

1 **Title:** Low Levels of Nucleotide Diversity in *Crocodylus moreletii* and Evidence of
2 Hybridization with *C. acutus*.

3
4 **Running Title:** Diversity and Hybridization in *C. moreletii*

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6 **Authors:** David A. Ray^{1,2,*}, Jennifer A. Dever³, Steven G. Platt^{4,5}, Thomas R.
7 Rainwater⁶, Adam G. Finger⁶, Scott T. McMurry⁶, Mark A. Batzer², Brady Barr⁷,
8 Peter J. Stafford⁸, Jenna McKnight⁹ and Llewellyn D. Densmore¹

9
10 1. Department of Biological Sciences, MS 3131, Texas Tech University, Lubbock,
11 TX, 79409, USA

12 2. Current Address: Department of Biological Sciences, Louisiana State University,
13 202 Life Sciences Building, Baton Rouge, LA, 70803, USA

14 3. University of San Francisco, Harney Science Center, Room 342, 2130 Fulton St.,
15 San Francisco, CA 94117, USA

16 4. Wildlife Conservation Society, 2300 Southern Blvd., Bronx, NY, 10460-1099,
17 USA

18 5. Current Address: Oglala Lakota College, P.O. Box 490, Kyle, SD, 57752, USA

19 6. The Institute of Environmental and Human Health, Box 41163, Texas Tech
20 University, Lubbock, TX 79409-1163, USA

21 7. National Geographic Society, 1145 17th St. NW, Washington, DC, 20036, USA

22 8. The Natural History Museum, Cromwell Road, London SW7 5BD, UK

23 9. Department of Biology, University of Miami, Coral Gables, FL 33124, USA

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26
27 ***Corresponding author:** David A. Ray
28 Department of Biological Sciences
29 Louisiana State University
30 202 Life Sciences Building
31 Baton Rouge, LA 70803
32 Phone: 225-578-7103
33 Fax: 225-578-7113
34 E-mail: daray@lsu.edu
35

1 **Abstract**

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

1 **Introduction**

2 The International Union for the Conservation of Nature and Natural Resources
3 (IUCN) Crocodile Specialist Group (CSG) recently emphasized the need for
4 population genetic surveys of several crocodilian species, including some considered
5 to be critically endangered (Ross 1998). The objectives of these surveys are to obtain
6 basic information on phylogeography, population structure and migration patterns, to
7 gather data on paternity patterns and the related issues of sperm storage and sperm
8 competition, and to examine suspected introgression from widespread crocodilians
9 into the genomes of more restricted species (e. g., invasion by the American crocodile
10 [*Crocodylus acutus*] into Cuban crocodile [*C. rhombifer*] habitat and resulting
11 hybridization, see Ramos et al. 1994). This information can be used to implement
12 more effective conservation strategies for crocodilians.

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

1 In contrast, mitochondrial DNA (mtDNA) sequence data sets have not been
2 extensively employed in population-level studies of crocodylians. Most studies using
3 mitochondrial data sets have focused on phylogenetic questions rather than
4 population structure or phylogeography (Densmore & Owen 1989, Densmore &
5 White 1991, Gatesy & Amato 1992, Ray et al. 2001). This is somewhat surprising
6 since the population structure of many other vertebrates have been examined using
7 DNA sequences from coding (Donovan et al. 2000, Fleischer et al. 2001, Hoffman &
8 Baker 2001, Kotlik & Berrebi 2001, Nielson et al. 2001), and non-coding regions of
9 the mitochondrial genome (Lahanas et al. 1994, Walker & Avise 1998, Cicero and
10 Johnson 1998, Barrowclough et al. 1999, Roman et al. 1999, Vila et al. 1999, Milot et
11 al. 2000, Rooney et al. 2001, Jensen-Seaman & Kidd 2001, Dawson et al. 2001). The
12 non-coding sequences are often shown to be the most variable portion of the genome
13 (McMillan & Palumbi 1997, Baker & Marshall 1997, but also see Randi & Lucchini
14 1998, Crochet & Desmarais 2000), suggesting that the control region may be a useful
15 marker for studies of crocodylian populations.

16 Recently, Glenn et al. (2002) published data examining mitochondrial control
17 region haplotype distributions in *A. mississippiensis*. They found a very low level of
18 nucleotide diversity - only three haplotypes spread across the southeastern United
19 States. These data fit a well-documented trend of low levels of molecular variation
20 for several markers (Gartside et al. 1977, Menzies et al. 1979, Adams et al. 1980,
21 Glenn et al. 1998), but contradict the most recent analyses with microsatellites (Davis
22 et al. 2002). A potential weakness of this study is that only 25 individuals were
23 examined (Glenn et al. 2002). For researchers to accurately judge the levels of

1 mtDNA variation that are expected in crocodylian populations, studies of other
2 species and larger sample sizes are required.

