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Low Mitochondrial DNA Variability in the Captive Breeding Population of the Critically Endangered Orinoco Crocodile (*Crocodylus intermedius*) from Colombia

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***Abstract.*—The Orinoco Crocodile (*Crocodylus intermedius*) is a critically endangered crocodile species found only in the Orinoco River basin in Colombia and Venezuela, in northwestern South America. It has mostly failed to recover from human exploitation despite decades of protection and management efforts designed to promote species recovery. Efforts to conserve the Orinoco Crocodile include protection of populations in the wild as well as captive breeding/rearing programs. Here, we used mitochondrial DNA sequences to assess genetic variability in the Orinoco Crocodile captive-breeding population managed by the Estación de Biología Tropical "Roberto Franco" (EBTRF). We recovered four closely related mtDNA haplotypes (differing by no more than two mutational steps) in 27 wild-caught crocodiles representing the range of the species in Colombia. We suggest that the unstructured haplotype network reveals that the Orinoco Crocodile can be managed as a single genetic unit in Colombia. We found some limited evidence of geographical structuring, with a single haplotype (Cin3) restricted to individuals from the northwestern part (Cravo Norte, Department of Arauca) of the species distribution in Colombia. We recovered two of the four haplotypes found in the 27 wild-caught crocodiles in living captive-bred individuals (F1 and F2). Haplotype Cin2, recovered in the female founder Dabeiba, predominated (accounting for almost 91%) among living captive-bred individuals. All remaining captive-bred individuals surveyed contained haplotype Cin1, corresponding to the female founder Lizeth. We suggest that loss of haplotype diversity in the ETBRF captive population is likely if steps are not taken to retain haplotype diversity.**

Key Words.—captive breeding; conservation genetics; genetic diversity; mtDNA; management unit

Introduction

The Orinoco Crocodile (*Crocodylus intermedius*; Fig. 1) is a critically endangered crocodile species found only in the Orinoco River basin in Colombia and Venezuela, in northwestern South America (Seijas et al. 2010). Once numbering in the hundreds of thousands, the Orinoco Crocodile was hunted to near extinction in the early- to mid-1900s as a result of overseas demand for crocodile skins (Medem 1981, 1983). During the peak period of extraction, between 3,000 and 4,000 skins were reportedly sold daily in the town of San Fernando de Apure, the center of the skin trade in Venezuela (Medem 1983). In spite of protection of the species in both Colombia and Venezuela since the 1970s, Orinoco Crocodile populations have largely failed to recover (Morales-Betancourt et al. 2014). Habitat alteration or destruction, as well as intentional (e.g., egg collection) or accidental human-related mortality (e.g., drowning in fishing nets) continue to threaten the species (Morales-Betancourt et al. 2013a). The Orinoco Crocodile is currently listed as Critically Endangered by the International Union for the Conservation of Nature (IUCN) and included in Appendix I of CITES (IUCN 2015). At national or regional levels, the Orinoco Crocodile is listed as endangered in Venezuela (Seijas et al. 2015), while in Colombia it is listed as critically endangered (Morales-Betancourt et al. 2015).

In Colombia, the first field surveys to document the precarious state of the Orinoco Crocodile were carried out in the mid-1970s, with an estimated population size of only about 280 adult individuals across the Colombian Llanos Savanna region (a vast region of tropical grassland situated to the east of the Andes in Colombia and Venezuela; Medem 1981). Subsequent field surveys indicated little or no population recovery (Lugo 1996; Barahona and Bonilla 1999; Rodríguez 2000; Ardila-Robayo et al. 2002, 2010; Castro et al. 2012). Remaining Orinoco Crocodiles in Colombia are thought to be reduced to only a few isolated remnant populations located in the departments of Arauca and Meta (Morales-Betancourt et al. 2013a), with probably fewer than about 250 crocodiles remaining in Colombia.

