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Research Article

Hope is the last thing lost: Colombian captive-bred population of the critically endangered Orinoco crocodile (*Crocodylus intermedius*) is a genetic reservoir that could help to save the species from extinction

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Abstract

A purpose of *ex-situ* populations is the preservation of genetic variation, but this is a challenging task since genetic diversity is commonly lost through each generation, and so the establishment of management guidelines should be a high priority. Fifty years ago, the National University of Colombia began a breeding program in the Roberto Franco Tropical Station (in Villavicencio, Meta) to conserve the critically endangered Orinoco crocodile *Crocodylus intermedius*. Despite the large number of individuals raised and kept in captivity, the Station has not been able to release individuals due to a lack of a complete genetic characterization that could determine whether the population is genetically viable. In this study we used a panel of 17 microsatellite loci to overcome this problem. We estimated from the founder animals and the live crocodiles the inbreeding, heterozygosities, the number of alleles, and their richness, and frequencies to understand the effects of managing a captive breeding program without considering genetic profiles. Our results revealed that the living population maintains much of its founder diversity with high levels of heterozygosity and low overall inbreeding, making it suitable for maintaining captive breeding and for implementing wild releases. We estimated the individual genetic diversity of the living crocodiles, as well as their relationships. This information, combined with the size, sex, and location, allowed us to propose combinations and to restructure the breeding groups. We demonstrated that molecular data could be used to improve the management of *ex-situ* conservation programs well beyond what could be achieved with pedigree information alone.

Key words: Critically endangered species, *ex-situ* conservation, genetic diversity, microsatellites, population genetics

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Introduction

Despite *in-situ* conservation representing the most effective way to protect endangered species, *ex-situ* conservation programs and reintroduction of captive-bred animals have become an important tool for managing the same spe-

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cies (Witzenberger and Hochkirch 2011). And in many cases these programs might be the only way to save them from extinction (Bertorelle et al. 2009). Nowadays, the aim of the *ex-situ* conservation centers goes beyond the survival of individuals targeting the conservation of genetic diversity (Ramírez et al. 2006). The probability of species long-term survival will be increased by efforts to restore as much genetic variation as possible through the production and reintroduction of offspring with high genetic diversity, capable of resisting and adapting to the environmental pressures of natural habitats (Araki et al. 2007; Goncalves da Silva et al. 2010). Nevertheless, this is not always an easy task, and several management considerations must be contemplated.

Despite detailed studbooks being the simplest means for the proper management of captive populations, the correct parental allocation of individuals is not always possible without the use of molecular data, since pedigree information is often insufficient to select the best breeding pairs (Tzika et al. 2008). Additionally, founders are assumed to be unrelated (i.e., founder assumption), although this is not always true (e.g., individuals born of the same brood) and this may lead to an underestimation of relatedness, resulting in incorrect management decisions (Russello and Amato 2004). Genetic information can guide the choice of individuals with the lowest mean kinship and highest diversity as parents of subsequent generations, reducing the overall level of relatedness, maximizing founder representation, and minimizing the expression of deleterious alleles in inbred animals (Montgomery et al. 1997).

The Orinoco crocodile (*Crocodylus intermedius*. Fig. 1) is endemic to the Orinoco Basin in Colombia and Venezuela, being considered as the most endangered and least-studied of the New World crocodilians (Ross 1998; Antelo et al. 2010; Seijas et al. 2010; Moreno-Arias and Ardila-Robayo 2020; Parra-Torres et al. 2020). It is one of the largest species of crocodiles, with males reaching lengths of up to seven meters. Sexual maturity is typically attained by females between seven and ten years of age, while males reach sexual maturity between nine and twelve years (Thorbjarnarson 1987; Garcés-Restrepo et al. 2014). During the 20th century, commercial hunting of the Orinoco crocodile, motivated by the high demand for its skin, brought the species to the brink of extinction and consequently, the species is categorized as Critically Endangered (CR) in the IUCN Red Book List (IUCN 2020). Currently the population status of the species is unknown, and the last censuses reported a general trend of poor recovery or population decline (Medem 1981; Lugo 1996; Seijas et al. 2010; Espinosa-Blanco and Seijas 2012; Babarro 2014; Parra-Torres et al. 2020).

To tackle this situation, two direct conservation strategies have been suggested and followed in Colombia. First, its protection has been legally regulated by prohibition decrees and through practices of improvement and protection of its habitats (Castro Casal et al. 2013). Second, as in other crocodilian species (e.g., *Alligator sinensis*; Xu et al. 2005), a captive breeding program was established in 1971 by Federico Medem at the Roberto Franco Tropical Biological Station (EBTRF by its acronym in Spanish) of the National University of Colombia located in Villavicencio, Meta department. Since 1998 the EBTRF has been part of the National Program for the Conservation of the Orinoco Crocodile (PROCAIMAN; see MAM 2002), and it represents the largest and only stock of individuals of the species in Colombia, allegedly containing more crocodiles than those found in the wild in the country (Posso-Peláez et al. 2018). Currently it contains more than

Figure 1. Adult female of *Crocodylus intermedius* at the Roberto Franco Tropical Biological Station. Photograph MVR.

600 individuals distributed in five different locations known as *ex-situ* subpopulations: Piscilago, Wisirare, Merecure, Ocarros, and EBTRF, being the last hope for the recovery of the species in the country. Nevertheless, despite the variety of ages and the large number of individuals raised and kept in captivity, the EBTRF has been unable to reintroduce animals due to the lack of a robust and conclusive genetic characterization that determines whether the population is genetically viable and has no signs of inbreeding (see MAM 2002). Therefore, it is unknown if the individuals have enough genetic diversity and are genetically adequate for release and if the individuals can be used to maintain and increase the *ex-situ* population´s genetic variability. In addition, due to the extended time that the program has been in operation, it is unknown whether there has been a loss of diversity.