3 Morelet's crocodile (*Crocodylus moreletii*) is one of two crocodile species to
4 occur in Mexico, Guatemala, and Belize. *C. moreletii* typically inhabits freshwater
5 wetland habitats while *C. acutus* is restricted to coastal mainland habitats and
6 offshore islands (Platt, 1996, Platt & Thorbjarnarson 2000a, b). Both species were
7 subjected to extensive hunting pressures during the middle of the 20th century leading
8 to drastic population declines (Charnock-Wilson 1970, Platt 1996, Platt &
9 Thorbjarnarson 2000a,b, Ross 1998). Both are currently considered endangered by
10 the IUCN and listed on Appendix I of CITES (Platt & Thorbjarnarson 2000a, b, Ross
11 1998).

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

21 Our study expanded on Dever et al. (2002) by incorporating mitochondrial
22 control region sequence data for a representative subsample of the animals originally
23 examined and for additional animals from several more distant locales, including

1 southern Belize, Mexico and Guatemala (n = 140). Our objectives were to 1)
2 examine intraspecific variation within the mitochondrial control region and
3 investigate its utility as a phylogeographic marker using *Crocodylus moreletii* as a
4 model and 2) use the mitochondrial data to evaluate the hypothesis proposed by
5 Dever et al. (2002) that Morelet's crocodile populations follow an isolation-by-
6 distance model of genetic differentiation. After collecting preliminary data, we noted
7 several individuals bearing haplotypes closely resembling those found in *C. acutus*
8 than in *C. moreletii*. Thus, we examined *C. moreletii* populations for evidence of
9 hybridization with *C. acutus* by identifying haplotype differences.

10 **Methods**

11 Sampling and Data Collection

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

1 crocodile (*C. niloticus*) were obtained from GenBank (accession numbers AF460218,
2 AF460207, AF460214 and AF460211, respectively).

3 Amplification of an ~540 bp DNA fragment (starting 6 bp within the tRNA-
4 Pro gene and ending just 3' of CSB-1 in domain III of the mitochondrial control
5 region) was performed using protocols and primers as previously described in Ray &
6 Densmore (2002). Following amplification, PCR products were purified using the
7 Qiagen PCR purification kit (Qiagen Inc., Valencia CA). Chain termination
8 sequencing reactions were performed on both strands using the PCR primers and
9 chromatograms were obtained using ABI 310 and 3100 genetic analyzers. Sequences
10 were visualized, aligned and edited with the program BioEdit (Hall 1999). All
11 sequences have been added to GenBank under accession numbers AY136686 -
12 AY136738 and AY341444 - AY341530. Haplotype definitions and distributions
13 have been deposited at the Population Genetics Database
14 (<http://seahorse.louisiana.edu/PGDB/>).

15 Data Analysis

16 PAUP* v4.0b10 (Swofford 1998) was used to generate HKY85 (Hasegawa et
17 al. 1985) genetic distances for the mtDNA sequences as suggested by a likelihood
18 ratio test implemented using Modeltest 3.06 (Posada and Crandall 1998) We
19 employed Arlequin v2.000 (Schneider et al. 2000) to perform analyses of haplotype
20 frequencies, to obtain estimates of F_{ST} , to perform tests of population subdivision via
21 AMOVA, and to perform Mantel's tests (Mantel 1967) with both distance and F_{ST}
22 estimates. Arlequin also was used to perform mismatch analyses (Rogers &
23 Harpending 1992), and to estimate haplotype and nucleotide diversities (Nei 1987, p.

1 180 and 257). A median-joining network of haplotypes was constructed using
2 Network (Bandelt et al. 1999, www.fluxus-engineering.com)

3 **Results**

4 One hundred forty initial mtDNA control region sequences ranging from 534
5 to 537 bp were obtained. The relative lack of insertion/deletion events (6) made
6 alignment of the sequences straightforward. Among the crocodiles sampled, there
7 were 45 polymorphic sites and 20 unique haplotypes (A-T). A median joining
8 analysis of the haplotypes yielded a network that clearly clusters the haplotypes into
9 two clades separated by 21 sequence changes (fig. 2). Haplotypes in clade 2 are
10 primarily from animals collected at Banana Bank Lagoon, but this clade is also
11 comprised of samples from New River, New River Lagoon and Indian Creek.

