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GENÉTICA DE LA CONSERVACIÓN DE LA MAYOR POBLACIÓN CAUTIVA DEL CRÍTICAMENTE AMENAZADO CAIMÁN DEL ORINOCO (*Crocodylus intermedius*): UNA CONTRIBUCIÓN PARA SU SUPERVIVENCIA

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Universidad Nacional de Colombia

Facultad de Ciencias, Departamento de Biología

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A mis padres por apoyarme y acompañarme durante todo el camino

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Resumen

Genética de la conservación de la mayor población cautiva del críticamente amenazado caimán del Orinoco (*Crocodylus intermedius)*:
Una contribución para su supervivencia.

En el último siglo ha habido un aumento en el número de especies amenazadas y la conservación a través de programas de cría en cautiverio se ha vuelto crucial para su supervivencia. Una de las principales consideraciones para el diseño de programas de reproducción es la preservación de la variabilidad genética que proporciona la materia prima para la adaptación. Si el manejo se basa solo en los pedigrí registrados, la información puede estar incompleta o inexacta y puede llevar a una subestimación de las relaciones de parentesco. Las acciones de manejo incorrectas pueden alterar la viabilidad de las reintroducciones debido a la pérdida de diversidad y depresión genética de la población de origen. En esta tesis de Maestría, utilizamos un sistema de 17 loci de microsatélites para caracterizar la variación genética de la mayor población *ex-situ* del críticamente amenazado *Crocodylus* intermedius en Colombia a cargo de la Estación de Biología Tropical Roberto Franco (EBTRF) con el objetivo de proponer pautas de manejo y evaluar reintroducciones pasadas y futuras. En el Capítulo 1 comparamos los índices genéticos de las poblaciones Fundadora y Viva y encontramos que los cocodrilos vivos mantienen gran parte de la diversidad fundadora, altos niveles de heterocigosidad y una baja consanguinidad. En el Capítulo 2 desarrollamos una poderosa herramienta que combina información de parentesco, diversidad individual, edad, sexo, tamaño y ubicación de los cocodrilos vivos por medio de la cual construimos combinaciones de individuos para planificar futuros grupos reproductores que maximicen la diversidad genética de la población. Proponemos diferentes núcleos reproductivos y demostramos que los datos moleculares pueden ser utilizados para mejorar la gestión del programa mucho más allá de lo que se puede lograr solo con la información del pedigrí. Para proporcionar información sobre el componente genético de los individuos liberados y sugerir mejoras en las reintroducciones, en el Capítulo 3 evaluamos la composición genética de cuatro grupos de cocodrilos reintroducidos y de los juveniles que serán liberados. Proponemos que, a corto plazo, las reintroducciones solo se realicen en lugares donde se tenga la certeza de que las poblaciones se han extinguido por completo. En caso de que la especie esté presente, antes de implementar medidas de reintroducción, es necesario evaluar con precisión su perfil genético y su situación, así como estimar el tamaño de la población.

Palabras clave: microsatélites, variabilidad genética, potencial evolutivo, cría en cautividad, diversidad individual, reintroducción.

Abstract

Conservation genetics of the largest captive population of the critically endangered Orinoco crocodile (*Crocodylus intermedius*): A contribution for its survival.

During the last century, many species have become endangered, and conservation through captive breeding programs has become crucial for their survival. One of the primary considerations for the design of reintroduction programs is the preservation of genetic variability, which provides the raw material for adaptation. If management is based only on recorded pedigrees, information may be incomplete or inaccurate and may lead to an underestimation of relatedness. Incorrect management actions can alter the viability of reintroductions due to the loss of genetic diversity and genetic depression of the source population. In this Master thesis, we used a 17 microsatellite loci system to characterize the extent of the genetic variation of the biggest *ex-situ* population of the critically endangered *Crocodylus intermedius* in Colombia in charge of the Roberto Franco Tropical Biology Station (EBTRF) aiming at proposing management guidelines and at assessing past and future reintroductions. In Chapter 1 we compared genetic indexes of the Founder and Alive populations and we found that the living crocodiles maintain much of the founder diversity, high levels of heterozygosity, and a low overall inbreeding. In Chapter 2 we developed a powerful tool that combined information of relatedness, individual diversity, age, sex, size, and location of the living crocodiles that allowed us to build combinations of individuals to plan future breeding groups that maximize the population´s genetic diversity. We propose different reproductive nuclei, and we demonstrate that molecular data can be used to improve the management of the program, well beyond of what can be achieved with pedigree information alone. To provide insights on the genetic component of the released individuals and to suggest the improvement of crocodile's reintroductions, in Chapter 3 we evaluated the genetic composition of four groups of crocodiles already reintroduced and of juveniles to be released. We propose that in the short term, reintroductions should only be carried out in places where it is certain that the populations have become completely extinct. In case the species is present before implementing reintroduction measures it is necessary to accurately assess its genetic profile and situation as well as to estimate population size.

Key words: microsatellites, genetic variability, evolutionary potential, captive breeding, individual diversity, reintroduction.

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Introduction

Time is running out for many of the world's animal species. Processes such as habitat loss, species introduction, overexploitation, pollution, and climate change, combined with stochastic factors, are the main drivers of species loss (Primack, 2002; Bertorelle *et al.,* 2009). By 2020, 9677 species of vertebrates were listed by the International Union for the Conservation of Nature (IUCN) as vulnerable, endangered, or critically endangered, which represents 18% of the total number of species evaluated. Facing this situation, Conservation Biology emerges as a 'crisis discipline' that combines ecology, taxonomy, genetics, and other areas of knowledge to stem the rapid rise of species loss, supporting decision-making and seeking for the protection of threatened species (Bertorelle *et al.,* 2009).

Conservation genetics

Conservation biology requires an efficient, cheap, and rapid method to obtain the information necessary for the implementation of conservation strategies, being population genetics one of those powerful instruments (Bertorelle *et al.,* 2009). With the use of mathematical models and molecular genetic data, it is possible to estimate crucial parameters for the evaluation of the health of natural populations and their long-term viability, such as effective population size, abundance, population fragmentation, gene flow, genetic drift, genetic diversity, sex ratio, patterns of mate choice, relatedness, effective and sex-specific dispersal rates, levels of inbreeding, introgressive exchange, viable population size, breeding system, effects of bottlenecks and structure (Bertorelle *et al.,* 2009).

