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# Low levels of nucleotide diversity in *Crocodylus moreletii* and evidence of hybridization with *C. acutus*

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**Abstract**

Examinations of both population genetic structure and the processes that lead to such structure in crocodilians have been initiated in several species in response to a call by the IUCN Crocodile Specialist Group. A recent study used microsatellite markers to characterize Morelet's crocodile (*Crocodylus moreletii*) populations in north-central Belize and presented evidence for isolation by distance. To further investigate this hypothesis, we sequenced a portion of the mitochondrial control region for representative animals after including samples from additional locales in Belize, Guatemala and Mexico. While there is limited evidence of subdivision involving other locales, we found that most of the differentiation among populations of *C. moreletii* can be attributed to animals collected from a single locale in Belize, Banana Bank Lagoon. Furthermore, mitochondrial DNA sequence analysis showed that animals from this and certain other locales display a haplotype characteristic of the American crocodile, *C. acutus*, rather than *C. moreletii*. We interpret this as evidence of hybridization between the two species and comment on how these new data have influenced our interpretation of previous findings. We also find very low levels of nucleotide diversity in *C. moreletii* haplotypes and provide evidence for a low rate of substitution in the crocodilian mitochondrial control region. Finally, the conservation implications of these findings are discussed.

## Introduction

The International Union for the Conservation of Nature and Natural Resources (IUCN) Crocodile Specialist Group (CSG) recently emphasized the need for population genetic surveys of several crocodilian species, including some considered to be critically endangered (Ross 1998). The objectives of these surveys are to obtain basic information on phylogeography, population structure and migration patterns, to gather data on paternity patterns and the related issues of sperm storage and sperm competition, and to examine suspected introgression from widespread

crocodilians into the genomes of more restricted species (e.g., invasion by the American crocodile [*Crocodylus acutus*] into Cuban crocodile [*C. rhombifer*] habitat and resulting hybridization, see Ramos et al. 1994). This information can be used to implement more effective conservation strategies for crocodilians.

Microsatellite data have been informative in accomplishing several of these goals. Examination of microsatellite loci in American alligator (*Alligator mississippiensis*) populations has documented population structure (Glenn et al. 1998, Davis et al. 2001a) and demonstrated multiple paternity (Davis et al. 2001b). Microsatellites developed for the genus *Crocodylus* by FitzSimmons et al. (2001) have the potential to be at least as useful. These loci not only amplify DNA in many crocodilian species, but are often highly variable (Dever and Densmore 2001). These loci have also been used to identify captive hybrids of *C. rhombifer*, the Siamese crocodile (*C. siamensis*), and the Estuarine crocodile (*C. porosus*) to prevent their re-introduction to the wild (FitzSimmons et al. 2002).

In contrast, mitochondrial DNA (mtDNA) sequence data sets have not been extensively employed in population-level studies of crocodilians. Most studies using mitochondrial data sets have focused on phylogenetic questions rather than population structure or phylogeography (Densmore and Owen 1989; Densmore and White 1991; Gatesy and Amato 1992; Ray et al. 2001). This is somewhat surprising since the population structure of many other vertebrates have been examined using DNA sequences from coding (Donovan et al. 2000; Fleischer et al. 2001; Hoffman and Baker 2001; Kotlik and Berrebi 2001; Nielson et al. 2001), and non-coding regions of the mitochondrial genome (Lahanas et al. 1994; Walker and Avise 1998; Cicero and Johnson 1998; Barrowclough et al. 1999; Roman et al. 1999; Vila et al. 1999; Milot et al. 2000; Rooney et al. 2001; Jensen-Seaman and Kidd 2001; Dawson et al. 2001). The non-coding sequences are often shown to be the most variable portion of the genome (McMillan and Palumbi 1997; Baker and Marshall 1997; but also see Randi and Lucchini 1998; Crochet and Desmarais 2000), suggesting that the control region may be a useful marker for studies of crocodilian populations.

Recently, Glenn et al. (2002) published data examining mitochondrial control region haplotype distributions in *A. mississippiensis*. They found a very low level of nucleotide diversity – only three haplotypes spread across the southeastern United States. These data fit a well-documented trend of low levels of molecular variation for several markers (Gartside et al. 1977; Menzies et al. 1979; Adams et al. 1980; Glenn et al. 1998), but contradict the most recent analyses with microsatellites (Davis et al. 2002). A potential weakness of this study is that only 25 individuals were examined (Glenn et al. 2002). For researchers to accurately judge the levels of mtDNA variation that are expected in crocodilian populations, studies of other species and larger sample sizes are required.

Morelet's crocodile (*Crocodylus moreletii*) is one of two crocodile species to occur in Mexico, Guatemala, and Belize. *C. moreletii* typically inhabits freshwater wetland habitats while *C. acutus* is restricted to coastal mainland habitats and offshore islands (Platt 1996; Platt and Thorbjarnarson 2000a,b). Both species were subjected to extensive hunting pressures during the middle of the 20th century leading to drastic population declines (Charnock–Wilson 1970; Platt 1996; Platt and Thorbjarnarson 2000a,b; Ross 1998). Both are currently considered endangered by the IUCN and listed on Appendix I of CITES (Platt and Thorbjarnarson 2000a,b; Ross 1998).