Efforts to conserve the Orinoco Crocodile include protection of populations in the wild as well as captive breeding/rearing programs (Seijas et al. 2010; Morales-Betancourt et al. 2013a). In Colombia, there are currently two captive populations for the Orinoco

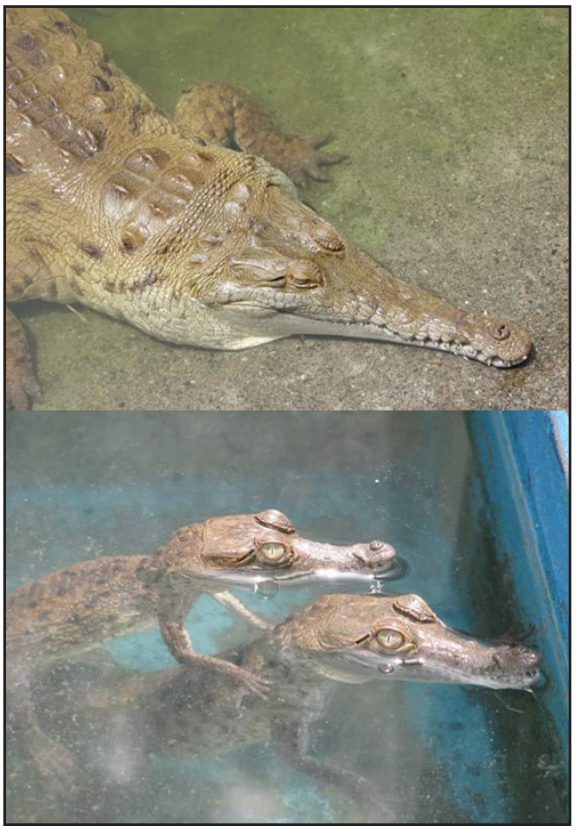

Figure 1. (Top) Adult Orinoco Crocodile (*Crocodylus intermedius*) in the Estación de Biología Tropical Roberto Franco (EBTRF), Facultad de Ciencias, Universidad Nacional de Colombia, and (Bottom) juvenile captive-bred Orinoco Crocodiles in the EBTRF, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá, D.C. (Photographed by Carolina Ibáñez).

Crocodile, both funded by private or public institutions with interests in the survival of the species (Morales-Betancourt et al. 2013b). The most important of these is maintained and managed by the Estación de Biología Tropical Roberto Franco (EBTRF), Facultad de Ciencias, Universidad Nacional de Colombia (Ardila-Robayo et al. 2010; Seijas et al. 2010). To date several small-scale releases of captive-bred and captive-reared Orinoco Crocodiles have been conducted in Colombia. These crocodiles have been released into areas where crocodiles have been extirpated or where they exist at critically low levels. As the number of individuals in the wild continues to decline, the release of captive-bred individuals is regarded as an important strategy to prevent local extinction and promote population recovery in the wild.

Despite major efforts to conserve the Orinoco Crocodile, genetic information on this species is strikingly sparse. Nothing is known about the extent of intra-population and inter-population genetic variation. The little that is known is based on genetic surveys of animals from the EBTRF Orinoco Crocodile captive population. For example, Bejarano Mora (2001) analyzed AFLP markers in a survey of Orinoco Crocodiles from the EBTRF population, reporting little evidence of genetic differentiation. Cuervo-Alarcón and Burbano-Montenegro (2012) examined variation at nine microsatellite loci in a survey of Orinoco Crocodiles from the EBTRF population. Levels of polymorphism in 53 wild captive adult Orinoco Crocodiles were generally low (1–5 alleles) and almost all loci surveyed exhibited heterozygote deficiencies. The heterozygote deficiencies in the 53 wild captive adult individuals were provided as evidence of inbreeding in wild populations (Cuervo-Alarcón and Burbano-Montenegro 2012). Alternatively, the widespread heterozygote deficiencies detected in the 53 wild captive adult individuals could also result from the Wahlund effect, whereby the pooling of wild individuals from variable sources or subpopulations with different allele frequencies results in widespread heterozygote deficits (a possibility the authors failed to consider). Given interest in reintroduction and population augmentation strategies to aid recovery of the Orinoco Crocodile in Colombia, there exists an increasing pressure to document genetic variation and population genetic structure in the Orinoco Crocodile to develop informed management practices.