The availability and application of molecular tools for biodiversity conservation have advanced considerably over the last 20 years, but microsatellites are still the most used tool for population genetics (Witzenberger & Hochkirch, 2011). Even though single nucleotide polymorphisms (SNPs) can have a higher precision (Roques *et al.,* 2019) microsatellites offer a cost advantage which is particularly important in research with low budgets. The potential analytical range of microsatellites extends from species to the community level and on the spatial/temporal scale; additionally, they are useful in population analyses, which include paternity (Lafferrier *et al.,* 2016), kinship (Recino-Reyes *et al.,* 2020), effects of reduced population sizes (Bishop *et al.,* 2009) and effects of

reintroduction and restocking (Rodriguez *et al.*, 2011). Currently, the principal disadvantage of microsatellites is the limited primers availability for many groups, a step that can be time-consuming and expensive. Fortunately, the potential for cross-species amplification – the amplification using microsatellite primers developed for a species in nearby taxa – and the constant increase of primers development, considerably reduce costs and allow the use of already designed tools.

One of the main uses of microsatellites is for the estimation of genetic diversity, one of the most important attributes of any population, defined as the variation in the amount of genetic information within and among individuals of a population, species, assemblage, or community (United Nations, 1992). The evaluation of this parameter in natural and captive populations is important to characterize the population structure (Witzenberger & Hochkirch, 2013), history (Karsten *et al.,* 2011), and hybridity (Weaver *et al.,* 2008). Additionally, it is a crucial parameter for taking decisions in breeding programs developed for the reintroduction of individuals (Lapbenjakul *et al.,* 2017).

Ex-situ populations and their utility for endangered species conservation

Despite *in situ* conservation represents by far the most effective way to protect endangered species, *ex-situ* conservation of captive-bred animals has become an important tool to protect endangered species, and in many cases the only way to save them from extinction (Frankham *et al.*, 2007, Bertorelle *et al.,* 2009, Witzenberger & Hochkirch, 2011). For this purpose, captive breeding programs can serve for the establishment and conservation of a healthy and self-sustaining population, and the formation of a captive stock that resemble wild populations as closely as possible for being a source for reintroductions (Frankham 2008; Goncalves da Silva *et al.* 2010; Witzenberger & Hochkirch, 2011). Nevertheless, if there is no adequate management, *ex-situ* populations can be affected by various phenomena that can alter the viability of reintroductions, such as the genetic adaptation to captivity, the genetic depression due to inbreeding and the loss of genetic diversity that occur from the moment of the foundation since the gene pool of the wild population is only represented by the individuals used in the foundation process (Frankham *et al.,* 2007, Witzenberger & Hochkirch, 2011).

Population genetics can assist captive conservation programs by providing tools that allow the formulation of guidelines that reduce the loss of genetic diversity to the maximum.

Variation estimated with molecular markers can be used to reconstruct pedigrees, assess founder relationships (Gautschi *et al.* 2003), identify genetically important individuals (Rusello & Amato, 2004), and compare wild and captive populations (Spitzweg *et a.,* 2018). Considering that in many cases the kinship relationships are not known, molecular genetic analyses guarantee the design of more strategical crosses (Witzenberger & Hochkirch, 2011).

Crocodylus intermedius conservation program in Colombia

Crocodilians are an ancient and successful group, considered the most economically valuable reptiles, with a global trade of skin, meat, tourism, and trophy hunting that generate economically sustainable-use programs that have underpinned many of the conservation projects since the 1980s (Caldwell, 2017; Somaweera *et al.*, 2018). However, the increase of human hunting pressures has led to significant declines in their populations worldwide. Colombia, the most diverse country in crocodilians species of the world (Morales-Betancourt *et al*., 2013), has two species classified with some degree of threat: *Crocodylus acutus* as Vulnerable (VU) and *Crocodylus intermedius* as Critically Endangered (CR) (IUCN, 2020).

Crocodylus intermedius has suffered a profound decline in their populations during the XX century, caused by commercial hunting and collection of eggs for local consumption (Castro-Casal *et al.,* 2013). Historically, the species was widely distributed throughout the Orinoco Basin in Colombia and Venezuela, inhabiting almost all large rivers (Medem, 1981). The intense hunting carried out between 1930 and 1960 in the Llanos of Colombia and Venezuela driven by the commercial trade for its skin nearly led to its extinction (Castro *et al.,* 2012). In Colombia, between 252,300 and 254,000 skins were traded during the hunting period, and between 1929 and 1934 it is estimated that 850,000 skins were exported from Venezuela (Medem, 1981; Medem, 1983; Castro *et al.,* 2012). Around 1940, the skin market began to decline due to the decrease of the species' populations, although opportunistic hunting continued (Medem, 1981; Thorbjarnarson, 1987; Castro-Casal *et al.,* 2013). Regardless of the scarce and imprecise information that exists, it has been estimated that between two and three million of skins could have been exported, although this number could be considerably higher (Thorbjarnarson, 1987; Antelo, 2008; Castro-Casal *et al.*, 2013).

To prevent the extinction of the species and to promote populations recovery, integrating it into the regional economic and cultural systems, the Ministry of the Environment (MMA by its acronym in Spanish), the Alexander von Humboldt Biological Resources Research Institute (IAvH) and the National University of Colombia (UNAL) formulated the National Program for the conservation of the Orinoco Crocodile (PROCAIMÁN) in 1998 (posteriorly updated in 2002). One of the conceived strategies of the program is the repopulation of natural habitats, where the Roberto Franco Tropical Biology Station (EBTRF), of the Faculty of Sciences of the National University of Colombia, located in Villavicencio, Meta, plays a crucial role keeping and breeding individuals in captivity (Ardila-Robayo *et al.* 2010, Posso-Peláez *et a*l., 2018).

