RESEARCH ARTICLE

Evolutionary History of Cuban Crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* Inferred From Multilocus Markers

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ABSTRACT

Among crocodilians, *Crocodylus rhombifer* is one of the world's most endangered species with the smallest natural distribution. In Cuba, this endemic species coexists with the American crocodile (*Crocodylus acutus*). Hybridization between these two species is well known in captivity and might occur in the wild, but has never been demonstrated genetically. Here, we combined molecular data with environmental, geographic, and fossil data to infer the evolutionary history of *Crocodylus* in the Cuban Archipelago, and to evaluate genealogical support for species boundaries. We analyzed seven microsatellite loci plus DNA sequence data from nuclear (RAG-1) and mitochondrial (cytochrome *b* and cytochrome oxidase I) genes from 89 wild-caught individuals in Cuba, Grand Cayman Island, Jamaica, and Central America, and two samples from zoo collections. Microsatellites showed evidence of introgression, suggesting potential hybridization among Cuban groups. In Cuba, *C. acutus* contained one mitochondrial DNA (mtDNA) haplotype, whereas *C. rhombifer* contained two haplotypes. MtDNA data showed that *C. acutus* is paraphyletic with respect to *C. rhombifer*, revealing 1% sequence divergence between species within Cuba vs. 8% divergence between Cuban forms and mainland *C. acutus*. We suggest that hybridization has been a historical as well as a current phenomenon between *C. acutus* and *C. rhombifer*. These findings suggest that long-term conservation of crocodiles in Cuba will require identification of genetically pure and hybrid individuals, and a decrease in anthropogenic activities. We also recommend more extensive morphological and genetic analyses of Cuban population to establish clear boundaries of the hybrid zone between *C. acutus* and *C. rhombifer*. *J. Exp. Zool. 315:358–375, 2011.*

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Hybridization plays a dual role in evolutionary biology and conservation. Evolutionarily, hybridization and introgression may contribute to genetic variability and increase fitness in small populations or they may result in the melding of two previously distinct evolutionary lineages (Grant and Grant, '92; Clarke et al., '98; Zimmer, 2002; Coyne and Orr, 2004a). On the other hand, hybridization can result in decreased fitness of hybrids contributing to maintain distinctive genetic lineages. Some lineages may tolerate a substantial amount of hybridization without losing their morphological or genetic distinctiveness, whereas in other cases morphological intermediates are formed (Mavárez et al., 2006). In the case of unidirectional hybridization or introgression, one lineage may cause the extinction of the other (Lynch and Walsh, '98; Coyne and Orr, 2004a).

Hybridization and introgression represent important issues in the conservation of biodiversity, in particular when discussing conservation of large and charismatic fauna, such as crocodylians (Ross, '98; Dyke et al., 2008). Among crocodylian species, hybrids may be detected occasionally based on morphological characters, but such data may be unreliable given that morphological variation may also result from phenotypic plasticity. Moreover, morphological characters typically do not allow one to determine whether an individual is a first generation hybrid, a backcross, or a later generation hybrid. Distinguishing among these categories is critical in conservation biology because each of these dictates the particular conservation strategy that should be implemented to protect or recover the pure parental populations (Campton, '87; Smith, '92; Leary et al., '96).

Molecular genetic markers can facilitate the identification of parental vs. hybrid individuals in wild and captive populations, as well as characterize population structure, allowing wildlife managers to assign unknown individuals to their geographical source population. Characterizing intraspecific genetic variation also helps captive breeding programs avoid out-crossing of divergent lineages (Densmore and Ray, 2001; MacGregor, 2002) and improve the efficiency of reintroduction programs (Densmore and Ray, 2001; Venegas-Anaya, 2001; Venegas-Anaya et al., 2008). Effective and long-term conservation of crocodylians will, therefore, benefit significantly from the identification of genetically pure and hybrid populations, and from the identification of any potentially unique intraspecific evolutionary lineages.

Although the systematics of New World *Crocodylus* remains unclear, the most accepted taxonomy divides the genus into four species: *Crocodylus acutus*, Cuvier (1807); *Crocodylus moreletii*, Duméril and Duméril (1851); *Crocodylus rhombifer*, Cuvier (1807); and *Crocodylus intermedius*, Graves (1819) (Densmore, '83; Medem, '83; Brochu, 2000). All four species are Neotropical lowland inhabitants, reaching a maximum elevation of 400 m; the latter two species are Cuban and South American endemics, respectively. Although the Cuban crocodile (*C. rhombifer*) is currently restricted to Cuba, fossil records from the Grand Cayman Islands and the Bahamas indicate that this species was more widespread earlier in the Quaternary (Varona, '66, '86; Morgan et al., '93; Franz et al., '95; Steadman et al., 2007). Morelet's crocodile (*C. moreletii*) is restricted to the Yucatan Peninsula, including parts of Mexico, Belize, and Guatemala. The American crocodile (*C. acutus*) is an abundant and widespread crocodylian found throughout much of the coastal regions of the Neotropics, from Mexico to northern Peru on the Pacific, and from the Gulf Coast of the United States to northwestern Venezuela on the Atlantic, including many islands of the Caribbean (Brazaitis, '73; Thorbjarnarson, '89, Platt and Thorbjarnarson, 2000; Cedeño-Vázquez et al., 2006).

In Cuba, *C. rhombifer* and *C. acutus* were subjected to extensive hunting pressures from the middle of the 19th century through to the 1960s, resulting in drastic population declines of both species (Rodríguez Soberón et al., 2000). Although the *C. acutus* population has recovered and is now found in most of the costal areas of Cuba, for *C. rhombifer* the last demographic estimate of population size was approximately 3,000 individuals, including 1,000 females (Ramos-Targarona et al., '94; Ramos-Targarona, 2000; Rodríguez-Soberón et al., 2000), all restricted to one freshwater location: the Zapata Swamp along the southern coast of western Cuba (Fig. 1).

Crocodylus rhombifer is primarily a freshwater species, although there are historic reports from brackish water areas along the Bay of Pigs (Gundlach, 1880). They prefer wetland habitats located on floodplains and depressions with some tidal influence, where the maximum height above sea level is only about 10 m. This species is a terrestrial predator in the low fresh or brackish water and peat soil of the Zapata Swamp. The forest in these marshy areas has created a peat swamp where low levels of oxygen decomposition in soil and water is characteristic. High levels of dissolved organic material and different grades of salinity are ecological factors that contribute to the spatial distribution of vegetation in the Zapata Swamp. Because of the challenging ecological conditions of the peat wetlands, where the

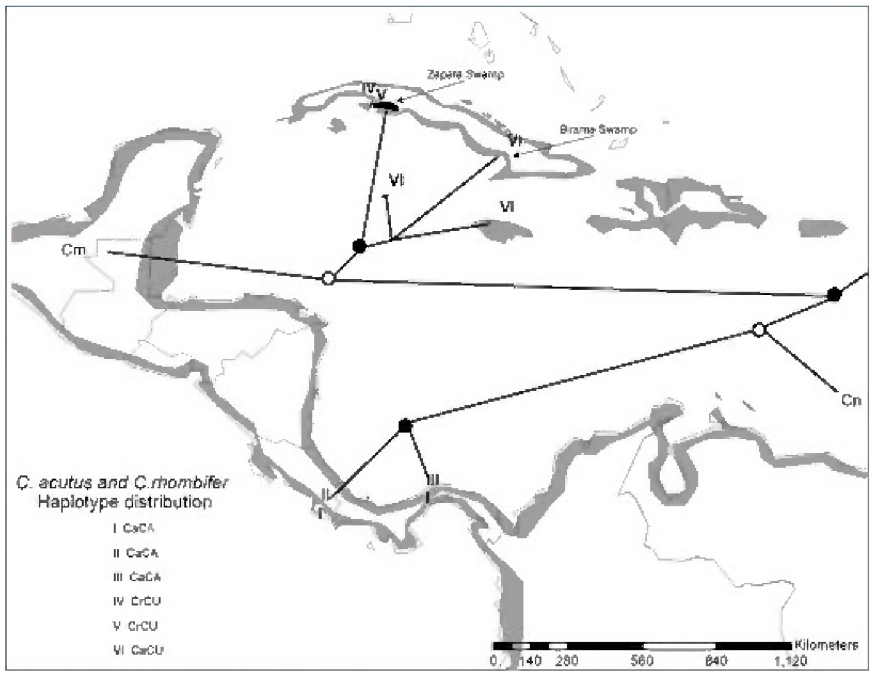

Figure 1. Distribution of *Crocodylus acutus* and *Crocodylus rhombifer* including sampling localities and mtDNA haplotypes in each locality. The *C. acutus* distribution is represented in light gray and the *C. rhombifer* distribution is represented in black. Haplotypes are connected by a cladogram based on the maximum likelihood topology. Branch lengths do not represent genetic distance; see Figure 5. Black dots represent nodes with high support and white dots represent nodes with low support. The arrows indicate the Zapata Swamp and the Birama Swamp locations. Abbreviations used in the figure indicate: Cn, *Crocodylus niloticus*; CaCU, Cuban *Crocodylus acutus*; CrCU, *Crocodylus rhombifer*; Cm, *Crocodylus moreletii*; CaCA, *Crocodylus acutus* of Central America.

