Animal Conservation (2004) **7**, 265–272 © 2004 The Zoological Society of London. Printed in the United Kingdom DOI:10.1017/S136794300400143X

Genetic diversity and population structure of Amazonian crocodilians

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(Received 29 July 2003; accepted 12 January 2004)

Abstract

We used the mitochondrial cytochrome *b* gene to study the population genetic structure of *Melanosuchus niger* (Brazil: Negro and Purus Rivers, Lake Janauacá; French Guiana: Kaw River swamps), and *Caiman crocodilus* (Brazil: Purus River, Lake Janauacá; French Guiana: Kaw River swamps). We found 10 haplotypes in *M. niger* and 9 haplotypes in *C. crocodilus*. Nested clade analysis indicated that isolation-by-distance was an important population dynamic in *M. niger*, but was unable to differentiate between isolation-by-distance, historical fragmentation or range expansion in *C. crocodilus*. Fu's *Fs* statistic supported the hypothesis of a demographic expansion in one out of four and two out of three sampled localities of *M. niger* and *C. crocodilus*, respectively. Populations of *M. niger* in central Amazonia also appeared to show differentiation that was correlated with water type. These results are compatible with the life-style of these two crocodilians; *C. crocodilus* is a habitat generalist and appears to disperse rapidly to newly available habitats, while *M. niger* is a more sedentary habitat specialist. Both species appear to be recovering from unregulated over-harvesting, however, their responses are life-history and, potentially, ecologically-dependent.

INTRODUCTION

Once extremely abundant in the rivers, lakes and swamps of South America, uncontrolled commercial hunting for their hides has relegated the black caiman (*Melanosuchus niger*) and the spectacled caiman (*Caiman crocodilus*) to islands of residual populations in parts of their former distributions. Millions of skins of these two commercially valuable crocodilian species were harvested from the 1930s onward by foreign trading companies, resulting in a precipitous decline, particularly of *M. niger*, throughout the Amazon basin (Medem, 1983; Rebêlo & Magnusson, 1983). Hunters initially concentrated on *M. niger*, and only later once its stocks became depleted did they also start commercial hunting of *C. crocodilus*. *Melanosuchus niger* was, therefore, much more severely affected and became listed under the International Union for Conservation of Nature and Natural Resources (IUCN) conservation dependent status category. Despite Brazilian federal laws that prohibit all commercial hunting (Law No. 5.197/67), illegal hunting continues throughout the year (Da Silveira & Thorbjarnarson, 1999), potentially threatening the survival of both species. While historical hunting was primarily for the skin trade, current hunting of crocodilians is mainly for meat fuelled by local demand (Da Silveira & Thorbjarnarson, 1999). In French Guiana, *M. niger* is also protected (Ministerial Decree, 15 May 1986). However, unlike the situation in Brazil, here most of its range is within a nature reserve system and, as a result, poaching is currently limited. *Caiman crocodilus* is not protected by law at present. However, both species need to be protected and managed to ensure recovery. The formulation and implementation of conservation policies needs to not only concentrate on the recovery of both species, but also to take into account local socioeconomic needs.

Ecological studies suggest that these two crocodilians are very different in terms of life-history and reproductive patterns (Da Silveira, Magnusson & Campos, 1997; Da Silveira & Magnusson, 1999), and may therefore need to be managed differently. However, nothing is known about the genetic diversity, metapopulation structure, or other population genetic indicators of either species. These data are vital for the conservation management of wild populations, for the management of captive breeding efforts, as well as for mitigating and preventing fitness losses associated with the isolation and decline of populations. There is compelling evidence that loss of fitness is associated with declines in genetic variability of natural populations and that genetic diversity and correspondingly fitness can recover with the restoration of gene flow (Flagstad *et al*., 2003; Randi *et al*., 2003). We therefore tested to see whether *M. niger* and *C. crocodilus*

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are genetically structured, whether they are genetically depleted as a result of over-exploitation during the 20th century and if they now show signs of recovery.