The *ex-situ* population of the EBTRF originated in 1970, when Federico Medem motivated by the notable population decline of the species, began the formation of the breeding center (Lugo, 1995). That year, the first pair was established with a seven-year-old male named Polo coming from Puerto Alicia, near Puerto López, Meta River, Meta department, and a female, named Dabeiba, from Puerto López, Río Meta, Meta department. In 1975, another male named Custodio arrived from San Carlos de Guaroa, Río Metica, Meta department, and in 1976 an old female named Lizeth arrived, coming from Charco Gaitán, Humea River, Meta department. In 1986, a male named Pancho arrived from Caño Yatea, Bocas del Guachiría, Meta River, Casanare department. In 1979, the Lizeth-Custodio and Dabeiba-Polo reproductive pairs were established, and although the first nesting occurred in 1986, only until 1991 the first offspring were obtained, with the implementation of a room with controlled humidity and temperature. Between 1986 and 1996, 25 donated and confiscated individuals arrived at the Station. Today, many of the confiscated individuals and the five founding crocodiles are dead but they left offspring that generated most of the station's population, and in 2004 the first F2 generation was obtained. Currently, there are 593 crocodiles under the Station care of which more than 90% are the product of *ex-situ* reproductive events: 361 distributed at the station itself in Villavicencio, 19 in Merecure Agrological Park in Villavicencio, 203 in Wisirare Park in Orocué – Casanare department, five in Ocarros BioPark in Villavicencio, and five in Piscilago conservation Park in Nariño – Cundinamarca department. Together, these individuals probably exceed the number of crocodiles known in wildlife in all the distribution range of the species in Colombia (Posso-Peláez *et al.,* 2018).

Despite the diversity of ages and the large number of crocodiles that are kept in captivity, the Station has not been able to make the number of releases that are expected in accordance with its potential and the measures established in the conservation plan. This is due to several reasons; firstly, before 2004 the Station did not have the guidelines for the management of animals such as filling databases with the information of every individual, a posture control, or monitoring of offspring and reproduction among others (Maldonado & Ardila, 2004). Without this information, it is not possible to establish the individuals eligible to be released. Moreover, in the National Program for the Conservation of the species (PROCAIMÁN), it is established that a genetic characterization of the crocodiles of the Station needs to be made to implement management that maintains and increases genetic variability, as well as a genetic characterization of the specimens to release (MMA, 2002). Although there is a genetic study (Cuervo, 2010; Cuervo-Alarcón & Burbano-Montenegro, 2012.) this one was hampered by some fundamental problems that make the results not conclusive enough to allow the appropriate genetic management that would permit the releases.

Frankham *et al.* (2007) established that a captive breeding and reintroduction program can be viewed as a process involving six stages: 1. the decline of the wild population, 2. the foundation of a captive population, 3. the growth of the captive populations to a secure size, 4. the management of the captive population over generations, 5. the selection of individuals for reintroduction, and 6. the management of the reintroduced population (Frankham *et al*, 2007). Considering that in Colombia the captive breeding conservation program of *C. intermedius* is stagnant in stage four, in this Master thesis we aimed at performing a conservation genetics study of the captive population in charge of the EBTRF using microsatellites molecular markers, allowing to advance in stages four, five and six mentioned above. First, we present a Chapter that evaluates the loss of genetic diversity since the foundation and the genetic potential currently available. Second, we present a Chapter where we formulated guidelines and recommendations to preserve the current genetic diversity as much as possible by determining the best breeding combinations. This section also determines the relationship between the founders and captive-bred individuals. Finally, the third Chapter focuses on releases, where we identify the most distant captive-bred offspring for reintroduction and evaluate the genetic profile of individuals released in four different rivers. The study also presents a reflective discussion on the future of the

captive breeding program and the enormous potential it has for repopulation aimed at the recovering of the wild populations of *C. intermedius* in Colombia.

Chapter 1

Captive bred populations of the critically endangered Orinoco Crocodile (*Crocodylus intermedius*) are genetic reservoirs to save the species from the extinction in Colombia.

1.1 Abstract

Ex-situ conservation programs and reintroduction of captive-bred animals have become an important tool to protect endangered species, and an example of this is the captive breeding program of *Crocodylus intermedius* in the Roberto Franco Tropical Station (EBTRF) in Colombia. Despite the large number of individuals kept in captivity, the Station has not been able to release individuals in part by the lack of a genetic characterization that determines the current genetic potential of the population. In this study, we used a panel of 17 microsatellites loci to estimate the number of alleles, allelic richness, allelic frequencies, inbreeding, and heterozygosities of the Founder and Alive crocodiles to understand at the genetic level, the effect of managing for 50 years a captive breeding program without considering genetic profiles. Our results revealed that despite having lost 7.5% of the diversity in terms of the number of alleles, the EBTRF living population maintains much of its founder diversity, high levels of heterozygosity, and a low overall inbreeding, making it suitable for maintaining captive breeding and making wild releases. However, some alleles are present in very low frequencies, so management measures should not only seek to maintain high levels of heterozygosity but also to prioritize the reproduction of individuals that have rare alleles in order not to lose them.

Key words: *ex-situ* conservation, microsatellites, allelic richness, genetic diversity loss.

1.2 Introduction

In the last century, several species became endangered and require intensive management to ensure their survival. Although *in-situ* conservation represents the most effective way to protect endangered species, *ex-situ* conservation programs and reintroduction of captive-bred animals have become an important tool to protect endangered species (Witzenberger & Hochkirch, 2011), and in many cases, such programs are the only way to save them from extinction (Bertorelle *et al.,* 2009). However, nowadays the aim of the *ex-situ* conservation programs goes beyond the survival of the individuals and targets the conservation of the genetic diversity over long periods (Ramirez *et al.,* 2006).

In captive breeding and liberation conservation programs, genetic diversity is a primary component of adaptive evolution and is essential to ensure the evolutionary potential that allows populations to adapt to changing environments (Frankham *et al.* 2002). The loss of genetic diversity occurs from the moment of foundation, since the gene pool of the captive population is only represented by the individuals used in the foundation process, and if there is not adequate management of the population, there may be a differentiation of the captive population with respect to the wild, causing harmful effects at the time of reintroductions (Frankham *et al.,* 2002). Different strategies can be implemented to minimize the loss of genetic diversity in captive populations. One of them is the use of polymorphic molecular markers such as microsatellites, which framed in the theory of population genetics can provide insights into processes such as the change and loss of genetic variability that is difficult or impossible to study via traditional approaches (Alcaide *et al.,* 2009).