Cuban crocodile nests and hunts its typical prey, the abundant Hutia of Zapata (*Capromys pilorides*). This habitat is different from the rest of Cuba, with extreme air temperature and higher annual precipitation (Varona, '66; Thorbjarnarson, '92: Ramos-Targarona, Tabares, and Rodríguez-Soberón, personal communication). The Cuban crocodile is considered the most morphologically, ecologically, and behaviorally distinct taxon among all *Crocodylus* species. Thorbjarnarson et al. (2008) considered these differences to be a result of adaptive evolution in Cuba and adjacent Caribbean islands, where ancestral *C. rhombifer* became a terrestrial or semi-terrestrial predator in the Pleistocene.

Crocodylus acutus is mainly a coastal/brackish water species, particularly where its distribution overlaps that of other large freshwater crocodylians (*Alligator mississippiensis* or *C. moreletii*). When not in sympatry with other crocodylians, *C. acutus* is known to inhabit freshwater inland wetlands. In the area of the Zapata Swamp, *C. acutus* and *C. rhombifer* are believed to be separated primarily by habitat, with *C. rhombifer* in the interior, shallow freshwater wetlands, particularly in areas where there are low bushy growths of red mangrove (*Rhizophora mangle*). *Crocodylus acutus* is found primarily in coastal sites or offshore cays (Thorbjarnarson, '89; Mazzotti et al., 2007a,b; Cedeño-Vázquez et al., 2008).

Hybridization between *C. rhombifer* and *C. acutus* has been shown to occur in captive populations, as confirmed by recent genetic analyses (Weaver et al., 2008). In Cuba, these two species are known to occur sympatrically in some areas of the Zapata Swamp, and natural hybridization zone is suspected based on the morphotypes found, but hybridization has not been demonstrated with DNA evidence (Varona, '66; Ramos-Targarona et al., '94; Rodríguez-Soberón, '97). Even though *C. rhombifer* is among the most morphologically distinct members of the genus *Crocodylus*, testing the hypothesis of hybridization using morphological data has been difficult, because in Cuba suspected hybrid individuals may express a mosaic of intermediate characters of parental phenotypes (Ramos-Targarona, 2006). Hybridization may be one of the most important threats to *C. rhombifer*, along with illegal hunting and habitat modification. As a result, genetic characterization of wild-caught individuals of both species and suspected hybrids is urgently needed.

Microsatellite markers developed for the genus *Crocodylus* (FitzSimmons et al., 2001) and mitochondrial DNA (mtDNA) sequence data have proven useful in the study of crocodile population genetics and have been used successfully to identify hybrids in other systems (Dever and Densmore, 2001; Fitzsimmons

et al., 2002; Hekkala, 2004; Ray et al., 2004; Russello et al., 2006; Rodríguez et al., 2007, 2008; Weaver et al., 2008). In this study, we examine two localities containing crocodiles in the Cuban archipelago. The Zapata Swamp locality includes two sites containing *C. rhombifer* and suspected hybrids. There are reports of *C. acutus* in the area, but none were found in the Zapata Swamp during this study. The Birama Swamp population is comprised of only *C. acutus* individuals. The goals of this study were to genetically characterize the population of *C. rhombifer* and suspected hybrids from the Zapata Swamp population, characterize *C. acutus* from the Birama Swamp population, and to assess the utility of nuclear and mitochondrial markers in genetically identifying morphological entities. Additionally, we inferred the evolutionary history of *C. rhombifer*, *C. acutus* and suspected hybrids in Cuba.

METHODS

Field Methods

Eighty-nine samples were collected from Cuba, Central America (Costa Rica and Panama), Grand Cayman Island, Jamaica, and from North American zoos (Appendix 1). Each sample consisted of a piece of a scale clipped from an animal's tail. Samples from the wild were taken from adults, subadults, and neonates from different nests to avoid underestimation of genetic variability. Individuals were classified as *C. rhombifer*, *C. acutus*, or as suspected hybrids based on external characters, including: body size, head size, general coloration, interocular distance, relationship between interocular distance and the distance from the infraorbital bridge to the snout, and shape and size of squamosal crest (Cuvier, 1807; Varona, '66, '86; Alvarez del Toro, '74; Ross, '98; Ernst et al., '99). Crocodiles were sampled from three locations in the Cuban Archipelago: two sites within the Zapata Swamp (Maneadero and La Zanja del Brigadista) and one site in the Birama Swamp (Jobabo). Nine samples of *C. rhombifer* were collected from Zanja del Brigadista during January 2001. Individuals sampled in Maneadero and Zanja del Brigadista were obtained in the years 2002 and 2007, and were classified as *C. rhombifer* ($n = 17$) or suspected hybrids ($n = 3$) based on morphology. All individuals from the Birama Swamp population ($n = 46$ collected in 2002 and 2007) were classified as *C. acutus* based on morphology. In addition, we collected *C. acutus* from Central America ($n = 18$), Grand Cayman Island ($n = 1$), Jamaica ($n = 2$), and two North American zoos ($n = 2$; CrDL179 and CrDL182). Zoo samples were previously analyzed by Weaver et al. (2008). We also included and reanalyzed the mtDNA data of Weaver et al. (2008), consisting of 43 sequences of cytochrome *b* (Cyt *b*) for *C. acutus*, *C. rhombifer*, and interspecific hybrids obtained from zoo collections (GenBank accession numbers EU034541 to EU034580). We include in the analyses *C. moreletii* from Guatemala ($n = 1$), *Crocodylus johnstoni* ($n = 1$), *Crocodylus porosus* ($n = 2$, one sample from zoo collection and one sequence from GenBank accession numbers NCC008143), *Crocodylus palustris* ($n = 1$), *Crocodylus niloticus* ($n = 5$, four samples from zoo collection and one sequence from GenBank accession number AJ810452). One individual of *Osteolaemus tetraspis* was used as outgroup.

Laboratory Methods

DNA Isolation. Total DNA was isolated from ethanol-preserved tail scale tissue for 89 individuals by standard proteinase K, phenol–chloroform extraction (Sambrook et al., '89). Purified DNA was dialyzed to eliminate excess salt using a silicon membrane (Spectrum, molecular porous membrane tubing MWCO: 12,000–14,000) in TE buffer (Tris-HCl pH 8.4 10 mM, EDTA 1 mM); the buffer was replaced three times after 1, 12, and 24 hr. The quality of DNA extraction was screened using 0.8% agarose gels stained with ethidium bromide.

Microsatellite DNA Amplification. We screened allelic diversity for 89 samples at seven polymorphic microsatellite loci, using the following published primers: Cj18F, Cj18R, Cj35F, Cj35R, Cj109F, Cj109R, Cj119F, Cj119R, Cj128F, Cj128R, Cj127F, Cj127R, Cj131F, and Cj131R (Dever and Densmore, 2001; FitzSimmons et al., 2001). Each locus was amplified by Polymerase Chain Reaction (PCR) in 7 μL reactions, containing 1 × PCR buffer (Qiagen, Valencia, CA), 0.2 μM dNTPs, 0.4 μM M13-tailed forward primer, 0.05 μM reverse primer, 0.4 μM differentially labeled M13 primer 5′-CACGACGTTGTAAAACGAC-3′, 0.19 U/μL TaqDNA polymerase (Qiagen), 1 μM $MgCl_2$, and 0.7 ng/μL DNA. The forward and reverse set of primers used was Cj18, Cj35, Cj109, Cj119, Cj127, Cj128, and Cj131 (FitzSimmons et al., 2001). PCR conditions were: 94°C (5 min), 25 cycles of 94°C (45 sec), annealing temperature: 50°C for Cj128, 54°C for Cj35, Cj109, Cj119, Cj128, Cj131, and 60°C for Cj127 (45 sec), 72°C (1 min), followed by 72°C (10 min). All PCR products were evaluated using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The allele sizes were measured using GeneMapper v4.0 software (Applied Biosystems).