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

1 The average HKY85 genetic distance estimate among all unique *C. moreletii*-
2 like haplotypes was 0.0062 ± 0.0087 , and among all *C. acutus*-like haplotypes was
3 0.0048 ± 0.0040 . The average genetic distance between *acutus*-like and *moreletii*-
4 like unique haplotypes was 0.0553 ± 0.0049 . When each haplotype was examined for
5 geographic distribution (table 1), one, A, was found to be present in all sampled
6 locales. Haplotype A was found at a high frequency (≥ 0.40) in all locales except
7 Banana Bank Lagoon where haplotype B (*C. acutus*-like) predominated. The
8 remaining haplotypes were, for the most part, single examples scattered throughout
9 the range. Within-population haplotype diversity ranged from zero at Gold Button
10 Lagoon to 0.413 ± 0.097 in New River Lagoon (table 2). Overall haplotype diversity
11 (h) for the 140 original samples was 0.502 ± 0.048 and 0.251 ± 0.055 for *C.*
12 *moreletii*-like haplotypes only. Overall nucleotide diversity (π) was 0.013 ± 0.007
13 and 0.00072 ± 0.00073 for *C. moreletii*-like haplotypes.

14 After incorporating all individuals for which control region sequence was
15 available, our estimate of F_{ST} was 0.28 (see table 3 for complete F_{ST} listings). We
16 suspected, however, that any population genetic structure found that involved the
17 Banana Bank population may have resulted from either invasion of *C. acutus* into
18 habitat considered typical of *C. moreletii* and subsequent misidentification of these
19 animals or from the inclusion of *C. moreletii*-*C. acutus* hybrids in our sample.
20 Therefore, we performed two sets of analyses on the data set. One set incorporated
21 all data from every animal collected. The second set of analyses involved the
22 removal of any samples in which the *C. acutus*-like haplotype had been identified.
23 This dual pattern of data analysis is discussed in each relevant table and will be

1 followed for the remainder of the paper. An unfortunate consequence of removing
2 individuals bearing the *C. acutus*-like haplotype from the second set of analyses was
3 to substantially reduce sample sizes at Banana Bank Lagoon (from 17 to 2), Irish
4 Creek (5 to 3), and Indian Creek (3 to 1). Individuals from New River and New
5 River Lagoon were also removed but sample sizes remained reasonable (21 and 31,
6 respectively).

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

14 An examination of the possible correlation between genetic and geographic
15 distances using Mantel test and incorporating all sequences did not support a model
16 of isolation by distance (correlation coefficient (r) = -0.137, $p = 0.750$). While
17 removing *C. acutus*-like haplotypes from analyses of populations increased the
18 correlation between geography and genetic similarity, results were not significant ($r =$
19 0.0918, $p = 0.334$).

20 **Discussion**

21 Diversity Estimates

22 This study represents only the second analysis of crocodilian mitochondrial
23 control region DNA to be performed from a population genetic perspective. It is also

1 the first to use mtDNA as a tool for such studies in a member of *Crocodylus*. The
2 only previous examination of a crocodylian was performed by Glenn et al. (2002) for
3 the American alligator. In that study, levels of haplotype and nucleotide variation in
4 *A. mississippiensis* were low ($h = 0.313$, $\pi = 0.00034$). Our estimates of nucleotide
5 diversity are twice as high as those reported by Glenn et al. (2002) but, with the
6 exception of some turtles (Avise et al. 1992, Lahanas et al. 1994, Roman et al. 1999,
7 Encalada et al. 1996), are still very low in comparison to estimates in other
8 vertebrates (Cicero & Johnson 1998, Barrowclough et al. 1999, Vila et al. 1999, Milot
9 et al. 2000, Dawson et al. 2001, Jensen-Seaman & Kidd 2001). Nucleotide diversity
10 increases to levels comparable to other vertebrates only when *C. acutus*-like
11 haplotypes are included.

12 The distribution of *A. mississippiensis* is more extensive than that of *C.*
13 *moreletii* and thus greater nucleotide variation in the alligator might be expected.
14 However, as Glenn et al. (2002) suggested, a population bottleneck 21,000 to 1500
15 years ago (Jackson 2000, Waters et al. 2000) may have played a role in reducing
16 nucleotide diversity since the lower effective population size of mtDNA would
17 impact this genome more severely than the nuclear genome (Maruyama & Fuerst
18 1984, 1985). The pattern of variation found at polymorphic microsatellite loci in *C.*
19 *moreletii* (Dever et al. 2002) is similar to that found in American alligators (Glenn et
20 al. 1998) in that there are high levels of variation at polymorphic loci, an observation
21 consistent with the hypothesis of a recent bottleneck.