The Orinoco Crocodile (*Crocodylus intermedius*) is endemic to the Orinoco Basin in Colombia and Venezuela, being one of the most endangered species among the 23 extant crocodilians species of the world, and it is considered as the most threatened vertebrate species in the Neotropics (Moreno-Arias & Ardila-Robayo, 2020). During the 20th century, commercial hunting of the Orinoco Crocodile motivated by the high demand for its skin brought it to the brink of extinction. Currently, the population status of the species is unknown and last censuses report a general trend of poor recovery or population decline (Medem, 1981; Lugo 1996; Seijas *et al.* 2010; Espinosa-Blanco & Seijas 2012; Babarro, 2014; Parra-Torres *et al.,* 2020). Thus, the Orinoco Crocodile is considered as “Critically Endangered” by the International Union for the Conservation of Nature (IUCN) and it is included in

Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; Castaño-Mora, 2002).

Protection measures were contemplated since 1970s in Colombia and Venezuela by setting an indefinite ban on the exploitation of the species (Godshalk 1982, Catro-Casal *et al.*, 2013). At the same time, motivated by the critical situation of *C. intermedius*, Federico Medem started the principal captive breeding program for the conservation of the crocodile in Colombia at the Roberto Franco Tropical Biology Station (EBTRF) of the Faculty of Sciences of the National University of Colombia located in Villavicencio - Meta department. In the 50 years of the program, the crocodile population of the EBTRF grew from the reproduction of 26 confiscated individuals and five wild crocodiles to the actual size of almost 600 individuals distributed in five different locations known as *ex-situ* subpopulations: Piscilago, Wisirare, Merecure, Ocarros, and the EBTRF.

Since 1998 the EBTRF is part of the National Program for the Conservation of the Orinoco Crocodile (PROCAIMÁN; MAM, 2002) as it represents the largest stock of individuals of the species in Colombia, probably containing more crocodiles than those found in the wild (Posso-Peláez *et al.,* 2018). Nevertheless, despite the diversity of ages and the large number of individuals kept in captivity, the Station has not been able to release individuals principally by the lack of a genetic characterization that determines the most apt individuals to be released, as well as a management that maintains and increases the population´s genetic variability. In addition, due to the long time that the program has been in operation, it is not known the loss of diversity that could have been generated from the moment of the foundation in 1970 to today.

Therefore, in this study, we aimed at evaluating and comparing genetic diversity parameters of Founder and Alive individuals of *C. intermedius* in the EBTRF. For this, we used a panel of 17 microsatellites loci to estimate allelic richness, frequencies, and heterozygosities in living and founder crocodiles, to understand at the genetic level the effect of managing a captive breeding program without considering the genetic profile of the individuals and the population.

1.3 Methods

1.3.1 Sampling

Since 2004 tissue samples have been taken from most of the crocodiles 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. Since the Station does not have a database where the information of the individuals is reported, a search was made according to the archive records deposited in the Station's file to know about the origin and status of the crocodiles. All the animals are microchipped allowing individual identification.

In total, the study included 461 individuals. The complete dataset comprised 37 crocodiles from wild origin either acquired through direct captures (with known geographical origin) or seizures from wildlife and breeding centers. These included five of the six dead wild founders (Lizeth, Dabeiba, Pancho, Custodio, and Juancho, except for Polo who died in 1998), two dead individuals of Vichada River, six young individuals of Cravo Norte River (one dead and five with 11 years old in 2021), and 24 seized individuals (13 dead). The remaining 424 samples corresponded to captive offspring (F1 and F2, Figure 1).

1.3.2 Laboratory procedures and genotyping

Genomic DNA was extracted from preserved tissue using the Invisorb® Spin Tissue Mini Kit (Stratec) following manufacturer protocols. Since no specific primers for microsatellite loci of *C. intermedius* are available, 17 primers developed for other species of the genus and already evaluated for cross-amplification by Laferriere *et al.,* (2016) were used in the present study. To amplify microsatellite DNA, four PCRs multiplex were performed (Table 1) using the Multiplex PCR kit MyTaq™ HS Mix (Bioline, USA). 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 that 0.4 µL were added), a final concentration of 4ng/ µL of DNA and the excess of ultra-pure water to complete. 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 (Table 1) 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 using as reference the alleles reported by Laferriere *et al.,* (2016). Genetic laboratory work was conducted at the Molecular Ecology Laboratory of the Genetics Institute, National University of Colombia in Bogotá.

1.3.3 Data Analysis

For the estimation of the genetic diversity, the EBTRF crocodile population was subdivided into two groups. The first group was composed of 37 F0 crocodiles coming from natural populations or confiscated, who represent the genetic potential that the Station has had since it was founded. The second group contained 440 individuals including F0, F1, and F2 generations distributed in the different *ex-situ* subpopulations of the station, which represent the current potential diversity of the EBTRF. We took this division considering that the presence of both young and adult individuals in each generation and the low reliability of the archive records, prevented subdividing the population by generations.

To estimate null allele frequencies at each locus on the whole dataset we used FreeNA (Chapuis & Estoup, 2007) and CERVUS 3.0.7 (Kalinowski *et al.,* 2007) software, and null alleles were considered when the frequency was higher than 0.05 in both programs results. 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; Bonferroni corrections were applied for both calculations. The number of alleles per locus (nA), allelic richness (AR), allelic frequencies and inbreeding coefficient (F_{IS}) were calculated in 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 & Rousset, 1995). Statistical significance differences for allelic richness, Ho, and F_{IS} between population subdivisions were tested with 15,000 permutations in FSTAT 2.9.3.2 (Goudet, 2001). Allele dropout was estimated using MICRO-CHECKER 2.2.3 (van Oosterhout *et al*., 2004).

1.4 Results

The 17 microsatellite loci were successfully amplified for 458 of the 461 available samples from crocodiles of the EBTRF *ex-situ* population. For the other three samples, between one and six loci were not obtained. One of the 17 loci resulted monomorphic (CpP1610) in both population divisions and there was no evidence for loci with null allele frequencies or for allele dropout.