Mitochondrial and Nuclear DNA Sequencing. From 38 samples, the complete Cyt *b* gene was amplified by PCR in two overlapping pieces, using two primer pairs: L14212 (5′-TTG GGC TTT AGA CCA AGA CC-3′), developed by Weaver and Venegas-Anaya (data not published), with CB3H (5′-GGC AAA TAG GAA RTA TCA -3′) (Palumbi, '96), and L14849 (5′- TCC TCC ACG AAC GCG GAR C-3′) with H15453 (5′-CCK TCC AYY TCT GTC TTA CAA G-3′) (Venegas-Anaya, 2001). We also amplified a 548 base pair (bp) fragment from the 3′ end of the cytochrome oxidase I (COI) gene using the primer pair COIa (5′-AGT ATA AGC GTC TGG GTA GTC -3′) with COIf (5′-CCT GCA GGA GGA GGA GAY CC-3′) (Kessing et al., '89). Finally, we amplified an 808 bp fragment of the nuclear gene, RAG-1, using the primers RAG1L-450 and RAG1R-1262 designed by Venegas-Anaya et al. (2007). The PCR conditions common to the three markers were as follows: 1 × PCR

buffer (Qiagen), 1 μM dNTPs, 1 μM $MgCl_2$, 0.1 × Q buffer, 0.5 μM each primer, Qiagen 0.03 U/μL Taq Polymerase, 1.12 ng/μL DNA sample, and sterile water added to volume up to 25 μL. All PCR amplifications were performed on Biometra thermocyclers. The PCR programs for Cyt *b*, COI, and RAG-1 varied as follows: Each started with a denaturing step for 2 min at 94°C (Cyt *b* and COI) or 3 min. (RAG-1). This was followed by 35 cycles (Cyt *b* and COI) or 30 cycles (RAG-1) of 94°C for 45 sec, 48 (Cyt *b* and COI) or 60°C (RAG-1) for 45 sec, and 72°C for 1.5 min (Cyt *b* and COI) or 1 min (RAG-1). A final extension step was performed at 72°C for 10 min. Big Dye terminators were used in the sequencing reaction (Applied Biosystems). Purified sequencing reaction products, obtained using Sephadex® G-50 (Sigma-Aldrich(R) Corporation, Nasdaq: SIAL) columns, were run on an Applied Biosystems 3130xl automated sequencer following manufacturer's protocols.

Analytical Methods

Microsatellite DNA Analysis. Genotypes were generated at seven microsatellite loci for the 89 individuals, including 66 Cuban crocodiles from the Birama Swamp (46 *C. acutus*) and the two Zapata Swamp sites (3 suspected hybrids and 17 *C. rhombifer*). We also genotyped one sample from Grand Cayman Island and two samples from Jamaica collected as *C. acutus*, 18 samples of *C. acutus* from Central America, plus 2 samples from North American zoos collected as *C. rhombifer*.

FSTAT statistical package version 2.93 (Goudet, 2001) was used to calculate the number and frequency of alleles at each microsatellite locus, the proportion of loci that were heterozygous (direct count heterozygosity, H_o), the average heterozygosity (H_s), and total heterozygosity (H_T) per locus. The F_{IS} (inbreeding coefficient within populations) values and significance levels (Bonferroni corrected given 21 tests to $\alpha = 0.00238$) were assessed through 21,000 randomizations of alleles. We also calculated pairwise F_{ST} between populations and tested the null hypothesis of $F_{ST} = 0$. Multilocus genotypes were randomized 20 times among pairs of samples and significance was assessed after the Bonferroni correction. An exact test for linkage disequilibrium among all pairs of loci, Nei's ('83) coefficient of intrapopulation gene variation (G_{ST}), and Nei's coefficient of interpopulation variation (D_{ST}) were also calculated using FSTAT. The Ewens–Watterson neutrality test (Ewens, '72; Watterson, '78) for the seven microsatellite loci was evaluated with the program ARLEQUIN (Excoffier and Schneider, 2005). We also conducted an AMOVA (Excoffier et al., '92) to measure genetic variation within and among the two *C. acutus* populations and *C. rhombifer*.

A model-based clustering method implemented in STRUCTURE 2.2 (Pritchard et al., 2000; Falush et al., 2007) was used to infer population structure and to identify distinct genetic populations, migrants, and admixed individuals. We estimated the optimal number of genetic clusters, *K*, based on the genotype data and assigned individuals probabilistically to particular clusters. Simulations were run using a significance level $\alpha = 0.01$. We also used the software, POPULATIONS v1.2.28 (Langella et al., 2001), to estimate Nei's genetic distance D_A, among individuals (Nei et al., '83), which were then used to compute a phenogram of genetic similarity among individuals by the neighbor-joining method (Saitou and Nei, '87).

Mitochondrial and Nuclear DNA Analysis. DNA sequences were edited, using Sequencher version 4.5 (Gene Codes, Ann Arbor, MI), and aligned using MEGA 4.0 (Tamura et al., 2007) with default parameter values. DNA sequences were aligned against the complete *C. niloticus* mitochondrial genome, translated into amino acids and inspected for premature stop codons or introns in order to detect pseudogenes in MacClade version 4.1 (Maddison and Maddison, 2005). All DNA sequences were deposited in GenBank (Table 1). We did not perform an Incongruence Length Difference test owing to doubts surrounding its utility (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002), and instead assessed potential data incongruence in phylogenetic reconstructions by a visual inspection of single-gene phylogenies.

After initial phenetic analyses using the neighbor-joining algorithm (BioNJ) (Saitou and Nei, '87; Gascuel, '97), a phylogeny was inferred using the maximum likelihood (ML) criterion (Felsenstein, '81), as implemented in PAUP* (Swofford, 2003) for the mtDNA data and nuclear data, separately and combined. We selected the best-fit models of DNA sequence evolution for the two sets of DNA data using the AIC as implemented in Modeltest version 3.7 (Posada and Crandall, '98). We used heuristic searches with TBR branch swapping in the ML analyses. We also conducted Bayesian MCMC phylogenetic inference (Rannala and Yang, '96; Yang and Rannala, '97) using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). For the Bayesian analysis, we ran parallel MCMCs with eight Metropolis-coupled chains each for 5,000,000 generations, sampling trees every 1,000 generations. Sampled trees from the run were used to construct a 50% majority rule consensus tree, in which marginal posterior probabilities of each clade were estimated from the clade's proportional representation among the post-burnin samples. We considered a posterior probability of 95% or greater as significant support for a given clade. For Bayesian analyses, we partitioned the data by codon and selected the best-fit models of DNA sequence evolution for each data partition using MrModeltest version 2.2 (Nylander et al., 2004). Clade support was also evaluated using nonparametric bootstrap analysis (Felsenstein, '85), with each pseudoreplicate data set analyzed by the BioNJ method. For all phylogenetic analyses, *O. tetraspis* was assigned as outgroup taxa following Brochu (2000) and Ray et al. (2004).

We conducted a second ML phylogenetic analysis using only Cyt *b* sequences in which we combined our data with all unique haplotypes, reported by Weaver et al. (2008) in their study of

Table 1. List of samples amplified for COI and Cyt *b* genes in this study, and the first two letters of the sample number refer to the genus and species.

			Weaver et al. (2008),			GenBank	
Sample number	Morphotype	Haplotype	Haplotype	Longitude	Latitude	COI	Cyt *b*
CaCA50	*C. acutus*	I	N/A	−83.584931	8.83106	HQ594989	HQ595027
CaCA51	*C. acutus*	I	N/A	−83.584931	8.83106	HQ594990	HQ595028
CaCA52	*C. acutus*	II	N/A	−83.498	8.765	HQ594991	HQ595029
CaCA53	*C. acutus*	II	N/A	−83.498	8.765	HQ594992	HQ595030
CaCA54	*C. acutus*	III	N/A	−79.95	9.28	HQ594993	HQ595031
CaCA55	*C. acutus*	I	N/A	−79.95	9.28	HQ594994	HQ595032
CaCA56	*C. acutus*	III	N/A	−79.95	9.28	HQ594995	HQ595033
CaJM47	*C. acutus*	VI	β	−77.522	18.4464	HQ595003	HQ595041
CaJM48	*C. acutus*	VI	β	−77.522	18.4464	HQ595004	HQ595042
CaKI407	*C. acutus*	VI	β	−81.273	19.337	HQ595005	HQ595043
CaCU17	*C. acutus*	VI	β	−77.266	20.684	HQ594996	HQ595034
CaCU18	*C. acutus*	VI	β	−77.266	20.684	HQ594997	HQ595035
CaCU19	*C. acutus*	VI	β	−77.266	20.684	HQ594998	HQ595036
CaCU21	*C. acutus*	VI	β	−77.266	20.684	HQ594999	HQ595037
CaCU22	*C. acutus*	VI	β	−77.266	20.684	HQ595000	HQ595038
CaCU23	*C. acutus*	VI	β	−77.266	20.684	HQ595001	HQ595039
CaCU37	*C. acutus*	VI	β	−77.266	20.684	HQ595002	HQ595040
CrambCU11	Hybrid	IV	α	−81.658	22.403	HQ595009	HQ595047
CrambCU12	Hybrid	IV	α	−81.658	22.403	HQ595010	HQ595048
CrDL179	*C. rhombifer*	IV	α			HQ595019	HQ595057
CrDL182	*C. rhombifer*	IV	α			HQ595020	HQ595058
CrCU10	*C. rhombifer*	IV	α	−81.658	22.403	HQ595011	HQ595049
CrCU15	*C. rhombifer*	IV	α	−81.658	22.403	HQ595012	HQ595050
CrCU19	*C. rhombifer*	IV	α	−81.656	22.271	HQ595013	HQ595051
CrCU21	*C. rhombifer*	IV	α	−81.656	22.271	HQ595014	HQ595052
CrCU22	*C. rhombifer*	IV	α	−81.656	22.271	HQ595015	HQ595053
CrCU25	*C. rhombifer*	V	ß	−81.656	22.271	HQ595016	HQ595054
CrCU26	*C. rhombifer*	IV	α	−81.656	22.271	HQ595017	HQ595055
CrCU7	*C. rhombifer*	IV	α	−81.658	22.403	HQ595018	HQ595056
CmGT134	*C. moreletii*	VIII				HQ595007	HQ595045
Cn118	*C. niloticus*					HQ595021	HQ595059
Cn121	*C. niloticus*					HQ595022	HQ595060
Cn127	*C. niloticus*					HQ595023	HQ595061
Cn129	*C. niloticus*					HQ595024	HQ595062
Cj3	*C. johnstoni*					HQ595006	HQ595044
Cpo1	*C. porosus*					HQ595008	HQ595008
Cpa	*C. palustris*					HQ595025	HQ595063
OStr	*O. tetraspis*					HQ595026	HQ595064