Dever et al. (2002) initiated a population genetic analysis of *C. moreletii* in Belize using microsatellite markers in order to assess the genetic variability of Morelet's crocodile. Results of this study demonstrated levels of genetic variation in north-central Belize (average $H_O = 0.49$) comparable to the American alligator and revealed evidence for some population substructure ($R_{ST} = 0.1$). The structure observed was primarily due to the inclusion of animals from a single small population found in Banana Bank Lagoon near the Belize River at the southern end of the sampled range. Dever et al. (2002) suggested that the observed data could support an isolation by distance model of gene flow (Wright 1978).

Our study expanded on Dever et al. (2002) by incorporating mitochondrial control region sequence data for a representative subsample of the animals originally examined and for additional animals from several more distant locales, including southern Belize, Mexico and Guatemala ($n = 140$). Our objectives were to (1) examine intraspecific variation within the mitochondrial

control region and investigate its utility as a phylogeographic marker using *Crocodylus moreletii* as a model and (2) use the mitochondrial data to evaluate the hypothesis proposed by Dever et al. (2002) that Morelet's crocodile populations follow an isolation-by-distance model of genetic differentiation. After collecting preliminary data, we noted several individuals bearing haplotypes closely resembling those found in *C. acutus* than in *C. moreletii*. Thus, we examined *C. moreletii* populations for evidence of hybridization with *C. acutus* by identifying haplotype differences.

## Methods

### *Sampling and data collection*

As part of continuing studies of Morelet's crocodile populations, we have sampled several locales (Figure 1) previously reported by Dever et al. (2002). We also sampled crocodiles at two additional sites in Belize: Crooked Tree Wildlife Sanctuary and the Macal River. All new samples were collected during the spring and summer of 2001 and 2002 using the methods described in Dever and Densmore (2001). Sample sizes for each locale can be found in Table 1. Total genomic DNA was extracted from blood samples using the Gentra Puregene isolation kit (Gentra Systems, Minneapolis, MN). Total genomic DNA was also isolated from skin clips of animals captured in the states of Peten, Guatemala and Tabasco, Mexico, using standard PCI protocols (Sambrook et al. 1989). For outgroup comparisons, control region sequences from the American crocodile (*Crocodylus acutus*), the Orinoco crocodile (*C. intermedius*), the Cuban crocodile (*C. rhombifer*), and the Nile crocodile (*C. niloticus*) were obtained from GenBank (accession numbers AF460218, AF460207, AF460214 and AF460211, respectively).

Amplification of an ~540 bp DNA fragment (starting 6 bp within the tRNA-Pro gene and ending just 3′ of CSB-1 in domain III of the mitochondrial control region) was performed using protocols and primers as previously described in Ray and Densmore (2002). Following amplification, PCR products were purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia CA). Chain termination sequencing reactions were performed on both strands using the PCR primers and chromatograms were

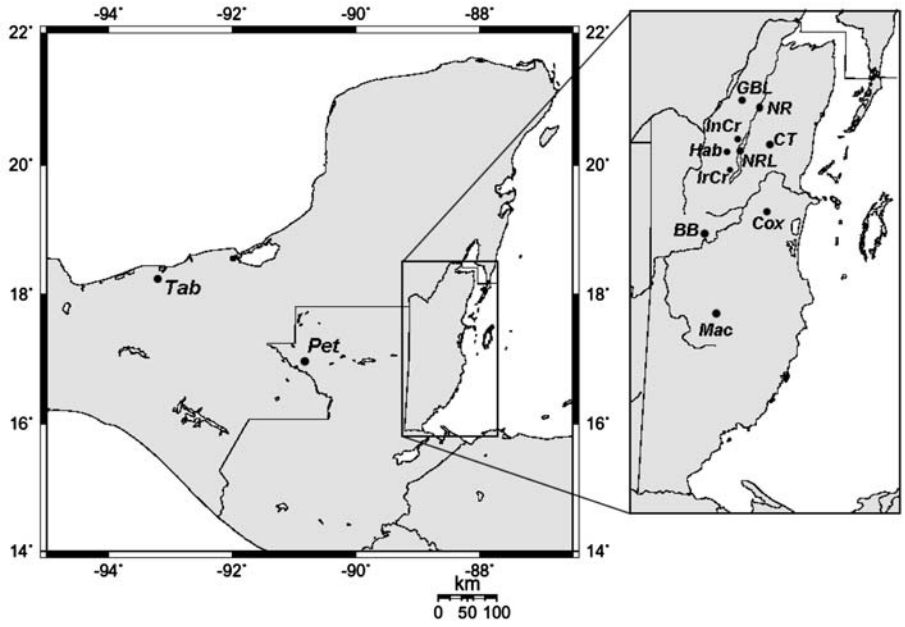


*Figure 1.* Map of sampling locales. BB = Banana Bank Lagoon, Cox = Cox Lagoon, CT = Crooked Tree Wildlife Sanctuary, GBL = Gold Button Lagoon, Hab = Habanero Lagoon, InCr = Indian Creek, IrCr = Irish Creek, Mac = Macal River, NR = New River, NRL = New River Lagoon, Pet = Peten, Guatemala, Tab = Tabasco, Mexico.

