya, 2001). *Caiman latirostris* is found in the Paraná and São Francisco River basins, and *C. yacare* mainly occurs in the Pantanal and Bolivian basins but also extends along the Madeira River into the Amazon basin. *Caiman crocodilus* is the most widely distributed species, found from southern Mexico south to the Amazon River basin (Ross, 1998). *Caiman crocodilus* has been classified into three subspecies in addition to the nominal subspecies. However, the only analysis that investigated morphological differentiation among regions of occurrence, and thus the validity of these subspecies, failed to show any consistent differences among the subspecies, rejecting their validity (Busack and Pandya, 2001). *Caiman yacare*, which sometimes is included as a subspecies of *C. crocodilus*, was significantly differentiated from *C. crocodilus* at a series of morphological and morphometric traits (Busack and Pandya, 2001).

Populations of *C. crocodilus* became severely threatened by the hide trade between 1960 and 1969, when more than 1.5 million skins were exported legally from the Brazilian Amazon (Smith, 1980). Harvest started focusing on *C. crocodilus* when commercially more desirable species, such as *Melanosuchus niger*, became too severely depleted to be harvested profitably. A little more than two decades thereafter, in studies conducted between 1993 and 1996 in the Jaú National Park, Rebêlo and Lugli (2001) found little demographic evidence of past overexploitation. The authors attributed the apparent well being of *C. crocodilus* in this area to a demographic recovery from past overexploitation. A pattern of demographic recovery is also observed in other regions of Amazônia (George Rebêlo personal communication).

Little is known about population genetic structuring and gene-flow patterns of *C. crocodilus*. Up to now, the only population genetic study is that of Farias *et al.* (2004) which investigated *C. crocodilus* from two localities in Brazil (Piagaçu-Purus Reserve and Janauacá Lake) and one locality in French Guiana (Approuague River). The authors found a signal of population expansion and high levels of genetic polymorphism in all three populations. They also found significant genetic differentiation between French Guiana and Brazil. However, the sampling scheme of Farias *et al.* (2004) was inadequate to discriminate among alternative historical processes underlying the observed differentiation between French Guiana and Brazil. It was also unable to test the hypothesis of panmixia within the Amazon basin.

The objective of this study was to quantify genetic variability and its spatial distribution in *C. crocodilus*. We used these patterns to test two specific hypotheses: 1) have *C. crocodilus* populations experienced a demographic and

genetic recovery, as hypothesized by Rebêlo and Lugli (2001) and Farias *et al.* (2004); and 2) does *C. crocodilus* of the Amazon basin form a panmictic population, as alluded to in Farias *et al.* (2004) and observed in other large Amazonian vertebrates (Cantanhede *et al.*, 2005; Hrbek *et al.*, 2005).

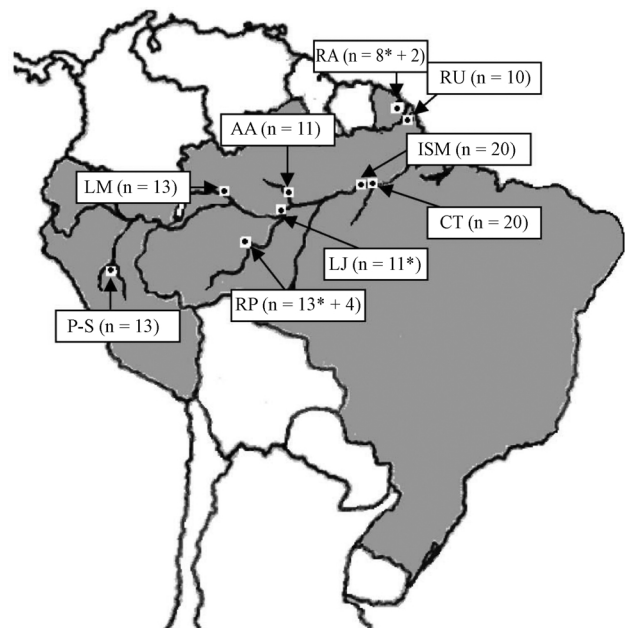
## Materials and Methods

### Samples

Samples of caudal scutes were collected from 125 individuals at nine localities during the years 2002, 2003 and 2004. The nine localities were: Approuague River (Kaw Swamps N.R.) in French Guiana; Uaçá River (A.I. Uaçá) in Amapá State, Brazil; São Miguel Island and the Tapará Community in Pará State, Brazil; the Anavilhanas Archipelago (E.E. Anavilhanas), Janauacá Lake, lower Purus River (Piagaçu-Purus RDS) and Mamirauá Lake (Mamirauá RDS) in Amazonas State, Brazil; and Pacaya-Samiria National Reserve in Peru (Figure 1). The majority of the samples was collected at night, and samples were preserved in 95% ethanol at ambient temperature until being processed in the laboratory.