In this study we used a panel of 17 microsatellite loci to genetically characterize the *ex-situ* population of the EBTRF and to tackle the previously described issues. We estimated allelic richness, frequencies, and heterozygosities in living and founder crocodiles to understand at the genetic level the effects of managing a captive breeding program without considering the genetic profile of the individuals and the population. Based on this data, we also estimated relationships of living individuals and developed recommendations for the combination of breeding groups.

Materials and methods

Sampling

Since 2004 tissue samples have been taken from most of the individuals comprising the *ex-situ* population in charge of the EBTRF. Scales and muscle samples were preserved in pure ethanol and kept at -20 °C until processing. We searched EBTRF records to clarify the geographic origins, status, and current location of each crocodile. All the animals were microchipped for individual identification.

In total, we included 551 individuals in the study. The complete dataset includes 40 crocodiles that were wild in origin (Suppl. material 1: appendix S1). They were

either acquired through direct captures as wildlife, from the breeding center or as confiscations. These animals included 24 seized individuals whose provenance was unknown (13 were dead in 2021), nine young individuals from Cravo Norte River (by 2021 one was dead and eight were 11 years old), and seven dead wild founders from the Cuisiana River, the Metica River, the Humea River, the Meta River, the Guachiría River, and the Vichada River. The remaining samples corresponded to 458 individuals of the captive offspring (F1 and F2) and 53 released crocodiles.

From the living individuals we evaluated, 82% belonged to the captive breeding program from five subpopulations: 316 were from the main headquarters of the program at the EBTRF in Villavicencio, Meta department; 19 were from the Parque Agroecológico Merecure in Puerto López, Meta department; five were from the Bioparque los Ocarros in Villavicencio, Meta department; four were from the Aquatic and Conservation Park Piscilago in Nilo, Cundinamarca department; and 127 were from the Parque Ecotemático Wisirare in Orocué, Casanare department. The remaining samples were from the two largest subpopulations: 44 from the EBTRF and 56 from Wisirare.

Laboratory procedures and genotyping

Genomic DNA was extracted from preserved tissue using the Invisorb Spin Tissue Mini Kit (Stratec) following manufacturer protocols. Seventeen microsatellite primers developed for other species of the genus and already evaluated for cross amplification by Lafferriere et al. (2016) were used. We implemented four PCRs multiplex using the Multiplex PCR kit MyTaq HS Mix (Bioline, USA) M1: CpP302, CpP305, CpP314, CpP1409, and CpP3216; M2: Cj16, Cj122, and Cu5123; M3: Cj18, Cj109, C391, CUJ131 and M4: Cj101, Cj127, Cp801, and CpDi13. Reactions were prepared in a final volume of 10 µL including: 5 µL of MyTaq HS Mix, 0.2 µL of 10X each primer (except for Cj122 and Cj109 with 0.4 µL added), a final concentration of 4 ng/ µL of DNA and the excess of ultra-pure water. Thermocycling conditions were as follows: a preliminary denaturation stage at 95 °C for 4 minutes, followed by 30 denaturation cycles at 95 °C for 30 seconds, two different annealing temperatures (57 °C for M1, M2 and M4 and 60 °C for M3) for 45 seconds and extension at 72 °C for 30 seconds, ending with a temperature of 72 °C for 5 minutes. Fragment lengths were determined using an ABI 3500 genetic analyzer. For this purpose, 1 µl of the PCR product was diluted in 99 µl water; 1 µl of this dilution was mixed with 8.5 µl Hi-Di Formamide (Applied Biosystems), 0.25 µl water and 0.25 µl GeneScan-600 LIZ Size Standard (Applied Biosystems). The Gene-Mapper 3.7 (Applied Biosystems Foster City, CA) and Osiris 2.13.1 (NCBI) software were used for scoring fragment lengths. A single person carried out the visualization and determination of allele sizes. Genetic laboratory work was conducted at the Molecular Ecology Laboratory of the Genetics Institute, National University of Colombia in Bogotá.

Data analysis

Estimation of loss of genetic diversity

To evaluate any loss of genetic diversity, the EBTRF crocodilian population was subdivided into two groups. The first group was composed of 40 F0 dead and alive crocodiles representing the genetic potential that the station has had

since it was founded. The second group contained 468 live individuals, including F0, F1 and F2 distributed in the different *ex-situ* subpopulations, representing the current potential diversity of the population. Null allele frequencies at each locus on the whole dataset were estimated using the software FreeNA (Chapuis and Estoup 2007) and CERVUS 3.0.7 (Kalinowski et al. 2007), and null alleles were considered when the frequency was higher than 0,05. Allele dropout was estimated using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004).

For the F0 population and the whole living population the number of alleles per locus (nA), allelic richness (AR), allelic frequencies and inbreeding coefficient (F_{IS}) were calculated using the software FSTAT 2.9.3.2 (Goudet 2001). F_{IS} significance for excess and defect of heterozygous was evaluated in Genepop 4.7.5 (p-value < 0.005; Raymond and Rousset 1995). Statistically significant differences for AR, Ho and F_{IS} between population subdivisions were tested with 15,000 permutations in FSTAT 2.9.3.2 (Goudet 2001). We removed F0 individuals from the living crocodilians to maintain independence between both groups. Expected heterozygosities (He) and observed heterozygosities (Ho) were estimated using ARLEQUIN 3.5.1.2 (Excoffier et al. 2005). The same software was used to test for Hardy Weinberg equilibrium (HWE) and linkage equilibrium and Bonferroni corrections were applied to both calculations.