It is important that all species of conservation concern receive genetic assessments to document the existence of genetic structuring that could play a role in developing management guidelines (DeSalle and Amato 2004). In conservation terms, the delineation and identification of appropriate units for conservation is considered critical to the establishment of scientifically managed conservation programs for threatened or managed species (Moritz 1999; Fraser and Bernatchez 2001; DeSalle and Amato 2004). Although specific criteria for the delineation and identification of conservation units is widely debated (for a review, see Fraser and Bernatchez 2001), the basic principle is to identify phylogenetic, population, or other units of practical significance that represent important evolutionary components of the species (Moritz 2002; Avise 2005). The recognition of genetic variation worthy of separate conservation consideration is also important as a guide to the management of captive breeding populations as well as to the translocation of individuals between areas for reintroduction or population augmentation purposes.

Mitochondrial DNA (mtDNA) has proved to be especially useful in providing insights into the existence of genetic structuring or historical divisions relevant for conservation below the species level (Avise 2004, 2005). This is because the rate of mutation fixation and allele frequency differences, due to complete or partial reproductive isolation of populations resulting from genetic drift, are greater for mtDNA than for nuclear loci (Avise 2004). Furthermore, while highly

polymorphic microsatellite loci can be used to detect very recent reductions in gene flow among populations from differences in the distribution of alleles, the high mutation rate typically observed at microsatellite loci can obscure genetic structure over greater spatial and evolutionary scales due to rapid allele turnover and convergent evolution (homoplasy; Zink et al. 2010, 2011). Thus, it useful to compare data from mtDNA and nuclear loci. Additionally, the high number of alleles typically observed at microsatellite loci means that a large number of samples are required to describe differences in allele frequencies among populations, a condition typically not achievable in analyses of critically endangered species such as the Orinoco Crocodile.

Systematic collection over the years of tissue samples from crocodiles held in the EBTRF Orinoco Crocodile captive population has produced more than 25 genetic samples from individuals either acquired through direct captures (with known geographical origin) or wildlife seizures (from information provided by the EBTRF; hereafter referred to as wild-caught specimens). These samples represent much of the range of the species in Colombia. While it would perhaps be unexpected to find major genetic subdivisions in the Orinoco Crocodile given the relatively small continental area occupied by the species, a first step in developing an informed conservation program for this critically endangered species is to confirm whether it should be managed as one or more genetic units. Furthermore, previously reported microsatellite data cannot be interpreted correctly without knowing for certain how many historical genetic units might exist in the species. The main objectives of this study were to examine whether recently isolated evolutionary divisions were overlooked in the studies of Bejarano Mora (2001) and Cuervo-Alarcón and Burbano-Montenegro (2012), as well as gain insights into the existence and pattern of genetic variation in the Orinoco Crocodile in the wild in Colombia. This was achieved by a survey of mtDNA variation in samples of wild-caught specimens from the EBTRF Orinoco Crocodile captive population.

Materials and Methods

Study population.—The captive population of Orinoco Crocodiles at EBTRF originated from wildlife seizures by the former Instituto de Recursos Naturales INDERENA (Colombia) and donations by the Peace Corps between 1974 and 1976 from the departments of Vichada and Casanare (Ardila-Robayo et al. 2010). Captive breeding was initiated in the 1970s with two pairs of wild-caught crocodiles (Lugo Rugeles 1995; Ardila-Robayo et al. 2010). However, it was not until 1991 that captive breeding efforts were successful (Lugo Rugeles 1995). Subsequent additions to the EBTRF captive population have been made to increase the captive stock. The major part of the current captive population of Orinoco Crocodiles at EBTRF is made up of decedents (F1 and F2) of a small number of original founder individuals originating from the rivers Humea, Metica, and Meta in the municipalities of Puerto López and San Carlos de Guaroa, Department of Meta (from information provided by the EBTRF).

Specimens and samples.—We received tissue samples from 164 Orinoco Crocodiles from the EBTRF captive population. From information provided by the EBTRF, we identified 27 crocodiles as wild in origin (hereafter referred to as wild-caught specimens); either acquired through direct captures (with known geographical origin) or wildlife seizures. These included tissue samples from 18 deceased specimens, including the two female founder individuals named Lizeth (Meta, River Humea) and Dabeiba (Puerto López, River Meta), and the two male founder individuals named Custodio (San Carlos de Guaroa, River Metica) and Pancho (Caño Yatea, Casanare), as well as the wild-caught male named Juancho (Rito Maní). Tissue samples of the deceased male named Polo were not available for analysis. The remaining 137 samples corresponded to captive offspring (F1 and F2).

