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research paper

Mitogenomic investigation reveals a cryptic lineage of *Crocodylus* in Cuba

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ABSTRACT.—The American crocodile, *Crocodylus acutus* (Cuvier, 1807), is the most widely distributed crocodylian in the Americas, and coexists with the endemic and critically endangered Cuban crocodile, *Crocodylus rhombifer* Cuvier, 1807. Although these species are morphologically distinguishable, previous studies have shown that they are more genetically related to each other than either of them are to continental *C. acutus*. Here, we characterize the mitochondrial genome of Cuban *C. acutus* and analyze the resulting data relative to previously published whole mitochondrial genomes to reconstruct patterns of variation and phylogenetic placement within *Crocodylus*. We sequenced 13,776 basepairs, representing 82% of the entire genome including five (COI, COII, ATP8, ND3, ND4L) of the 13 protein-coding genes and 16 of the 22 tRNAs. Independent gene analysis of nucleotide diversity and genetic distance of Tamura-Nei demonstrated that the 16S rRNA, 12S rRNA, and COI genes are the most conserved in *Crocodylus*, while ND6 was the most variable (approximately 9%). Phylogenetic analysis confirmed that Cuban *C. acutus* forms a well-supported sister relationship with *C. rhombifer*, in contrast to continental *C. acutus* that clusters with *Crocodylus intermedius* Graves, 1819. The results are consistent with the hypothesis that Antillean *C. acutus* represents a cryptic lineage with genetic divergence at the species level. The ability to fully evaluate the taxonomic status of the Caribbean lineage of *C. acutus* still requires more comprehensive population samplings across the range as well as nuclear DNA sequence data. Of more immediate consequence, our results provide important information to be integrated into current *Crocodylus* conservation strategies in Cuba.

The genus *Crocodylus* is comprised of 12 species, four of which inhabit the Americas, including the widespread *Crocodylus acutus* (Cuvier, 1807) and three endemic species (*Crocodylus rhombifer* Cuvier, 1807—Cuba; *Crocodylus moreletii* Duméril and Bibron, 1851—Central America; *Crocodylus intermedius* Graves,

1819—Venezuela, Colombia). The Cuban crocodile (*C. rhombifer*) has the smallest geographical distribution in the Americas, currently restricted to approximately 300 km^2 in Zapata Swamp in the province of Matanzas (Tabet et al. 2014). In contrast, the American crocodile (*C. acutus*) has the widest distribution, found from the southern tip of Florida to the Atlantic and Pacific coasts, from Mexico to the north of South America, and from Caribbean islands such as Cuba, Jamaica, and Hispaniola (Tabet et al. 2014, Budd et al. 2015). The American crocodile is sympatric with the Cuban crocodile in Zapata Swamp, while it occurs in allopatry in the rest of the coastal areas of the island (Milián-García et al. 2015). In the Cuban archipelago, it can be found in 60 localities distributed in 11 provinces, including Isla de la Juventud as well as in adjacent northern and southern cays of the island (Tabet et al. 2014).

The populations of Cuban *Crocodylus* have declined as a result of intense hunting pressure since the middle of the 19th century. Currently, the main threats for the wild populations of Cuban *Crocodylus* are: illegal hunting, habitat loss/modifications, and anthropogenic hybridization (Tabet et al. 2014, Milián-García et al. 2015). The endemic *C. rhombifer* is considered the most endangered *Crocodylus* in the Americas, listed as "Critically Endangered" by the International Union for the Conservation of Nature (IUCN), while *C. acutus* is considered "Vulnerable" (IUCN 2016).

Although the two traditionally recognized species inhabiting Cuba are considered morphologically distinguishable, they are genetically more closely related to each other than they are to mainland populations of *C. acutus* (Milián-García et al. 2011). In addition, further in situ studies of *C. rhombifer* and *C. acutus* populations suggest that the American crocodile inhabiting Cuba is a cryptic lineage morphologically similar to *C. acutus* (Weaver et al. 2008, Milián-García et al. 2011, Rodriguez et al. 2011, Milián-García et al. 2015). Extensive hybridization between populations of Cuban *Crocodylus* has also been detected, and may be a key modulator of the degree of genetic differentiation that has resulted in the formation of three major lineages: *C. rhombifer*, Cuban *C. acutus,* and mainland *C. acutus* (Milián-García et al. 2015).

The recognition of cryptic species has significant implications for the taxonomy and conservation of crocodylians (Eaton et al. 2009, Hekkala et al. 2011, Shirley et al. 2014). This information is critical for developing comprehensive management plans that take into account all existing diversity and evolutionary potential to help ensure the persistence of these species. It has been suggested that the failure to recognize unique lineages and distinct populations results in inaccurate assessments of global biodiversity, in general (Cunningham et al. 2016), and crocodylians, specifically (Hekkala et al. 2015). Genetic data have provided enhanced resolution for the detection and characterization of cryptic species diversity in a range of taxa (Hebert et al. 2004, Russello et al. 2005, Hekkala et al. 2011), including the use of whole mitochondrial genome sequencing (i.e., mitogenomics) for "super-DNA-barcoding" and subsequent population genetic (Shamblin et al. 2015) and phylogenetic analysis (Gillett et al. 2014, Gómez-Rodríguez et al. 2015).

Here, we conducted a mitogenomic investigation of *Crocodylus* populations inhabiting Cuba to: (1) characterize, for the first time, the mitochondrial genome of Cuban *C. acutus*; (2) determine the phylogenetic position of Cuban *C. acutus* relative to whole mitochondrial genomic data previously collected for all recognized species of *Crocodylus* (Meredith et al. 2011); and (3) explore implications for taxonomy and *Crocodylus* conservation in Cuba.

Materials and Methods

Data Collection.—We sampled a fragment of a caudal scale from one *C. acutus* individual from the most abundant on-island population of the American crocodile located in the Monte Cabaniguán Wildlife Refuge, Birama Swamp, Las Tunas Province (20°40′28.1″N, 77°17′18.7″W). This population, located on the eastern side of Cuba, has not historically overlapped with *C. rhombifer*. Moreover, previous studies of crocodiles in Birama Swamp have morphologically characterized all sampled individuals (n = 69) as *C. acutus* and have reconstructed the same mitochondrial DNA haplotype across cytochrome oxidase I (COI), cytochrome b (cyt b), and the D-loop (Milián-García et al. 2011, 2015). Consequently, we are confident that individuals from this location are not admixed and felt justified in sequencing a single exemplar individual.