Assessment of parental veracity

To assess the veracity of the provenance of the captive bred individuals registered in the records, we ran a parental pairs analysis with known sexes using the likelihood-based approach implemented in the software CERVUS 3.0.7 (Kalinowski et al. 2007). Two levels of confidence were set at 80% (relaxed) and 95% (strict). Positive LOD scores (the logarithms of the likelihood ratios) and Delta scores were compared to identify the most likely parents for each offspring. We evaluated separately five groups: 1) seven individuals registered as coming from the founding couple Pancho/Dabeiba; 2) two individuals registered as coming from Ocarros; 3) 54 individuals registered as coming from Merecure; 4) 90 individuals registered as coming from Piscilago; and 5) 246 individuals registered as coming from Wisirare. To determine samples' distributions and using the potential fathers by location for each of the groups, we ran simulations of 10,000 offspring genotypes, each at a sampling rate of 100% and with a proportion of mistyped loci set at 0.01. Determinations were made conforming to the established sets with the location and origin of the individuals.

Assessment of founder assumption

Relationships among the founder crocodiles were inferred using ML-RELATE (Kalinowski et al. 2006), a maximum likelihood-based software that estimates relatedness coefficients (r) for each pair of individuals and provides the relationships that have the highest likelihood for each pair of individuals (half-sibling, full-sibling, and unrelated). The coefficient goes from zero (i.e., individuals not related) to one (i.e., identical genotypes). We evaluated relatedness in two groups: nine individuals from Cravo Norte River and 12 individuals seized from the Rango Rudd hatchery.

Management formulations

To facilitate the development of management guidelines, the living crocodile population was subdivided into five groups according to the location of individuals in the subpopulations (i.e., EBTRF, Ocarros, Piscilago, Wisirare, and Merecure). The number of alleles per locus (nA) and allelic frequencies were calculated for each group using FSTAT 2.9.3.2 (Goudet 2001). Using this information, we genetically identified key individuals with rare alleles.

We estimated inbreeding coefficients at the individual level for each of the living and dead crocodiles using the GENHET 2.3 R script (Coulon 2010). We calculated the homozygosity by loci (HL), a homozygosity index that correlates with the inbreeding coefficient and weighs the contribution of each locus depending on their allelic variability (Aparicio et al. 2006). This allowed us to identify the most genetically diverse individuals. Homozygosity by loci indices for founders and live crocodiles (disregarding founders that may be alive to maintain sample independence) were compared using a U Mann Whitney test.

Relationships among all the living crocodiles were inferred using ML-RELATE (Kalinowski et al. 2006). The relatedness index allowed us to evaluate the relationships of the crocodiles that are alive and have reproduced. To guide the choice of reproductive pairs that will generate neonates with high genetic diversity, the *r* and the HL indexes, and the presence of alleles detected at low frequencies (see below), were combined with additional information from each single crocodile (i.e., size, age, sex, origin, current location), to propose options of viable crosses with reproductive, non-related, and highly diverse individuals from the five subpopulations already established and from two newly formed reproductive nuclei integrated at the Universidad de los Llanos in Villavicencio, Meta.

Results

The 17 microsatellite loci were successfully amplified for 548 of the 551 individuals. Between one and six loci failed to amplify for the other three samples. Locus CpP1610 resulted as monomorphic and therefore it was removed from the analyses. There was no evidence for null alleles or for allele dropout.

Estimation of loss of genetic diversity

Our data set represents 82% of the living crocodiles of the Station and 91% of the F0 population. Of the missing founders, three corresponded to juveniles from Cravo Norte that had not reproduced to date and only one founder breeder from which no tissue sample was taken. A total of 72 alleles were revealed: 69 in F0 crocodiles and 65 in live crocodiles (89.9% of the F0 alleles, Table 1). The F0 population had eight private alleles while the live population had three, suggesting that in the living population we found wild individuals or non-genotyped parents. The average number of alleles per locus, the average allelic richness, the expected heterozygosity, and the inbreeding coefficient F_{IS} were higher in the F0 population than in the live population (Table 1). Fourteen loci in the live population showed significant deviation from the Hardy-Weinberg equilibrium. No significant linkage disequilibrium was found between pairs of loci. In the F0

population two loci had deviations in the F_{IS} coefficient for heterozygous defect and one locus for heterozygous excess. The live population showed deviations in the F_{IS} coefficient in seven loci for heterozygous excess (Table 1).

Even though the live population showed a higher Ho than the F0 population, differences between each group were not significant (H0 p = 1). Likewise, although the F0 crocodiles showed generally higher AR and F_{IS}, differences were statistically not significant (AR p = 0.332, FIS p = 0.332). Although there were loci where allele frequencies did not change considerably between F0 and the live populations (e.g., CpP3216, Cj127; Table 2), there were other loci that showed strong changes, and even a loss of alleles (e.g., Cj109, Cj18, Cj391, Cpp801; Table 2).

Assessment of parental veracity and the founder assumption test

Of the 399 individuals evaluated, 325 (81.5%) had potential fathers in the subpopulation of origin registered; and for 74 (18.5%) individuals the physical registry does not correctly indicate the origin of these crocodiles (Table 3). When testing the relationships between the founding crocodiles that came from the same sites, we found that several were related (Table 4). Although the nine individuals from Cravo Norte showed unrelatedness in some cases, most were related as half and full-siblings. We found a similar result for the seized crocodiles from the Rango Rudd hatchery.

Table 1. Genetic diversity of the F0 and live populations of *Crocodylus intermedius* in the Roberto Franco Tropical Biological Station. N – sample size; nA – alleles per locus; AR – allelic richness; Ho – observed heterozygosity; He – expected heterozygosity; HWE – Hardy-Weinberg equilibrium; F_{IS} – inbreeding coefficient; * Significance for heterozygous defect; ** Significance for heterozygous excess.