Molecular methods.—We extracted genomic DNA from tissue samples using proteinase K digestion and guanidine thiocyanate/silica extraction following Hoyos et al. (2017). We used polymerase chain reaction (PCR) to amplify the mitochondrial cytochrome b (cyt b) and cytochrome c oxidase subunit I (COI) genes following Bloor et al. (2015). Both mitochondrial genes were amplified in all individuals. We amplified the mitochondrial cyt b gene using the primers 5′-AAT TCC CAT TAT TCT CAC TTG G-3′ and 5′-TTG GGA AGG TGT GTG TAT TCC-3′ (Bloor et al. 2015), and the mitochondrial COI gene using the primers 5′-CGA GTT TGC AGT TCG TCG TG-3′ and 5′-AGC ATG TCG TAT TGC GGT TG-3′ (Bloor et al. 2015). These mitochondrial genes have been used to uncover surprising levels of genetic diversity in the sister species *Crocodylus acutus* (Bloor et al. 2015). Cycling conditions included an initial 2 min at 94° C, followed by 34 cycles of 30 sec at 94° C, 30 sec at 55° C, and 2 min at 72° C, with a final step of 72° C for 10 min. We purified PCR products using ethanol precipitation. For bidirectional sequencing of PCR products, we used the same primers used for PCR amplification and Big-Dye DNA sequencing chemistry (Applied Biosystems, Foster City, California, USA). Resultant reaction products

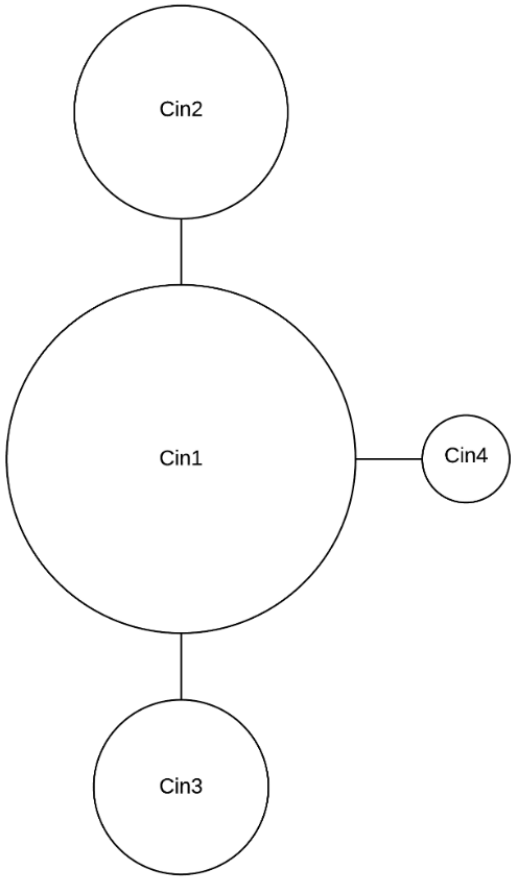

Figure 2. Unrooted cladogram based on the 95% probability of parsimony procedure (Templeton et al. 1992) to show relationships among wild-caught Orinoco Crocodile (*Crocodylus intermedius*) haplotypes from the EBTRF captive-breeding program. All branches are of unit length (one mutational step). Open circles represent observed haplotypes; areas of circles are proportional to the number observed for each haplotype.

were run on an ABI 3500 Genetic Analyzer automated sequencer (Applied Biosystems). We confirmed all polymorphisms by re-amplification and sequencing.

Analyses.—We assembled and edited sequence traces using the program CodonCode Aligner version 4.2 (CodonCode Corporation; www.codoncode.com). We obtained 1,200 bp of complete cyt b gene sequence and 1,557 bp of complete COI gene sequence for all individuals. Unique haplotype sequences have been added to GenBank under accession numbers KX454157- KX454164 (Table 1). We translated DNA sequences into amino acid sequences using the program MEGA version 6 (Tamura et al. 2013) to check for the presence of premature stop codons. We used an unrooted parsimony network based on a statistical parsimony procedure (Templeton et al. 1992) with the 95% probability of parsimony criterion as implemented in the program TCS version 1.21 (Clement et al. 2000) to reconstruct genealogical relationships among sequences. This approach estimates the maximum number of substitutions to connect two haplotypes with 95% confidence under parsimony and performs better when inferring relationships among haplotypes that differ by a small number of differences.