DNA was extracted using the NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) commercial kit following manufacturer's instructions. The mitochondrial genome sequences of *C. rhombifer* (GenBank accession numbers NC024513 and JF502247) served as a template for the design of specific oligonucleotides (27 pairs). The primers were designed using Primer 3 (http://primer3.ut.ee/). Each set of oligonucleotides generated a product of approximately 600 basepairs (bp) that overlaps by approximately 100 bp with adjacent fragments. PCR reactions were performed in a total volume of 15 µl containing: 1 µl of DNA (20-50 ng), 1× Taq DNA Polymerase VWR Master Mix (Tris-HCl pH 8.5, $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.2% Tween® 20, 0.4 mM of each dNTP, 0.2 µM of each oligonucleotide, and 0.2 units μl^{-1} of *Taq* Polymerase VWR) (VWR Chemicals, Belgium). Cycling conditions were as follows: 94 °C for 2 min followed by 35 cycles of 94 °C (45 s), annealing temperature (45 s), and 72 °C (1 min 30 s) followed by a final extension step at 72 °C for 10 min. Optimal annealing temperatures varied depending on each oligonucleotide pair, ranging from 40 to 52 °C. The oligonucleotides sequences and corresponding annealing temperatures are shown in Online Appendix 1.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following manufacturer's instructions. All PCR products were sequenced using a Sequencer Beckman Coulter 8800 and the GenomeLab™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, Inc., Brea, CA).

Data Analysis.—DNA sequences were edited and aligned using Sequencher v5.4.1 (Gene Codes, Ann Arbor, MI). Alignments were performed using the mitochondrial genome of *C. rhombifer* as a reference. A partial mitochondrial genome for Cuban *C. acutus* was obtained by joining the consensus sequence of each fragment as implemented in Geneious 8.1.2 (Biomatters, Ltd.). Preliminary annotation of each sequence was done using the mitochondrial genome annotation server MITOS (Bernt et al. 2013). Annotations were manually validated by comparison to the *C. rhombifer* reference using the ClustalW algorithm as implemented in MEGA version 7 (Kumar et al. 2016). Partial mitochondrial genome of Cuban *C. acutus* is available in Genbank accession number xxxxxxx.

The number of polymorphic sites and nucleotide diversity (π) (Nei 1987) were calculated using DNASP v5 (Librado and Rozas, 2009). In each case, a pairwise comparison of genomes for the sequence of Cuban *C. acutus* was also conducted with continental *C. acutus* sequences and other *Crocodylus* species available on

GenBank: *Crocodylus palustris* Lesson, 1831 (GU144286, Feng et al. 2010), *C. moreletii* (HQ585889, Meganathan et al. 2011), *Crocodylus mindorensis* Schmidt, 1935 (GU144287, Feng et al. 2010), *Crocodylus johnsoni* Krefft, 1873 (HM488008, Meganathan et al. 2011), *Crocodylus porosus* Schneider, 1801 (AJ810453, Janke et al. 2005), *Crocodylus novaeguineae* Schmidt, 1928 (HM636896, Man et al. 2011), *Crocodylus siamensis* Schneider, 1801 (EF581859, Srikulnath et al. 2012), *C. intermedius* (JF502242, Meredith et al. 2011), *C. rhombifer* (NC024513), *Crocodylus niloticus* Laurenti, 1768 (JF502246, Meredith et al. 2011), *Crocodylus suchus* Geoffroy, 1807 (JF502243, Meredith et al. 2011), *C. acutus* (JF502241, Meredith et al. 2011).

Similarly, the genetic distances between all pairs of genomes were calculated using the Tamura-Nei (Tamura and Nei 1993) model of nucleotide substitution in MEGA v7 (Kumar et al. 2016), as selected according to the Bayesian information criterion (BIC).

The sequence of each tRNA identified in the mitochondrial genome of Cuban *C. acutus* was transcribed using the Geneious 8.1.2 (Biomatters, Ltd.). The secondary structure and sequence of the anticodon were identified using tRNAscan-SE v1.21 (Lowe and Chan 2016) maintaining default parameters. The entire sequence obtained for the five genes encoding proteins (COI, COII, ATP8, ND3, and ND4L) was translated into the amino acid sequence using MEGA v7 (Kumar et al. 2016).

Maximum likelihood and Bayesian phylogenetic reconstruction were used to infer the phylogenetic placement of Cuban *C. acutus*. The analyses included 23 *Crocodylus* mitochondrial genomes (partial or complete): *C. acutus* (HM636894, JF502241), *C. intermedius* (HM636895, JF502242), *C. johnsoni* (HM488008); *C mindorensis* (GU144287), *C. moreletii* (HQ585889); *C. niloticus* (AJ810452, DQ273697, JF502245, JF502246), *C. suchus* (JF502243, JF502244); *C. novaeguineae* (HM636896, JF502240), *C. palustris* (GU144286, HM488007), *C. porosus* (AJ810453, DQ273698), *C. rhombifer* (JX292787, JF502247), *C. siamensis* (DQ353946, EF581859). In addition, *Mecistops cataphractus* (Cuvier, 1825) (EF551000) and *Osteolaemus tetraspis* Cope, 1861 (AM493868, EF551001) mitogenomic sequences were used as outgroups. Sequences were aligned using the MUSCLE algorithm and default parameters as implemented in Geneious 8.1.2 (Biomatters, Ltd.).

A maximum likelihood phylogenetic tree was constructed using PHYML (Guindon et al. 2010) and assuming the model of nucleotide substitution HKY85, selected according with the Akaike information criterion as implemented in MODELTEST (Posada and Crandall 1998). Nodal support was evaluated using bootstrap analysis and 1000 replicates. A Bayesian inference phylogenetic tree was also constructed using MrBayes v3.2.6 (Huelsenbeck and Ronquist 2001) and the HKY85 model of nucleotide substitution. Four simultaneous chains were run for a total of 1.0×10^7 generations, each one using a random tree as the starting point and sampling a tree every 1000 generations for a total of 10,000 trees explored. The first 5000 trees were discarded (burn-in) and the remaining were used to construct a majority-rule consensus tree and derive posterior probability values.