Locus	Null alleles	F0 population (total alleles = 69)								Live population (total alleles = 65)							
		N	nA	Private alleles	AR	Ho	He	HWE	F_{IS}	N	nA	Private alleles	AR	Ho	He	HWE	F_{IS}
CpP3216	No	40	2	–	1.984	0.475	0.481	Yes	0.012	468	2	–	1.971	0.561	0.460	**No**	-0.217**
CpP305	No	40	3	–	2.877	0.600	0.664	Yes	0.097	468	3	–	2.566	0.530	0.588	**No**	0.100
CpP1409	No	40	3	–	2.198	0.375	0.445	Yes	0.159	468	3	–	2.622	0.650	0.565	**No**	-0.152**
CpP302	No	40	5	–	3.646	0.750	0.693	Yes	-0.084	468	5	–	3.481	0.750	0.707	**No**	-0.062**
CpP314	No	40	3	–	2.795	0.550	0.619	Yes	0.113	468	3	–	2.874	0.639	0.663	**No**	0.034
Cj16	No	40	5	1	3.168	0.600	0.596	Yes	-0.007	468	4	–	2.721	0.620	0.561	**No**	-0.102
CU5123	No	40	4	–	3.058	0.800	0.682	Yes	-0.175**	468	4	–	3.292	0.741	0.689	**No**	-0.079**
Cj122	No	40	5	–	4.064	0.700	0.781	Yes	0.105	468	5	–	3.942	0.816	0.771	**No**	-0.057
Cj18	No	40	5	1	3.404	0.775	0.702	Yes	-0.106	468	5	1	3.071	0.635	0.612	**No**	-0.040
CUJ131	No	40	4	1	2.325	0.400	0.492	Yes	0.189*	468	3	–	2.009	0.560	0.502	Yes	-0.114**
Cj109	No	40	6	2	3.543	0.675	0.716	Yes	0.059	468	4	–	3.266	0.786	0.699	**No**	-0.123**
Cj391	No	40	10	2	4.546	0.675	0.806	Yes	0.164*	468	8	–	2.859	0.583	0.537	**No**	-0.089
CCj101	No	40	3	–	2.184	0.575	0.529	Yes	-0.087	468	4	1	2.151	0.596	0.485	**No**	-0.230
CpDi13	No	40	2	–	1.969	0.475	0.453	Yes	-0.050	468	3	1	2.057	0.506	0.479	Yes	-0.053
Cj127	No	40	3	–	1.291	0.075	0.074	Yes	-0.017	468	3	–	1.828	0.344	0.299	**No**	-0.152**
CpP801	No	40	6	–	3.500	0.725	0.703	Yes	-0.032	468	6	–	2.974	0.637	0.582	**No**	-0.094
Mean			4.313		2.910	0.577	0.590		0.019		4.063		2.730	0.622	0.575		-0.013
SD			1.991		0.871	0.187	0.179		0.086		1.482		0.610	0.117	0.118		0.059

Table 2. Allelic frequencies of 16 polymorphic microsatellite loci in F0 and live populations of *Crocodylus intermedius* in the Roberto Franco Tropical Biological Station. [a] Private allele in that population; [b] Private allele in that subpopulation; [c] Alleles with low frequencies.

Locus	Allele	F0 population (N = 40; nA = 69)	Live population					
			Total (N = 465, nA = 65)	EBTRF (N = 314, nA = 63)	Ocarros (N = 5, nA = 52)	Piscilago (N = 4, nA = 43)	Wisirare (N = 127, nA = 53)	Merecure (N = 18, nA = 49)
CpP3216	137	0.613	0.643	0.642	0.900	0.625	0.638	0.667
	141	0.388	0.357	0.358	0.100	0.375	0.362	0.333
CpP305	176	0.325	0.103	0.080	0.100	0.375	0.169	0.000
	192	0.413	0.435	0.482	0.800	0.500	0.303	0.444
	196	0.263	0.461	0.438	0.100	0.125	0.528	0.556
CpP1409	245	0.263	0.286	0.299	0.200	0.750	0.248	0.250
	249	0.700	0.578	0.605	0.700	0.250	0.512	0.611
	253	0.038	0.135	0.096	0.100	0.000	0.240	0.139
CpP302	194	0.500	0.431	0.422	0.700	0.750	0.429	0.472
	196	0.138	0.173	0.164	0.100	0.000	0.197	0.194
	200	0.150	0.133	0.140	0.100	0.125	0.134	0.056
	202	0.138	0.017 [c]	0.022	0.100	0.000	0.000	0.028
	208	0.075	0.245	0.252	0.000	0.125	0.240	0.250
CpP314	254	0.525	0.367	0.433	0.400	0.375	0.217	0.278
	258	0.238	0.351	0.330	0.300	0.250	0.382	0.556
	262	0.238	0.283	0.237	0.300	0.375	0.402	0.167
Cj16	141	0.125	0.053	0.064	0.200	0.250	0.000	0.194
	151	0.038 [a]	0.000	0.000	0.000	0.000	0.000	0.000
	167	0.600	0.592	0.596	0.700	0.625	0.587	0.528
	171	0.175	0.286	0.291	0.100	0.125	0.283	0.278
	173	0.063	0.069	0.049	0.000	0.000	0.130	0.000
CU5123	204	0.250	0.246	0.260	0.200	0.375	0.201	0.333
	214	0.025	0.094	0.111	0.100	0.000	0.039	0.222
	216	0.375	0.216	0.221	0.600	0.375	0.181	0.222
	220	0.350	0.444	0.408	0.100	0.250	0.579	0.222
Cj122	378	0.175	0.156	0.169	0.200	0.375	0.126	0.056
	380	0.175	0.310	0.275	0.200	0.125	0.406	0.278
	386	0.350	0.178	0.215	0.400	0.250	0.051	0.417
	390	0.163	0.092	0.080	0.100	0.125	0.138	0.000
	392	0.138	0.263	0.261	0.100	0.125	0.280	0.250
Cj18	203	0.000	0.005 [a,c]	0.008 [b,c]	0.000	0.000	0.000	0.000
	207	0.300	0.209	0.231	0.400	0.250	0.122	0.361
	209	0.163	0.157	0.140	0.000	0.000	0.228	0.028
	211	0.425	0.560	0.572	0.600	0.750	0.520	0.611
	213	0.100	0.069	0.049	0.000	0.000	0.130	0.000
	215	0.013 [a]	0.000	0.000	0.000	0.000	0.000	0.000
CUJ131	185	0.650	0.517	0.463	0.400	0.500	0.685	0.222
	189	0.013 [a]	0.000	0.000	0.000	0.000	0.000	0.000
	191	0.300	0.481	0.537	0.400	0.500	0.311	0.750
	193	0.038	0.002 [c]	0.000	0.200	0.000	0.004	0.028
Cj109	372	0.213	0.338	0.347	0.400	0.375	0.307	0.361
	374	0.238	0.072	0.045	0.100	0.000	0.130	0.139
	376	0.013 [a]	0.000	0.000	0.000	0.000	0.000	0.000
	382	0.100	0.231	0.240	0.100	0.125	0.213	0.222
	384	0.425	0.359	0.368	0.400	0.500	0.350	0.278
	388	0.013 [a]	0.000	0.000	0.000	0.000	0.000	0.000
Cj391	153	0.350	0.646	0.611	0.300	0.500	0.728	0.722
	157	0.075	0.017 [c]	0.025	0.000	0.000	0.000	0.028
	159	0.013 [a]	0.000	0.000	0.000	0.000	0.000	0.000
	161	0.013	0.001 [c]	0.000	0.100	0.000	0.004	0.000
	169	0.050	0.098	0.126	0.100	0.000	0.016	0.250
	171	0.013	0.003 [c]	0.005 [b]	0.000	0.000	0.000	0.000