Results

Mitochondrial DNA sequence variation was very low; of the 2,757 sites surveyed for all samples, only three were variable (Table 1). One substitution in COI resulted in a change in amino acid. We did not observe stop codons or indels, which suggests that our sequences are authentic mitochondrial copies and not nuclear insertions. The three variable sites defined four haplotypes (Table 1). The haplotype network generated from these sequences revealed a single group of closely related haplotypes separated by relatively small number of differences among haplotypes for wild-caught individuals (Fig. 2). A similar lack of diversity was identified in captive individuals (Table 1). Intraspecific variation was very low, with a maximum of two differences between haplotypes (0.07% maximum sequence divergence, uncorrected). Among the 27 wild-caught crocodiles, the most common haplotype was Cin1, which was found in 16 individuals (including the two founder individuals Pancho and Lizeth as well as the wild caught-wild male specimen Juancho). We recovered haplotype Cin2 in 6 wild-caught individuals (including the female founder individual Dabeiba), haplotype Cin4 in the single wild-caught individual corresponding to the founder individual Custodio, and haplotype Cin3 in the four wild-caught individuals from Cravo Norte (Department of Arauca). Of the 137 offspring analyzed, 119 contained haplotype Cin2, whereas only 18 contained haplotype Cin1 (Table 1).

We recovered the most common haplotype for wild-caught samples (Cin1) in individuals from the departments of Meta and Casanare from the western part of the species distribution in Colombia. The geographic distribution of haplotype Cin2 overlapped with the distribution of Cin1, being found in the Department of Meta extending to the eastern part of the species range into the Department of Vichada. Haplotype Cin4 was detected in a single individual from the Department of Meta. These three haplotypes had completely or incompletely overlapping distributions. In contrast, haplotype Cin4, recovered only in four individuals from Cravo Norte in the Department of Arauca in the northern part of the species range in Colombia, did not overlap with any other haplotypes. Therefore, the mitochondrial sequence data provide some evidence for minor genetic differentiation among Orinoco Crocodile populations.

Table 1. Variable positions for the four mitochondrial haplotypes found within the 27 wild-caught Orinoco Crocodiles (*Crocodylus intermedius*) from the EBTRF captive-breeding program based on complete cytochrome b (cyt b: position 372) and cytochrome oxidase I (COI: positions 1158 and 1501) gene sequences. GenBank accession numbers are given for haplotype sequences. The frequency of each haplotype found in the 27 captive-wild and 137 captive-bred offspring is also given. One substitution (position 1501) in COI resulted in a change in amino acid.

	variable sites			haplotype frequencies		
phaplotype	372	1158	1501	wild-caught (n = 27)	captive-bred offspring (n = 137)	GenBank accessions (cyt b, COI)
Cin1	G	G	C	16	18	KX454161, KX454157
Cin2	.	A	.	6	119	KX454162, KX454158
Cin3	.	.	T	4	0	KX454163, KX454159
Cin4	A	.	.	1	0	KX454164, KX454160