Results

Sequencing of the 23 overlapping fragments from the mitochondrial genome of Cuban *C. acutus* generated a total of 13,776 bp (Fig. 1). This represents 82% of the complete mitochondrial genome relative to the *C. rhombifer* reference genome

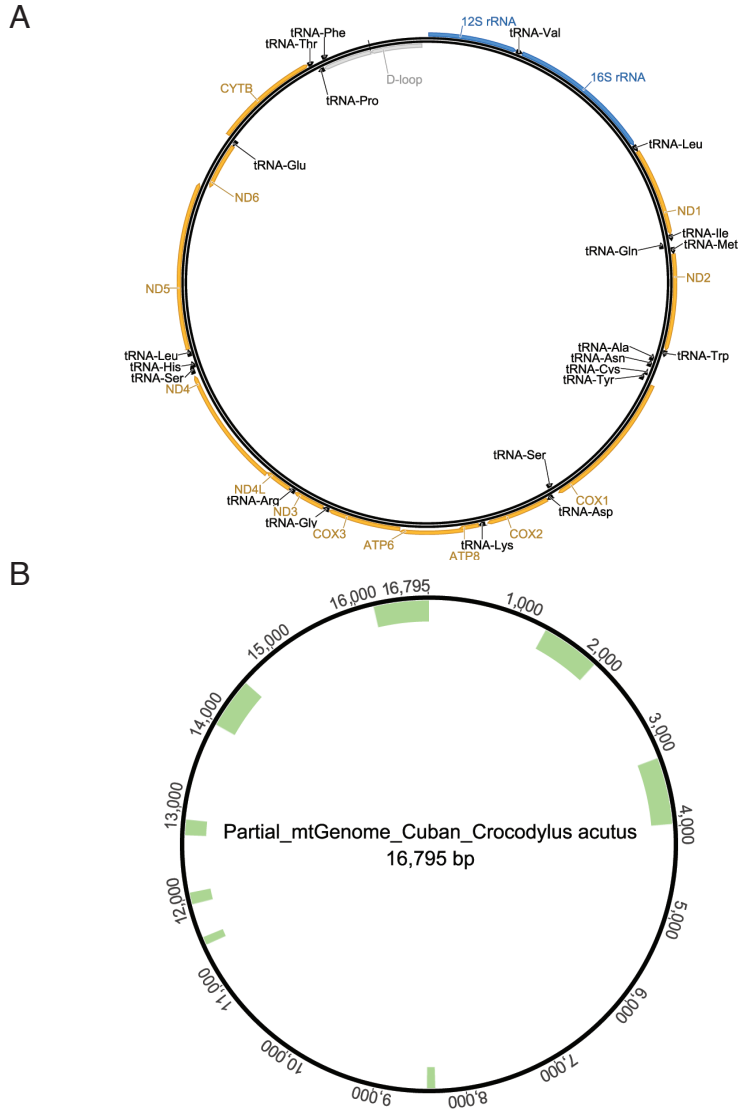

Figure 1. (A) Gene arrangement and organization of the mitochondrial genome in Crocodylia. (B) Representation of the partial mitochondrial genome obtained in the present study for Cuban *Crocodylus acutus*. Non-sequenced fragments are highlighted in green (online color figure).

(16,795 bp). Five (COI, COII, ATP8, ND3, ND4L) of the 13 protein-coding genes and 16 of the 22 tRNAs were sequenced in their entirety. The $tRNA^{Phe}$ / $tRNA^{SerII}$, ribosomal RNA 12S / 16S, and the D-loop were partially sequenced. No information was obtained for $tRNA^{Gln}$, $tRNA^{Glu}$, $tRNA^{Ile}$, and $tRNA^{Met}$. Most genes were encoded in the heavy chain, except for ND6, $tRNA^{Glu}$, $tRNA^{Ser}$, $tRNA^{Tyr}$, $tRNA^{Cys}$, $tRNA^{Ala}$, and $tRNA^{Gln}$ genes that were encoded in the light chain.

As expected, the mitochondrial genome of Cuban *C. acutus* shares the typical arrangement of genes observed in crocodylians (Fig. 1). This includes the characteristic variations in the arrangement of some genes encoding tRNAs compared to other vertebrates, while maintaining the same organization of protein-coding genes found

in mammals, amphibians, and fishes. The $tRNA^{Phe}$ gene is located on the 5' end of the control region, forming a group with $tRNA^{Pro}$ and $tRNA^{Thr}$ genes (TPF group) with the control region adjacent to the 12S rRNA gene. The $tRNA^{Ser}$ and $tRNA^{His}$ also are in a different order compared to other vertebrates, forming the cluster $tRNA^{Ser}$, $tRNA^{His}$, $tRNA^{Leu}$ (SHL) instead of the more common arrangement in vertebrates (HSL). In addition, it has a different arrangement for ND6/cyt b than found in the mitochondrial genome of birds, and has longer noncoding regions between the genes encoding proteins and tRNA, compared to that observed in mammals, amphibians, and fishes.

The longest noncoding regions located between genes encoding tRNAs and protein-coding genes were found between cyt b and $tRNA^{Thr}$ (46 bp), and ND4 and $tRNA^{SerII}$ (31 bp). Other similar spacer regions were detected between 1 and 21 bp. The nucleotide composition was asymmetric (31.0% A, 28.8% C, 15.0% G, 25.2% T) with an overall GC content of 43.8%.

Gene Variation at the Mitochondrial Level.—Genes encoding proteins and ribosomal RNA were sequenced at 87% of full length on average, ranging from 60% to 100% coverage (Table 1). Independent gene analysis of nucleotide diversity and genetic distance were performed for each gene of Cuban *C. acutus* relative to the corresponding sequences previously published for 12 species within the genus (Online Appendix 2). The most conserved genes were 16S rRNA, 12S rRNA, and COI, with nucleotide diversity values of 0.038, 0.04, and 0.058, respectively. The greatest variability was found in the genes encoding the subunits of NADH dehydrogenase I, with an average nucleotide diversity of 0.09 for ND5 and ND6. The control region, typically the most variable part of the vertebrate mitochondrial genome, had an average nucleotide diversity of 0.06 across *Crocodylus* (Table 1).

Protein-coding Genes.—For the genes COII, ND4L, and ATP8, all of the observed nucleotide changes were transitions, mainly in the third codon position. Changes were synonymous for the primary sequence of the proteins. The ND3 gene exhibited a transition in the second position of a codon that generated a conservative amino acid change; the amino acid serine was replaced by asparagine, both uncharged polar amino acids. For the COI gene, 11 transitions and two transversions were detected, four of which were non-synonymous. The amino acid change isoleucine to methionine was conservative since both are nonpolar, while the changes from alanine to threonine, phenylalanine to serine, and phenylalanine to leucine were non-conservative.

Phylogenetic Analysis.—Cuban populations of *C. rhombifer* and *C. acutus* (0.009) possessed the lowest levels of mitochondrial genomic divergence of any pairwise comparison between recognized *Crocodylus* species (Table 2). This is in contrast to the larger value of genetic differentiation detected between mainland populations of *C. acutus* relative to Cuban *C. acutus* (0.054) and Cuban *C. rhombifer* (0.054), respectively.