A total of 69 alleles were observed in the sampled individuals but the number differed between both subpopulations: 67 in F0 crocodiles and 64 in Alive crocodiles, representing 92.5% of the F0 alleles (Table 2). The F0 population presented five private alleles while the Alive population presented two (Table 3), suggesting that in the living population we found wild individuals or with non-genotyped parents. The number of alleles per locus ranged from 2 to 9 in the F0 population with a mean of 4.2, and from 2 to 8 with a mean of 4 in the Alive population. The allelic richness varied between 2 and 5.391 (averaging 3.853) in the F0 population and between 2 and 4.955 (averaging 3.369) in the Alive population. Some alleles had considerably higher frequencies than others (e.g., 337 vs. 341/343 in locus Cj127, Table 3). Although there were loci where allele frequencies did not change considerably between F0 and Alive populations (e.g., CpP3216, CUJ131, CpDi13), there were other loci that showed strong changes, and even loss of alleles (e.g., Cj109, Cj18, Cj391, Cpp801).

The level of observed heterozygosity (Ho) varied between 0.081 and 0.784 (averaging 0.573) in the F0 population and between 0.339 and 0.813 (averaging 0.617) in the Alive population. The expected heterozygosity (He) varied between 0.080 and 0.807 (averaging 0.587) in the F0 population and from 0.297 and 0.769 (averaging 0.574) in the Alive population. The inbreeding coefficient F_{IS} varied between -0.168 and 0.198 (averaging 0.025) in the F0 population and between -0.195 and 0.122 (averaging -0.075) in the Alive population.

Six loci in the F0 population and 14 loci in the Alive population showed significant deviation from the Hardy-Weinberg equilibrium after Bonferroni corrections, and the six F0 loci in deviation were shared with the Alive loci in deviation. No significant linkage disequilibrium was found between pairs of loci. The F0 population showed deviations in the F_{IS} coefficient in one locus for defect of heterozygous, and in five loci por excess of heterozygous. In the Alive populations two loci presented deviations in the F_{IS} coefficient for defect of heterozygous (Table 2). Even though the Alive 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 allelic richness and F_{IS} compared to the Alive ones, differences were statistically not significant (AR p = 0.169, F_{IS} p =1).

1.5 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, as most of the studies are conducted in mammals and birds (Witzenberger & Hochkirch, 2011). The results presented here are pivotal for the feasibility of the captive breeding program of the Orinoco Crocodile in Colombia, 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, making it suitable for maintaining captive breeding and making wild releases.

Howbeit, the EBTRF population covers a very restricted range of the historical natural distribution of the species in Colombia, and key individuals (e.g., from Vichada department) presented rare alleles, suggesting that the genetic diversity of the station does not cover the unknown threatened possible diversity available in the wild. For this reason, it is necessary and urgent to evaluate wild populations, as well as to enrich the diversity of the Station´s population by bringing wild individuals coming from unsampled sites (e.g., Guayabero / Duda / Lozada Rivers) and ensuring their reproduction.

1.5.1 Genetic diversity of captive population of EBTRF

The expected heterozygosity obtained in the currently living crocodiles of the EBTRF is similar and even higher to that reported for other wild populations of species of the genus *Crocodylus*. An He of 0.552 has been reported for *C. moreletii* (McVay *et al.* 2008), 0.572 for *C. acutus* (Mauger *et al*., 2017), 0.579 for *C. porosus* (Isberg *et al*., 2004), 0.45 for *C. niloticus* (Hekkala *et al.,* 2010) and between 0.47 and 0.66 for captive populations of *C. rhombifer* (Weaver *et al.,* 2008). This shows that although the captive population of Orinoco Crocodile experienced an allele loss compared with the Founder population, it maintains an important part of the variability in terms of heterozygosity, presenting genetically viable individuals to be reproduced and used for conservation and management purposes.

Our results showed that there is no statistical difference between the observed heterozygosity values and the allelic richness between the Alive and the Founder populations. However, a decrease in variability is detected by the loss of alleles (Table 3). This phenomenon had already been reported in captive populations of the Jamaican yellow boa *Chilabothrus subflavus* (Tzika *et al.,* 2008), where a loss of genetic diversity is detected in the first generations by the allelic richness and not by the heterozygosities, reflecting the limited efficiency of tests based on heterozygosity variations to detect recent inbreeding (Luikart e*t al.*, 1998). Such consequence has been related to the differential reproduction of the individuals (Tzika et al., 2008). A similar situation was detected in the EBTRF, where the variations in allele frequencies showed that only few reproduced founders segregated alleles to the next generation. This result is also confirmed by the archive records of the individuals and the information provided by the personnel of the station. Also, heterozygosity is important for the short-term success of captive populations but is an overly optimistic estimate of the effects of a bottleneck in the long term, since little heterozygosity is expected to be lost (Nei et al., 1975; Allendorf, 1986). Therefore, the loss of alleles is a more appropriate measure to evaluate the loss of genetic diversity since it will have a significant effect on the future adaptability and survival of species in the wild (Allendorf & Luikart, 2007, Jamieson & Lacy, 2012). Consequently, knowing the genetic profile of individuals is crucial to develop strategies to prevent genetic loss, since for example one founder from Vichada River (microchip 95919774) did not reproduce causing the loss of four alleles in the current captive population.

According to our inbreeding results, most of the living crocodiles are not related (Table 2). This is because the few breeding pairs comprised unrelated and genetically diverse individuals. Furthermore, there has not been a generational turnover that may cause the reproduction between relatives. It is noteworthy that the inbreeding coefficient is higher in F0 than in the Alive population, and that in the living population we have deviations in five loci due to excess heterozygotes while in the F0 population there is only none. This may be because initially there were many confiscated individuals that came from the same breeding farm, which were possibly related to each other. However, most of these crocodiles did not reproduce and, if they did, they were combined with wild or seized crocodiles, generating a decrease in the F_{IS} of the living population and even an excess of variability.

When comparing with the F0 population, two unique alleles were found in the Alive one. Firstly, allele 203 of locus Cj18 is present in five individuals that came from eggs collected

in Merecure, one of the *ex-situ* subpopulations of the Program. Information provided by one of the Station's oldest officials (Willington Martínez) suggested that there was an F0 in Merecure, probably dead and not genotyped, that could probably be the source of this allele. Additionally, allele 354 of the CCj101 locus was found in nine juvenile crocodiles from Wisirare that came from wild collected eggs.