### Laboratory protocol

Total genomic DNA was extracted using a standard phenol/chloroform method and precipitated with 70% ethanol (Sambrook *et al.*, 1989). The mitochondrial cytochrome



**Figure 1** - Geographic distribution of the nine localities analyzed in this study. N corresponds to the number of individuals sampled. Sampled localities and their geographic coordinates are: Approuague River - AR (4°40' N & 52°10' W); Uaçá River - UR (3°45' N & 51°36' W); São Miguel Island - SMI (0°77' S & 97°65' W); Tapará Community - TC (0°77' S & 97°65' W); Anavilhanas Archipelago - AA (2°32' S & 60°15' W); Janauacá Lake - JL (3°26' S & 60°17' W); Purus River - PR (4°43' S & 62°21' W); Mamirauá Lake - ML (2°59' S & 64°53' W) and Pacaya-Samiria - P-S (4°19' S & 76°55' W).

*b* gene was amplified via Polymerase Chain Reaction (PCR) using the primers L14254 (5'-ATGACCCACCAACTACG AAAAT-3') from Glenn *et al.* (2002) and H15982 (5'-TCC CTGCTTTGGTAGCCAGG-3') from Farias *et al.* (2004).

PCR reactions were carried out in a final volume of 25  $\mu$ L and contained 11.7  $\mu$ L of ddH<sub>2</sub>O, 3  $\mu$ L of MgCl<sub>2</sub> (25mM), 2.5  $\mu$ L of dNTPs (10 mM), 2.5  $\mu$ L of 10x buffer (100 mM Tris-HCl, 500 mM KCl), 2  $\mu$ L of each primer (2  $\mu$ M), 0.3  $\mu$ L of Taq DNA Polymerase (5 U/ $\mu$ L) and 1  $\mu$ L of DNA (concentration varied between 50 ng and 100 ng). PCR conditions were as follows: denaturation at 92 °C for 35 s, primer annealing at 55 °C for 35 s, and primer extension at 72 °C for 90 s; these three steps were repeated 35 times, and followed by a final extension at 72 °C for 5 min. Purification of products was done using the GFXTM PCR DNA Kit (Amersham Bioscience, São Paulo) following the manufacturer's protocol.

Purified PCR products were sequenced directly. Each reaction contained 4  $\mu$ L of amplified DNA product (~30 ng), 2  $\mu$ L of primer (L14254 for the 5' segment of the amplified DNA fragment, and L14731 (5'-TCGTGCCAT GAATTTGAG-3') from Glenn *et al.* (2002) as an internal primer for the 3' portion of our DNA fragment), 2  $\mu$ L of 5x replacement buffer (400 mM Tris-HCl pH 9.0, 10 mM MgCl<sub>2</sub>) and 2  $\mu$ L of DYEnamic ET Dye Terminator mix (Amersham Bioscience, São Paulo). Cycle sequencing PCR conditions were as follows: denaturation at 93 °C for 15 s, primer annealing at 50 °C for 35 s, and primer extension at 60 °C for 120 s; these three steps were repeated 35 times. Resulting fluorescently labeled product was precipitated using a mixture of 70% ethanol and 175 mM ammonium acetate. Precipitated DNA product was resuspended in Hi-Di Formamide, and resolved on a MegaBACE 1000 automatic DNA analysis system (Amersham Bioscience, São Paulo) using the manufacturer's recommended settings.

## Data verification

Identity of the 125 DNA products was verified by comparing the data with cytochrome *b* sequences of *Alligator mississippiensis* (AF318548-AF318557) (Glenn *et al.*, 2002), *Melanosuchus niger* and *Caiman crocodilus* (AY462456-AY462487) (Farias *et al.*, 2004), and *C. crocodilus* (NC002744) (Janke *et al.*, 2001) deposited in GenBank. Sequences were aligned by eye in the program BioEdit (Hall, 1999), and conceptually translated into amino acids. The 1085 bp alignment did not show insertions or deletions, and translation produced no unexpected stop codons.

## Intraspecific analytical methods

Relative contributions of historical and ongoing processes are not easy to distinguish, thus various strategies

have been proposed (Templeton *et al.*, 1987; Bernatchez, 2001). In this study we used the Nested Clade Analysis (NCA) developed by Templeton and colleagues (Templeton and Sing, 1993; Templeton *et al.*, 1995; Templeton, 2001; 2004). The program TCS 1.18 (Clement *et al.*, 2000) was used for haplotype network estimation following the cladogram estimation rules laid out in Templeton *et al.* (1992) and elaborated in Templeton (1998; 2004). The program Geodis 2.0 (Posada *et al.*, 2000) was used to test significant changes in haplotype and nested clade geographic distribution relative to other haplotypes and nested clades within their higher-level nesting clades (Templeton *et al.*, 1995). The program PAUP\* 4.0b10 (Swofford, 2002) was used to estimate a Neighbor Joining tree based on  $F_{ST}$  values.