Locus	Allele	F0 population (N = 40; nA = 69)	Live population					
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Cj391	173	0.163	0.033 [c]	0.041	0.100	0.500	0.000	0.000
	175	0.175	0.191	0.178	0.200	0.000	0.252	0.000
	179	0.125	0.01 [c]	0.013	0.200	0.000	0.000	0.000
	183	0.025 [a]	0.000	0.000	0.000	0.000	0.000	0.000
CCj101	354	0.000	0.012 [a,c]	0.003	0.000	0.000	0.035	0.000
	356	0.513	0.625	0.634	0.600	0.375	0.587	0.833
	358	0.025	0.012 [c]	0.016	0.000	0.000	0.004	0.000
	360	0.463	0.352	0.347	0.400	0.625	0.374	0.167
CpDi13	358	0.000	0.011 [a]	0.013	0.000	0.125	0.000	0.028
	360	0.663	0.624	0.613	0.600	0.625	0.650	0.556
	362	0.338	0.366	0.374	0.400	0.250	0.350	0.417
Cj127	337	0.963	0.817	0.820	1.000	0.875	0.815	0.750
	341	0.013	0.004 [c]	0.003	0.000	0.000	0.000	0.056
	343	0.025	0.178	0.177	0.000	0.125	0.185	0.194
CpP801	166	0.050	0.002 [c]	0.002	0.100	0.000	0.000	0.000
	170	0.063	0.168	0.177	0.100	0.000	0.146	0.222
	174	0.013	0.001 [c]	0.002 [b]	0.000	0.000	0.000	0.000
	178	0.338	0.167	0.170	0.300	0.125	0.177	0.000
	182	0.413	0.599	0.557	0.400	0.750	0.677	0.778
	186	0.125	0.063	0.092	0.100	0.125	0.000	0.000

Table 3. Number of individuals with registered provenance with and without potential parents in each assigned subpopulation. N – sample size.

	Dabeiba-Pancho (N = 7)	Ocarros (N = 2)	Merecure (N = 54)	Piscilago (N = 90)	Wisirare (N = 246)
Individuals with potential parents	7	1	53	62	202
Individuals without potential parents	0	1	1	28	44

Table 4. Coefficient of relationship and possible relationships within the founder crocodiles from Cravo Norte River (a) and Rango Rudd hatchery (b). Relationships: U = unrelated; HS = half sibling; FS = full sibling.

a. Cravo Norte

	575	579	581	584	592	593	1021	1072	1266			
575	–											
579	0.125 HS	–										
581	0.000	0.650 FS	–									
584	0.406 HS	0.000	0.000	–								
592	0.178 HS	0.614 FS	0.747 FS	0.000	–							
593	0.116 HS	0.445 FS	0.508 FS	0.000	0.544 FS	–						
1021	0.000	0.282 HS	0.401 HS	0.050 U	0.398 FS	0.483 FS	–					
1072	0.042 U	0.310 FS	0.395 HS	0.000	0.500 FS	0.417 HS	0.601 FS	–				
1266	0.000	0.269 U	0.369 FS	0.011 U	0.349 HS	0.285 HS	0.464 FS	0.592 FS	–			

b. Rango Rudd hatchery

	105	106	122	127	128	156	162	163	213	214	215	385
105	–											
106	0.575 FS	–										
122	0.500 FS	0.200 HS	–									
127	0.000	0.085 U	0.500 FS	–								
128	0.576 FS	0.243 HS	0.294 HS	0.302 FS	–							
156	0.142 HS	0.151 HS	0.353 HS	0.500 FS	0.000	–						
162	0.304 HS	0.275 HS	0.787 FS	0.366 HS	0.220 HS	0.272 HS	–					
163	0.000	0.000	0.402 HS	0.451 FS	0.102 U	0.264 HS	0.261 HS	–				
213	0.492 FS	0.306 FS	0.180 HS	0.172 U	0.006 U	0.283 HS	0.205 HS	0.000	–			
214	0.173 HS	0.132 HS	0.642 FS	0.321 HS	0.000	0.547 FS	0.615 FS	0.170 HS	0.248 HS	–		
215	0.000	0.000	0.434 FS	0.392 HS	0.000	0.714 FS	0.317 FS	0.213 HS	0.034 U	0.694 FS	–	
385	0.028 U	0.000	0.5 FS	0.303 HS	0.500 FS	0.000	0.330 HS	0.627 FS	0.027 U	0.188 HS	0.000	–