The last two letters indicate the geographic origin of the sample using ISO 3166 country codes, except for the ones from Central America in which the last two letters indicate Central American origin. Unique haplotypes are arbitrarily numbered. Specific designations are based on morphological observations taken in the field. Geographic coordinates are in decimal degrees.

captive *C. rhombifer*. We expected that the inclusion of the samples analyzed by Weaver et al. (2008) would provide us with a more complete picture of the genetic status of *C. rhombifer* in the wild, because the zoo animals studied in Weaver et al. (2008) were all wild-caught or the F1 progeny of individuals caught at least 50 years ago.

To estimate divergence times among taxa from the combined mtDNA dataset, we first tested whether the data conformed

to a clock-like model of evolution using a likelihood ratio test of the ML tree vs. a ML clock-enforced tree (Felsenstein, '81; Page and Holmes, '98). We then used published fossil data to calibrate the ages of the nodes on our ML tree. The age of the most recent common ancestor (MRCA) of *O. tetraspis* and *Crocodylus* species, which formed the root of our molecular phylogeny, was constrained assuming a minimum age of 19 and 12 million years based on the fossil record (Brochu, 2000; Ray et al., 2004).

RESULTS

Genetic Characterization of Cuban Populations

Microsatellite Variation. All microsatellite loci were polymorphic among the samples from Cuba and showed allelic variation among and within the two localities, Birama Swamp (*C. acutus*) and Zapata Swamp (*C. rhombifer* and suspected hybrids) (Fig. 2). Eighty-three alleles were found at the seven loci. Thirty nine alleles were found in the two Zapata Swamp sites and 44 alleles in the Birama Swamp. *Crocodylus acutus* and *C. rhombifer* shared 30 alleles among the seven loci, independent of sampling site. The average of total heterozygosity (H_t) and intrapopulation genetic diversity (H_s) were 0.788 and 0.618, respectively (Table 2). The coefficient of genetic differentiation among population (G_{ST}) varied from 0.024 to 0.378, with a mean of 0.216. The results indicated that 21.6% of total genetic diversity is among populations, with 78.4% representing intrapopulation genetic diversity (Table 2).

The test for linkage disequilibrium among loci was non-significant in all pairwise comparisons indicating independent segregation of alleles in each population ($P > 0.05$). The Ewens–Watterson neutrality test (Ewens, '72; Watterson, '78) failed to reject the neutral hypothesis of the distribution of allelic frequencies for the two populations under study, Birama Swamp (*C. acutus* from Cuba, $P = 0.904$) and the combined Zapata Swamp (*C. rhombifer* and suspected hybrids, $P = 0.906$). No significant deviation from Hardy–Weinberg equilibrium was detected for any locus in the Birama and Central American population. However, in the Zapata Swamp locality, the overall F_{IS} value was significant following sequential Bonferroni correction (Table 3).

Genetic Variability. Within Cuba, observed (H_o) and expected (H_e) heterozygosity were not significantly different in either species, nor did H_o differ significantly between species (Table 4). The average number of alleles per locus was 6.3 and 5.6 for *C. acutus* and *C. rhombifer*, respectively. When comparing Cuban *C. acutus* vs. *C. rhombifer*, the two groups were significantly differentiated at microsatellite loci ($F_{ST} = 0.337$, $P = 0.0233$). The F_{ST} values between Cuban *C. acutus* and Central American *C. acutus* and *C. rhombifer* and *C. acutus* of Central America were 0.26456 ($P = 0.0033$) and 0.28868 ($P = 0.0033$), respectively; *P*-values were obtained after 300 permutations; the corresponding adjusted nominal level (5%) for multiple comparisons was 0.016667.

Cluster Differentiation. Three clusters were identified by the individual-based Bayesian method implemented in STRUCTURE 2.2 (Fig. 3): Cuban *C. acutus* cluster, Central American *C. acutus* cluster, and *C. rhombifer* cluster. Forty-two of 46 Cuban *C. acutus* formed a genetic cluster with 0.9–1.0 certainty of inclusion. Four Cuban *C. acutus* showed posterior probability values ranging from 0.054 to 0.113 of assignment to the Central American *C. acutus* cluster, and may be potential hybrids. Additionally, the individual from Grand Cayman was also assigned as hybrid with 0.556 probability value of belonging to Cuban *C. acutus* cluster and 0.441 of belonging to *C. rhombifer*, suggesting recent migration. The genotype of one out of two individuals from Jamaica was assigned with a posterior probability > 0.90 to the Central American *C. acutus* cluster (Fig. 3). The two individuals from zoo collections were identified morphologically and genetically as *C. rhombifer* by Weaver et al. (2008). They were assigned in our analysis as Central American *C. acutus* and *C. rhombifer* admixed individuals with a posterior probability > 0.078 to belong to the Central American *C. acutus* cluster.

Eighteen alleles were diagnostic for Cuban *C. acutus*, 24 alleles for *C. rhombifer*, and 29 alleles for Central American *C. acutus* (Table 5; Fig. 2). Among the three individuals classified morphologically as suspected hybrids, one individual (Cramb8) showed combination of diagnostic alleles from parental species (*C. rhombifer* and Cuban *C. acutus*). Also, Cramb12 showed a combination of Central American diagnostic allele with one allele common to the entities under study (Fig. 2); this is considered genetic evidence of hybrid status with the two *C. acutus* group.

Similar to the STRUCTURE analysis, the neighbor-joining phenogram based on Nei's genetic distance D_A (Nei et al., '83) among 89 Caribbean and Central American individuals showed three main groups (Tables 3, 4; Fig. 4). The Central American group included all *C. acutus* from Central America plus one genotype from Jamaica, the CaJM48, and three individuals from the Cuban *C. acutus* (CaCU1, CaCU18, and CaCU10). The *C. rhombifer* group included all *C. rhombifer* and hybrids genotypes from Cuba, the second *C. rhombifer* genotype originally collected as a *C. acutus* morphotype in Jamaica (CaJM47), the Grand Caiman Island genotype (CaKI48), one Cuban *C. acutus* genotype (CaCU11), and the genotypes of the two zoo individuals (CrDL179 and CrDL182). Cuban *C. acutus* group included all Cuban *C. acutus* genotypes, all from the Cuban Birama Swamp population.