22 The mismatch distribution calculated from the mitochondrial sequence data,
23 however, does not support the bottleneck scenario (fig. 4). The plot for only *C.*

1 *moreletii*-like data is consistent with a population that has been at equilibrium in the
2 recent past (cf. fig. 2 of Rogers & Harpending 1992) and thus suggests that a
3 population bottleneck similar to the one hypothesized for *A. mississippiensis* is
4 unlikely. Adding haplotypes characteristic of the American crocodile causes the plot
5 to mimic those encountered when there is incomplete lineage sorting. It is indeed
6 possible that paraphyly caused by the retention of two haplotypes which were
7 characteristic of the common ancestor of both *C. acutus* and *C. moreletii* exists in *C.*
8 *moreletii* (see Funk and Omland 2003). We do not discount this scenario. However,
9 it is clear that this level of sequence divergence (5.53%) is more typical of
10 comparisons between well-defined species of *Crocodylus* (avg HKY85 divergence =
11 6.46%; see Table 4 and Ray et al. 2001).

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

20 Using sequences taken from Ray & Densmore (2002) we estimated the
21 sequence divergence between *Osteolaemus* and all members of *Crocodylus*
22 (accession numbers AF460207-AF460215, AF461417 and AF460218) using the
23 HKY85 model and incorporating a gamma correction of 0.1911 as suggested by

1 Modeltest v3.06 (Posada and Crandall 1998). The average divergence was calculated
2 to be 0.208. Brochu (2000, 2001) has suggested a minimum divergence between
3 *Osteolaemus* and *Crocodylus* at 19 mya. After assuming this divergence time, we
4 arrived at a substitution rate of 1.09×10^{-8} . This value is 5.4 times lower than the
5 value typically cited for mammals (5.9×10^{-8} ; Brown et al. 1982). Assuming a more
6 recent divergence for the genus *Crocodylus* of 12 mya (Brochu, pers. comm.) and
7 calculating the mean divergence (0.199) between *C. cataphractus* and other members
8 of the genus allows us to arrive at an estimated rate of 1.66×10^{-8} , 3.6 times lower
9 than the standard mammalian rate. If either of these rates of change is accurate, lower
10 estimates of mtDNA nucleotide diversity may be expected when using control region
11 sequences to examine crocodylian populations.

12 Population Structure

13 The initial F_{ST} estimate (0.28) raised the possibility that there may be
14 significant subdivision among the locales sampled. It should be noted, however, that
15 with the exception of Peten in Guatemala, Banana Bank Lagoon is the only locale to
16 show high levels of differentiation from other sampled locales (table 3). This is
17 similar to results that Dever et al. (2002) recovered using nine microsatellite loci.
18 While it is true that a reduction in statistical power probably accompanied our switch
19 to a mtDNA marker, adding sequence data may have clarified why this population
20 appeared to fit the isolation by distance model in the original microsatellite analyses
21 (Dever et al. 2002). We hypothesize that the introgression of *Crocodylus acutus*
22 genes into several populations (see below), especially in Banana Bank Lagoon, may
23 have confounded the situation with regard to the microsatellite data. When we

1 removed the haplotypes characteristic of *C. acutus* from the data set, our estimate of
2 F_{ST} was reduced to 0.06. Thus, the isolation by distance model of genetic
3 differentiation may not be appropriate for explaining population patterns in *C.*
4 *moreletii* and further investigation is warranted.

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

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

22 Possible Hybridization

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

21 The average genetic distance between the clades 1 and 2 of figure 2 was
22 0.055, a value typical of interspecific comparisons in other species of *Crocodylus*
23 (Ray et al. 2001, and Table 4). Haplotypes in the lower clade include animals from

1 Banana Bank Lagoon, within the Belize River drainage, and populations associated
2 with the New River drainage of north-central Belize. The presence of *C. acutus*
3 haplotypes in presumed *C. moreletii* habitat raises the question of whether or not the
4 animals with *C. acutus* haplotypes are hybrids, immigrant American crocodiles from
5 the coastal regions of Belize, or both.

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

17 Obviously, a more extensive study of the morphology of animals considered
18 potential hybrids must be performed. Our results, however, do suggest hybridization.
19 The identification of *Crocodylus acutus* haplotypes in or close to main river channels
20 suggests an avenue for introgression into *C. moreletii* habitat. Three of the suspected
21 hybrids collected in the New River drainage were males nearing sexual maturity (total
22 length [TL]= 167 cm to 189 cm, Platt & Thorbjarnarson 2000b), suggesting that male
23 *C. acutus* could have traveled inland from the coast via New River or that hybrid

1 offspring from a *C. acutus* female migrated into the area. While the former situation
2 is possible, SGP, TRR, and AGF have collected Morelet's crocodiles along the New
3 River for over ten years (~400 animals) and have found no evidence of purebred *C.*
4 *acutus* invading this river system, however it is possible that some *C. acutus*
5 immigrants were missed. We suggest that a more likely explanation is backcrossing
6 between hybrids formed initially near the coast and purebred *C. moreletii* farther
7 inland. Regardless of their origin both their size and presence in *C. moreletii* habitat
8 suggest that they may eventually establish territories as breeding adults and that any
9 future offspring from these individuals and *C. moreletii* females in the area will be
10 hybrids.