Management formulations

The number of alleles differed among the five subpopulations (Table 2). As expected, the largest subpopulations (EBTRF and Wisirare) showed unique alleles and the highest number of alleles (63 and 53 alleles, respectively), while the population with the lowest number of alleles was Piscilago (43 alleles). Although the other subpopulations did not show unique alleles, they did have alleles at very low frequencies (e.g., allele 203 locus Cj18 was present in five individuals or allele 193 locus Cj131 in only three individuals; Suppl. material 1: appendix S2). We identified and prioritized the management guidelines of 76 individuals that contained those rare alleles with low frequencies. The individual diversity (HL) of the living individuals that make up the entire *ex-situ* population varied between 0.075 and 0.947. However, 95.5% of the individuals that had an index lower than 0.6 and 74.8% were grouped between 0.2 and 0.5 (Suppl. material 1: appendix S3). No differences were found between the HL of founding crocodiles and living crocodiles (p-value = 0.292; $\alpha = 0.05$).

We found that in some cases current combinations of individuals are not the most appropriate when considering their genetic profiles. For example, the EBTRF represents the largest of the *C. intermedius* subpopulations and contains 97% of the alleles from the entire captivity program including 55 priority crocodiles and three unique alleles; but the subpopulation has no active reproductive nucleus. Piscilago has an F0 priority male in an isolated tank only for exhibition. The three males found in Ocarros are priority crocodiles since they have scarce alleles, but two of them are related to the females located there and they have not contributed to the growth of the captive population. Since genetic parameters for the selection of reproductive individuals must be urgently considered, we proposed changes and reorganized crocodiles in the subpopulations with combinations that guarantee the recovery of rare alleles and minimize the mean kinship. All the parental combinations were assembled by the combination of the *r* and HL indexes with important complementary information regarding every single crocodile (i.e., size, age, sex, origin, current location, capacity of the tanks). Using this information, we considered the priority crocodiles identified with the allele frequencies, combining them with unrelated crocodiles of reproductive age that showed the lower HL. We also considered whether the selected individuals had the appropriate size and health status, as well as if they had normal growth according to the growth model estimated for the EBTRF.

We reorganized the individuals that make up the reproductive nucleus of Ocarros and the two nuclei of Piscilago, and we selected the individuals of the two new nuclei from the Universidad de los Llanos. In Wisirare we proposed not to make changes considering that transport to Wisirare is complex, and since we found an unrelated kinship level and a low HL in the individuals that made up the breeding stock. For now, we recommend considering only the six reproductive nuclei mentioned above (Table 5). Once the individuals are reorganized, we provided program officials with the necessary information to establish new pairs in EBTRF and Merecure that will depend on the capacity of the tanks. We selected six males not suitable for reproduction (with no priority and high HL) to be taken to Piscilago (one individual) and to the Hacienda Nápoles Park in Puerto Triunfo, Antioquia (five individuals). These individuals are to take part in environmental education and awareness-raising, but not for reproduction.

Table 5. Past and present reproductive combinations for four *ex-situ* subpopulations of *Crocodylus intermedius* in Colombia. The values in parentheses represent the homozygosity by loci for each individual. The values in the table represent the relatedness (relationship) coefficient between both individuals compared. Females are in the rows, males in the columns. Individuals in bold represent priority crocodiles. Relationships: U Unrelated; HS Half sibling; FS Full sibling.

	Ocarros				**Piscilago**				
Past Situation	Unique tank				Tank 1		Tank 2 (isolated)		
	F/M	**154 (0.195)**	**156 (0.478)**	**157 (0.610)**	F/M	**214 (0.456)**	**213 (0.351)**		
	155 (0.453)	0	0	0	115 (0.233)	0	0		
	158 (0.226)	0.144 HS	0	0.14 HS	**118 (0.351)**	0.248 HS	1 FS		
Present combinations	F/M	**156 (0.478)**			Tank 1		Tank 2	Tank 3	
	155 (0.453)	0			F/M	**214 (0.459)**	193 (0.599) Isolated	F/M	**213 (0.351)**
	158 (0.226)	0			115 (0.233)	0		**238 (0.323)**	0
	172 (0.245)	0			258 (0.203)	0		239 (0.319)	0
	272 (0.239)	0			345 (0.306)	0		268 (0.289)	0
	Universidad de los Llanos				**Wisirare**				
Present combinations	Tank 1		Tank 2		Unique tank				
	F/M	**579 (0.303)**	F/M	**157 (0.610)**	F/M		385 (0.405)	389 (0.189)	
	174 (0.384)	0	194	0	384 (0.441)		0	0	
	203 (0.429)	0	240	0	387 (0.310)		0	0	
	255 (0.347)	0	256	0	388 (0.292)		0	0	
	262 (0.387)	0	257	0	391 (0.171)		0	0.173 U	
	274 (0.274)	0	270	0	392 (0.265)		0	0	
	276 (0.417)	0	275	0					
	286 (0.305)	0	332	0					
	290 (0.339)	0	450	0					
	576 0.309)	0	577	0.01 U					

Finally, we selected 84 juvenile individuals that have the genetic, size, health, and age requirements for release (sizes less than 2.5 m and HL < 0.6), and we moved them to a tank under semi-natural conditions in Merecure park.