Phylogenetic Results

Cyt b, COI, and RAG-1 Sequence Data. RAG-1 showed no variation among all samples of *C. acutus* and *C. rhombifer*; therefore, we excluded these data from further analyses. As expected, we observed no phylogenetic conflict between our mitochondrial genes, and therefore combined COI and Cyt *b*

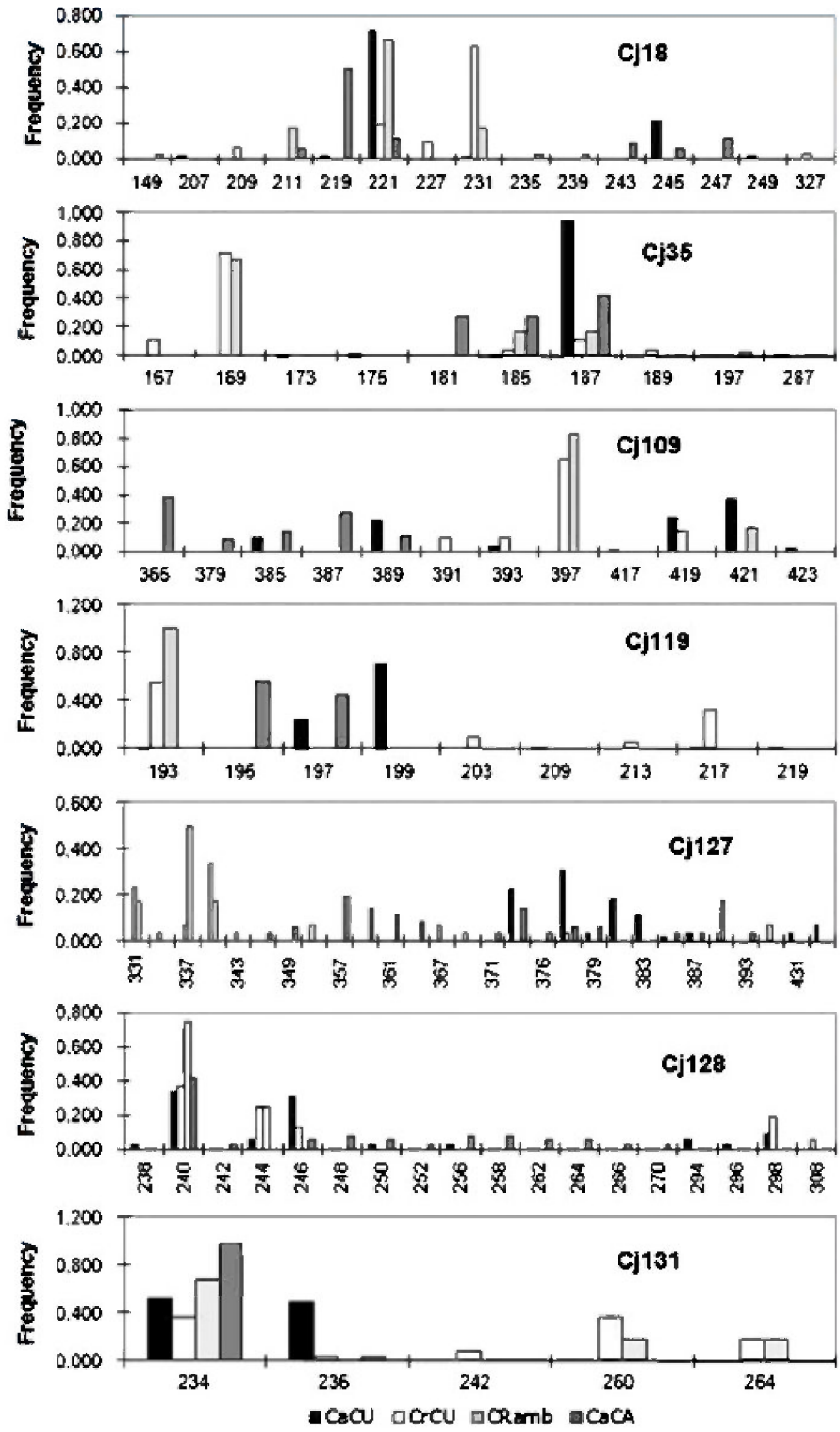

Figure 2. Allele frequency distributions for seven microsatellite loci and the four groups under study from the Cuban Archipelago and Central America: Cuban *C. acutus* (CaCU) in black, *C. rhombifer* (CrCU) in white, suspected hybrids in light gray, and *C. acutus* from Central America (CaCA) in dark gray.

sequences from each individual into a single haplotype for all further analyses. Among the 38 concatenated mitochondrial sequences generated in this study we found 14 haplotypes. Among the 29 *C. acutus* and *C. rhombifer* samples, we recovered six mitochondrial haplotypes (Table 1). Within Cuba, we found three mitochondrial haplotypes: two in *C. rhombifer* and suspected hybrids (haplotypes IV and V) and one in *C. acutus* (haplotype VI; Table 1; Figs. 1, 4). Samples from Jamaica and Grand Cayman Island corresponded to haplotype VI. American zoo samples corresponded to haplotype IV (CrDL179 and CrDL182). Among all 1,746 mitochondrial characters, 163 were uninformative and 296 characters were parsimony informative. Between haplotypes IV and V (CrCU25), we found five variable characters and all of them were uninformative.

The best-fit model of evolution selected by AIC in Modeltest 3.7 for the mitochondrial combined data set was GTR+G (a general time reversible 6-parameter model with rate variation among sites; Tavaré, '86). The combined mitochondrial data were partitioned by codon for ML and Bayesian analyses and the best-fit models selected by AIC in MrModeltest were GTR+G, HKY+I (Hasegawa et al., '85 with proportion of invariant sites; Hasegawa et al., '85), and GTR+I+G (a general time reversible 6 parameter model with proportion of invariant sites and rate variation among sites; Waddell and Steel, '97) for first, second, and third codon position, respectively. For the mitochondrial combined data set, the three phylogenetic inference criteria produced similar topologies. We decided to use as our point of reference the combined mitochondrial ML tree in the following results (Fig. 5).

All mtDNA sequences from New World *Crocodylus* were separated from *O. tetraspis* by an average model-corrected genetic distance of 0.56±0.100. In general, resolution within the group was poor and support for internal nodes was low. Neotropical *Crocodylus* forms a polyphyletic group that also includes *C. niloticus*. Within Neotropical *Crocodylus*, we observed two well-supported clades (Fig. 5) corresponding to a *C. acutus* Central American clade and the Caribbean clade. The Caribbean clade included Cuba, Jamaica, and Grand Caiman Island samples (posterior probability [pp] = 1, bootstrap = 99%). The Central American clade clustered all *C. acutus* from Central America and was represented by three haplotypes (pp = 1, bootstrap = 99%). The average mitochondrial model-corrected genetic distance

Table 2. Number of unique alleles obtained (N_A) per locus and Nei's estimators of heterozygosity for seven microsatellite loci from Cuban and Central American populations of crocodiles.

Locus	N_A	H_s	H_t	D_{ST}	G_{ST}
Cj18	15	0.589	0.804	0.216	0.268
Cj35	10	0.427	0.686	0.260	0.378
Cj109	12	0.690	0.879	0.190	0.216
Cj119	9	0.533	0.818	0.285	0.348
Cj127	28	0.862	0.940	0.078	0.083
Cj128	18	0.794	0.814	0.020	0.024
Cj131	5	0.432	0.575	0.143	0.249
Overall		0.618	0.788	0.170	0.216

H_t is total heterozygosity, H_s is intrapopulation genetic diversity, D_{ST} is Nei's coefficient of interpopulation variation, and G_{ST} is component H_t explained by D_{ST} (Nei's coefficient of intrapopulation gene variation).

Table 4. Observed heterozygosity (Ho), expected heterozygosity (He) with the corresponding *t* standard error (SE) and F_{IS} statistic (Wright, '65) for two Cuban and Central American populations of *Crocodylus*.

Population	Ho/He	SE	F_{IS}
Birama Swamp	0.601/0.555	0.331/0.253	−0.087
Zapata Swamp	0.486/0.658	0.236/0.132	0.268
Central America	0.579/0.620	0.113/0.105	0.094

Table 3. F_{IS} and *P*-values per locus within populations as measures of the deviation from Hardy–Weinberg equilibrium for seven microsatellite loci from two Cuban and Central American populations of crocodiles $\alpha = 0.00238$ and 21,000 randomizations.

	Birama Swamp		Zapata Swamp		*C. acutus*, Central America	
locus	F_{IS}	*P*-value	F_{IS}	*P*-value	F_{IS}	*P*-value
Cj18	0.119	0.1933	0.140	0.2857	0.167	1.000
Cj35	−0.026	1.000	0.564	0.0043	−0.214	0.4463
Cj109	−0.094	0.9071	−0.069	0.8098	−0.185	0.0000
Cj119	0.286	0.0331	0.716	0.0010	0.350	0.1473
Cj127	−0.108	0.9483	0.373	0.0007	0.400	0.9696
Cj128	−0.280	1.000	−0.114	0.8571	0.044	0.9531
Cj131	−0.275	0.9810	0.326	0.0395	0.000	0.1142
All	−0.087	0.9779	0.268	0.0002	0.094	0.041