MATERIALS AND METHODS

Tissue samples were collected from the tail scales obtained during the marking of individuals. Scales were preserved in 95% ethanol and, once in the laboratory, stored in a freezer. Brazilian samples of *M. niger* were collected from Anavilhanas Archipelago ($n = 17$), Lake Janauacá (Solimões-Amazonas River, $n = 8$) and Rio Purus ($n = 8$), and from the Kaw Swamps Reserve in French Guiana ($n = 13$). Samples of *C. crocodilus* were collected from Lake Janauacá ($n = 11$) and Rio Purus ($n = 13$), and Approuague River in French Guiana ($n = 8$). Collecting localities are shown in Fig. 1. Tissue samples were digested using a Proteinase K/SDS solution, followed by phenol–chloroform extraction, the addition of 5 M NaCl and a final 70% ethanol precipitation of DNA product (Sambrook, Fritsch & Maniatis, 1989).

The mitochondrial cytochrome *b* gene was selected because of its ability to resolve questions of population structure among many taxonomic groups (Avise, 2000). We also chose sequence information since we wished to test hypotheses of population structure with no *a priori* assumption of a population structure (Templeton, Routman & Phillips, 1995; Templeton, 1998). Nearly complete cytochrome *b* was amplified using the polymerase chain reaction (PCR) using primer L14254 (5′-ATGACCCACCAACTACGAAAAT-3′) from Glenn *et al*. (2002) and a primer H15990 (5′-TTAGAAYGTCGGCTTTGG-3′) designed specifically for this project; L14254 and L14731 (5′-TGTCGTGCCATGAATTTGAG-3′) from Glenn *et al*. (2002) were used for sequencing. PCR was performed in 25 µl reaction volumes containing 13.75 µl of ddH_2O, 2.5 µl of 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 5.0 µl of primer mix (0.2 µM each forward and reverse primer), 2.0 µl of dNTP mix (200 µM each dNTP), 0.5 U of Promega Taq DNA Polymerase and about 10 ng of DNA. The cycling protocol was as follows: denaturation at 94°C for 35 s, annealing at 50°C for 35 s and extension at 72°C for 90 s, repeated for 35 cycles. Sequencing reactions were performed according to the manufacturers recommendation using the Terminator Cycle Sequencing Kit (Amersham Pharmacia, São Paulo, Brazil), and resolved on a MegaBACE automated sequencer (Amersham Pharmacia, São Paulo, Brazil). Homologous protein-coding regions were aligned manually and confirmed by translating the DNA data into putative amino acid sequences using the programme BioEdit (Hall, 1999).

DATA ANALYSIS

A number of statistical methods have been developed to infer the historical processes that have shaped the observed patterns of genetic distribution and diversity. A haplotype network for each species was reconstructed using the programme TCS version 1.13 (Clement, Posada & Crandall, 2000), which implements the algorithm described by Templeton, Crandall & Sing (1992). Nested clade analysis (NCA) was implemented using the approach of nested design described by Templeton & Sing (1993) to test for historical and recurrent population events without an *a priori* assumption of population structure. We used the programme GeoDis version 2.0 (Posada, Crandall & Templeton, 2000) to test for the geographical association of clades and nested clades using a permutation contingency analysis (Templeton & Sing, 1993); distances among localities were calculated as distance along the river course except in the case of the French Guiana population, where distances to other populations were calculated from geographical coordinates. Population subdivision and structure was also examined using pair-wise population F_{ST} tests (Cockerham & Weir, 1993) and an analysis of molecular variance (AMOVA: Excoffier, Smouse & Quattro, 1992) as implemented in the programme ARLEQUIN (Schneider, Roessli & Excoffier, 2000).

Genetic diversity was measured by haplotype number, observed homozygosity (Chakraborty, 1990) and gene diversity, which is defined as the probability that two randomly chosen haplotypes are different in the sample (Nei, 1987). The gene diversity is essentially equivalent to the expected heterozygosity in diploid data. The diversity index theta (θ) was calculated as described by Watterson (1975). The genetic equilibrium of mtDNA alleles was tested using Tajima's D test (Tajima, 1989) and the more powerful *Fs* test (Fu, 1997). Although these tests were formally designed to test for selection, a significant deviation from genetic equilibrium in mtDNA alleles is most probably the result of recent population expansions or bottlenecks in situations where no selective advantage among haplotypes exists (Hartl & Clark, 1997). We used the programme Fluctuate (Kuhner, Yamato & Felsenstein, 1998) to arrive at a maximum likelihood estimate of population growth rate; we assumed a mutation rate of 1×10^{-8} per site per generation, which is the generally accepted mitochondrial DNA mutation rate (Li, 1997).