Discussion

This study represents one of the few examples of the application of genetic tools for the management of captive-bred populations of endangered reptiles (Witzenberger and Hochkirch 2011), and these results are pivotal for the feasibility of the breeding program of the Orinoco crocodile in Colombia and as a strategy for its conservation. Our results revealed that the EBTRF living population maintains much of its founder diversity, high levels of heterozygosity, and a low overall inbreeding, and make it suitable for maintaining captive breeding and allowing wild releases.

Genetic diversity of the captive population of EBTRF

The expected heterozygosity obtained in the currently living crocodiles of the EBTRF is similar and even higher than that reported for wild populations of other species of the genus *Crocodylus*, evaluated with the same loci (e.g., Isberg et al. 2004; McVay et al. 2008; Mauger et al. 2017). This shows that, although the captive population of the Orinoco crocodile experienced an allele loss compared with the founder population, it maintains an important part of the variability in

terms of heterozygosity, with genetically viable individuals to be reproduced and used for conservation and management. This is because few breeding pairs comprised unrelated and genetically diverse individuals and there has not been a generational turnover that might result in reproduction between relatives.

Our results showed no statistical difference between observed heterozygosity and the allelic richness between the live and the founder populations. However, a decrease in variability was detected by the loss of alleles (Table 2). This phenomenon has already been reported in *ex-situ* population of other reptiles: in a captive population of the northern river terrapin *Batagur baska*, the first generation maintained only 73% of the founder alleles, while there were no significant differences in the heterozygosities between founders and the first generation (Spitzweg et al. 2018). Furthermore, in captive populations of the Jamaican yellow boa *Chilabothrus subflavus*, a loss of genetic diversity due to the differential reproduction of individuals was detected in the first generations by the allelic richness and not by the heterozygosities (Tzika et al. 2008). This reflects the limited efficiency of tests based on heterozygosity variations to detect recent inbreeding (Luikart et al. 1998; Tzika et al. 2008). A similar situation was detected in the EBTRF, where variations in allele frequencies showed that only a few reproducing founders segregated alleles to the next generation. Consequently, knowing the genetic profile of individuals is crucial for developing strategies that prevent genetic loss.

One of the objectives of captive breeding programs is to guarantee the survival of the offspring, which can be compromised by phenomena such as inbreeding and captive adaptation (Farquharson et al. 2021). We found that the inbreeding coefficient is higher in F0 than in the live population and in the living population we have deviations in seven loci due to excess heterozygotes while in the F0 population there are no loci with this deviation. This may be because, initially, many confiscated individuals came from the same breeding farm and thus may be closely related. However, most of these crocodiles did not reproduce; and, if they did, they were combined with wild or seized crocodiles, decreasing the F_{IS} of the living ones. For the future management of the program, generational change must be considered since species' response to captivity adaptation may differ in the first generation in relation to subsequent generations (Farquharson et al. 2021). Captive adaptation can improve fitness at the population level in the captive environment, but when animals are returned to the wild, captive adaptations can be maladaptive and contribute to the poor success of reintroduction programs (Frankham 2008; Jule et al. 2008).

The captive breeding program of the EBTRF plays a key role in Orinoco crocodile conservation. Nonetheless, management of these captive populations was not guided by the standards necessary to conserve and maximize genetic diversity, despite the previous recommendation for genetic monitoring (see Williams and Osentoski 2007). Furthermore, genetic management has been recommended within the actions framed in PROCAIMAN (MAM 2002). The only genetic characterization of the EBTRF *ex-situ* population was carried out by Cuervo-Alarcón and Burbano-Montenegro (2012). In this study, the sampling coverage of the captive population was limited; of the seven wild founders, only one was used in the analysis. We genotyped the same crocodiles and obtained different numbers of alleles for the same loci (e.g., 16 alleles at locus Cj16 versus five in our study). Furthermore, their division of the samples in two groups based on age is inadequate, returning inconsistent results with an admixture of

F0, F1, and F2 generations in the established groups, as well as an erroneous determination of crocodiles with wild origin.

The only other study of *C. intermedius* population genetics considering wild individuals was carried out in Hato El Frío in Venezuela by Lafferriere et al. (2016) and aimed at reporting multiple paternity in the species. When comparing the EBTRF population with El Frío Biological Station population, the Venezuelan individuals have a greater diversity in terms of allele composition with 90 alleles in the 17 loci. It is remarkable that the locus CpP1610 was monomorphic in our study while in Venezuela it was polymorphic with two alleles, but with one allele more frequent than the other (Lafferriere et al. 2016). However, the overall He / Ho level in the EBTRF was a little higher than in Venezuela (0.622 / 0.575 vs 0.524 / 0.544). This difference was probably due to different ways in which individuals reproduced since even though the Venezuelan individuals were born from reintroduced individuals, they follow the principles of a natural population; while, in the EBTRF they have been dependent on arbitrary human management that has reproduced the same individuals without a generational change. Finally, unlike the Venezuelan population that did not show significant deviations from Hardy-Weinberg expectations at any locus (Lafferriere et al. 2016), in the EBTRF we found deviations in 14 loci for live crocodiles. These deviations were expected since the animals originated from a few breeding pairs crossed without scientific basis or management.

Management guidelines

The breeding program for *Crocodylus intermedius* in Colombia aims to preserve and increase as much as possible the current genetic diversity and to produce neonates with the highest genetic diversity possible to support management actions (MAM 2002). Based on our data, we suggest that selective breeding should be implemented, and some mating combinations should be avoided. To achieve this goal, we proposed a robust system of 16 polymorphic microsatellite loci for estimating the relationship, the individual diversity and rarity of the living crocodiles. This, combined with information of age, size, sex, and location, allowed us to design combinations for planning breeding groups in each subpopulation. The tool enables a simultaneous maximization of genetic diversity, combining non-related diverse individuals and individuals containing rare alleles to achieve a genetic gain by minimizing the relationships between the individuals combined and guaranteeing no loss of alleles in the following generations.