In the Alive population, some alleles have very low frequencies (e.g., allele 203 at locus Cj18; allele 193 at locus Cj131; alleles 157, 161, 171, 173, and 179 at locus Cj391; Table 3). In that sense, to maximize the genetic variability of the station, two management goals need to be considered: first, to maintain high levels of heterozygosity by combining unrelated genetically variable individuals, and second to prioritize individuals that have rare alleles to not lose them. Considering that the captive population is not completely hosted in Villavicencio, it is necessary to evaluate the genetic profile of each *ex-situ* subpopulation to identify crocodiles with rare alleles to restructure the breeding pairs. We are sure that with the introduction of a breeding strategy that considers the genetic profile of each individual and combines less related crocodiles, the percentage of genetic diversity preserved can be significantly increased.

1.5.2 Genetic diversity respect to wild populations

The single study of *C. intermedius* population genetics considering wild individuals was carried out in Hato El Frío in Venezuela by Laferriere *et al.* (2016). When comparing EBTRF population with El Frío Biological Station population, the Venezuelan individuals have a greater diversity in terms of alleles composition, with 90 alleles in the 17 loci (an average of 5.3 alleles per locus) compared to 69 in the EBTRF (a maximum average of 4.19 alleles per locus). 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 strongly predominant over the other. This considerable difference is probably because the founding crocodiles in the EBTRF were few, especially those from the wild, causing a genetic bottleneck during the foundation process.

Contrarily, the He / Ho level in the EBTRF was a little higher than in Venezuela (0.617 / 0.574 vs 0.524 / 0.544 respectively). This difference was probably due to the different ways in which individuals are reproducing; 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, which has reproduced the same individuals without a generational change.

Finally, unlike the Venezuelan population which did not present significant deviations from Hardy-Weinberg expectations at any locus, in the EBTRF we found deviations in six loci for the F0 group and 14 loci for live crocodiles. The deviation in F0 is to be expected since they do not make up a population but constitute a group of individuals extracted from different natural populations, confiscated or from hatcheries. For the living crocodile population, deviations from the equilibrium are also expected since the animals originated from a few breeding pairs crossed without scientific basis or management.

1.5.3 Previous genetic assessment of EBTRF

The captive breeding program of the EBTRF plays a key role in the Orinoco Crocodile conservation in Colombia. Nonetheless, management of these captive population was not guided with the standards necessary to conserve and maximize genetic diversity, even if the genetic monitoring of individuals was recommended more than one decade ago (Williams & Osentoski, 2007). According to the review of the archive records, the pedigree information or the relationship of the breeders has not been fully considered for the management of the animals. Additionally, given the overpopulation in the EBTRF, the spatial disposition of the individuals has been done only considering the size of the crocodiles and the capacity of the tanks.

The only genetic characterization of the EBTRF *ex-situ* population was carried out by Cuervo (2010). However, this first genetic attempt was hampered by some fundamental problems that made the results not suitable for use in the crocodile population management. Firstly, the sampling coverage of the captive population was limited, and this was not evaluated in the interpretations. For example, of the five wild founders mentioned above, only one was used in the analysis, and of the 32 crocodiles reported as F0 according to archive records until 2010, only 17 were included. Secondly, Cuervo (2010) used polyacrylamide gels to genotype individuals, but this method can give a weak resolution in the identification of alleles. We genotyped the same crocodiles and obtained different numbers of alleles for the same loci: Cuervo (2010) found four alleles at locus Cj18 (six in our study), five alleles at locus Cp305 (three in our study), 16 alleles at locus Cj16 (five in our study), 15 alleles at locus CpP302 (four in our study) and one at locus Cj131 (three in our study). Furthermore,

the artificial division of the samples used by Cuervo (2010) in two groups based on age is inadequate, returning inconsistent results. The F0 group, which corresponded to the founders, was made up of adult individuals; and the F1 group, was made up of juveniles. This last generation was divided into two groups based on the results obtained: those born in captivity from adults and those juveniles that are presumed to have been collected from clutches in the wild. However, justifying a population division based solely on the age is wrong since it is known that there are F1 individuals born in 1991 or 1994, and by 2010 potential parents of many crocodiles could have died, leading to a poor estimation of the juveniles from the wild. This shows an admixture of F0, F1, and F2 generations in established groups, as well as a wrong determination of crocodiles with wild origin. Under these results, Cuervo (2010), proposes that the profile of the wild juveniles could be representing the wild populations, but they are reflecting the profile of the station. Therefore, to obtain congruent interpretations and not make unsubstantiated assumptions it is recommended to combine the genetic information, the information of origin of each crocodile even if it is incomplete, and the knowledge provided by the people who have worked in the program.

Finally, knowing that the *ex-situ* population managed by the EBTRF has a high genetic potential that can be used for the recovery of wild populations of *C. intermedius*, in Chapter 2 we will evaluate the genetic profile of each individual that makes up the *ex-situ* population to develop management strategies. These will consider: the location of the individuals in the *ex-situ* subpopulations, the identification of the most diverse juvenile crocodiles to be released and the determination of the most appropriate viable breeding combinations that maximize genetic diversity.

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1.7 Tables and Figures

Figure 1. Adult individuals of *Crocodylus intermedius* hosted at Roberto Franco Tropical Biology Station in Villavicencio, Meta department.

Table 1. Primer sequence information and distribution in each PCR Multiplex.