The number of segregating sites between sequences (*S*), Nei's (1987) nucleotide diversity ( $\pi$ ), Nei's (1987) gene diversity ( $\hat{H}$ ), and Watterson's (1975) theta ( $\theta$ ) were calculated using the programs Arlequin ver. 2000 (Schneider *et al.*, 2000) and DnaSP (Rozas *et al.*, 2003). These programs were also used to compute pair-wise  $F_{ST}$  statistics (Weir and Cockerham, 1984), Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992), and tests of selective neutrality of Fu (1997) and Tajima (1989). Fu's  $F_s$  is in general more powerful than the test of Tajima in detecting demographic events.

Wright's inbreeding coefficient ( $F$ ), the classic population genetic measure, was used to characterize intrapopulation variation and differentiation between populations. We used the method of Cockerham and Weir (1993) to estimate  $F_{ST}$ . Statistical significance of  $F$  values was estimated using bootstrapping implemented in Arlequin 2000 (Schneider *et al.*, 2000), and adjusted using the method of Bonferroni for multiple comparison (Rice, 1989). We tested the hypothesis of isolation by distance using the Mantel test (Mantel, 1967) implemented in the program Arlequin ver. 2000 (Schneider *et al.*, 2000), estimating the significance of correlation between matrix of  $\ln F_{ST}$  values and between-locality river distances with 10000 permutations.

Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) tests if molecular variation is non-randomly distributed among user-defined or natural 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 Raymond and Rousset's test of exact population differentiation (Raymond and Rousset, 1995).

**Results**

We sequenced 1085 base pairs (bp) of the mitochondrial cytochrome *b* gene in 125 individuals sampled from nine localities (Figure 1). We found a total of 38 haplotypes (Tables 1 and 2) that included one common haplotype; this

haplotype (H1) is the most frequent one and is widely distributed, two characteristics that are representative of a most likely ancestral haplotype (Castellose and Templeton, 1994). The conceptual translation of the 1085 bp fragment in the program BioEdit (Hall, 1999) resulted in a sequence of 361 amino acids without unexpected stop codons, con-

**Table 1** - Variable sites in the 1085 bp fragment of the mitochondrial cytochrome *b* gene of *Caiman crocodilus*. A total of 41 sites were variable resulting in 38 haplotypes. N indicates the number of individuals in which a particular haplotype was found. Haplotypes are deposited in GenBank under accession numbers DQ246626 to DQ246663.

Haplotype	Position of a nucleotide change	N
	00111 000001122223344555566666777888889999000 01367263444659791467801269129033571347115 33201748039682849020408546193103205612473	
H1	ACTGTCGAAGGCACGCGACCCATTTCATGCATCCCTTCCCC	63
H2	. . . . . C . . . . .	1
H3	. . . . . T . . . . .	1
H4	. . . . . G . . . . .	1
H5	. . . . . A . . . . .	1
H6	. . . . . T . . . . . G . . . . .	1
H7	. . . . . G . . . G . . . . .	1
H8	. . . . . . . . . . . C . . . . .	1
H9	. . C . . . . . . . . . . C . . . . .	3
H10	. . . . . T . . . . . C . . . . .	1
H11	. . . . . T . . . . . C . . . . .	2
H12	. T . . . . . T . . . . .	1
H13	. . . . . C . . . . .	1
H14	. T . . . . .	1
H15	. . . . . G . . . . .	9
H16	. T . . T . . . . .	1
H17	. . . . . T . . . . .	1
H18	. . . . . C . . . . .	1
H19	. . . . . A . . . . .	1
H20	. . . . . A . . . . .	1
H21	. . . . . . . . . . T	2
H22	. . . . A . . . . . C . . . . .	1
H23	G . . . . . T . . . . .	13
H24	. . C . . . . .	1
H25	. . . . G . . . . .	1
H26	. . . C . . . . . C . . . . .	1
H27	. . . . . C . . . . .	2
H28	. . . . . C . . . T . .	1
H29	. . . . . TTT . C . . . . .	1
H30	. . . . . C . . . . .	1
H31	. . . . . G . . . . .	1
H32	. . . . . G . . . . T . . . . .	1
H33	. . . . . A . . . . .	1
H34	. . . . T . . . . .	1
H35	. . . . . C . . . . .	1
H36	. . . . . C . . . . .	1
H37	. . . . . G . . . . .	1
H38	. . . . . G . A . . . . .	1