Maximum likelihood and Bayesian phylogenetic trees including Cuban *C. acutus* and whole mitochondrial genomes of 12 other *Crocodylus* species recovered the same tree topology, which was largely consistent with broad-scale patterns previously obtained by Meredith et al. (2011). Specifically, a monophyletic Indo-Pacific clade was reconstructed with high support, with African species clustering with the New

Table 1. Length of the ribosomal genes, protein-coding and control region of the mitochondrial genome expressed in basepairs (bp) and the percentage of each gene sequenced for *Crocodylus acutus* of Cuba. Genes were arranged in descending order of their average nucleotide diversity and genetic distance of Tamura-Nei, estimated for all members recognized to date within the genus *Crocodylus*.

Gene	bp	Percent sequenced	Average Π	Percent	Average genetic distance
ND6	530	71	0.092	9.20	0.106
ND5	1,861	84	0.091	9.09	0.101
ND1	963	62	0.084	8.40	0.093
ND4L	294	100	0.081	8.08	0.089
Cyt b	1,165	73	0.081	8.06	0.089
ND2	1,056	87	0.080	7.97	0.087
ATP6	697	93	0.078	7.82	0.087
ND4	1,374	97	0.073	7.33	0.082
COX3	786	98	0.072	7.21	0.078
ND3	348	100	0.068	6.85	0.074
ATP8	162	100	0.067	6.74	0.075
COX2	684	100	0.065	6.47	0.069
COX1	1,558	100	0.058	5.83	0.063
D-loop	1,073	49	0.060	6.00	0.062
12S_ARNr	985	92	0.040	4.00	0.042
16S_ARNr	1,593	60	0.038	3.80	0.040

World clade as previously described (Man et al. 2011, Oaks 2011). Within the New World clade, mainland *C. acutus* and *C. intermedius* were also found to be sister taxa (Meredith et al. 2011, Oaks, 2011). Importantly, Cuban *C. acutus* and *C. rhombifer* form a monophyletic group relative to all other species found in the Americas, with significant nodal support (posterior probability > 0.99) (Fig. 2). This result renders *C. acutus* paraphyletic, with the Cuban *C. acutus* more closely related to *C. rhombifer* than to mainland *C. acutus* populations.

Discussion

Description of the Mitochondrial Genome of Cuban *Crocodylus acutus*.—Mitochondrial genome sequences have been important for investigating certain conflicts in crocodylian systematics (Janke et al. 2005, Srikulnath et al. 2012), as well as to show patterns of significant intra- and interspecific diversification (Eaton et al. 2009, Shirley et al. 2014, Bloor et al. 2015). Here, we followed up on previous research that revealed unique lineages within Cuban *Crocodylus*, corresponding to the endemic Cuban crocodile (*C. rhombifer*) and a cryptic lineage morphologically similar to the American crocodile (*C. acutus*) (Weaver et al. 2008, Milián-García et

Table 2. Estimates of Tamura-Nei genetic distances (Tamura and Nei 1993) by pairs of representative mitochondrial genomes for Cuban *Crocodylus acutus*, mainland *Crocodylus acutus* and *Crocodylus rhombifer*.

Species 1	Species 2	Genetic distance
Cuba *C. acutus* (present study)	*C. rhombifer*	0.009
Cuba *C. acutus* (present study)	Mainland *C. acutus*	0.054
Mainland *C. acutus*	*C. rhombifer*	0.054

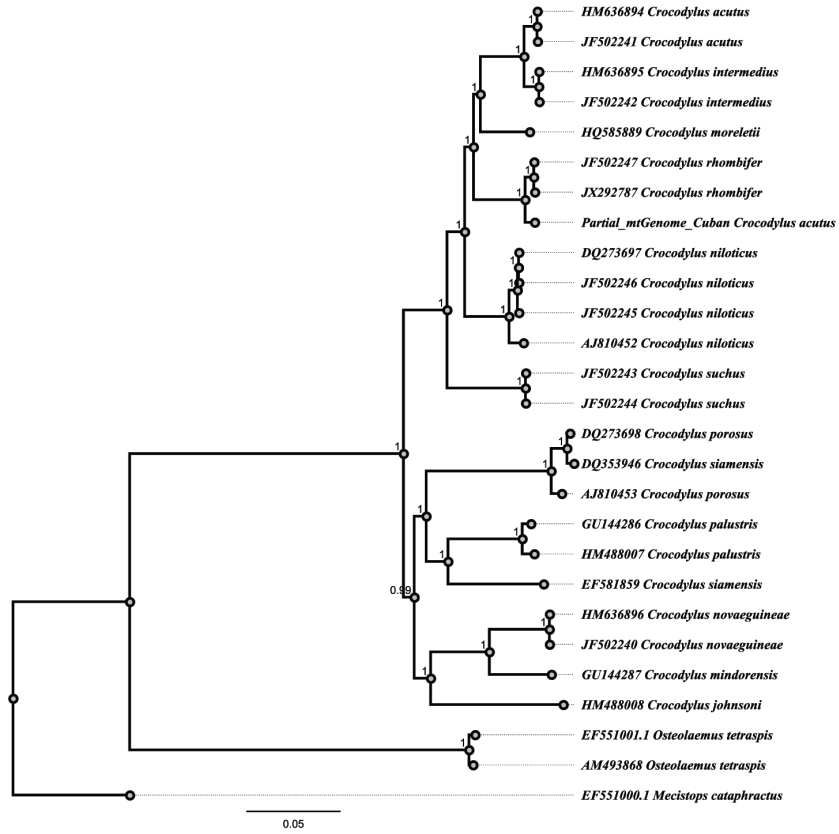

Figure 2. Bayesian phylogenetic tree based on the resulting partial or complete mitochondrial genome sequences for all known *Crocodylus* species. The mitogenomes employed have been identified by their accession number to the GenBank database, and the partial mitochondrial genome of Cuban *C. acutus* (*Crocodylus acutus*_Cuba) generated in the present study has been included. *Mecistops cataphractus* and *Osteolaemus tetraspis* were used as outgroups to root the tree. The numbers on the nodes indicate posterior probability values.

al. 2011, 2015). The sequencing of the mitochondrial genome for this cryptic lineage is of great importance for both resolving *Crocodylus* systematics analysis and for better understanding patterns of intraspecific genetic diversity throughout the range of this taxon previously described as *C. acutus*.