*Table 1.* Haplotype distribution for mtDNA control region sequences

| Haplotype | Sample location | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BB (17) | Cox (6) | CT (3) | GBL (13) | Hab (11) | InCr (3) | IrCr (5) | Mac (11) | NR (23) | NRL (38) | Pet (5) | Tab (5) |
| A | 2 | 5 | 2 | 13 | 9 | 1 | 2 | 9 | 19 | 29 | 2 | 4 |
| *B* | *14* | – | – | – | – | *2* | 2 | – | – | *4* | – | – |
| *C* | – | – | – | – | – | – | – | – | – | *2* | – | – |
| *D* | *1* | – | – | – | – | – | – | – | – | – | – | – |
| E | – | 1 | – | – | – | – | – | – | – | – | – | – |
| F | – | – | 1 | – | – | – | – | – | – | – | – | – |
| G | – | – | – | – | 1 | – | – | – | – | – | – | – |
| H | – | – | – | – | 1 | – | – | – | – | – | – | – |
| I | – | – | – | – | – | – | 1 | – | – | – | – | – |
| J | – | – | – | – | – | – | – | 1 | – | – | – | – |
| K | – | – | – | – | – | – | – | 1 | – | – | – | – |
| L | – | – | – | – | – | – | – | – | 1 | – | – | – |
| M | – | – | – | – | – | – | – | – | 1 | – | – | – |
| N | – | – | – | – | – | – | – | – | – | 1 | – | – |
| O | – | – | – | – | – | – | – | – | – | 1 | – | – |
| *P* | – | – | – | – | – | – | – | – | – | 1 | – | – |
| Q | – | – | – | – | – | – | – | – | – | – | 1 | – |
| R | – | – | – | – | – | – | – | – | – | – | 1 | – |
| S | – | – | – | – | – | – | – | – | – | – | 1 | – |
| T | – | – | – | – | – | – | – | – | – | – | – | 1 |

Sample sizes from each locale are indicated in parentheses.

obtained using ABI 310 and 3100 genetic analyzers. Sequences were visualized, aligned and edited with the program BioEdit (Hall 1999). All sequences have been added to GenBank under accession numbers AY136686–AY136738 and AY341444–AY341530. Haplotype definitions and distributions have been deposited at the Population Genetics Database (http://seahorse.louisiana.edu/PGDB/).

### *Data analysis*

PAUP* v4.0b10 (Swofford 1998) was used to generate HKY85 (Hasegawa et al. 1985) genetic distances for the mtDNA sequences as suggested by a likelihood ratio test implemented using Modeltest 3.06 (Posada and Crandall 1998) We employed Arlequin v2.000 (Schneider et al. 2000) to perform analyses of haplotype frequencies, to obtain estimates of $F_{ST}$, to perform tests of population subdivision via AMOVA, and to perform Mantel's tests (Mantel 1967) with both distance and $F_{ST}$ estimates. Arlequin also was used to perform mismatch analyses (Rogers and Harpending 1992), and to estimate haplotype and nucleotide diversities (Nei 1987, p.180 and 257). A median-joining network of haplotypes was constructed using Network (Bandelt et al. 1999, www.fluxus-engineering.com)

## Results

One hundred forty initial mtDNA control region sequences ranging from 534 to 537 bp were obtained. The relative lack of insertion/deletion events (6) made alignment of the sequences straightforward. Among the crocodiles sampled, there were 45 polymorphic sites and 20 unique haplotypes (A–T). A median joining analysis of the haplotypes yielded a network that clearly clusters the haplotypes into two clades separated by 21 sequence changes (Figure 2). Haplotypes in clade 2 are primarily from animals collected at

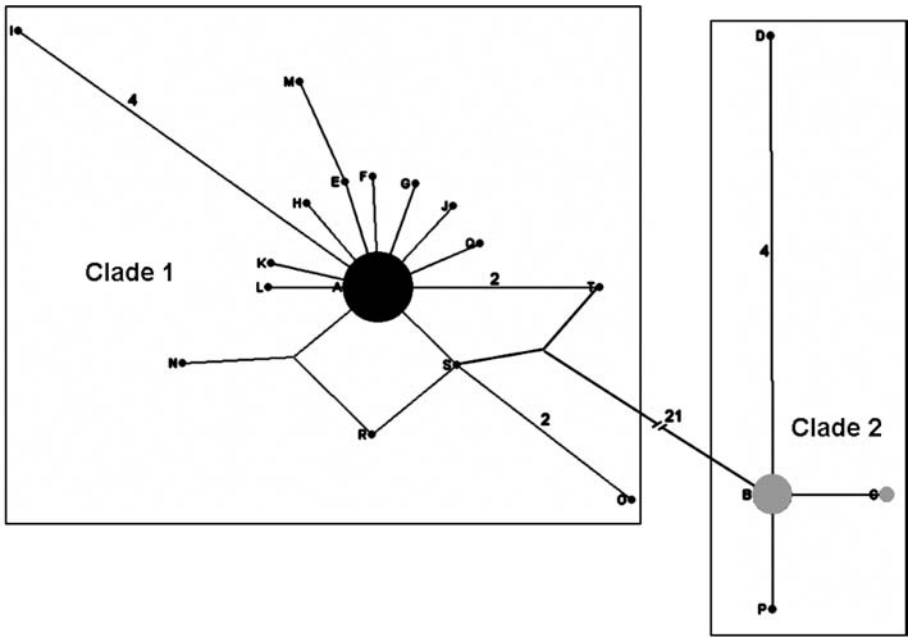


*Figure 2.* Median-joining network of *C. moreletii*-like and *C. acutus*-like haplotypes.Connections between haplotypes indicate single sequence differences unless otherwise indicated.