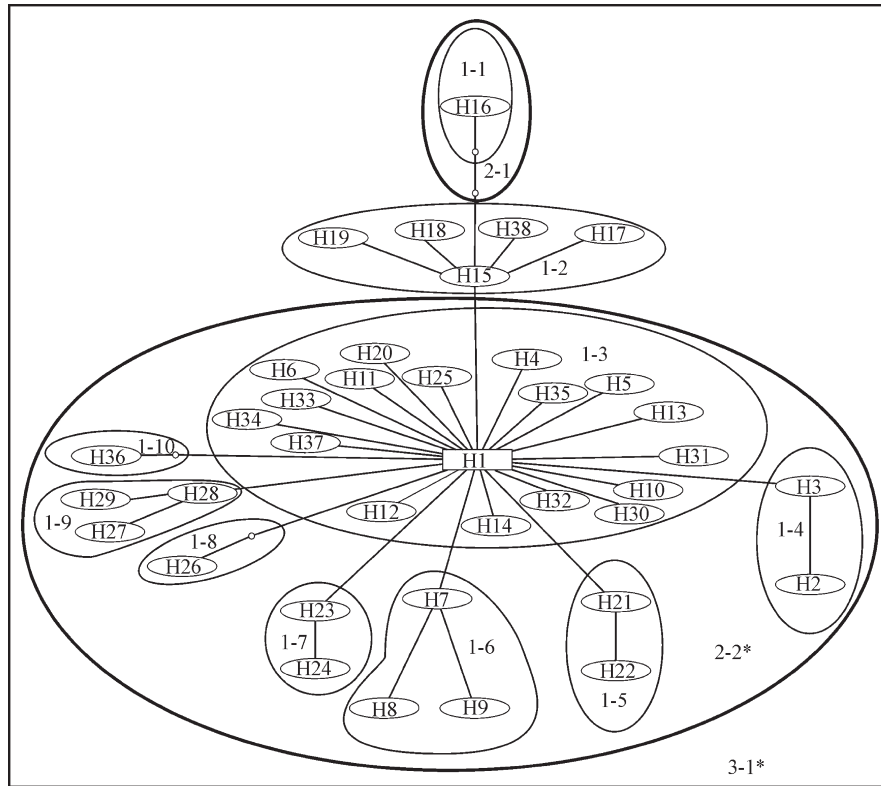
firming that we have not amplified and sequenced nuclear pseudogenes. We also found an incomplete stop codon at the end of cytochrome *b* characteristic of crocodylians (Glenn *et al.*, 2002). A characteristic mtDNA anti-G bias (Zhang and Hewitt, 1996) was observed in all sequences. Haplotypes are deposited in GenBank under the accession numbers DQ246626 to DQ246663.

In the NCA (Templeton *et al.*, 1995) we encountered two levels at which we could not reject the null hypothesis of no association of geographic distance and distribution of

genetic diversity. Nesting scheme and significant levels are shown in Figure 2. Using the 14 July 2004 NCA interpretational key (see <http://darwin.uvigo.es/software/geodis.html>), we inferred continuous range expansion, long distance colonization or past fragmentation in nesting level 3-1 (Table 3). This inference pertains to the contrast between sampling localities from the Amazon basin and those outside the Amazon basin. For localities from within the Amazon basin we infer continuous range expansion at level 2-2 (Table 3).

**Table 2** - Haplotype frequencies of the mitochondrial cytochrome *b* gene in sampling localities of *C. crocodilus*.

Haplo- types	Brazil							Peru	French Guiana	Total
	Purus River	Janauacá Lake	Anavilhanas Archipelago	Mamirauá Lake	Tapará Community	São Miguel Island	Uaçá River	Pacaya- Samíria	Approuague River	
H1	15	5	4	3	18	12	-	6	-	63
H2	-	-	-	-	-	-	-	1	-	1
H3	-	-	-	-	-	-	-	1	-	1
H4	-	-	-	-	-	1	-	-	-	1
H5	-	-	-	-	1	-	-	-	-	1
H6	-	-	-	1	-	-	-	-	-	1
H7	-	-	-	-	-	-	1	-	-	1
H8	-	-	-	-	-	-	1	-	-	1
H9	-	-	-	-	-	-	3	-	-	3
H10	-	-	-	1	-	-	-	-	-	1
H11	-	-	-	2	-	-	-	-	-	2
H12	-	-	1	-	-	-	-	-	-	1
H13	-	-	1	-	-	-	-	-	-	1
H14	-	-	1	-	-	-	-	-	-	1
H15	-	-	-	-	-	-	3	-	6	9
H16	-	-	-	-	-	-	1	-	-	1
H17	-	-	-	-	-	-	-	-	1	1
H18	-	-	-	-	-	-	-	-	1	1
H19	-	-	-	-	-	-	1	-	-	1
H20	-	1	-	-	-	-	-	-	-	1
H21	-	2	-	-	-	-	-	-	-	2
H22	-	1	-	-	-	-	-	-	-	1
H23	1	1	-	1	-	5	-	5	-	13
H24	-	-	-	1	-	-	-	-	-	1
H25	1	-	-	-	-	-	-	-	-	1
H26	-	1	-	-	-	-	-	-	-	1
H27	-	-	2	-	-	-	-	-	-	2
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	-	-	-	-	-	1	-	-	-	1
H37	-	-	-	-	-	1	-	-	-	1
H38	-	-	-	-	-	-	-	-	1	1
Total	17	11	11	13	21	20	10	13	9	125



**Figure 2** - Most parsimonious network of 38 mtDNA haplotypes detected in the sample of 125 individuals of *C. crocodilus*. Lines represent one mutational step, circles represent haplotypes. The one square represents the most likely ancestral haplotype. Empty circles represent inferred, but not detected haplotypes. \* indicates a significant nesting level inferred in NCA.