In crocodylians, tRNA genes and protein-encoding genes are separated by spacers that are longer than in other vertebrates (Janke et al. 2005). This organization is largely a result of the rearrangement of the tRNAs. An example is the non-coding region that is shared between *Alligator* and *Crocodylus* positioned at the 5' end of $tRNA^{Thr}$. This spacer is between 32 and 68 bp (Glenn et al. 2002), with Cuban *C. acutus* exhibiting one 46 bp in length that is likely the result of the movement of $tRNA^{Phe}$, as has been found in *Alligator mississippiensis* (Daudin, 1802) (Janke and Arnarson 1997). Furthermore, *Alligator* and *Crocodylus* have a 23–30 bp spacer between ND4 and $tRNA^{SerII}$ genes, while it is 264 bp in *Caiman* and 31 bp in Cuban *C. acutus*. This contrasts with birds and other reptiles that have no more than three nucleotides separating these coding regions (Kumazawa and Nishida 1995).

In terms of base composition, Cuban *C. acutus* exhibited a high abundance of the "A" nucleotide, similar to other species of the genus. In contrast, other members of Crocodylia, such as *Alligator sinensis* Fauvel, 1879 and *Paleosuchus palpebrosus* (Cuvier, 1807), have an abundance of the "C" nucleotide (Meganathan et al. 2010). Overall, the GC content was found to be lower than the AT content, which is a common feature of the mitochondrial genomes in crocodylians.

Gene Variation at the Mitochondrial Level in *Crocodylus*.— Mitochondrial ribosomal and protein-coding genes have been widely used as molecular markers in studies of population genetics and phylogenetics (Milián-García 2008, Milián-García et al. 2011, Oaks 2011, Patwardhan et al. 2014). Although these markers have resolved relationships at higher taxonomic level within the order Crocodylia, they are unable to provide the resolution necessary to discern recent relationships within *Crocodylus* (Oaks 2011, Srikulnath et al. 2015). These results demonstrate the need to better understand patterns of intraspecific differentiation within the members of the genus, before establishing interspecific relationships.

In the present study, we found that the most conserved regions within *Crocodylus* are the ribosomal genes (approximately 4%) and COI (5.83%), which is consistent with data from other members of this order. Similarly, the greatest variability was observed for ND5 and ND6 (approximately 9%), but not for ATPase genes. Comparing protein-coding genes and ribosomal RNA for seven species within the order, COI was the most conserved gene while ND6 was the most variable. The variability of the rRNA was low, but higher than COI (Li et al. 2007). Similarly, Srikulnath et al. (2012) reports that COI and cyt b genes are conserved while ND and ATPase genes are more variable.

When analyzing a short region of the COI gene in 23 species of five families of vertebrates (Crocodylidae, Alligatoridae, Bovidae, Suidae, Cercopithecidae), intraspecific polymorphism was generally low, with an average of 0.24%. By contrast, the average genetic divergence when comparing species of the same genus was 9.8%, with crocodylians the lowest at 6.5% (Eaton et al., 2010). This average value of nucleotide divergence for crocodylians is consistent with previous analyses of representatives from the family Crocodylidae and Alligatoridae (Eaton et al. 2010). Our analysis revealed a lower value (5.83%) for the genus *Crocodylus*.

The ND6-cyt b gene has been considered an effective marker for phylogenetic studies of the order Crocodylia, especially for comparing closely related taxa (McAliley et al. 2006). The results obtained in this study show that, for *Crocodylus*, ND5-ND6 are useful regions for examining variability within the genus. When comparing haplotype pairs, these genes showed the highest nucleotide diversity values, even at the intraspecific level (Online Appendix 2).

In addition to the coding sequences, the mitochondrial genome of vertebrates has the largest non-coding region in the form of the control region, which is generally the most variable part of the genome. Variation within the control region can take the form of nucleotide substitutions, as well as changes in length due to short insertions/deletions and in the number of tandemly repeated sequences (Ray and Densmore 2002). In the present study, analysis of the partial control region sequences revealed an average nucleotide diversity of 6%, which was lower than that of certain protein-coding genes such as ND5, ND6, ND4L, ND1, and cyt b. While the control region typically is the most variable part of mtDNA, the absence of domain III in our sequence may be influencing the lower value obtained with respect to the rest of mitochondrial fragments.

TRANSFER RNA.—Some vertebrate mitochondrial tRNAs exhibit unusual features. In most, the size of the arm D is variable and is even absent in $tRNA^{Ser}$ (AGY), which is common to all vertebrates. With this exception, the remaining mitochondrial tRNA structure may take the typical cloverleaf and stabilization requires less tertiary interactions compared to cytoplasmic tRNAs. The $tRNA^{Lys}$ of marsupials and placental mammals have a reduced D arm and $tRNA^{Ser}$ (UCN) consists of six nucleotides at the anticodon arm, instead of five. In contrast to birds, these two tRNAs have cloverleaf canonical structures in reptiles and amphibians (Pereira 2000). The only conserved features are the general structure of the anticodon region and the presence of the CCA end that is not encoded in mtDNA, but is post-transcriptionally added (Fernández-Silva et al. 2003).

For the genus *Crocodylus*, all tRNAs adopt the cloverleaf secondary structure except $tRNA^{Ser}$ (AGY) (Li et al. 2007, Ji et al. 2008, Meganathan et al. 2011). In the present study, all 16 completely sequenced tRNAs exhibited this structure and were similar to those previously reported (Online Appendix 3).

The high degree of conservation in the primary structure of tRNAs is indicative of the importance of folding for function. While there may be differences in the nucleotide sequence, they all can be virtually folded in the same conformation in inverted L-shaped, except for differences in the anticodon arm and aminoacyl end (Krebs et al. 2014). In our study, it was verified that nucleotide changes observed in the tRNAs with respect to the *C. rhombifer* reference were not in the anticodon loop and did not affect the expected folding. This is important since the structure of this molecule determines its function as an adapter during synthesis of proteins.

PROTEIN-CODING GENES.—The protein subunits encoded in the mitochondrial genome are essential for the proper assembly and activity of the complexes of the oxidative phosphorylation system (Fernández-Silva et al. 2003). This implies that changes in the gene sequence should not affect the structure and function of the protein subunit, since the main function of mitochondria is to produce the required cellular ATP via the oxidative phosphorylation system (Fernández-Silva et al. 2003).

In three mitochondrial protein-coding genes (COII, ATP8, and ND4L) analyzed, it was found that nucleotide changes do not generate variation in the primary structure of the protein, while in the ND3, there was a conservative nonsynonymous substitution that does not severely affect the structure and function of the protein. In COI, one conservative and three non-conservative changes were identified that could influence the structure and function of the protein. The effects of these changes in the protein subunit COI should be further evaluated by studying their secondary and tertiary folding. This is especially important, as a previous analysis of the 13 protein-coding genes in six species of *Crocodylus* suggested that the level of evolutionary pressure acting on these genes could be attributed to negative or purifying selection (Meganathan et al. 2011).