RESULTS

A total of 47 individuals of *M. niger* from four locations and 32 individuals of *C. crocodilus* from three locations were analysed. The final *M. niger* alignment comprised 871 positions of partial sequence of the cytochrome *b* gene defining 10 haplotypes separated by 11 segregating sites (Fig. 2(a)). Sequence divergence ranged from 0 to 0.69%. The final *C. crocodilus* alignment comprised 1142 positions of nearly complete sequence of cytochrome *b* defining 9 haplotypes separated by 9 segregating sites (Fig. 2(b)). Sequence divergence ranged from 0 to 0.38%. The genetic diversity of cytochrome *b* in these two species is relatively high compared to the observed genetic diversity of the American alligator *Alligator mississippiensis* (Table 1: *A. mississippiensis* data from

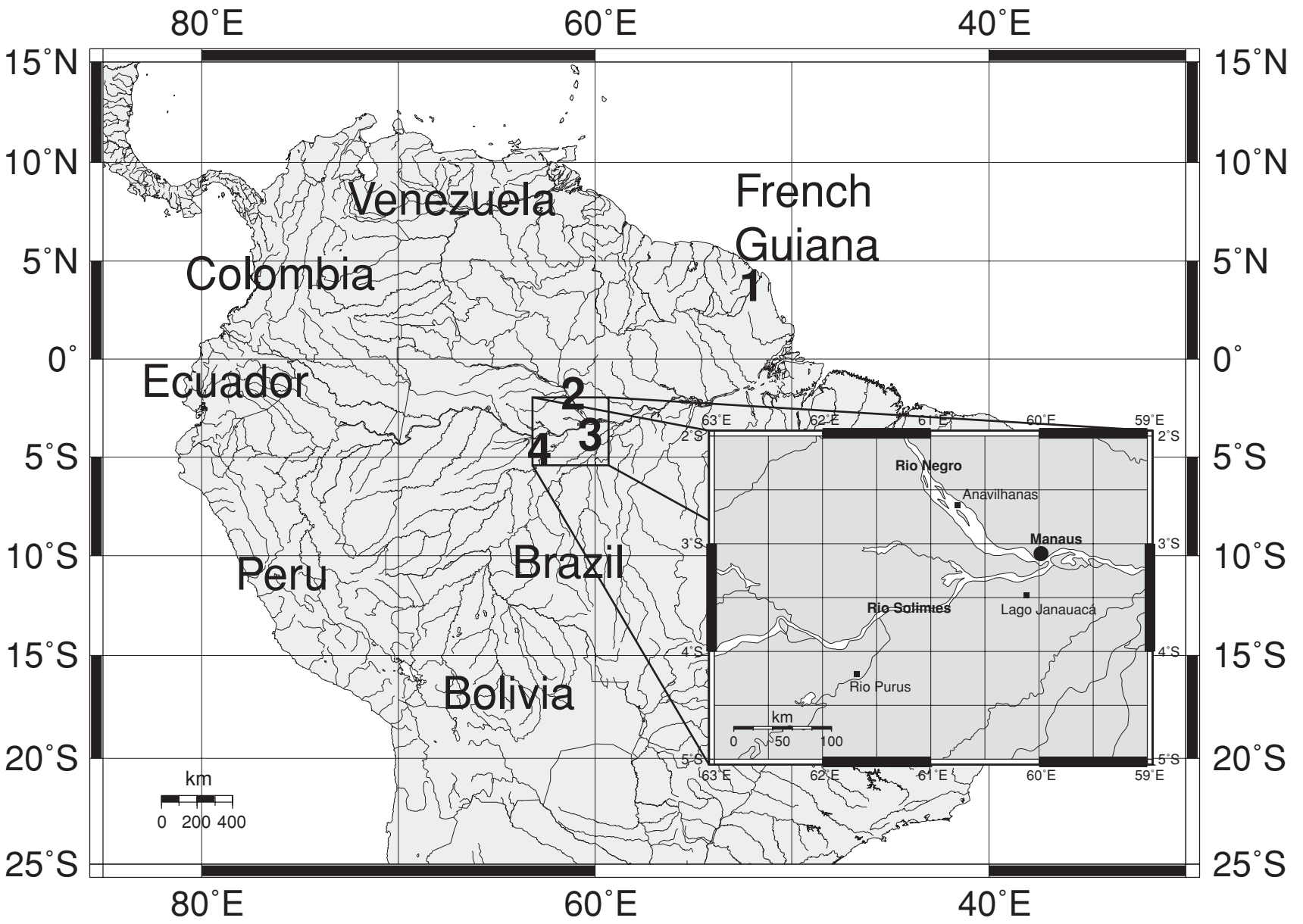

Fig. 1. A map of South America with the approximate collecting localities indicated. Locations are: 1, Kaw Swamps, French Guiana; 2, Anavilhanas, Brazil; 3, Janauacá, Brazil; 4, Purus, Brazil.