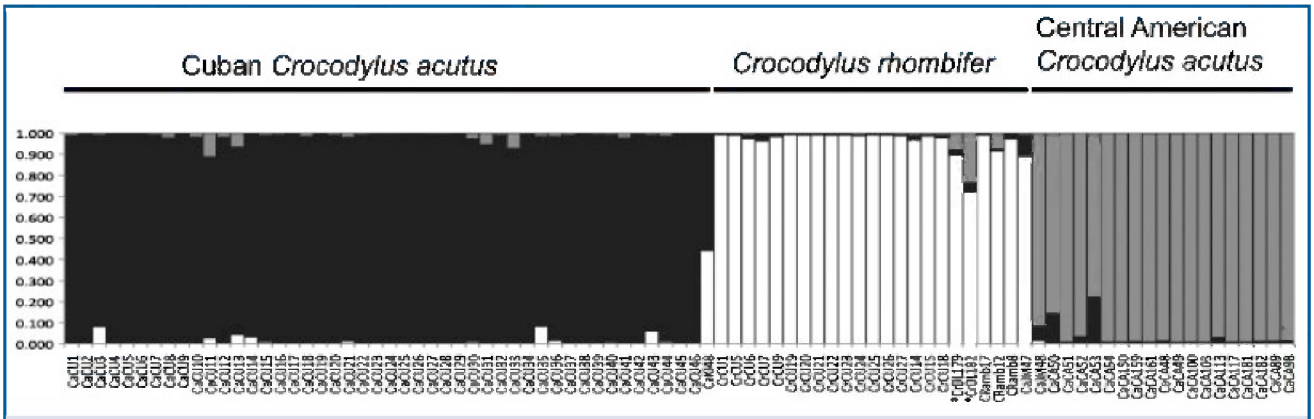

Figure 3. STRUCTURE 2.2 bar plot that represents the model-based clustering of individual genotypes. Individuals from Cuba, Grand Caiman Island, Jamaica, Panama, Costa Rica, and zoo collections are represented by the vertical bar, which represents the populations estimated membership. Cuban *C. acutus* (CaCU) in black, *C. rhombifer* (CrCU) in white, and Central America *C. acutus* (CaCA) in dark gray. Admix individuals are represented by colored broken bars. Samples from zoo collections are marked with an asterisk (*).

Table 5. Distribution of diagnostic alleles per locus in the three crocodile entities under study.

	Cj131	Cj128	Cj127	Cj119	Cj109	Cj35	Cj18
Diagnostic alleles							
C. acutus ($n = 46$)	–	238	381	199	417	173	207
		294	383	209	421	175	249
		296	431	219	423	287	
			437				
Suspected hybrids ($n = 3$)	234	240	331	193	397	169	211
	260	244	337		421	185	221
	264		339			187	231
			391				
C. rhombifer ($n = 17$)	242	308	331	203	391	167	209
	260		335	213	397	169	227
	264		337			189	327
			339				
			343				
			351				
			367				
			369				
			391				
			425				
C. acutus Central America ($n = 18$)	–	242	347	195	365	181	149
		248	349		379	197	211
		252	357		387		235
		258	359				239
		262	361				243
		264	365				247
		266	371				
		270	376				
			393				

Shaded areas indicate combination of diagnostic alleles in suspected hybrids from purebred populations.

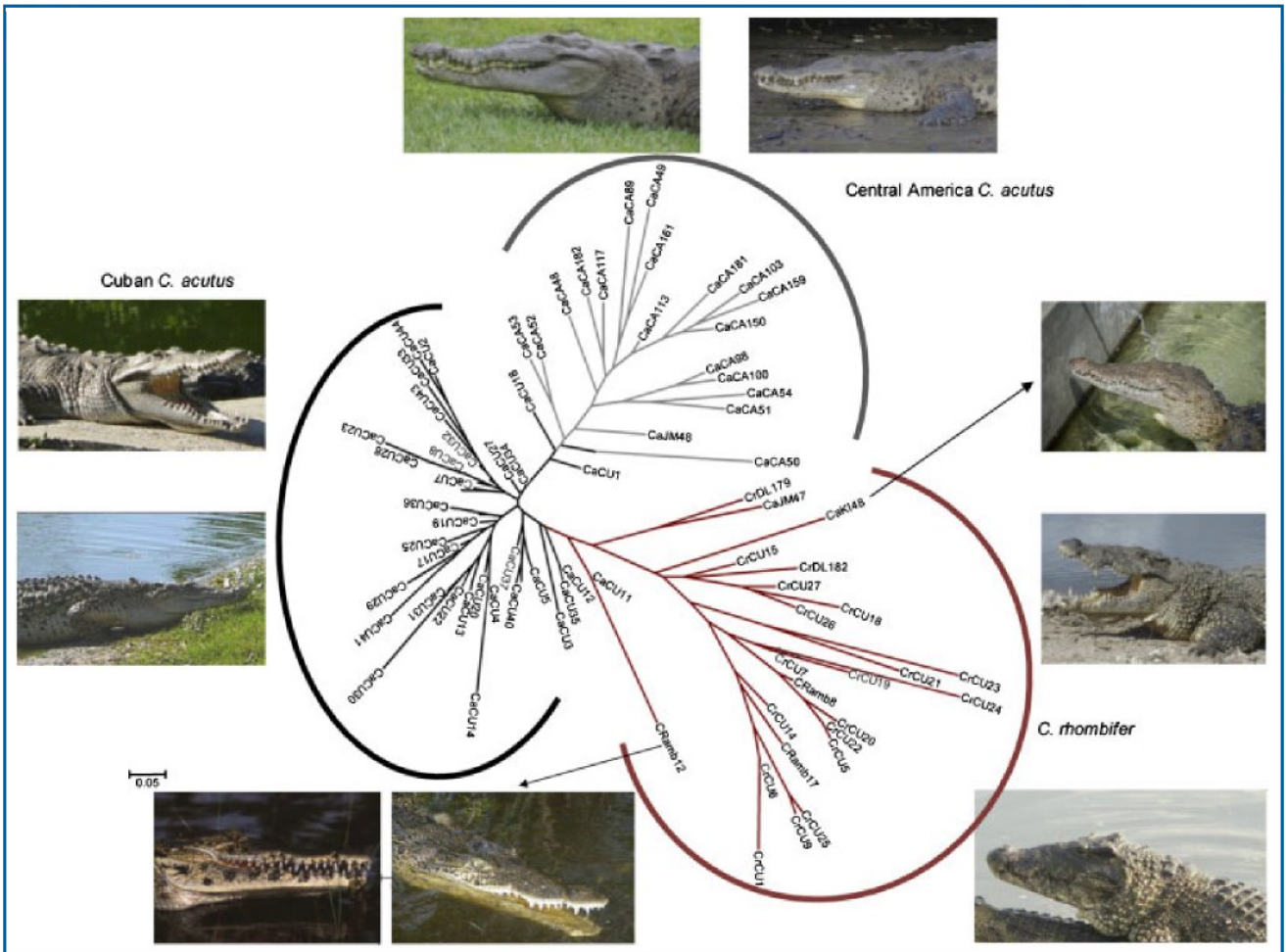

Figure 4. Neighbor-joining tree based on individual microsatellite (Nei,'83) genetic distances. The letters on branch tips represent individual's identification: Cuban *Crocodilus acutus* (CaCU#) in the black group, *C. rhombifer* and suspected hybrids (CrCU# and CrambCU#, respectively) in the dark burgundy group, and Mesoamerican and Caribbean *C. acutus* (CaCA#, CaJA#, and CaKI#) in the light gray group. Pictures next to each clade show representative morphotypes within each group and include morphotypes from either wild or captive individuals.

between these two clades was 0.080±0.0006. The Central American clade did not include the *C. acutus* individual from Grand Cayman Island or the two individuals from Jamaica as we expected. In contrast, microsatellite results grouped one of the Jamaican individuals (CaJM48) within the Central American *C. acutus* group (Figs. 4, 5). The Caribbean clade contained two subclades, the Cuban *C. acutus* clade and the *C. rhombifer* clade. The *C. rhombifer* clade included all individuals from the Zapata Swamp localities (*C. rhombifer* and hybrids) and samples from zoo collections (haplotypes IV and V) (pp = 1.00, bootstrap = 74%). When we excluded sample CrCu25 from the analysis (haplotype V), the stability of the clade increased (bootstrap 98%). Cuba *C. acutus* clade contained haplotype VI, representing all *C. acutus* samples from Cuba, Grand Cayman Island, and both Jamaican samples (putative *C. acutus*) (pp = 1.00, bootstrap = 99%).

Our mtDNA data revealed an important conflict between the current taxonomy and our molecular assessment of *Crocodylus*, in that *C. acutus* is distinctly paraphyletic with respect to *C. rhombifer* (Fig. 5). The mean distance between the two Caribbean clades was 0.01±0.0008 (pp = 1.00, bootstrap = 100%) (Table 6; Fig. 5).

When we included the published Cyt *b* sequence data from captive populations, we found that the entire haplotype network of Weaver et al. (2008) formed part of our Caribbean clade. The β haplotype (843 bp) of Weaver et al. (2008) was identical to our Cuban *C. acutus* haplotype VI, and their α haplotype was identical to our unique *C. rhombifer* haplotype IV. Weaver et al. (2008) did not report our haplotype V (Table 1; Fig. 5).