**Table 3** - Results of Nested Clade Analysis (NCA) of *C. crocodilus*. Level refers to the nesting clades shown in Figure 2. Only those nesting clades that show genetic or geographic variation are reported. Permutational  $\chi^2$  probability is assessed by randomly permuting the lower level clade categories within the nesting clade versus geographical locality 10000 times. Inferences are based on the 14 July 2004 key provided on the GeoDis 2.0 website (<http://darwin.uvigo.es/software/geodis.html>).  $H_0$  = no association of haplotypes with geography.

Level	$\chi^2$	Prob	Interpretation
1-2	3.6111	0.7111	$H_0$ not rejected
1-3	118.9418	0.0458	$H_0$ not rejected (no significant nested contrasts within this nesting clade)
1-7	6.4615	0.2845	$H_0$ not rejected
2-2	229.5434	0.0000	continuous range expansion
3-1	111.6078	0.0000	continuous range expansion, long distance colonization or past fragmentation

Hierarchical AMOVA analysis (Excoffier *et al.*, 1992) implemented in the program Arlequin ver. 2000 (Schneider *et al.*, 2000) was used to investigate differentiation between the sampling localities of the Amazon basin and those of the Atlantic coast drainages. Results show that 35.30% of variation occurs between the two groups, 6.97% occurs among localities within the two groups, and 57.73%

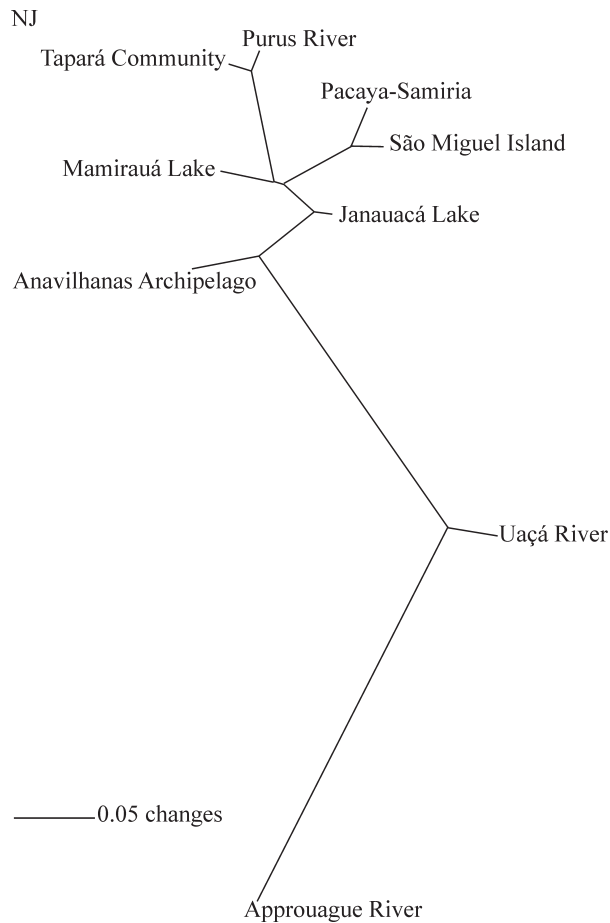
occurs within sampling localities. The genetic difference between the Amazon basin and the Atlantic coast drainages is significant ( $F_{CT} = 0.3530$ ,  $p = 0.023$ ), and is graphically illustrated in Figure 3.

A second AMOVA analysis concentrated exclusively on the Amazon basin. When the Amazon basin was treated as one group, 8.39% of the observed genetic variation occurred between localities, and 91.61% within localities. However, among locality differentiation is significant ( $F_{ST} = 0.0839$ ,  $p < 0.001$ ). Global test of exact population differentiation (Raymond and Rousset, 1995) also supported the hypothesis of differentiation among localities ( $p < 0.001$ ). We tested if this differentiation may be due to isolation-by-distance by testing for significant association of geographic distance and genetic divergence of sampled localities using the permutational procedure of Mantel (1967). Results of the Mantel test indicate that isolation-by-distance is not a significant structuring factor, neither for all populations analyzed ( $r = 0.4621$ ,  $p = 0.058$ ), nor for the Amazon basin only ( $r = 0.1036$ ,  $p = 0.354$ ). Estimates of the gene flow parameter  $Nm$  derived from  $F_{ST}$  values indicate that high levels of genetic exchange exist between nearly all sampled localities (Table 4).

Analyses of mutation-drift equilibria (Tajima, 1989; Fu, 1997) indicate that almost all sampled localities are in a genetic equilibrium (Table 5). Only for the Anavilhanas

Archipelago, the Mamirauá Lake and the Taparú Community does Fu's  $F_s$  test show a significant genetic disequilibrium. However, when the Amazon basin is treated as one

large population, both statistics indicate a significant genetic disequilibrium (Tajima's  $D = -2.548$ ,  $p < 0.0001$ ; Fu's  $F_s = -30.965$ ,  $p < 0.0001$ ).



**Figure 3** - Unrooted neighbor-joining  $F_{ST}$  topology of *C. crocodilus* sampling localities. Branch lengths are proportional to genetic distances.