Banana Bank Lagoon, but this clade is also comprised of samples from New River, New River Lagoon and Indian Creek.

Other crocodylian control region sequences ranged from 533 bp in *Crocodylus niloticus* to 534 bp in both *C. intermedius* and *C. rhombifer* to 536 bp in *C. acutus*. The inclusion of outgroup taxa in a neighbor-joining tree of unique haplotypes (Figure 3) strongly suggested that all of the haplotypes present in clade 2 had originated in *C. acutus* and not *C. moreletii* (bootstrap = 95%). This was verified by incorporating ten additional sequences from *C. acutus* (accession numbers AY568308–AY568317). These sequences, obtained from animals originating from the west coast of Mexico to Florida were not substantially different from the original *C. acutus* reference sequence – five haplotypes (U–Y) with an average of 1.82 differences between them. Therefore, we designated each unique haplotype as either *C. moreletii*-like or *C. acutus*-like.

The average HKY85 genetic distance estimate among all unique *C. moreletii*-like haplotypes was 0.0062 ± 0.0087, and among all *C. acutus*-like haplotypes was 0.0048 ± 0.0040. The average genetic distance between *acutus*-like and *moreletii*-like unique haplotypes was 0.0553 ± 0.0049. When each haplotype was examined for geographic distribution (Table 1), one, A, was found to be present in all sampled locales. Haplotype A was found at a high frequency (≥0.40) in all locales except Banana Bank Lagoon where haplotype B (*C. acutus*-like) predominated. The remaining haplotypes were, for the most part, single examples scattered throughout the range. Within-population haplotype diversity ranged from zero at Gold Button Lagoon to 0.413 ± 0.097 in New River Lagoon (Table 2). Overall haplotype diversity ($h$) for the 140 original samples was 0.502 ± 0.048 and 0.251 ± 0.055 for *C. moreletii*-like haplotypes only. Overall nucleotide diversity ($\pi$) was 0.013 ± 0.007 and 0.00072 ± 0.00073 for *C. moreletii*-like haplotypes.

After incorporating all individuals for which control region sequence was available, our estimate of $F_{ST}$ was 0.28 (see Table 3 for complete $F_{ST}$ listings). We suspected, however, that any population genetic structure found that involved the Banana Bank population may have resulted from either invasion of *C. acutus* into habitat considered typical of *C. moreletii* and subsequent misidentification of these animals or from the inclusion of *C. Moreletii–C. acutus* hybrids in our sample. Therefore, we performed two sets of analyses on

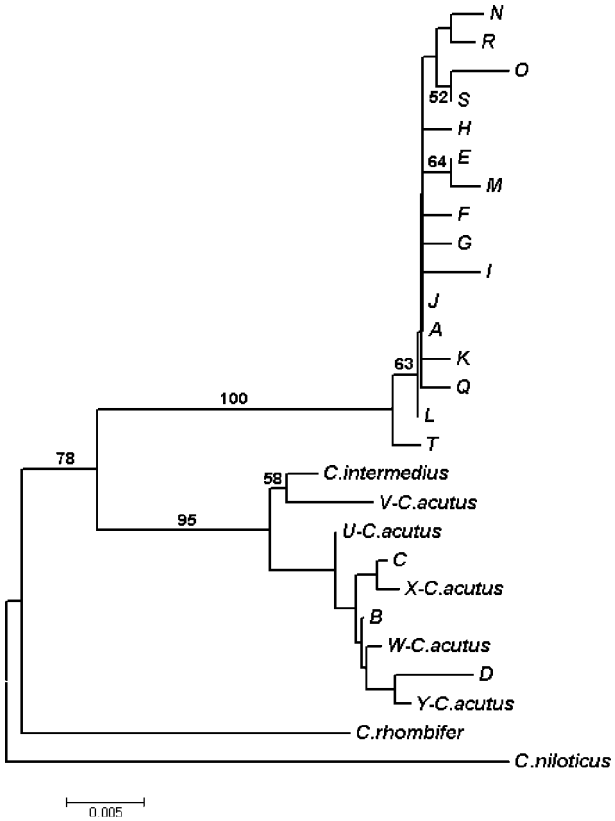


*Figure 3*. Neighbor-joining tree of unique haplotypes and outgroup sequences generated using HKY85 genetic distances. Numbers at nodes indicate bootstrap values (1000 replicates).