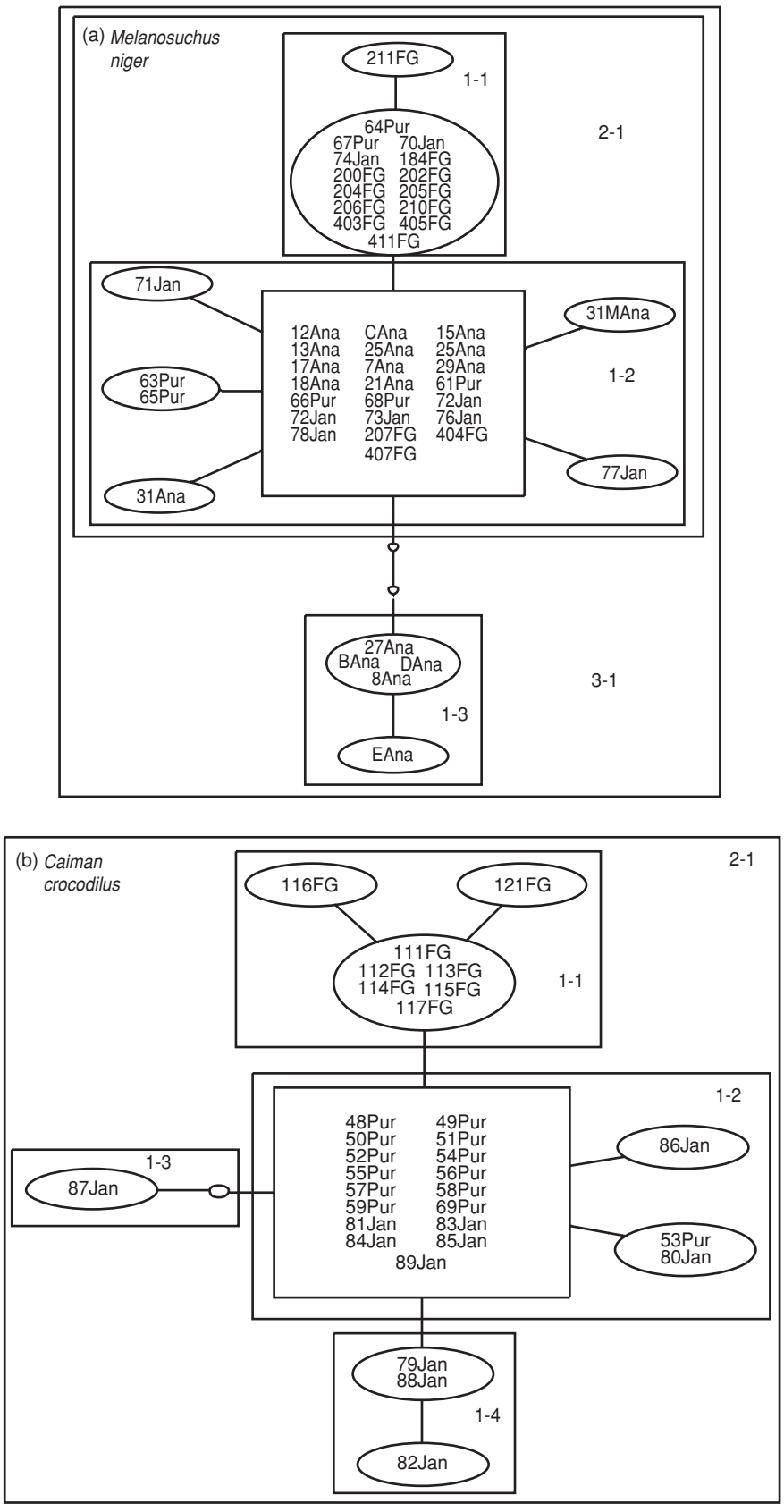

Fig. 2. A minimum spanning network and corresponding nested design for the cytochrome *b* haplotypes of (a) *Melanosuchus niger* and (b) *Caiman crocodilus*. Each oval represents a haplotype and the size of the oval is proportional to the number of individuals of that particular haplotype. Haplotype designations are: Ana, Anavilhanas; Jan, Janauacá; Pur, Purus; FG, French Guiana.