11 The existence of potential hybrids at Banana Bank could also be explained by
12 backcrossing. All crocodiles captured at this locale were juveniles (TL = 54 – 99
13 cm). Most were approximately the same size, with only a few being slightly larger
14 than the others. The small size of these animals does not lend support to a long (~36
15 km overland or ~67 km via the Belize River) migration from the coast. Instead, these
16 size measurements suggest these are members of a single or two successive cohorts
17 from at least one breeding female. We cannot determine if the maternal parent(s) was
18 “pure” *Crocodylus acutus* but the lack of definitive morphological characters
19 identifying animals 4575 and 4577 (both bear *C. acutus*-like haplotypes but display
20 primarily *C. moreletii* scalation) suggest that if they are hybrids, the cross-species
21 interaction may have occurred two or more generations previous to this one.
22 Backcrossing between the initial hybrids and *C. moreletii* could erase most of the
23 phenotypic characters passed from the *C. acutus* ancestor while allowing the

1 haplotype to remain intact through maternal transmission. It may be that some low
2 level of hybridization has always occurred where the two species are sympatric and
3 that what we have observed is typical of the species' interaction.

4 Conclusions and implications

5 There are several potentially critical conservation issues that must be
6 considered in light of our findings. Platt & Thorbjarnarson (1997) observed possible
7 hybrids of *C. acutus* and *C. moreletii* in the coastal regions of southern Belize, but not
8 in central or northern Belize. Our discoveries of *C. acutus* haplotypes in Banana
9 Bank and the New River drainage indicate that there may be considerably more
10 genetic contact between these species than was previously recognized. *Crocodylus*
11 *moreletii* has here-to-fore been considered one of the least endangered of those
12 crocodylians currently listed on Appendix I of CITES and Platt & Thorbjarnarson
13 (2000b) suggested that an endangered classification is no longer warranted. If
14 hybridization is indeed occurring, however, it may be that genetically pure *C.*
15 *moreletii* in Belize are rarer than previously assumed and it is of the utmost
16 importance that this species not be removed from Appendix I until the degree of
17 interspecific genetic contact has been accurately assessed. Those Morelet's crocodile
18 populations that have been identified as genetically pure (e.g., the Macal River
19 system in Belize and possibly the Guatemalan and Mexican populations) should also
20 be vigorously protected.

21 A potentially more important issue, however, is the threat introgression from
22 *C. moreletii* may represent to Belizean *C. acutus* populations. The recovery of *C.*
23 *acutus* from the hunting pressures of the 1950's and 1960's has been slower than for

1 *C. moreletii* (Platt & Thorbjarnarson 2000a). Thus, it is likely that high levels of
2 hybridization represent a larger danger to the genetic integrity of *C. acutus* than to *C.*
3 *moreletii* in Belize. It is therefore critical that a comparable study of nuclear and
4 mitochondrial markers in *C. acutus* be performed to determine if the introgression
5 observed is “one-way” or “two-way” in nature. A study of microsatellite variation in
6 Central American *C. acutus* populations is currently being conducted by LDD that
7 and colleagues should help provide an answer to this question.

8 The results of our study indicate that while there is some minor evidence of
9 population differentiation in *Crocodylus moreletii* the issue deserves more attention
10 and it is critical that future studies increase sampling outside of Belize. Dever et al.
11 (2002) attributed the differentiation between Banana Bank Lagoon and those
12 populations in North-central Belize to isolation-by-distance. The mtDNA data
13 presented herein indicate that this level of differentiation may be due (at least in part)
14 to the introduction of foreign alleles from *C. acutus* into the Banana Bank Morelet’s
15 crocodile population. This result emphasizes the need for multiple markers in such
16 studies, even if sampled populations are *a priori* thought to be “pure”. Furthermore,
17 local conservation and management efforts designed for either *C. moreletii* or *C.*
18 *acutus* should incorporate protocols that allow the genetic heritage of their animals to
19 be accurately determined. It is now clear that range alone cannot always accurately
20 predict the genetic makeup of crocodile populations found in every locality.