*Table 2*. Haplotype ($h$) and nucleotide ($\pi$) diversity estimates for *C. moreletii* mtDNA control region sequences

| Locale[a] | All data | |
|---|---|---|
| | Haplotype diversity | Nucleotide diversity |
| Banana Bank | 0.324 ± 0.136 | 0.0091 ± 0.0045 |
| Cox Lagoon | – | – |
| Crooked Tree | – | – |
| Gold Button Lagoon | 0 | 0 |
| Habenero | 0.346 ± 0.172 | 0.0007 ± 0.0004 |
| Indian Creek | – | – |
| Irish Creek | – | – |
| Macal River | 0.182 ± 0.021 | 0.0003 ± 0.0003 |
| New River | 0.324 ± 0.124 | 0.0064 ± 0.0036 |
| New River Lagoon | 0.413 ± 0.097 | 0.0123 ± 0.0031 |
| Peten, Guat. | – | – |
| Tabasco, Mex. | – | – |
| Overall | 0.502 ± 0.048 | 0.251 ± 0.055 |

[a] Locales with $n < 10$ are excluded from individual estimates of diversity but included in overall

*Table 3.* Pairwise estimates of $F_{ST}$ for sampled populations

| | BB | Cox | CT | GBL | Hab | InCr | IrCr | MR | NR | NRL | Pet | Tab |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Banana Bank Lag. | – | −0.304 | −0.200 | 0.000 | −0.276 | 0.000 | −0.200 | −0.276 | −0.308 | −0.317 | −0.017 | −0.290 |
| Cox Lagoon | 0.638 | – | −0.059 | 0.139 | −0.068 | −1.000 | −0.059 | −0.068 | −0.021 | 0.029 | 0.094 | −0.097 |
| Crooked Tree | 0.568 | −0.059 | – | 0.490 | −0.024 | −1.000 | −0.200 | −0.024 | 0.156 | 0.283 | −0.090 | −0.104 |
| Gold Button Lag. | 0.795 | 0.139 | 0.490 | – | 0.069 | 0.000 | 0.490 | 0.069 | −0.004 | −0.021 | 0.481 | 0.207 |
| Habanero Lag | 0.632 | −0.068 | −0.024 | 0.069 | – | −0.900 | −0.024 | −0.045 | −0.010 | 0.027 | 0.141 | −0.071 |
| Indian Creek | −0.102 | 0.372 | 0.143 | 0.817 | 0.398 | – | −1.000 | −0.900 | −0.950 | −0.967 | −0.500 | −1.000 |
| Irish Creek | 0.188 | 0.167 | −0.014 | 0.558 | 0.210 | −0.235 | – | −0.024 | 0.156 | 0.283 | −0.090 | −0.104 |
| Macal River | 0.632 | −0.068 | −0.024 | 0.069 | −0.045 | 0.398 | 0.210 | – | −0.010 | 0.027 | 0.141 | −0.071 |
| New River | 0.626 | −0.053 | 0.016 | 0.029 | −0.032 | 0.417 | 0.245 | −0.032 | – | −0.015 | 0.333 | 0.002 |
| New River Lag. | 0.541 | 0.038 | −0.020 | 0.060 | −0.014 | 0.292 | 0.152 | −0.014 | −0.017 | – | 0.451 | 0.069 |
| Peten, Guatemala | 0.467 | 0.094 | −0.090 | 0.481 | 0.141 | 0.073 | −0.012 | 0.141 | 0.202 | 0.151 | – | 0.044 |
| Tabasco, Mexico | 0.619 | −0.097 | −0.104 | 0.207 | −0.071 | 0.311 | 0.118 | −0.071 | −0.053 | −0.046 | 0.044 | – |

Estimates incorporating all sequences are located below the diagonal. Estimates above the diagonal were calculated after removing *C. acutus*-like haplotypes. Estimates with *P*-values ≤ 0.05 are italicized.

the data set. One set incorporated all data from every animal collected. The second set of analyses involved the removal of any samples in which the *C. acutus*-like haplotype had been identified. This dual pattern of data analysis is discussed in each relevant table and will be followed for the remainder of the paper. An unfortunate consequence of removing individuals bearing the *C. acutus*-like haplotype from the second set of analyses was to substantially reduce sample sizes at Banana Bank Lagoon (from 17 to 2), Irish Creek (5 to 3), and Indian Creek (3 to 1). Individuals from New River and New River Lagoon were also removed but sample sizes remained reasonable (21 and 31, respectively).

Removing individuals with *C. acutus*-like haplotypes reduced $F_{ST}$ to 0.06 but three population pairs remained that continued to show high pairwise $F_{ST}$ values. Comparisons between the Peten, Guatemala population and New River, New River Lagoon, and Gold Button Lagoon were significantly different from those expected under a null hypothesis of no population differentiation when *C. acutus*-like haplotypes are not considered. Values ranged from 0.333 (NR versus Peten, $P = 0.028$) to 0.481 (GBL versus Peten, $P = 0.010$).

An examination of the possible correlation between genetic and geographic distances using Mantel test and incorporating all sequences did not support a model of isolation by distance (correlation coefficient $(r) = -0.137$, $P = 0.750$). While removing *C. acutus*-like haplotypes from analyses of populations increased the correlation between geography and genetic similarity, results were not significant ($r = .0918$, $P = 0.334$).

## Discussion

### *Diversity estimates*

This study represents only the second analysis of crocodilian mitochondrial control region DNA to be performed from a population genetic perspective. It is also the first to use mtDNA as a tool for such studies in a member of *Crocodylus*. The only previous examination of a crocodilian was performed by Glenn et al. (2002) for the American alligator. In that study, levels of haplotype and nucleotide variation in *A. mississippiensis* were low ($h = 0.313$, $\pi = 0.00034$). Our estimates of nucleotide diversity are twice as high as those reported by Glenn et al. (2002) but, with the exception of some turtles (Avise et al. 1992; Lahanas et al. 1994; Encalada et al. 1996; Roman et al. 1999), are still very low in comparison to estimates in other vertebrates (Cicero and Johnson 1998; Barrowclough et al. 1999; Vila et al. 1999; Milot et al. 2000; Dawson et al. 2001; Jensen-Seaman and Kidd 2001). Nucleotide diversity increases to levels

comparable to other vertebrates only when *C. acutus*-like haplotypes are included.