Table 1. Patterns of genetic diversity for Amazonian crocodiles

Species	Population	*n*	No. of haplotypes	Nei's gene diversity	Watterson's θ (S)	Observed homozygosity	Tajima's D test	Fu's *Fs* test
Melanosuchus niger black caiman	Anavilhanas (2)	17	5	0.625 ± 0.111	1.775	0.375	−0.186	−0.038
	Purus (4)	8	3	0.714 ± 0.123	0.771	0.285	0.414	−0.071
	Janauacá (3)	8	4	0.754 ± 0.139	1.157	0.250	−0.812	−1.387*
	Fr. Guiana (1)	14	3	0.473 ± 0.136	0.629	0.527	−0.532	−0.465
	All	**47**	**10**	**0.715 ± 0.049**	**2.491**	**0.285**	**−1.330***	**−3.856***
Caiman crocodilus spectacled caiman	Purus (4)	13	2	0.154 ± 0.126	0.322	0.846	−1.149	−0.537
	Janauacá (3)	11	6	0.800 ± 0.114	2.049	0.200	−1.359	−2.662*
	Fr. Guiana (1)	8	3	0.464 ± 0.200	0.771	0.536	−1.310	−0.998
	All	**32**	**9**	**0.692 ± 0.079**	**2.235**	**0.308**	**−1.612***	**−4.885***
Alligator mississipiensis American alligator	**All**	**25**	**2**	**0.153 ± 0.092**	**0.265**	**0.920**	**−1.158**	**−1.061**

Population samples are numbered as shown in Fig. 1.
N, sample size for each location.
*, significance level $P < 0.05$.
Data for *A. mississippiensis* are from Glenn *et al*. (2002).

Glenn *et al*., 2002), the only other crocodilian species with sequence data to be studied extensively and on a comparable geographical scale. Sequence divergence in a sample of 25 alligators from its complete range varied from 0 to 0.14%. As in other crocodilians (Glenn *et al*., 2002), *M. niger* and *C. crocodilus* do not have a complete stop codon at the end of the cytochrome *b* gene and they have a non-coding spacer that is 70 and 68 base-pairs (bp) long, respectively, between cytochrome *b* and $tRNA^{Thr}$. The sequences used in population analyses have been deposited in GenBank (accession numbers: AY462456-AY462533).

Genetic patterns of both species are summarised in Table 1. The mean frequency base composition in *M. niger* is 28% A, 24% T, 35% C and 13% G, while in *C. crocodilus* it is 31% A, 25% T, 33% C and 11% G, confirming a slight under-representation of guanine, as is normally observed in the mitochondrial genome (Zhang & Hewitt, 1996). In *M. niger* two common haplotypes predominate, while in *C. crocodilus* only one common haplotype predominates; all others represent low-frequency haplotypes or singletons.

Table 2. Nested contingency analysis of geographical association (Templeton *et al.* 1995) of *Melanosuchus niger* (a) and *Caiman crocodilus* (b)

Clade	Chi-square statistic	Probability	Interpretation
Melanosuchus niger (**a**)			
1-1	0.3896	1.0000	H_0 not rejected
1-2	18.0084	0.2927	H_0 not rejected
2-1	19.4857	0.0002	Restricted gene flow with isolation-by-distance
Entire cladogram	9.3023	0.0280	Restricted gene flow with isolation-by-distance
Caiman crocodilus (**b**)			
1-2	2.2883	0.4353	H_0 not rejected
Entire cladogram	39.5636	0.0000	Insufficient genetic resolution to discriminate between isolation-by-distance, historical fragmentation and range expansion

Clade numbers refer to the nesting clades I shown in Fig. 2.
Permutational chi-square probability is assessed by randomly permuting the lower level clade categories within the nesting clade versus geographical locality 10 000 times.
Interpretation is based on Templeton *et al*. (1995).
H_0 = no geographical association of haplotypes.