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1 **Figure Legends:**

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

7

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

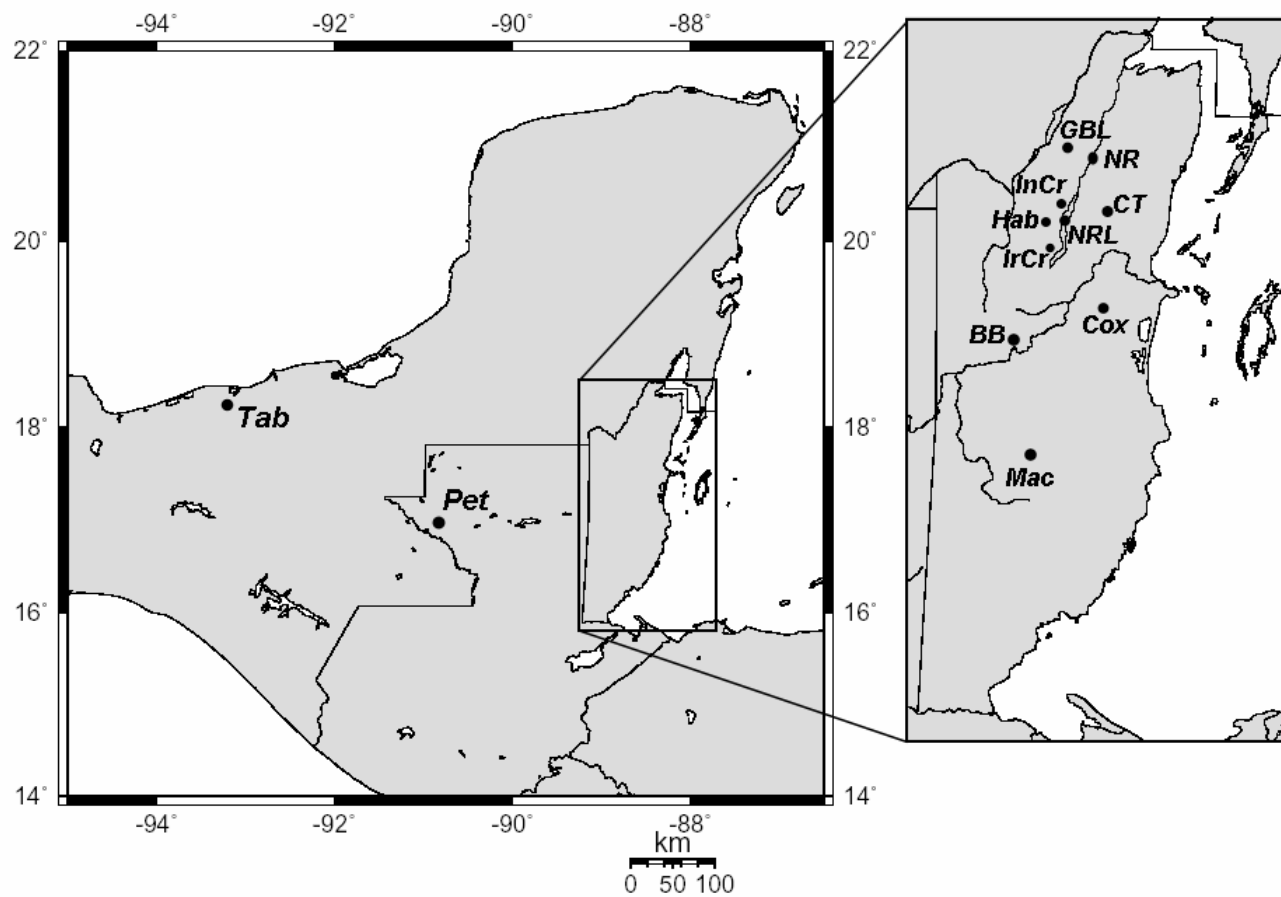
11

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

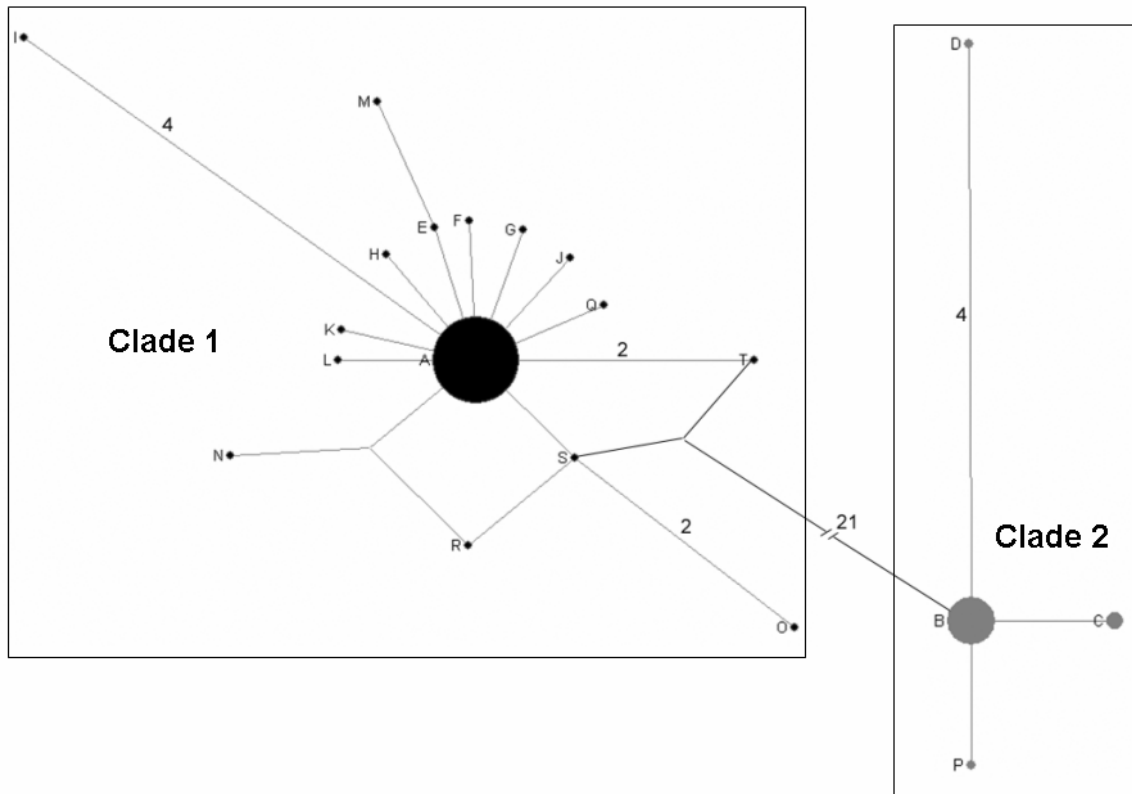
15

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