## Discussion

### Cytochrome *b* polymorphism and genetic equilibria tests

Gene diversity encountered in the present study was high ( $\hat{H} = 0.733$ ; 1085 bp), and comparable to the values ( $\hat{H} = 0.692$ ; 1142 bp) found in the study of Farias *et al.* (2004). These values are much higher than the value observed for *Alligator mississippiensis* ( $\hat{H} = 0.153$ ; 1317 bp) by Glenn *et al.* (2002). These values indicate that *Caiman crocodilus* populations retain high levels of genetic diversity in spite of historical events which reduced its population size. Neither the hypothesized climatic changes in the Amazon basin (Ab'Saber, 1977), nor recent commercial overexploitation (Smith, 1980; Da Silveira and Thorbjarnarson, 1999) appear to have affected the gene diversity of *C. crocodilus* populations. In contrast to *C. crocodilus*, the only other alligatorid crocodilian for which a comparable data set has been generated, the American alligator *A. mississippiensis*, shows much lower gene diversity (Glenn *et al.*, 2002). This low gene diversity was attributed by the authors to severe reduction in population size during the Pleistocene, with a subsequent demographic expansion in the Holocene, but not to commercial overexploitation that also significantly reduced the census numbers of this species.

Of the nine localities studied, three (Table 5) show a significantly negative value for Fu's  $F_s$  test. Although this test was formally designed to test for selection, in the absence of selective advantage among haplotypes, a significant negative deviation from genetic equilibrium in

**Table 4** - Matrix of pair-wise  $F_{ST}$  values (below diagonal) and number of effective migrants ( $Nm$ ) between pairs of populations (above diagonal) separated by geographic distance (above diagonal in parentheses). \* Significant values after Bonferroni correction ( $p < 0.0014$ ).

Populations	Purus River	Janaucá Lake	Anavilhanas Archipelago	Mamirauá Lake	Taparú Community	São Miguel Island	Uaçá River	Pacaya-Samiria	Approuague River
Purus River	-	5.3113 (250 km)	2.4972 (255 km)	20.3905 (450 km)	$\infty$ (830 km)	13.0309 (860 km)	0.9608 (1720 km)	2.6401 (1450 km)	0.1873 (2050 km)
Janaucá Lake	0.0860	-	4.0000 (105 km)	16.2541 (590 km)	4.3075 (620 km)	6.7095 (590 km)	1.6876 (1700 km)	4.3917 (1670 km)	0.4983 (1750 km)
Anavilhanas Archipelago	0.1668	0.1111	-	5.5869 (605 km)	2.3885 (715 km)	2.7013 (645 km)	1.5888 (1800 km)	2.2512 (1655 km)	0.5211 (1970 km)
Mamirauá Lake	0.0239	0.0298	0.0821	-	9.4655 (1250 km)	38.7575 (1220 km)	1.9501 (2290 km)	14.1938 (1050 km)	0.6681 (2450 km)
Taparú Community	-0.0010	0.1040	0.1731*	0.0501*	-	5.2000 (30 km)	0.9052 (1080 km)	1.8365 (2300 km)	0.2019 (1220 km)
São Miguel Island	0.0369	0.0693	0.1561*	0.0127	0.0877	-	1.1213 (1110 km)	1.2256 (2330 km)	0.3608 (1250 km)
Uaçá River	0.3422*	0.2285*	0.2393*	0.2040*	0.3558*	0.3083*	-	1.2256 (3340 km)	1.6788 (170 km)
Pacaya-Samiria	0.1592	0.1022	0.1817*	0.0340	0.2139*	0.0040	0.2897*	-	0.3679 (3070 km)
Approuague River	0.7274*	0.5008*	0.4896*	0.4280*	0.7122*	0.5808*	0.2294	0.5760*	-

**Table 5** - Indexes of genetic diversity and test of populational equilibria. N = number of individuals sampled, hp = number of unique haplotypes observed, S = number of segregating (polymorphic) sites,  $\theta$  = Watterson's Theta based on S,  $\pi$  = Nei's nucleotide diversity,  $\hat{H}$  = Nei's gene diversity. \* Significant values after Bonferroni correction ( $p < 0.0056$ ).