RELATIONSHIPS BETWEEN CUBAN *CROCODYLUS* SPECIES.—Crocodylians have been considered a relatively homogeneous, ancient, and widely distributed group. Yet many studies have shown patterns of considerable intraspecific variation (Hekkala et al. 2010, Meredith et al. 2011, Milián-García et al. 2015) and diversification at the species level (Eaton et al. 2009, Hekkala et al. 2011, Shirley et al. 2014).

The natural history for species that inhabit large geographic regions may be complex, but it may help elucidate current patterns of biodiversity. The American

crocodile is the most widely distributed species among New World crocodiles. Given the wide geographical distribution and vulnerable conservation status (IUCN 2016) of *C. acutus*, it has been challenging to reconstruct range-wide estimates of genetic diversity and population structure (Bloor et al. 2015). Preliminary phylogeographic analysis for *C. acutus* revealed a significant degree of variation in the genetic structure of its populations (Weaver et al. 2008, Rodriguez et al. 2011), but these studies were based on limited character sampling (cyt b and CR), and more importantly, did not do include vouchered specimens of Cuban *C. acutus*.

Previous studies focusing on the Cuban populations of *Crocodylus* revealed that the lineages of the island are unique. The American crocodile inhabiting Cuba exhibits morphological and ecological characteristics of *C. acutus*, but is genetically more similar to the endemic Cuban crocodile, *C. rhombifer,* at the level of mtDNA (Milián-García 2008, 2015, Milián-García et al. 2011, 2014, 2015). It has been hypothesized that this close relationship may be the result of a mitochondrial capture event associated with past hybridization, proposing that haplotype β unique to Cuban *C. acutus* may represent a glimpse of ancient haplotype diversity in *C. rhombifer* (Rodriguez et al., 2011). However, there is no morphological or genetic evidence of hybridization event, presently or ancestrally, in any population other than Zapata Swamp. Furthermore, the mitochondrial capture hypothesis fails to explain why just the β haplotype is widespread in the Caribbean without any evidence of other haplotypes in the region. In the present study, we sampled *C. acutus* from the largest Cuban population in Birama Swamp, where it is allopatric relative to *C. rhombifer* and where all individuals previously studied have been classified genetically and morphologically as *C. acutus*. Moreover, there is no evidence to suggest that *C. rhombifer* and *C. acutus* ever overlapped in this location in eastern Cuba (Milián-García et al. 2015), further suggesting that our results are not confounded by mitochondrial capture.

Rather, we argue that the mitogenomic results reported here further suggest the existence of a cryptic lineage of the American crocodile currently inhabiting Cuba and possibly other locations in the Caribbean region (Milián-García et al. 2011, 2014, 2015). Although estimates of molecular divergence between members of the genus *Crocodylus* vary between clades—in part based on which molecular data are used—it has been reported that formally-recognized *Crocodylus* species generally exhibit <1% of intraspecific divergence, and between 2.5% and 7.5% interspecific divergence (Hekkala et al. 2011, Srikulnath et al. 2012). Cuban *C. acutus* and *C. rhombifer* showed an extremely low value of genetic distance (0.9%), especially since they are currently considered distinct species. In contrast, Cuban *C. acutus* possessed a genetic distance of 5.4% relative to continental *C. acutus* populations, which is similar to the expected differences between different species within *Crocodylus* (Ray et al. 2004).

The phylogenetic analysis allowed us to further examine the position of Cuban *C. acutus* relative to a broad-sampling of *Crocodylus* species with published mitochondrial genomes. The phylogenetic tree revealed a well-supported, monophyletic *Crocodylus*, as has been reported previously (Meredith et al. 2011, Oaks 2011) (Fig. 2). At a finer-level, Cuban *C. acutus* forms a well-supported sister relationship with *C. rhombifer*, in contrast to continental *C. acutus* that clusters with *C. intermedius* (posterior probability = 1, Fig. 2). These results based on whole or partial mitochondrial genomes are consistent with previously published analyses of cyt b, COI genes, and a fragment of the control region (Milián-García et al. 2011, 2015), but with greater nodal support.

That *C. rhombifer* and Cuban *C. acutus* are closely related but distinguishable morphologically is certainly a challenge for the taxonomy of the Cuban *Crocodylus* species. This pattern of cryptic diversity has been observed for other species of the order Crocodylia, including *C. niloticus* (Hekkala et al. 2011, Cunningham et al. 2016). Similarly, African crocodylians belonging to the genus *Osteolaemus* and *Mecistops* were previously considered a single species, but now have been proposed to contain three species within *Osteolaemus* and two in *Mecistops* (Eaton et al. 2009, Shirley et al. 2014). Morphology has not adequately identified many cryptic crocodylian species (Shirley et al. 2015). Despite these results based on mitogenomics, the ability to fully evaluate the taxonomic status of the Caribbean lineage of *C. acutus* still requires a more comprehensive population sampling across the range, as well as nuclear DNA sequence data (Milián-García et al. 2015).

The recognition of cryptic species in well-established crocodylians is not only of taxonomic significance, but also has important implications for species conservation. This is particularly vital for rare, exploited, or endangered species, since management plans may not adequately protect existing diversity and evolutionary potential (Eaton et al. 2009, Shirley et al. 2014). Identifying patterns of intraspecific genetic diversity, as well as appropriate management units, will be key elements in the conservation and management of widely distributed species, such as *C. acutus*, moving forward (Cunningham et al. 2016). In Cuba, there are at least two distinct genetic lineages differentiated from Mainland *Crocodylus* can be recognized. None of the threats to their persistence have been resolved, ratifying the Critically Endangered status of the *C. rhombifer*, and punctuating the need for an immediate evaluation of currently described Cuban *C. acutus* as a distinct entity from the continental form.