The distribution of *A. mississippiensis* is more extensive than that of *C. moreletii* and thus greater nucleotide variation in the alligator might be expected. However, as Glenn et al. (2002) suggested, a population bottleneck $21{,}000 \pm 1500$ years ago (Jackson 2000; Waters et al. 2000) may have played a role in reducing nucleotide diversity since the lower effective population size of mtDNA would impact this genome more severely than the nuclear genome (Maruyama and Fuerst 1984, 1985). The pattern of variation found at polymorphic microsatellite loci in *C. moreletii* (Dever et al. 2002) is similar to that found in American alligators (Glenn et al. 1998) in that there are high levels of variation at polymorphic loci, an observation consistent with the hypothesis of a recent bottleneck.

The mismatch distribution calculated from the mitochondrial sequence data, however, does not support the bottleneck scenario (Figure 4). The plot for only *C. moreletii*-like data is consistent with a population that has been at equilibrium in the recent past (cf. Figure 2 of Rogers and Harpending 1992) and thus suggests that a population bottleneck similar to the one hypothesized for *A. mississippiensis* is unlikely. Adding haplotypes characteristic of the American crocodile causes the plot to mimic those encountered when there is incomplete lineage sorting. It is indeed possible that paraphyly caused by the retention of two haplotypes which were characteristic of the common ancestor of both *C. acutus* and *C. moreletii* exists in *C. moreletii* (see Funk and Omland 2003). We do not discount this scenario. However, it is clear that this level of sequence divergence (5.53%) is more typical of comparisons between well-defined species of *Crocodylus* (avg. HKY85 divergence = 6.46%; see Table 4 and Ray et al. 2001).

Another possibility is that a recent event has reduced the nucleotide diversity via a selective sweep or some other event that mimics the effect of a sweep. This is a difficult hypothesis to test but we would expect that such a process would result in a mismatch distribution which mimics one expected after a bottleneck; again, the calculated distribution does not follow such a pattern. Finally, there may be a reduction in the substitution rate in the control region of mtDNA in crocodilians when compared with other vertebrates. The sequences presented here as well those of other crocodilians suggest that this may indeed be the case.

Using sequences taken from Ray and Densmore (2002) we estimated the sequence divergence between *Osteolaemus* and all members of *Crocodylus* (accession numbers AF460207–AF460215, AF461417 and AF460218) using the HKY85 model and incorporating a gamma correction of 0.1911 as suggested by Modeltest v3.06 (Posada and Crandall 1998). The average divergence was calculated to be 0.208. Brochu (2000, 2001) has suggested a minimum divergence between *Osteolaemus* and *Crocodylus* at 19 mya. After assuming

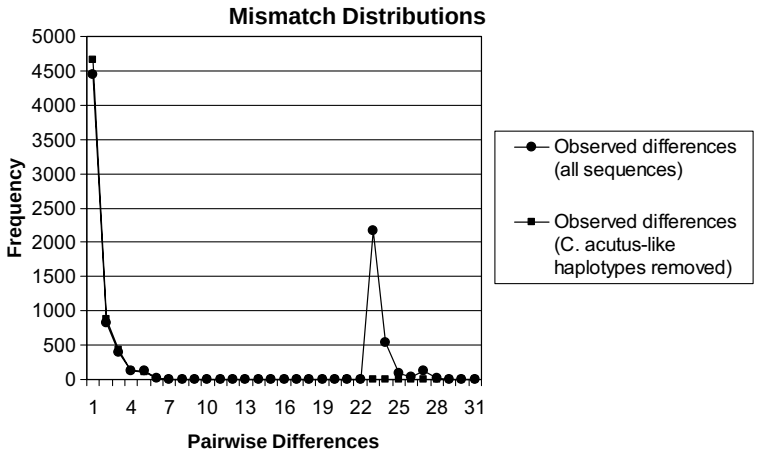


*Figure 4.* Mismatch distributions of pairwise sequence differences in mtDNA control region sequences from *C. moreletii*. The number of mismatches is given on the horizontal axis and the frequency in each category is represented on the vertical axis.

*Table 4*. Pairwise HKY85 distance estimates for comparisons among ten *Crocodylus* species using control region sequence data

| | *C. acu* | *C. min* | *C. nil* | *C. por* | *C. pal* | *C. mor* | *C. john* | *C. int* | *C. rhom* | *C. siam* |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. acutus* | – | | | | | | | | | |
| *C. mindorensis* | 0.062 | – | | | | | | | | |
| *C. niloticus* | 0.049 | 0.060 | – | | | | | | | |
| *C. porosus* | 0.061 | 0.074 | 0.063 | – | | | | | | |
| *C. palustris* | 0.054 | 0.081 | 0.068 | 0.054 | – | | | | | |
| *C. moreletii* | 0.034 | 0.079 | 0.059 | 0.066 | 0.073 | – | | | | |
| *C. johnsoni* | 0.081 | 0.065 | 0.074 | 0.081 | 0.084 | 0.101 | – | | | |
| *C. intermedius* | 0.008 | 0.069 | 0.052 | 0.056 | 0.059 | 0.030 | 0.086 | – | | |
| *C. rhombifer* | 0.038 | 0.074 | 0.054 | 0.070 | 0.065 | 0.052 | 0.086 | 0.043 | – | |
| *C. siamensis* | 0.065 | 0.083 | 0.065 | 0.070 | 0.054 | 0.078 | 0.084 | 0.070 | 0.075 | – |