According to the likelihood ratio test of rate homogeneity, our mitochondrial data failed to reject a molecular clock model of evolution. Using the split *O. tetraspis* and all *Crocodylus* as

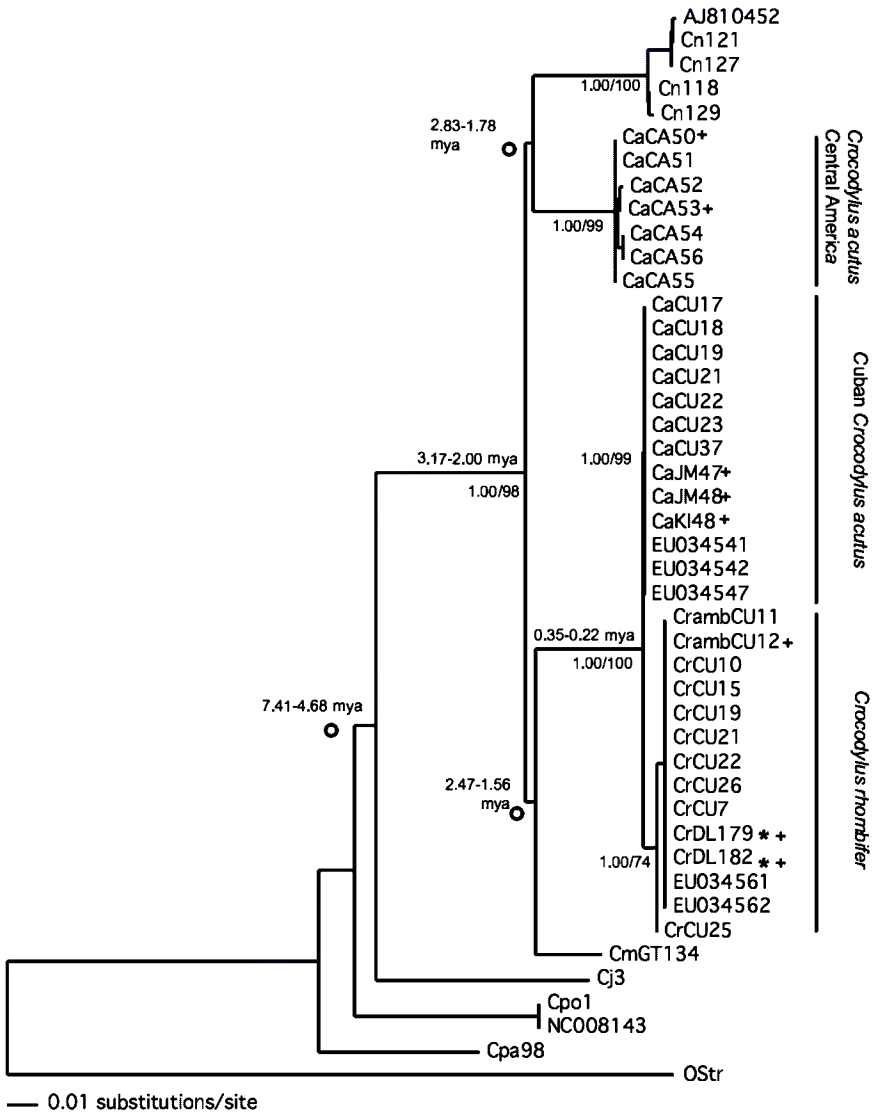

Figure 5. Maximum likelihood tree inferred for all mtDNA haplotypes obtained from Cuba, Jamaica, Grand Caiman Island, Panama, Costa Rica, Republic of Guinea, and zoo collections, including this study. Haplotypes consisted of two combined mtDNA sequences: 548 base pairs (bp) of COI and 1,200 bp of Cyt *b*. For each branch on the tree, statistical support is indicated by Bayesian marginal posterior probabilities (before the diagonal) and bootstrap values (after the diagonal). Three important branches with no support are indicated by white circles. Samples from zoo collection are marked by (*) and individuals assigned as a genetic hybrids by STRUCTURE are identify by (+). Estimated divergence times in millions of years ago (mya) are also indicated for major nodes. *Crocodylus rhombifer* clade would contain haplotype s from Weaver et al. (2008) (results not shown), here represented by three Cyt *b* sequences, GenBank accession numbers EU034541, EU034542, and EU034547. Cuban *Crocodylus acutus* clade would contain haplotype α from Weaver et al. (2008) (results not shown), here represented by two Cyt *b* sequences, GenBank accession numbers EU034561 and EU034562. Phylogeny was rooted with *Osteolaemus tetraspis* (19–12 mya).

calibration points, the MRCA of our Central American *C. acutus* and *C. niloticus* samples was dated between 2.83±0.35 and 1.78±0.22 million years ago (mya) and the MRCA of *C. moreletii* and the Caribbean clade samples was dated between 2.47±0.07 and 1.56±0.04 mya. We dated the MRCA of the Caribbean clade between 0.35±0.03 and 0.22±0.02 mya (Fig. 5).

DISCUSSION

Nuclear Genetic Variation Among Cuban Crocodiles

Examinations of diversity among Cuban populations of *Crocodylus* revealed two distinct groups (one assignable to *C. rhombifer* and the other to Cuban *C. acutus*), with evidence

Table 6. Model-corrected pairwise genetic distances among the three clades of New World *Crocodylus* inferred in this phylogenetic study, as well as time of divergence among Clades.

			Time of divergence	
	Divergent groups	Distance	*Osteolaemus tetraspis* and all other *Crocodylus* split at 19 mya	*Osteolaemus tetraspis* and all other *Crocodylus* split at 12 mya
OStr	CAmerica&Cn	0.56±0.1000	19.75±3.53	12.40±2.23
Cj	CAmerica&Cn	0.21±0.0300	7.41±1.06	4.68±0.67
CaCA&Cn	CaCU&CrCU&Cm	0.09±0.0100	3.17±0.35	2.00±0.22
CaCA	Cn	0.08±0.0100	2.83±0.35	1.78±0.22
CaCU&CrCU	Cm	0.07±0.0020	2.47±0.07	1.56±0.04
CaCA	CrCU	0.08±0.0005	2.82±0.02	1.78±0.01
CaCA	CaCU & CrCU	0.08±0.0006	2.82±0.02	1.78±0.01
CaCA	CaCU	0.08±0.0007	2.82±0.02	1.78±0.02
CaCU	CrCU	0.01±0.0008	0.35±0.03	0.22±0.02

Rates of divergence were calculated using fossil record: *Osteoloemus tetrospis* and all other *Crocodylus* minimum divergent time 19 mya and 12 mya (Brochu, 2000; Ray et al., 2004; Brochu, personal communication). The analysis also included the two unique Cyt *b* haplotypes published by Weaver et al. (2006). The genetic distances are based on combined COI and Cyt *b* sequences. OStr, *Osteoloemus tetrospis*; CAmerica, *Crocodylus* sp. from America included in this study; Cn, *Crocodylus niloticus*; Cj, *Crocodylus johnstoni*; CaCU, Cuban *Crocodylus ocutus*; CrCU, *Crocodylus rhombifer*; Cm, *Crocodylus moreletii*; CaCA, *Crocodylus ocutus* from Central America.

of hybridization in the Zapata Swamp where hybrid individuals express either intermediate parental phenotypes or a mosaic of parental characters.

Our microsatellite genetic analyses showed differentiation between the nuclear genomes, such that the two Cuban groups are distinctive yet more similar to each other than either is to Central American *C. acutus*. F_{ST} values among three populations were 0.337 ($P=0.0233$) between Cuban *C. acutus* and *C. rhombifer*, 0.26456 ($P=0.0033$) between Cuban *C. acutus* and Central American *C. acutus*, and 0.28868 ($P=0.0033$) between *C. rhombifer* and Central American *C. acutus*. These genetic results are surprising, given that pure *C. rhombifer* and Cuban *C. acutus* are morphologically distinct, whereas Cuban and Central American *C. acutus* are not. This pattern is consistent with the results of Weaver et al. (2008); using microsatellite data, they identified three very distinct groups among their *C. rhombifer* and *C. acutus* samples. For the Caribbean samples, they found two different genetic groups that they called α and β *C. rhombifer* independently of their morphotype. Their third group clustered together all *C. acutus* from the mainland. As did Weaver et al. (2008), we also found specific microsatellite alleles to characterize each cluster.

Population genetic analyses showed significantly positive F_{IS} values within *C. rhombifer*. Positive F_{IS} values within the Zapata Swamp have probability values lower than $\alpha=0.00238$, indicating a marginal statistically significant deficit of heterozygotes. This homozygosity could be caused by the presence of subdivision in the population into separate demes (Wahlund effect), given that the Zapata Swamp locality consisted of two sampling sites or by nonrandom mating (Allendorf and Luikart, 2007). Polygynic and territorial behavior, as well as highly restricted ecological requirements, could also contribute to inbreeding in the Zapata Swamp. *Crocodylus rhombifer* is both geographically and ecologically restricted, inhabiting). Only freshwater habitats (Ross, '98).