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Literature Cited

Bernt M, Donath A, Jühling F, Externbrink F, Florentz C, Fritzsch G, Pütz J, Middendorf M, Stadler PF. 2013. MITOS: improved de novo metazoan mitochondrial genome annotation. Mol Phylogenet Evol. 69:313–319. https://doi.org/10.1016/j.ympev.2012.08.023

Bloor P, Ibánez C, Viloria-Lagares TA. 2015. Mitochondrial DNA analysis reveals hidden genetic diversity in captive populations of the threatened American crocodile (*Crocodylus acutus*) in Colombia. Ecol Evol. 5:130. https://doi.org/10.1002/ece3.1307

Budd KM, Spotila JR, Mauger LA. 2015. Preliminary mating analysis of American crocodiles, *Crocodylus acutus*, in Las Baulas, Santa Rosa, and Palo Verde National Parks, Guanacaste, Costa Rica. South Am J Herpetol. 10:4–9. https://doi.org/10.2994/SAJH-D-14-00022.1

Cunningham SW, Shirley MH, Hekkala ER. 2016. Fine scale patterns of genetic partitioning in the rediscovered African crocodile, *Crocodylus suchus* (Saint-Hilaire, 1807). PeerJ. 4:e1901. https://doi.org/10.7717/peerj.1901

Eaton MJ, Martin A, Thorbjarnarson J, Amato G. 2009. Species-level diversification of African dwarf crocodiles (genus *Osteolaemus*): a geographic and phylogenetic perspective. Mol Phylogenet Evol. 50:496–506. https://doi.org/10.1016/j.ympev.2008.11.009

Eaton MJ, Meyers GL, Kolokotronis S-O, Leslie MS, Martin AP, Amato G. 2010. Barcoding bushmeat: molecular identification of Central African and South American harvested vertebrates. Conserv Genet. 11:1389–1404. https://doi.org/10.1007/s10592-009-9967-0

Feng G, Wu X, Yan P, Li X. 2010. Two complete mitochondrial genomes of *Crocodylus* and implications for crocodilians phylogeny. Amphib-Reptil. 31:299–309. https://doi.org/10.1163/156853810791769464

Fernández-Silva P, Enriquez JA, Montoya J. 2003. Replication and transcription of mammalian mitochondrial DNA. Exp Physiol. 88:41–56. https://doi.org/10.1113/eph8802514

Gillett CP, Crampton-Platt A, Timmermans MJ, Jordal B, Emerson BC, Vogler AP. 2014. Bulk de novo mitogenome assembly from pooled total DNA elucidates the phylogeny of weevils (Coleoptera: Curculionoidea). Mol Biol Evol. 31(8):2223–2237. https://doi.org/10.1093/molbev/msu154

Glenn TC, Staton JL, Vu AT, Davis LM, Bremer JRA, Rhodes WE, Brisbin IL, Sawyer RH. 2002. Low mitochondrial DNA variation among American alligators and a novel non-coding region in crocodilians. J Exp Zool. 294:312–324. https://doi.org/10.1002/jez.10206

Gómez-Rodríguez C, Crampton-Platt A, Timmermans MJ, Baselga A, Vogler AP. 2015. Validating the power of mitochondrial metagenomics for community ecology and phylogenetics of complex assemblages. Methods Ecol Evol. 6:883–894. https://doi.org/10.1111/2041-210X.12376

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 59:307–321. https://doi.org/10.1093/sysbio/syq010

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci USA. 101:14,812–14,817. https://doi.org/10.1073/pnas.0406166101

Hekkala E, Shirley MH, Amato G, Austin JD, Charter S, Thorbjarnarson J, Vliet KA, Houck ML, Desalle R, Blum MJ. 2011. An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile. Mol Ecol. 20:4199–4215. https://doi.org/10.1111/j.1365-294X.2011.05245.x

Hekkala ER, Amato G, DeSalle R, Blum MJ. 2010. Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations. Conserv Genet. 11:1435–1443. https://doi.org/10.1007/s10592-009-9970-5

Hekkala ER, Platt SG, Thorbjarnarson JB, Rainwater TR, Tessler M, Cunningham SW, Twomey C, Amato G. 2015. Integrating molecular, phenotypic and environmental data to elucidate patterns of crocodile hybridization in Belize. Roy Soc Open Sci. 2:150409. http://dx.doi.org/10.1098/rsos.150409

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics. 17(8):754–755. https://doi.org/10.1093/bioinformatics/17.8.754

IUCN (International Union for Conservation of Nature). 2016. IUCN Red List of Threatened Species. Version 2016. Available from: http://www.iucnredlist.org

Janke A, Arnarson U. 1997. The complete mitochondrial genome of *Alligator mississippiensis* and the separation between recent Archosauria (birds and crocodiles). Mol Biol Evol. 14(12):1266–1272. https://doi.org/10.1093/oxfordjournals.molbev.a025736

Janke A, Gullberg A, Hughes S, Aggarwal RK, Arnason U. 2005. Mitogenomic analyses place the gharial (*Gavialis gangeticus*) on the crocodile tree and provide Pre-K/T divergence times for most crocodilians. J Mol Evol. 61(5):620–626. https://doi.org/10.1007/s00239-004-0336-9

Ji X, Wu X, Yan P, Amato G. 2008. Complete sequence and gene organization of the mitochondrial genome of Siamensis Crocodile (*Crocodylus siamensis*). Mol Biol Rep. 35:133–138. https://doi.org/10.1007/s11033-007-9062-x

Krebs JE, Lewin B, Kilpatrick ST, Goldstein ES. 2014. Lewin's genes XI, Jones & Bartlett Learning, Burlington, Mass.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis v7.0 for bigger datasets. Mol Biol Evol. 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

Kumazawa Y, Nishida M. 1995. Variations in mitochondrial tRNA gene organization of reptiles as phylogenetic markers. Mol Biol Evol. 12(5):759–772.

Li Y, Wu X, Ji X, Yan P, Amato G. 2007. The complete mitochondrial genome of salt-water crocodile (*Crocodylus porosus*) and phylogeny of crocodilians. J Genet Genomics. 34:119–128. https://doi.org/10.1016/S1673-8527(07)60013-7

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25:1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Lowe TM, Chan PP. 2016. tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. Nucleic Acids Res. 44:W54–W57. https://doi.org/10.1093/nar/gkw413

Man Z, Yishu W, Peng Y, Xiaobing W. 2011. Crocodilian phylogeny inferred from twelve mitochondrial protein-coding genes, with new complete mitochondrial genomic sequences for *Crocodylus acutus* and *Crocodylus novaeguineae*. Mol Phylogenet Evol. 60:62–67. https://doi.org/10.1016/j.ympev.2011.03.029

McAliley LR, Willis RE, Ray DA, White PS, Brochu CA, Densmore LD 3rd. 2006. Are crocodiles really monophyletic?-Evidence for subdivisions from sequence and morphological data. Mol Phylogenet Evol. 39:16–32. https://doi.org/10.1016/j.ympev.2006.01.012

Meganathan PR, Dubey B, Batzer MA, Ray DA, Haque I. 2010. Molecular phylogenetic analyses of genus *Crocodylus* (Eusuchia, Crocodylia, Crocodylidae) and the taxonomic position of *Crocodylus porosus.* Mol Phylogenet Evol. 57:393–402. https://doi.org/10.1016/j.ympev.2010.06.011