Locus	Primer sequences (5'- 3')	Dye	Multiplex Mix	Annealing temperature (˚C)
CpP3216	F: CAGTCGGGCGTCATCAGATTAATTCATTGGCTCTC R: GTTTATGCCTTTGCCTTTAG	JOE	1	57˚C
CpP305	F: GTTTGTAGCTGGAACCTGATAGTG R: CAGTCGGGCGTCATCAGGTTAACACGTGGTAACTACA	HEX	1	57˚C
CpP1409	F: GTTTATGCCCTACTGGTTATCTATC R: CAGTCGGGCGTCATCAGGGAAGGGGATTTAATAAT	FAM	1	57˚C
CpP302	F: GTTTGGAACCCAAGAACTTACAAC R: CAGTCGGGCGTCATCATTGGGTTTAGTCAGCACATA	ROX	1	57˚C
CpP1610	F: CAGTCGGGCGTCATCATAGAGGGATTTTGACTGT R: GTTTGATTATTTTGTCTGGGTTCTT	ROX	1	57˚C
CpP314	F: GTTTGAAATGCCACTAATACACACA R: CAGTCGGGCGTCATCACCAATTCTTCAGGTCCTTAT	TAMRA	1	57˚C
Cj16	F: CATGCAGATTGTTATTCCTGATG R: TGTCATGGTGTCAATTAAACTC	JOE	2	57˚C
Cu5123	F: GGGAAGATGACTGGAAT R: AAGTGATTAACTAAGCGAGAC	HEX	2	57˚C
Cj122	F: GTTTCATGCTGACTGTTTCTAATCACC R: GGAACTACAATTGGTCAACCTCAC	ROX	2	57˚C
Cj18	F: ATCCAAATCCCATGAACCTGAGAG R: CCGAGTGCTTACAAGAGGCTGG	JOE	3	60˚C
CUJ131	F: GTCCCTTCCAGCCCAAATG R: CGTCTGGCCAGAAAACCTGT	TAMRA	3	60˚C
Cj109	F: GTATTGTCAACCCCACCGTGTC R: GTTTCCCCTCCACAGATTTACTTGC	TAMRA	3	60˚C
C391	F: ATGAGTCAGGTGGCAGGTTC R: CATAAATACACTTTTGAGCAGCAG	FAM	3	60˚C
Cj101	F: ACAGGAGGAATGTCGCATAATTG R: GTTTATACCGTGCCATCCAAGTTAG	FAM	4	57˚C
CpDi13	F: GTTTGTGTCAGCCTATACATGTT	HEX	4	57˚C

	R: CAGTCGGGCGTCATCAGTCTCAGAGTATGCCTAGAA			
Cj127	F: CCCATAGTTTCCTGTTACCTG R: GTTTCCCTCTCTGACTTCAGTGTTG	ROX	4	57˚C
CpP801	F: CAGTCGGGCGTCATCATTGGCATTAGATTGGTAGAC R: GTTTCTATGCCAAAGCTACAAC	TAMRA	4	57˚C

Table 2. Genetic diversity of the F0 and Alive populations of *Crocodylus intermedius* in the Roberto Franco Tropical Biology 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.

		F0 population (total alleles = 67)								Alive population (total alleles = 64)							
Locus	Null alleles	N	nA	Private alleles	AR	Ho	He	HWE	F_{IS}	N	nA	Private alleles	AR	Ho	He	HWE	F_{IS}
CpP3216	No	37	2	-	2.000	0.432	0.477	Yes	0.094	439	2	-	2.000	0.547	0.457	**No**	-0.195**
CpP305	No	37	3	-	3.000	0.595	0.665	Yes	0.107	439	3	-	2.973	0.51	0.581	**No**	0.122
CpP1409	No	37	3	-	2.870	0.351	0.419	Yes	0.163	439	3	-	2.996	0.658	0.568	**No**	-0.159**
CpP302	No	37	4	-	3.985	0.784	0.673	Yes	-0.168	439	4	-	3.995	0.754	0.700	**No**	-0.076**
CpP314	No	37	3	-	3.000	0.568	0.618	Yes	0.083	439	3	-	3.000	0.647	0.664	Yes	0.021
Cj16	No	37	5	1	4.824	0.595	0.548	**No**	-0.087	440	4	-	3.804	0.625	0.563	**No**	-0.112
CU5123	No	37	4	-	3.740	0.784	0.682	Yes	-0.152	440	4	-	3.983	0.734	0.690	**No**	-0.067
Cj122	No	37	5	-	5.000	0.784	0.801	Yes	0.022	439	5	-	4.955	0.813	0.769	**No**	-0.057
Cj18	No	37	5	1	4.483	0.757	0.700	Yes	-0.082	439	5	1	4.115	0.647	0.612	**No**	-0.057
CUJ131	No	37	3	-	2.870	0.378	0.488	**No**	0.226	439	3	-	2.155	0.542	0.505	**No**	-0.069
Cj109	No	37	6	2	4.966	0.703	0.694	**No**	-0.013	439	4	-	3.908	0.772	0.694	**No**	-0.112**
Cj391	No	37	9	1	7.616	0.676	0.807	**No**	0.164*	439	8	-	4.436	0.565	0.524	**No**	-0.078
CCj101	No	37	3	-	2.740	0.541	0.532	**No**	-0.017	440	4	1	2.661	0.602	0.484	**No**	-0.240
CpDi13	No	37	3	-	2.936	0.405	0.504	**No**	0.198*	440	3	-	2.813	0.489	0.509	**No**	0.035*
Cj127	No	37	3	-	2.226	0.081	0.08	Yes	-0.019	440	3	-	2.154	0.339	0.297	Yes	-0.144**
CpP801	No	37	6	-	5.391	0.730	0.712	Yes	-0.026	440	6	-	3.949	0.632	0.571	**No**	-0.109
Mean		4.19			3.853	0.573	0.587		0.025	4			3.369	0.617	0.574		-0.075
SD		1.759			1.414	0.200	0.178		0.122	1.461			0.889	0.120	0.116		0.089

* Significance for heterozygous defect

** Significance for heterozygous excess

Table 3. Allelic frequencies of 16 polymorphic microsatellite loci in F0 and Alive populations of *Crocodylus intermedius* in the Roberto Franco Tropical Biology Station.