Melanosuchus niger

The nested clade analysis (Templeton *et al*., 1995) of *M. niger* haplotypes revealed a significant association of clades and nested clades with geographical location in the nested clade categories, namely at the two-step clade 2-1 and the entire cladogram levels (Fig. 2(a); Table 2(a)). Following the key of Templeton *et al*. (1995), restricted gene flow with isolation-by-distance was inferred in both nested clade categories. Differentiation between populations was also inferred from Wright's *F* statistics (Wright, 1951); after sequential Bonferroni correction for multiple comparisons ($P = 0.008$), significant F_{ST} values were associated with geographical distance, i.e. the contrast between the Kaw Swamps in French Guiana and each of the three localities in Brazilian Amazonia. Significant differentiation at the $P = 0.05$ level was associated with water type, i.e. the contrast between the black-water igapó habitats of the Rio Negro Anavilhanas Archipelago and the white-water várzea habitats of the Solimões region represented by Rio Purus and Lake Janauacá. When grouped into more inclusive groups according to macrogeographical areas and water type, where the Solimões region comprised Rio Purus and Lake Janauacá, Rio Negro comprised the Anavilhanas

Table 3. Matrix of pair-wise F_{ST} values and geographical distances (in parentheses)

Population	Anavilhanas	Purus	Janauacá	French Guiana
Anavilhanas	–	–	–	–
Purus	0.16 (350 km)	–	0.07 (200 km)	0.78* (3700 km)
Janauacá	0.14 (150 km)	−0.02 (200 km)	–	0.51* (3500 km)
Fr. Guiana	0.42* (3450 km)	0.32* (3700 km)	0.28* (3500 km)	–

Values for *Caiman crocodilus* and *Melanosuchus niger* are shown above and below the diagonal, respectively
C. crocodilus was not sampled in the Anavilhanas Archipelago.
*, significance level $P < 0.008$.

archipelago, and the Guyana Shield comprised the Kaw Swamps of French Guiana, AMOVA indicated 70.69% of the genetic variation was attributable to variation within populations, –2.63% of the total variance referred to variance between populations within regions and 31.93% to variance between regions. Thus, although significant differentiation was observed at all hierarchical levels, the majority of the observed variation was within populations.

In addition to population structuring driven by isolation-by-distance and possibly also by water type, demographic expansion may also be an important population dynamic. While Tajima's (1989) D test suggests that *M. niger* populations are at a genetic equilibrium with respect to mtDNA alleles, the more sensitive *Fs* test of Fu (1997) was significantly negative for the Lake Janauacá sample, a result consistent with a hypothesis of a demographic expansion (Hartl & Clark, 1997). Results of the Fluctuate (Kuhner *et al*., 1998) analysis suggest that the current female inbreeding effective population size of *M. niger* is 5.73 $(\pm 0.60) \times 10^5$ individuals with a per generation growth of 0.0024%.

Caiman crocodilus

Similar to *M. niger*, *C. crocodilus* showed significant association of haplotypes with geography at the entire cladogram level (Fig. 2(b); Table 2(b)). The NCA inference key suggests that the geographical sampling scheme in this species is inadequate to discriminate between historical fragmentation, range expansion and isolation-by-distance. Pair-wise F_{ST} analysis shows that the contrast between the Appouague River, French Guiana and the two Brazilian populations from Lake Janauacá and Rio Purus is significant, while differentiation between Lake Janauacá and Rio Purus is non-significant (Table 3). AMOVA with two groups representing macrogeographical areas, where Rio Purus and Lake Janauacá comprised the Brazilian Amazon region and the Appouague River comprised the French Guiana Amazon region, attributed 57.95% of the variation to variance between regions, 3.19% of the total variance referred to variance between populations within regions and 38.86% to variance within populations.

Neutrality tests showed a significantly negative Fu's (1997) *Fs* in the Lake Janauacá and Approuague River populations, while the less powerful Tajima's (1989) D was non-significant. This pattern is consistent with populations undergoing a demographic expansion (Hartl & Clark, 1997) and is supported by the results of the programme Fluctuate (Kuhner *et al*., 1998), which suggests that the current female inbreeding effective population size of *C. crocodilus* is 2.57 $(\pm 0.26) \times 10^6$ individuals with a per generation growth of 0.0100%.