Meganathan PR, Dubey B, Batzer MA, Ray DA, Haque I. 2011. Complete mitochondrial genome sequences of three *Crocodylus* species and their comparison within the Order Crocodylia. Gene. 478:35–41. https://doi.org/10.1016/j.gene.2011.01.012

Meredith RW, Hekkala ER, Amato G, Gatesy J. 2011. A phylogenetic hypothesis for *Crocodylus* (Crocodylia) based on mitochondrial DNA: evidence for a trans-Atlantic voyage from Africa to the New World. Mol Phylogenet Evol. 60:183–191. https://doi.org/10.1016/j.ympev.2011.03.026

Milián-García Y. 2008. Caracterización genética de poblaciones de *Crocodylus* (Crocodylia: Crocodylidae) que habitan en Cuba: *C. rhombifer, C. acutus* y supuestos híbridos con el empleo de marcadores nucleares y mitocondriales. *In:* Bioquímica. Havana: University of Havana. p. 62.

Milián-García Y. 2015. Caracterización genética del género *Crocodylus* (Crocodylia: Crocodylidae) en Cuba mediante el empleo de marcadores nucleares y mitocondriales. *In:* Biochemistry. Havana: University of Havana. p. 100.

Milián-García Y, Ramos-Targarona R, Pérez-Fleitas E, Sosa-Rodríguez G, Guerra-Manchena L, Alonso-Tabet M, Espinosa-López G, Russello MA. 2015. Genetic evidence of hybridization between the critically endangered Cuban crocodile and the American crocodile: implications for population history and in situ/ex situ conservation. Heredity. 114:272–280. https://doi.org/10.1038/hdy.2014.96

Milián-García Y, Russello MA, Espinosa López G. 2014. Genética para la Conservación del género *Crocodylus* en Cuba: Pasado, presente y futuro. *In:* Los Crocodylia de Cuba. Ed. by U. d. Alicante. p. 340.

Milián-García Y, Venegas-Anaya M, Frías-Soler R, Crawford AJ, Ramos-Targarona R, Rodríguez-Soberón R, Alonso-Tabet MT, Thorbjamarson J, Sanjur OI, Espinosa-Lopéz G, et al. 2011. Evolutionary history of Cuban crocodiles *Crocodylus rhombifer* and *Crocodylus acutus* inferred from multilocus markers. J Exp Zool. 315A(6):358–375. https://doi.org/10.1002/jez.683

Nei M. 1987. Molecular evolutionary genetics. Columbia University Press. 512 p. https://doi.org/10.1002/ajpa.1330750317

Oaks JR. 2011. A time-calibrated species tree of Crocodylia reveals a recent radiation of the true crocodiles. Evolution. 65:3285–3297. https://doi.org/10.1111/j.1558-5646.2011.01373.x

Patwardhan A, Ray S, Roy A. 2014. Molecular markers in phylogenetic studies–a review. J Phylogenetics Evol Biol. 2:2. https://doi.org/10.4172/2329-9002.1000131

Pereira SL. 2000. Mitochondrial genome organization and vertebrate phylogenetics. Genet Mol Biol. 23(4):745–752. https://doi.org/10.1590/S1415-47572000000400008

Posada D, Crandall K. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14:817–818. https://doi.org/10.1093/bioinformatics/14.9.817

Ray DA, Densmore L. 2002. The crocodilian mitochondrial control region: general structure, conserved sequences, and evolutionary implications. J Exp Zool. 294:334–345. https://doi.org/10.1002/jez.10198

Ray DA, Dever JA, Platt SG, Rainwater TR, Finger AG, McMurry ST, Batzer MA, Barr B, Stafford PJ, McKnight J, et al. 2004. Low levels of nucleotide diversity in *Crocodylus moreletii* and evidence of hybridization with *C. acutus.* Conserv Genet. 5:449–462. https://doi.org/10.1023/B:COGE.0000041024.96928.fe

Rodriguez D, Forstner MRJ, Moler PE, Wasilewski JA, Cherkiss MS, Densmore LD III. 2011. Effect of human-mediated migration and hybridization on the recovery of the American crocodile in Florida (USA). Conserv Genet. 12:449–459. https://doi.org/10.1007/s10592-010-0153-1

Russello MA, Glaberman S, Gibbs JP, Marquez C, Powell JR, Caccone A. 2005. A cryptic taxon of Galapagos tortoise in conservation peril. Biol Lett. 1:287–290. https://doi.org/10.1098/rsbl.2005.0317

Shamblin BM, Dutton PH, Bjorndal KA, Bolten AB, Naro-Maciel E, Santos AJB, Bellini C, Baptistotte C, Marcovaldi MÂ, Nairn CJ. 2015. Deeper mitochondrial sequencing reveals cryptic diversity and structure in Brazilian green turtle rookeries. Chelonian Conserv Biol. 14:167–172. https://doi.org/10.2744/CCB-1152.1

Shirley M, Villanova V, Vliet K, Austin J. 2015. Genetic barcoding facilitates captive and wild management of three cryptic African crocodile species complexes. Anim Conserv. 18:322–330. https://doi.org/10.1111/acv.12176

Shirley MH, Vliet KA, Carr AN, Austin JD. 2014. Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proc Roy Soc B: Biol Sci. 281:2013–2483. http://dx.doi.org/10.1098/rspb.2013.2483

Srikulnath K, Thapana W, Muangmai N. 2015. Role of chromosome changes in *Crocodylus* evolution and diversity. Genomics Inform. 13:102–111. https://doi.org/10.5808/GI.2015.13.4.102

Srikulnath K, Thongpan A, Suputtitada S, Apisitwanich S. 2012. New haplotype of the complete mitochondrial genome of *Crocodylus siamensis* and its species-specific DNA markers: distinguishing *C. siamensis* from *C. porosus* in Thailand. Mol Biol Rep. 39:4709–4717. https://doi.org/10.1007/s11033-011-1263-7

Tabet MA, Targarona RR, Soberón RR, Thorbjarnarson J, Ferrer JB, Álvarez VB. 2014. Los Crocodylia de Cuba, Universidad de Alicante.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 10:512–526.

Weaver JP, Rodriguez D, Venegas-Anaya M, Cedeño-Vazquez JR, Forstner M, Densmore L III. 2008. Genetic characterization of captive Cuban crocodiles (*Crocodylus rhombifer*) and evidence of hybridization with the American crocodile (*Crocodylus acutus*). J Exp Zool. 309A(10):649–660. https://doi.org/10.1002/jez.471