Sequences are taken from Ray and Densmore (2002).

this divergence time, we arrived at a substitution rate of $1.09 \times 10^{-8}$. This value is 5.4 times lower than the value typically cited for mammals ($5.9 \times 10^{-8}$; Brown et al. 1982). Assuming a more recent divergence for the genus *Crocodylus* of 12 mya (Brochu, pers. comm.) and calculating the mean divergence (0.199) between *C. cataphractus* and other members of the genus allows us to arrive at an estimated rate of $1.66 \times 10^{-8}$, 3.6 times lower than the standard mammalian rate. If either of these rates of change is accurate, lower estimates of mtDNA nucleotide diversity may be expected when using control region sequences to examining crocodylian populations.

### *Population structure*

The initial $F_{ST}$ estimate (0.28) raised the possibility that there may be significant subdivision among the locales sampled. It should be noted, however, that with the exception of Peten in Guatemala, Banana Bank Lagoon is the only locale to show high levels of differentiation from other sampled locales (Table 3). This is similar to results that Dever et al. (2002) recovered using nine microsatellite loci. While it is true that a reduction in statistical power probably accompanied our switch to a mtDNA marker, adding sequence data may have clarified why this population appeared to fit the isolation by distance model in the original microsatellite analyses (Dever et al. 2002). We hypothesize that the introgression of *Crocodylus acutus* genes into several populations (see below), especially in Banana Bank Lagoon, may have confounded the situation with regard to the microsatellite data. When we removed the haplotypes characteristic of *C. acutus* from the data set, our estimate of $F_{ST}$ was reduced to 0.06. Thus, the isolation by distance model of genetic differentiation may not be appropriate for explaining population patterns in *C. moreletii* and further investigation is warranted.

Three of the 20 unique haplotypes were located exclusively in the Peten, Guatemala sample, raising the possibility that this population has differentiated genetically from the others. This population was indeed determined to be significantly different from New River, New River Lagoon and Gold Button Lagoon based on haplotype frequencies (Table 3). However, the distribution of these three haplotypes on the neighbor-joining tree did not support any phylogeographic pattern and results from the Mantel tests support this interpretation.

While it is interesting to note $F_{ST}$ reductions when comparing Banana Bank Lagoon, Peten and the remaining populations, these results must be interpreted with caution because of large differences in sample size between the two populations and others. This is especially true in the case of Banana Bank Lagoon after removal of the *C. acutus*-like haplotypes from the data set. These two factors introduce the specters of sampling bias and reduction in statistical power. Thus, the *C. moreletii*-like haplotype data appear to give no substantial evidence of phylogeographic structure and little information with regard to possible population substructuring in the Yucatan

Peninsula. However, the Guatemalan population should be further investigated.

*Possible hybridization*

Hybridization between *C. moreletii* and *C. acutus* has long been postulated (Ross and Ross 1974; Ross and Mayer 1983). Typically, specimens of *C. moreletii* have five to six scales in each transverse dorsal scale row and exhibit irregular scale groups on both the ventral and lateral surfaces of the tail (Brazaitis 1973; Ross and Ross 1974; Ross and Mayer 1983) while specimens of *C. acutus* exhibit fewer scales in each dorsal scale row and regular scale groups on the tail. Crocodiles with characteristics of both species have been reported from coastal regions of Mexico (Powell 1972) and Belize (Schmidt 1924; Abercrombie et al. 1980; Platt and Thorbjarnarson 1997; Sigler 1998). These individuals typically exhibit a reduced number of dorsal scales in each transverse row, and reduced or absent subcaudal scale irregularities. In an examination of museum specimens of *C. acutus* collected from throughout its range, Ross and Ross (1974) found irregular scale groups on the lateral surface of the tail only where *C. acutus* was sympatric with *C. moreletii* (Belize through Chiapas, Mexico), or where the population may have been influenced by feral *C. moreletii* (west coast of Mexico). The dorsal armor of the suspected hybrids resembled both parent species (Ross and Ross 1974). In addition, preliminary microsatellite analyses of sympatric animals from both species have suggested that hybridization may be occurring (E. Hekkala and G. Amato pers. comm.). Therefore, both Morelet's crocodile and American crocodile populations that have been characterized as 'pure' warrant vigorous protection.

The average genetic distance between the clades 1 and 2 of Figure 2 was 0.055, a value typical of interspecific comparisons in other species of *Crocodylus* (Ray et al. 2001, and Table 4). Haplotypes in the lower clade include animals from Banana Bank Lagoon, within the Belize River drainage, and populations associated with the New River drainage of north-central Belize. The presence of *C. acutus* haplotypes in presumed *C. moreletii* habitat raises the question of whether or not the animals with *C. acutus* haplotypes are hybrids, immigrant American crocodiles from the coastal regions of Belize, or both.