Our work is necessary and complements the previous data, since most captive breeding projects are not monitored genetically, and only recently attention has been paid to the pedigree or relatedness of breeders using conservation genetic approaches (e.g. Spitzweg et al. 2018). Furthermore, this is one of the few studies combining relatedness information with the homozygosity by loci, which can be very useful when the number of individuals involved is large, allowing discrimination only when the *r* index may not be enough.

Despite the living crocodiles of our sample retaining approximately 90% of the genetic diversity of the wild-caught founder individuals with the presence of three unique and 13 rare alleles, the difference in the number of alleles and the allele frequencies among the five subpopulations revealed that the diversity is unevenly distributed between groups. If no action is taken to balance this, the loss of rare genetic diversity in the next few generations could be drastic, jeopardizing

the viability of the program (Groombridge et al. 2012). To solve this, we explicitly recommend using the combination of genetic data with the information from the archive records so as not to rely solely on the latter; since, as we found when performing the parent pairs analysis, a large part of the archive files is wrong in determining the origin of the individuals. Finally, we explicitly recommend implementing conservation genetic assessments for other captive breeding projects to preserve maximum genetic diversity and to avoid inbreeding depression (recommended by Xu et al. 2005; Tzika et al. 2008; Spitzweg et al. 2018).

The EBTRF conservation program covers a very restricted range of the historical natural distribution of the species in Colombia, and key individuals (e.g., from Vichada department) had rare alleles, suggesting that the genetic diversity of the Station does not cover the unknown threatened possible diversity available in the wild. It is necessary and urgent to evaluate wild populations, as well as to enrich the diversity of the Station´s population by including wild individuals from unsampled sites (e.g., Guayabero / Duda / Lozada Rivers). These individuals must be genotyped to determine the presence of rare alleles, individual genetic diversity, and degree of relationship. As we demonstrated here, the basic assumption of unrelated founders may be incorrect, particularly given the often-imprecise nature of information on their origin (Gautschi et al. 2003). We recommended completing the dataset with the missing crocodile samples and including them in the management guidelines. In turn, it is necessary to genotype the crocodiles that are going to be born to have a complete genetic profile of the program, to evaluate future trends in allele frequencies and to restructure combinations if necessary.

The EBTRF contains the largest subpopulation (about 370 individuals), the largest number of tanks available, and a high genetic diversity involving three unique alleles. More than 150 crocodiles have passed through the EBTRF and have died from recent hatchlings to the first clutches of 1991 and the F0. After 2005, fewer eggs from the EBTRF were incubated since eggs from Wisirare, Piscilago and later Occarros began to be carried to the Station for incubation. Considering that the EBTRF subpopulation has the highest number of adult crocodiles with unique diversity, it is necessary to re-implement the breeding stock with these individuals. It is urgent to maintain a balance in the proportion of eggs incubated according to their origin and the number of parents that produce them. In the EBTRF we found juvenile individuals that we considered as priority because they contained alleles at low frequencies (Suppl. material 1: appendix S2). However, these individuals have not attained reproductive age, so we recommend keeping them until they can be included in the reproductive nuclei.

Future perspective

Through the implementation of the crosses proposed here, the program will ensure highly genetically variable offspring that preserve the available genetic diversity. By combining the offspring produced by different reproductive pairs, we will be able to form groups of unrelated and highly diverse individuals that, according to the requirements of natural populations, could be released into the wild.

This research supports the actions defined in PROCAIMAN to advance the recovery of populations of the Orinoco crocodile in Colombia. This is of urgent application since, even though management actions were established 20 years

ago, the natural situation of the species has apparently not changed or even has deteriorated (see Medem 1981; Lugo 1996; Seijas et al. 2010; Espinosa-Blanco and Seijas 2012; Babarro 2014; Parra-Torres et al. 2020). By using the genetic system developed here, it is urgent to genetically characterize wild populations to define whether they need genetic management. The evaluation of the captive populations together with natural populations, as well as demographic and ecological studies, should guide the recovery of eggs and hatchlings both *ex-situ* and *in-situ* and define a reintroduction protocol, including the monitoring of introduced and wild populations.

However, more support and research are needed to comply with what has been established in PROCAIMAN (MAM 2002). Currently, neither the population sizes nor important ecological characteristics are known, such as whether there is reproduction in wild populations, the magnitude of reproduction, physiological parameters associated with reproductive events, nesting times, demographic structure of populations, etc. As a crucial component for the success in the recovery of the species, focused environmental education and awareness initiatives must be developed. These efforts should aim to foster actions and establish an extensive dialogue with human communities regarding the coexistence of this Colombian crocodile and its recovery.

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Additional information

Conflict of interest

The author has declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: MVR, AMSG. Data curation: AMSG, MVR. Formal analysis: AMSG, MVR. Funding acquisition: AMSG, MVR. Investigation: MVR, AMSG. Methodology: AMSG, MVR. Project administration: AMSG, MVR. Resources: AMSG, MVR. Software: AMSG, MVR. Supervision: MVR, AMSG. Validation: AMSG, MVR. Visualization: AMSG, MVR. Writing – original draft: MVR, AMSG. Writing – review and editing: AMSG, MVR.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary information

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Data type: tables and figure (word document)

Explanation note: **appendix S1.** Provenance of the wild-born crocodiles. **appendix S2.** Information of priority crocodiles presenting alleles at low frequencies. **appendix S3.** Distribution of individual diversity (homozygosity by loci, HL) of the living crocodiles that make up the *ex-situ* population managed by the Roberto Franco Biological Tropical Station.

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