Discussion

Data from mtDNA sequencing suggest that Orinoco Crocodile genetic variability is extremely low. Within two of the most variable regions of the mitochondrial genome, we found only four mtDNA haplotypes among 27 wild-caught individuals representing the entire range of the species in Colombia, and a very low percentage (0.1) of variable sites in these variable mitochondrial DNA regions. Orinoco Crocodile mtDNA sequence variation can be directly compared to homologous sequence data obtained from other *Crocodylus* species. In an analysis of 40 individuals of the American Crocodile (*C. acutus*) from three captive populations in Colombia, the authors recovered 18 variable sites, resulting in eight haplotypes (Bloor et al. 2015). The American Crocodile is a close relative of the Orinoco Crocodile, and has a similar history of human exploitation leading to a well-documented reduction in population size. Furthermore, the mtDNA sequence divergence found here for the Orinoco Crocodile is also considerably lower than that reported for other mtDNA sequences from other *Crocodylus* species, including the *C. moreletii* (Ray et al. 2004), *C. rhombifer* (Weaver et al. 2008) and *C. porosus* (Luck et al. 2012). Whether this low level of mitochondrial variability in the Orinoco Crocodile specimens from Colombia represents the effects of a population bottleneck related to the well-documented reduction in population size for the species or historical long-term low levels of genetic variability is difficult to assess. The Orinoco River Basin is seemingly devoid of potentially significant geographical barriers to dispersal that could have promoted regional differentiation among populations of the Orinoco Crocodile. Thus, high levels of historical gene flow could explain the very close genetic relationships within the Orinoco Crocodile samples analyzed. However, it should be noted that we only included wild-caught specimens from Colombia, so further haplotype diversity could be detected in Venezuela. Comparison of mtDNA variability from pre-hunting populations and present-day populations would be the only way to separate these two alternative explanations.

Because of problems of sampling error, it is very likely that geographical distribution of haplotypes could be altered with additional data. However, examination of related information regarding the origin of wild-caught specimens provides some interesting insights into possible geographical structuring. The presence of apparently common haplotypes that are widespread with haplotypes confined to one or a few nearby areas (private haplotypes) is clearly of interest and suggestive a possible geographical structuring resulting from low or moderate contemporary gene flow between populations that are connected tightly in history. There are no obvious pronounced barriers to gene flow in the Orinoco River Basin. This suggests that possible geographical structuring is most likely the result of the current patchy distribution of the species and fixation of mtDNA haplotypes in small isolated populations. This could explain the apparently restricted distribution of haplotype Cin3 from Cravo Norte, although the limited sample size makes this hard to determine. Any proposal of geographical structuring in the Orinoco Crocodile should be taken with some caution until further data are obtained from throughout the species distribution (including Venezuela).

On the basis of the mtDNA network and limited geographic structure, we suggest that population management (including captive breeding and release) of the Orinoco Crocodile can operate free of genetic restrictions (at least in Colombia), focusing instead on demographic (including population size and breeding success) and ecological considerations, as well as population viability to maintain large populations of Orinoco Crocodile in several locations. However, the possibility of more recent population structure and isolation would have important implications for management planning because it would indicate that individuals are unlikely to recolonize an area rapidly after a local population crash and newly established populations are likely to respond independently to management actions. From this perspective, confirmation of the separation of the Cravo Norte population would seem to be of importance to management planning. Because mtDNA is maternally inherited, patterns of genetic structure may reflect female-specific aspects of dispersal and population history. If dispersal patterns

are similar in both females and males, then observed patterns of differentiation at mtDNA loci should provide an accurate record of population differentiation for the species. From this perspective, the results of ongoing release and satellite tracking studies for the Orinoco Crocodile will provide valuable information on dispersal patterns.

Finally, in terms of the composition of the preset-day EBTRF Orinoco Crocodile captive population, a single haplotype (Cin2) predominated, accounting for just over 91%, in living captive-bred individuals (F1 and F2). Future loss of mtDNA variability in the captive-breeding population at ETBRF is therefore predicted if steps are not taken to retain haplotype diversity. These should incorporate the inclusion of individuals from Cravo Norte (representing haplotype Cin3).

Acknowledgments.—This paper is dedicated to Maria Cristina Ardila Robayo (recently deceased), former Director of the Estación Tropical de Biologia Roberto Franco, who was a herpetologist and advocate of crocodilian conservation in Colombia. Although our time spent together was short, we will be eternally touched by her humanity, generosity, and persistence. The Ministerio de Ambiente y Desarrollo Sostenible (MADS) de Colombia project No. 38-2012 financed this project. Laboratory work was carried out under the permit Contrato de acceso a recursos genéticos No. 48 del 2012, para el proyecto "Aportes al conocimiento de manejo de la población ex situ del Caimán llanero *Crocodylus intermedius* en Colombia: caracterización genética de la población a cargo de la Universidad Nacional de Colombia a través de la Estación de Biología Tropical Roberto Franco."

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