While subadult animals of the two species are often difficult to distinguish from a distance in the field, ventral and nuchal scale patterns are generally reliable diagnostic characters (Brazaitis 1973). Unfortunately, voucher photographs taken of most animals were from inappropriate angles and these scale patterns were not always visible. We have, however, obtained photographs of several animals sampled from Banana Bank showing ventral scale patterns. Three animals (4575–4577) examined at Banana Bank in July 2002 were phenotypically similar to *C. moreletii*. Two of these animals had *C. acutus*-like haplotypes, 4575 and 4577. One crocodile (4675) exhibited a reduced number of scales ($< 5$, typical of *C. acutus*) in each transverse dorsal row, and irregular scale groups were present only on the lateral surface of the tail.

Obviously, a more extensive study of the morphology of animals considered potential hybrids must be performed. Our results, however, do suggest hybridization. The identification of *Crocodylus acutus* haplotypes in or close to main river channels suggests an avenue for introgression into *C. moreletii* habitat. Three of the suspected hybrids collected in the New River drainage were males nearing sexual maturity (total length [TL] = 167–189 cm, Platt and Thorbjarnarson 2000b), suggesting that male *C. acutus* could have traveled inland from the coast via New River or that hybrid offspring from a *C. acutus* female migrated into the area. While the former situation is possible, SGP, TRR, and AGF have collected Morelet's crocodiles along the New River for over ten years (~400 animals) and have found no evidence of purebred *C. acutus* invading this river system, however it is possible that some *C. acutus* immigrants were missed. We suggest that a more likely explanation is backcrossing between hybrids formed initially near the coast and purebred *C. moreletii* farther inland. Regardless of their origin both their size and presence in *C. moreletii* habitat suggest that they may eventually establish territories as breeding adults and that any future offspring from these individuals and *C. moreletii* females in the area will be hybrids.

The existence of potential hybrids at Banana Bank could also be explained by backcrossing. All crocodiles captured at this locale were juveniles (TL = 54–99 cm). Most were approximately the same size, with only a few being slightly larger than the others. The small size of these animals

does not lend support to a long (~36 km overland or ~67 km via the Belize River) migration from the coast. Instead, these size measurements suggest these are members of a single or two successive cohorts from at least one breeding female. We cannot determine if the maternal parent(s) was "pure" *Crocodylus acutus* but the lack of definitive morphological characters identifying animals 4575 and 4577 (both bear *C. acutus*-like haplotypes but display primarily *C. moreletii* scalation) suggest that if they are hybrids, the cross-species interaction may have occurred two or more generations previous to this one. Backcrossing between the initial hybrids and *C. moreletii* could erase most of the phenotypic characters passed from the *C. acutus* ancestor while allowing the haplotype to remain intact through maternal transmission. It may be that some low level of hybridization has always occurred where the two species are sympatric and that what we have observed is typical of the species' interaction.

## Conclusions and implications

There are several potentially critical conservation issues that must be considered in light of our findings. Platt and Thorbjarnarson (1997) observed possible hybrids of *C. acutus* and *C. moreletii* in the coastal regions of southern Belize, but not in central or northern Belize. Our discoveries of *C. acutus* haplotypes in Banana Bank and the New River drainage indicate that there may be considerably more genetic contact between these species than was previously recognized. *Crocodylus moreletii* has here-to-fore been considered one of the least endangered of those crocodilians currently listed on Appendix I of CITES and Platt and Thorbjarnarson (2000b) suggested that an endangered classification is no longer warranted. If hybridization is indeed occurring, however, it may be that genetically pure *C. moreletii* in Belize are rarer than previously assumed and it is of the utmost importance that this species not be removed from Appendix I until the degree of interspecific genetic contact has been accurately assessed. Those Morelet's crocodile populations that have been identified as genetically pure (e.g., the Macal River system in Belize and possibly the Guatemalan and Mexican populations) should also be vigorously protected.

A potentially more important issue, however, is the threat introgression from *C. moreletii* may represent to Belizean *C. acutus* populations. The recovery of *C. acutus* from the hunting pressures of the 1950s and 1960s has been slower than for *C. moreletii* (Platt and Thorbjarnarson 2000a). Thus, it is likely that high levels of hybridization represent a larger danger to the genetic integrity of *C. acutus* than to *C. moreletii* in Belize. It is therefore critical that a comparable study of nuclear and mitochondrial markers in *C. acutus* be performed to determine if the introgression observed is "one-way" or "two-way" in nature. A study of microsatellite variation in Central American *C. acutus* populations is currently being conducted by LDD that and colleagues should help provide an answer to this question.

The results of our study indicate that while there is some minor evidence of population differentiation in *Crocodylus moreletii* the issue deserves more attention and it is critical that future studies increase sampling outside of Belize. Dever et al. (2002) attributed the differentiation between Banana Bank Lagoon and those populations in North-central Belize to isolation-by-distance. The mtDNA data presented herein indicate that this level of differentiation may be due (at least in part) to the introduction of foreign alleles from *C. acutus* into the Banana Bank Morelet's crocodile population. This result emphasizes the need for multiple markers in such studies, even if sampled populations are *a priori* thought to be "pure". Furthermore, local conservation and management efforts designed for either *C. moreletii* or *C. acutus* should incorporate protocols that allow the genetic heritage of their animals to be accurately determined. It is now clear that range alone cannot always accurately predict the genetic makeup of crocodile populations found in every locality.

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