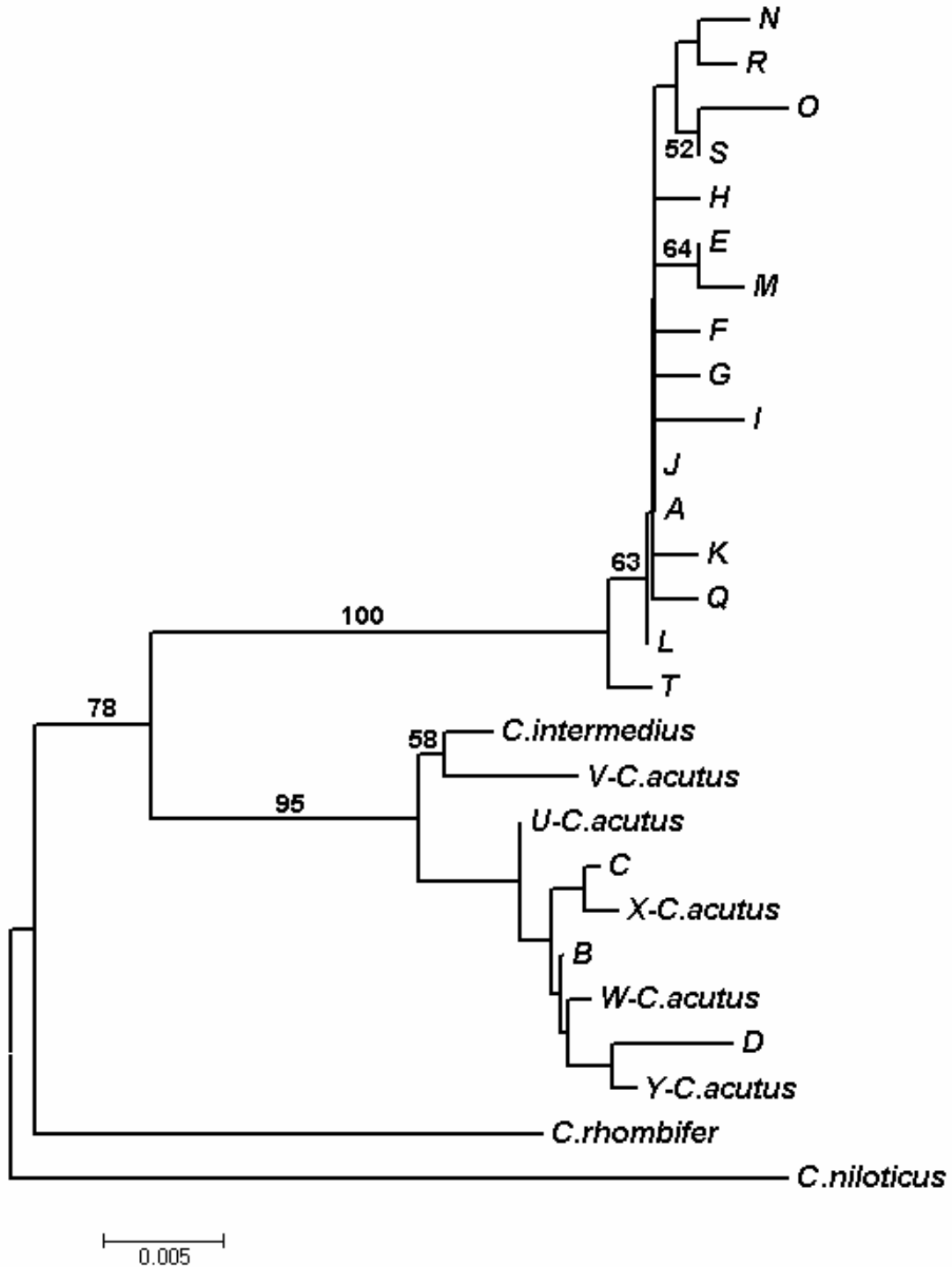
- 1 Figure 1. Range map of *Crocodylus moreletii* with sample locales indicated. BB = Banana Bank Lagoon, Cox = Cox Lagoon, CT =
- 2 Crooked Tree Wildlife Sanctuary, GBL = Gold Button Lagoon, Hab = Habanero Lagoon, InCr = Indian Creek, IrCr = Irish Creek,
- 3 Mac = Macal River, NR = New River, NRL = New River Lagoon, Pet = Peten, Guatemala, Tab = Tabasco, Mexico.