Identification of Hybridization Events Using Microsatellite Loci

Our microsatellite data indicated that one of the three suspected hybrids (CRamb12) based on morphology is the product of interspecific hybridization with a posterior probability of 0.075 to belong to the Central American *C. acutus* cluster and 0.915 to *C. rhombifer* (Fig. 3). On the other hand, the identification of diagnostic alleles in parental species and its combinations in the morphological hybrid CRamb8 revealed a molecular pattern of hybridization between *C. rhombifer* and Cuban *C. acutus*. This evidence has been corroborated in a parallel survey increasing both, sample size of hybrids and number of loci taking into account the limited evidence supported in only one morphological hybrid at one loci (data not shown). Given that hybrids share mitochondrial haplotypes with *C. rhombifer*, hybridization likely occurred in a female *C. rhombifer* breeding with male *C. acutus* (Epifanio and Philipp, 2001). Observations made on captive crocodiles suggest that hybridization occurs almost exclusively between female *C. rhombifer* and male *C. acutus* (Varona, '86; Rodríguez Soberón, 2000). The likelihood of unidirectional hybridization may be owing to two factors. First,

in the Zapata Swamp, there is a 2-month overlap in the courtship and mating period of Cuban *C. acutus* and *C. rhombifer* (Rodríguez Soberón, 2000). Second, in the contact zone between these two populations, there is a higher number of *C. acutus* individuals. As a result, at the peripatric zone, *C. acutus* males have a greater opportunity to breed with newly breeding *C. rhombifer* females than do *C. rhombifer* males. It is known that female crocodylians often have multiple mates, and perhaps the earlier mating with *C. acutus* allows sperm precedence.

Natural hybridization can be part of the evolutionary processes; however, the increase of anthropogenically mediated hybridization has been implicated as the cause of extinction of many taxa independent of their taxonomic status (species, subspecies, or locally adapted populations). Hybridization is also a serious conservation concern because it can go undetected, particularly if hybrids are difficult to differentiate morphologically, such as in the case of *C. rhombifer* and *C. acutus* (Rhymer and Simberloff, '96; Allendorf et al., 2001; Fitzsimmons et al., 2002; Allendorf and Luikart, 2007). Although the Central American and Cuban *C. acutus* are morphologically, behaviorally, and ecologically difficult to distinguish from each other, our mtDNA and microsatellite data established that these two groups are genetically quite diverged. Cuban *C. acutus*, Central American *C. acutus*, and *C. rhombifer* are clearly independently evolving lineages, despite some naturally or anthropogenic-mediated hybridization. Based on our evidence of hybridization between *C. rhombifer* and *C. acutus* in the wild and the numerical superiority and wider range of *C. acutus* relative to *C. rhombifer*, we strongly urge that efforts to avoid interspecific hybridization be taken into account in the conservation management plan for *C. rhombifer*.

Phylogeography

Combining our genetic data with observations on geological history and paleoclimatic conditions, we propose a phylogeographic scenario for the evolution of *Crocodylus* in Central America and the Caribbean that attempts to account for (1) the Pliocene divergence between Cuba and Central America, (2) the morphological similarity between *C. acutus* from Central America and Cuba, despite the fact that the latter population is much more closely related to *C. rhombifer*, (3) the divergence of *Crocodylus* lineages within Cuba, and finally, (4) instances of discordance between morphology and microsatellite genotypes.

We suggest that the ancestor of *C. rhombifer* arrived in Cuba during the late Pliocene, early Pleistocene (2.47–1.56 mya) through marine dispersal (e.g., Hass, '91; Hass and Hedges, '91; Hass et al., '93; Hedges, '96a,b), and subsequently acquired its distinctive morphology. The presence of shared haplotypes between mainland and Caribbean island populations of *Crocodylus* (Cedeño-Vázquez et al., 2008; Rodríguez et al., 2008; Weaver et al., 2008) suggests that migration of individuals between island and mainland may not be a rare event. The closest mainland localities to Cuba are the Yucatan Peninsula and Florida; although given the present and past ocean current patterns, South America is another possible point of origin (Guppy, '17; Lessios et al., '84; Lessios, '88; Schwartz and Henderson, '91; Henderson and Hedges, '95; Hedges, '96b; WWF, 2009).

The most parsimonious explanation for how Cuban *C. acutus* is much more closely related to *C. rhombifer*, yet morphologically resembles Central American *C. acutus*, could be through local adaptation of the *C. rhombifer* ancestor in Cuba. If the speciation event that gave rise to *C. acutus* and *C. rhombifer* (0.35–0.22 mya; Fig. 5) was linked to the colonization of a new niche by the ancestral *C. rhombifer*, one might expect extensive morphological and ecological divergence, whereas its sister lineage, the Cuban *C. acutus*, maintains the ancestral phenotype. When the rapidly evolving lineage is also a small geographic isolate, the scenario may be referred to as peripatric speciation (Coyne and Orr, 2004b).

Introgression of mtDNA from *C. rhombifer* into *C. acutus* could also explain the incongruence between mtDNA and morphology, as follows. Ancestral *C. rhombifer* arrived in Cuba roughly 2 mya and evolved its distinctive morphology. Before 0.35–0.22 mya, the ancestor of the Cuban *C. acutus* arrived and males crossed with female *C. rhombifer*, allowing introgression of mitochondrial haplotypes. Hybridization stops before 0.22 mya, and we are left with three distinct clusters of microsatellites, two phenotypes (*C. acutus* vs. *C. rhombifer*) and two main mtDNA lineages (mainland vs. Cuba). Hybridization with introgression has been reported in other species as a mechanism of speciation (Ferris et al., '83; Tegelstrom, '87; Hird and Sullivan, 2009; Larsen et al., 2010). Moreover, hybridization between *Crocodylus* species is a common event, both in the wild and in captivity (Fitzsimmons et al., 2002; Hekkala, 2004; Ray et al., 2004; Russello et al., 2006; Rodríguez et al., 2008; Weaver et al., 2008).

Taxonomic Implications

Regardless of the evolutionary origins of Cuban *Crocodylus*, clearly the molecular data is incongruent with the current taxonomic status of *C. acutus*, unless we accept paraphyletic species. In addition to the paraphyly of *C. acutus*, net genetic divergence of 8% or an estimated 2.82–1.78 mya is consistent with among-species comparisons (Ray et al., 2001, 2004). One of three possible taxonomic decisions would make *Crocodylus* species names correspond to the mtDNA clades revealed here. First, *C. rhombifer* (Cuvier, 1807) could be considered a junior synonym of *C. acutus* (Cuvier, 1807), perhaps relegated to subspecific status. Second, the Cuban population of *C. acutus* could be assigned to *C. rhombifer*, because our genetic data support a recent diversification of the Cuban clade (0.35 ± 0.003 mya) and because our data also showed evidence of recent hybridization. These two options would imply that the morphological differences between the two Cuban lineages are of little taxonomic importance. Third, the *C. acutus* population in Cuba could represent an undescribed species with minimal

morphological divergence, but substantial genetic divergence, from Central American populations. Unfortunately, we were unable to obtain molecular data from the type specimens of either species or the type locality of *C. acutus* (Haiti), so we cannot definitively establish conspecificity of specimens to the type *C. acutus* or *C. rhombifer* at this point.

Conclusions

Previous examinations have assumed that Cuban *C. acutus* and Central American *C. acutus* are conspecific based on morphological and behavioral characteristics. Our microsatellite nuclear data showed that these groups represent two very distinct populations. Moreover, the mtDNA data indicated that the Cuban *C. acutus* are more similar to *C. rhombifer* than to the Central American *C. acutus*. This could indicate an ancient introgression event between mainland *C. acutus* and *C. rhombifer*, or the rapid evolution of the *C. rhombifer* morphology and life history after roughly 3 million years of evolutionary quiescence in Cuban *C. acutus*. In either case, microsatellite and mtDNA data show that Cuba contains two distinct genetic lineages. Thus, two Evolutionarily Significant Units exist on the island of Cuba: *C. rhombifer* (Cuvier, 1807) and a second clade morphologically similar to *C. acutus* (Cuvier, 1807) that we call Cuban *C. acutus* for the time being. Furthermore, we found evidence for recent hybridization between *C. rhombifer* and Cuban *C. acutus* in the wild. No taxonomic changes are proposed here, because we suggest more genetic and morphological studies are necessary to more thoroughly understand the New World *Crocodylus* systematic relationships. We hope that our results will provide an initial scientific basis for further evaluations of the threatened populations of *C. rhombifer* and Cuban *C. acutus*, including additional studies of the combined genetic, morphological, and behavioral characters of these closely related endemic lineages.

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