Population	N	hp	S	$\theta$	$\pi$	$\hat{H}$	Tajima's <i>D</i> ( <i>P</i> value)	Fu's <i>F<sub>s</sub></i> ( <i>P</i> value)
Purus River	17	3	2	0.591588 ± 0.443049	0.000217 ± 0.000295	0.228 ± 0.129	-1.50358 (0.0513)	-1.68032 (0.0152)
Janauacá Lake	11	6	6	2.048503 ± 1.111063	0.001240 ± 0.000936	0.800 ± 0.114	-1.35867 (0.0902)	-2.66235 (0.0114)
Anavilhanas Archipelago	11	7	6	2.048503 ± 1.111063	0.001441 ± 0.001048	0.873 ± 0.089	-0.93702 (0.2029)	-3.70317* (0.0028)
Mamirauá Lake	13	10	10	3.222469 ± 1.531315	0.001654 ± 0.001146	0.949 ± 0.051	-1.76982 (0.0240)	-7.84985* (0.0001)
Taparú Community	21	4	3	0.845609 ± 0.540302	0.000276 ± 0.000336	0.271 ± 0.124	-1.72678 (0.0193)	-2.81979* (0.0014)
São Miguel Island	20	5	5	1.409348 ± 0.758839	0.000732 ± 0.000618	0.600 ± 0.101	-1.33438 (0.0874)	-1.71119 (0.0681)
Uaçá River	10	6	8	2.827886 ± 1.458992	0.002376 ± 0.001571	0.876 ± 0.085	-0.38158 (0.3735)	-1.18530 (0.1834)
Pacaya-Samíria	13	4	3	0.966741 ± 0.631371	0.000874 ± 0.000718	0.679 ± 0.089	-0.05895 (0.4706)	-0.62780 (0.2142)
Approuague River	9	4	3	1.103811 ± 0.738453	0.000614 ± 0.000587	0.583 ± 0.183	-1.51297 (0.0524)	-1.89165 (0.0124)
Amazon basin	105	29	31	5.931417 ± 1.748750	0.000878 ± 0.000671	0.635 ± 0.053	-2.54884* (<0.0001)	-30.96536* (<0.0001)
All samples	125	38	41	7.787209 ± 2.144140	0.001169 ± 0.000821	0.733 ± 0.042	-2.56818* (<0.0001)	-28.82952* (<0.0001)

mtDNA alleles is most probably the result of recent population expansion (Rand, 1996; Hartl and Clark, 1997). Fu's *F<sub>s</sub>* statistic is more sensitive to demographic events than is Tajima's *D* (Rand, 1996). Thus, the inference drawn from our analyses suggests that while the Anavilhanas and Mamirauá localities - both of which are strictly protected at the federal and state level, respectively - and the Taparú locality have experienced a recent population expansion, this expansion was not very strong, and has been registered only by the most sensitive statistic. Some areas, however, show very little genetic evidence of population expansion, or of census number increase. These areas include the Pacaya-Samíria National Reserve (BM, pers. obs.) and the Uaçá Indigenous Area (Ruffeil, 2004) where *C. crocodilus* remains a popular food item, and is harvested in significant numbers. When all sampled localities are analyzed as one population, both Tajima's *D* and Fu's *F<sub>s</sub>* statistics are significantly negative. This result suggests an overall population expansion of this species that also has been registered as growth in census numbers (Rebêlo and Lugli, 2001). Again, the signal is not very strong and is only observed when the statistical power of the tests is increased by analyzing all samples together.

The genetic signal of overall population expansion is compatible with historical data and current observations. *Caiman crocodilus* is a habitat generalist. It also has been much less affected by the commercial trade than other sympatrically occurring species, such as *Melanosuchus niger*, *Crocodilus intermedius* or *Crocodilus acutus*, being harvested in large numbers only after these latter species became too severely depleted to support commercial operations. Because of the lack of ecological specialization (Herron, 1994), *C. crocodilus* was able to expand into habitats previously occupied by sympatrically occurring species (Da Silveira *et al.*, 1997). Even when commercial hunters started harvesting the then plentiful *C. crocodilus* and precipitated its demographic decline, the present population is probably larger than were historical populations, which had

to co-exist with large numbers of well established crocodylian species (Ross, 1998). *Caiman crocodilus* also has, once again, expanded following global and local harvest moratoria and regulations, experiencing two cycles of recent expansion, with an intervening period of decline, and it is this second expansion we are observing in the current pattern of genetic diversity.

#### Inference of population genetic structure

A minimum-spanning haplotype network was nested into higher level nesting categories (Templeton *et al.*, 1992) and analyzed for non-random distribution of genetic diversity over geographic space (Templeton *et al.*, 1995). The Nested Clade Analysis (NCA) allows identification of population genetic structure and the discrimination of various historical and ongoing processes responsible for the current pattern of genetic structuring. Its greatest power lies in that it requires no *a priori* hypothesis of population structure. Once patterns are observed, they can then be tested further.

Using the NCA approach we observed two hierarchical levels which have a significantly non-random distribution of genetic diversity. Inferences from level 3-1 suggest that the main populational dynamics responsible for the observed genetic differentiation of the Atlantic drainage systems not connected to the Amazon basin and Amazon basin localities are continuous range expansion, long distance colonization, or past fragmentation. However, past fragmentation is not very likely due to the small number of mutational steps separating the Uaçá and Approuague Rivers haplotypes from haplotypes found in the Amazon basin. When only the Amazon basin is analyzed, the inference at level 2-2 is continuous range expansion. Thus, continuous range expansion is likely to be the main dynamic within the Amazon basin, but due to insufficient sampling, we cannot differentiate between continuous range expansion or long distance colonization as the main populational dynamic responsible for the observed genetic differentia-



tion between the Atlantic drainages not connected to the Amazon basin and Amazon basin sampling localities. Analysis of Molecular Variance (Excoffier *et al.*, 1992), as well as pair-wise  $F_{ST}$  values also support the inference that the Uaçá and Approuague Rivers are significantly differentiated from localities of the Amazon basin.