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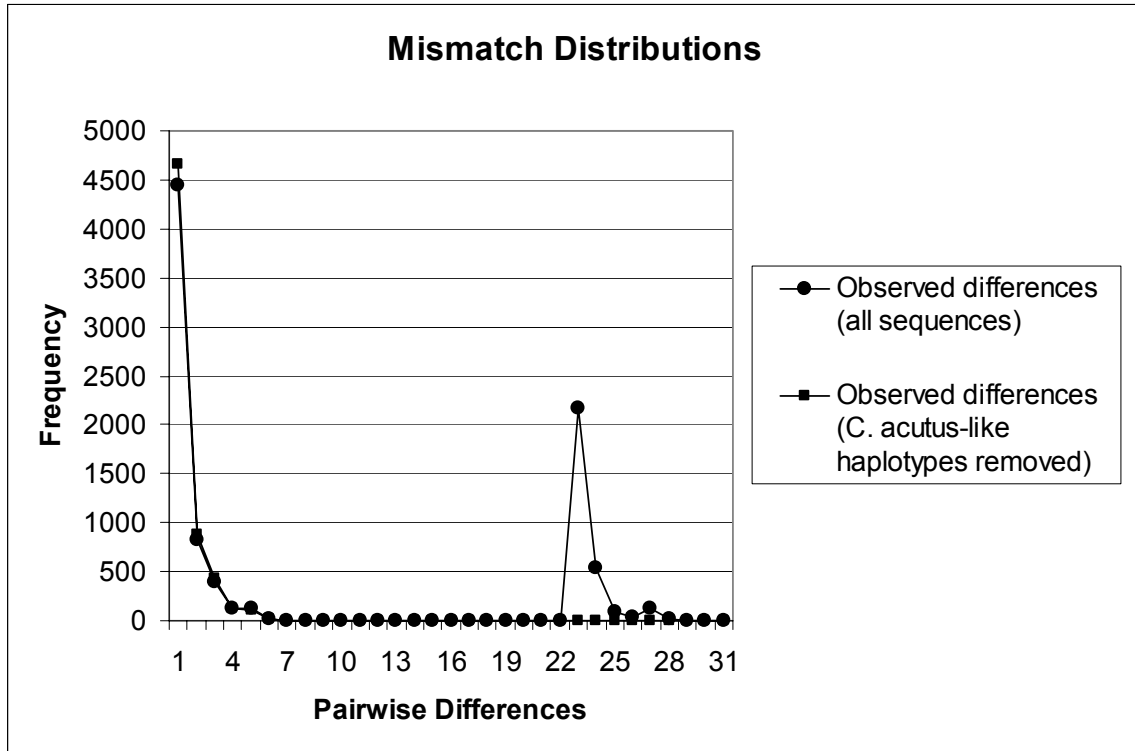


Table 1. Haplotype distribution for mtDNA control region sequences. Sample sizes from each locale are indicated in parentheses.

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	-	1	4	-	-
C	-	-	-	-	-	-	-	-	1	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

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

All Data		
Locale*	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

* Locales with $n < 10$ are excluded from individual estimates of diversity but included in overall estimates.

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

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

Table 4. Pairwise HKY85 distance estimates for comparisons among ten *Crocodylus* species using control region sequence data. Sequences are taken from Ray and Densmore (2003).

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