DISCUSSION

The two species analysed in the present study are very different in terms of habitat use; *M. niger* is a habitat specialist found primarily in várzea and igapó floodplains, associated lakes and in swamps, while *C. crocodilus* is a habitat generalist. The molecular data suggest that isolation-by-distance is a significant population structuring force acting on *M. niger*. Differentiation of populations inhabiting black-water and white-water regions of the Amazon basin may also be important. *Caiman crocodilus* shows significant differentiation between geographically distant populations, but our sampling scheme precludes us from making firm conclusions about the proximal causes of this differentiation. Both species showed non-significant differentiation between Lake Janauacá and Rio Purus (localities 3 and 4; Fig. 1), but a significant differentiation of the geographically distant and hydrologically isolated French Guiana (locality 1; Fig. 1).

A much more interesting and potentially important result is the apparent differentiation of the Anavilhanas Archipelago population of *M. niger* from the Lake Janauacá and the Rio Purus populations. These three populations are equidistant to each other (~200 km) and if one follows river courses, the Lake Janauacá population lies approximately half way between the Anavilhanas Archipelago and Rio Purus. It is possible that the observed differentiation could be driven by ecological and/or limnological differences between the black-water and white-water regions (Sioli, 1984). *Melanosuchus niger* inhabits the floodplains of central Amazonia. However, the black-water igapó floodplain is nutrient-poor and highly acidic, while the white-water várzea floodplain is nutrient-rich with near neutral pH; the Anavilhanas Archipelago is in a black-water system while both Lake Janauacá and Rio Purus are white-water systems. Although our data suggest that ecological and/or limnological requirements may have to be taken into account for future management of *M. niger*, the actual designation of different management and protection units will require additional data and studies.

Genetic diversity, as estimated by Nei's diversity index and Watterson's θ, is, in general, slightly higher in *M. niger* than in *C. crocodilus*. The French Guianan populations of both species show low levels of gene diversity compared to the Brazilian Amazon populations; these populations are hydrologically unconnected to the Amazon watershed and

may also have experienced greater hunting pressure. In comparison to cytochrome *b* data from *A. mississippiensis* sampled throughout its natural range (Glenn *et al*., 2002), both Amazonian species are genetically much more diverse than the American alligator (Table 1). It appears that the decimation of census numbers in the 20th century did not necessarily deplete the genetic diversity of these two Amazonian crocodilians to the extent that might have been expected. Furthermore, both species appear to be undergoing a demographic expansion in Lake Janauacá and the French Guianan population of *C. crocodilus* is also expanding. Conclusions drawn from genetic data are supported by census information (Da Silveira *et al*., 1997; Da Silveira & Thorbjarnarson, 1999). The observed population increases are expected for species released from hunting pressure.

As a habitat generalist, *C. crocodilus* is probably better poised for faster recovery than *M. niger*. This interpretation is supported by the results from the programme Fluctuate, which suggest that the current female inbreeding effective population size of *C. crocodilus* is approximately an order of magnitude larger than that of *M. niger* and that the *C. crocodilus* population is increasing by approximately an order of magnitude faster than the *M. niger* population. The preferred habitat of *M. niger*, the Amazonian várzea and igapó, is at present the most endangered Amazonian ecosystem (Goulding, Smith & Mahar, 2000). Coupled with heavier hunting pressure, the ability of *M. niger* to disperse into suitable habitat may limit its speed of recovery. Similarly, isolation-by-distance and, potentially, also local differentiation would seem to be a more important population structuring mechanism in *M. niger* than in the generalist *C. crocodilus*. Therefore, it is likely that *M. niger* will need a longer time to recover, but with continuous management the recovery of both species appears to be realistic.

Acknowledgments

This research was supported by the Federal University of Amazonas (to I.P.F.), the Wildlife Conservation Society (to R.D.S. and J.T.), the Sociedade Civil Mamirauá (to R.D.S.), the Kaw Swamps Natural Reserve and the French Ministry of Environment (to B.T.) and a National Science Foundation grant INT-0002213 (to T.H.). We thank Cláudia Pereira de Deus for assistance in the field and Jack Sites for helpful and insightful comments. Permission to conduct fieldwork and to collect tissue samples has been granted by IBAMA and the French Ministry of Environment.

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