A possible factor that could have contributed to this distribution of genetic diversity is the present day distribution of river basins relative to their paleogeographic positions. The direction of the inferred colonization or range expansion is from the Amazon basin into the coastal drainages of French Guiana and Amapá State of Brazil. This could have occurred during the last Pleistocene glacial maximum when sea levels were up to 200 m lower than present. The Amazon delta extended much further east than its present position, and many of the now isolated coastal drainages were connected to the Amazon basin via the delta of the Amazon River. This would have facilitated dispersal and colonization of new areas, now outside the Amazon basin, during the glacial maximum. Modern alligators are less tolerant to salt water than other crocodylians since they possess neither a tongue gland in their mouth cavities that excretes salt, nor a reno-cloacal complex adapted for the excretion of salt and conservation of fresh water (Taplin and Grigg, 1989). For this reason, salt water is considered a major barrier to dispersal of Alligatoridae (Brochu, 2001), and it is unlikely they would have colonized the French Guiana and Amapá coastal drainages recently.

NCA analyses within the Amazon basin indicate that continuous range expansion is the most likely processes responsible for the observed distribution pattern of genetic diversity. Both AMOVA and Raymond and Rousset's test of exact population differentiation reject the hypothesis of panmixia; however, the distribution of genetic diversity is not compatible with the model of isolation-by-distance. In spatial autocorrelation analysis (Koenig, 1999; Diniz-Filho and Telles, 2002) which tests the hypothesis of isolation-by-distance, geographic distances are partitioned into classes of connectivity or lack thereof at ever increasing distances. The spatial autocorrelation predicts elevated correlation at lower distances of connectivity with eventual leveling off, a pattern not observed in our data. The observed structure is therefore most likely the result of genetic subsampling of parental populations during periods of range expansion. However, range expansion did not proceed in a linear manner. Range expansion possibly proceeded locally as commercially more valuable species were being locally depleted by commercial hunters, and the resulting ecological space was being filled by an expanding *C. crocodilus* population. Alternatively, we may be observing a signature of coalescent processes in a species distributed over a large geographic area, thus a historical record rather than an ongoing process.

Another pattern which contributes to the rejection of panmixia within the Amazon basin is the significant differ-

entiation of the Anavilhanas locality from all but the geographically closest Mamirauá Lake, Purus River and Janauacá Lake localities. The locality from the Anavilhanas archipelago is the only one sampled from a black water system (Sioli, 1984). Black water systems are limnologically and ecologically differentiated from white water systems, often supporting different animal and plant communities (Sioli, 1984; Goulding *et al.*, 2003). There are a number of black water systems in the Amazon basin, but the Negro River is the largest. The observed differentiation of the Anavilhanas locality corroborates, in principle, the findings of Farias *et al.* (2004). Although Farias *et al.* (2004) did not include *C. crocodilus* from Anavilhanas in their analyses, they observed weak genetic differentiation between black water (Anavilhanas) and white water (rest of the Amazon basin) sampling localities of *Melanosuchus niger*, the other large alligatorid crocodylian found in Amazônia. Ecological differences between caiman populations occupying black water and white water habitats were also observed 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 that reduces genetic exchange between limnologically differentiated systems. The two other significant pair-wise  $F_{ST}$  comparisons observed within Amazônia occur between geographically distant localities.

The lack of pattern of genetic structuring among localities within the Amazon basin contrasts with the study of Verdade *et al.* (2002) who studied five geographically proximate populations of *Caiman latirostris* from the state of São Paulo. Based on an analysis of four microsatellite loci, Verdade *et al.* (2002) observed significant correlation between geographic and genetic distance. The habitat occupied by these populations is fragmented, which, combined with high mortality and low birth rates, should result in a low number of successfully dispersing individuals per generation leading to the pattern of isolation-by-distance (Verdade *et al.*, 2002). The fragmented and discontinuous habitat occupied by *C. latirostris* contrasts with what is essentially a continuous habitat of the Amazon basin available to *C. crocodilus*. Nevertheless, the fragmented populations of *C. latirostris* outside the core continuous habitat of the Pantanal basin show a certain degree of differentiation, which is a classic pattern of peripatric differentiation observed in diverse taxa (Mayr 1963).

## Acknowledgments

We would like to thank Sociedade Civil Mamirauá, The Mamirauá Institute, The Wildlife Conservation Society, The Nature Conservancy of Brazil and FAPEAM (Fundação de Amparo a Pesquisa no Estado do Amazonas) for financial support, RAN/IBAMA for permission to conduct field work, and CGEN/IBAMA for permission to conduct laboratory work. Renato Da Silveira, Pedro Alexandre Sampaio, Eduardo Matheus von Muhlen, Augusto Ruffeil

and Marcelo Crossa helped in the field, and Richard Bodmer facilitated fieldwork in Peru, and the comments of two anonymous reviewers improved this publication.

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Associate Editor: Sérgio Furtado dos Reis