Locus	Allele	F0 (N=37)	Alive (N=437)	Locus	Allele	F0 (N=37)	Alive (N=437)
CpP3216	137	0.622	0.646	**CUJ131**	185	0.649	0.500
	141	0.378	0.354		191	0.311	0.495
CpP305	176	0.297	0.094		193	0.041	0.005
	192	0.419	0.441	**Cj109**	372	0.216	0.346
	196	0.284	0.466		374	0.189	0.063
CpP1409	245	0.230	0.281		376	0.014 [a]	0.000
	249	0.730	0.577		382	0.095	0.230
	253	0.041	0.142		384	0.473	0.362
CpP302	194	0.459	0.432		388	0.014 [a]	0.000
	196	0.149	0.177	**Cj391**	153	0.338	0.653
	200	0.311	0.137		157	0.068	0.015
	208	0.081	0.253		161	0.014	0.007
CpP314	254	0.527	0.354		169	0.054	0.104
	258	0.216	0.362		171	0.014	0.003
	262	0.257	0.285		173	0.176	0.013
Cj16	141	0.081	0.056		175	0.189	0.195
	151	0.041 [a]	0.000		179	0.122	0.010
	167	0.649	0.588		183	0.027 [a]	0.000
	171	0.162	0.288	**CCj101**	354	0.000	0.010 [a]
	173	0.068	0.068		356	0.514	0.627
CU5123	202	0.243	0.241		358	0.027	0.011
	214	0.027	0.105		360	0.459	0.351
	216	0.392	0.209	**CpDi13**	358	0.054	0.045
	220	0.338	0.444		360	0.635	0.605
Cj122	378	0.189	0.154		362	0.311	0.350
	380	0.189	0.307	**Cj127**	337	0.959	0.819
	386	0.284	0.185		341	0.014	0.005
	390	0.189	0.082		343	0.027	0.176
	392	0.149	0.271	**CpP801**	166	0.054	0.002
Cj18	203	0.000	0.006[a]		170	0.068	0.181
	207	0.297	0.200		174	0.014	0.001
	209	0.149	0.162		178	0.311	0.162
	211	0.432	0.563		182	0.419	0.606
	213	0.108	0.069		186	0.135	0.047
	215	0.014[a]	0.000				

[a] Private allele for that locus in that population

Chapter 2

Conservation management guidelines for the breeding program of the Orinoco Crocodile (*Crocodylus intermedius*) in Colombia using a microsatellite marker system.

2.1 Abstract

Captive breeding and reintroduction have been essential tools to recover critically endangered species. A critical purpose of the *ex-situ* populations is the preservation of the genetic variation, but this is not an easy task since genetic diversity is commonly lost through each generation. Furthermore, this becomes a challenge when the *ex-situ* populations have been initiated from wild individuals coming from already genetically depauperate populations. Thus, the establishment of management guidelines of reproduction in such programs should be a high priority. Fifty years ago, the National University of Colombia began a breeding program for the conservation of the critically endangered Orinoco crocodile *Crocodylus intermedius.* In this *ex-situ* population, the information of every single individual was not rigorously compiled thought-out the development of the breeding program, restricting the establishment of reproductive combinations between unrelated individuals. Since the conservation of the genetic diversity depends mostly on the choice of the breeding individuals, we developed a powerful tool aimed at maximizing that diversity, based on the information coming from the genotyping of 16 microsatellite loci system. Our results allowed us to estimate the individual diversity of the living crocodiles, as well as the relationship between them. This information combined with information of age, sex, size, and location, allowed us to build combinations to plan future breeding groups that maximize the population´s genetic diversity. We propose different reproductive nuclei within six subpopulations that make up the program, and we demonstrate that molecular data can be used to improve management of the program well beyond what can be achieved with pedigree information alone.

Key Words: *ex-situ* conservation, population genetics, genetic diversity, unique alleles.

2.2 Introduction

The Orinoco caiman *Crocodylus intermedius* (Gray, 1819) is a true freshwater crocodile whose historical distribution covered all the lowlands of the Orinoco basin in Colombia and Venezuela (Medem, 1981). During the 20th century, the species suffered a profound decline throughout its distribution range caused mainly by commercial hunting, generating its current classification as critically endangered of extinction (CR) by the IUCN (2020). To tackle this situation, two direct conservation strategies have been suggested and followed in Colombia. Firstly, its protection has been legally regulated by prohibition decrees and through practices of improvement and protection of its habitats (Castro-Casal *et al.*, 2013). Secondly, as in other crocodilian species (e.g., *Alligator sinensis*; Xu *et al*., 2005), a captive breeding program has been established with the aim of recovering wild populations through the release of individuals (MMA, 2002).

The captive breeding program was established in 1971 by Federico Medem, and currently hosts almost 600 individuals. The main headquarters of the program and the largest number of crocodiles are in the Roberto Franco Tropical Biology Station in Villavicencio, Meta department, with 361 individuals. Additionally, there are other four support subpopulations located in the Parque Agroecológico Merecure in Villavicencio (Meta department) with 19 individuals, Bioparque los Ocarros in Villavicencio (Meta department) with five individuals, Parque Ecotemático Wisirare in Orocué (Casanare department) with 169 individuals and the Aquatic and Conservation Park Piscilago in Nariño (Cundinamarca department) with four individuals. Finally, the program plans to integrate the sixth subpopulation at the Universidad de los Llanos (Unillanos) also in Villavicencio, where tanks are currently being adapted.

One of the priorities in the *ex-situ* programs is the conservation of genetic variability through the production of offspring with high genetic diversity, since it is necessary that the juveniles born can resist and adapt to the environmental pressures of the habitat in which they are released (Araki *et al,* 2007). It is also important that bred individuals provide enough genetic diversity to the *in-situ* populations (i.e., *de novo* population or genetic reinforcement of already existing ones). Howbeit, one of the concurrent problems in captive breeding programs is the loss of the genetic diversity of the *ex-situ* populations (Witzenberger & Hochkirch, 2011). In the case of the captive breeding program of *C. intermedius*, although a good diversity was recorded in terms of heterozygosity, there was a loss of genetic diversity and a change in

allelic frequencies between the founder individuals and the current population (Chapter 1). This has been the result of establishing the reproductive nuclei without having a genetic basis, combining crocodiles based on the distribution of the individuals in the subpopulations considering only the age and size of the individuals, and the capacity of the tanks. In addition, although there is an archive record with the information of each individual, in many cases it is incomplete and does not contain a location control or monitoring of offspring and reproduction (Maldonado & Ardila, 2004).

The success of a reintroduction program is determined by the ability of individuals to reproduce and thrive (Dylan, 2008). Hence, if the aim of an *ex-situ* breeding program is to serve for the reintroduction of individuals to the natural habitat, the probability of species' long-term survival will be increased by efforts to restore as much genetic variation as possible (Goncalves *et al.,* 2010). Nevertheless, this is not always an easy task, and several management considerations must be considered. Although detailed studbooks constitute 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, and pedigree information is not necessarily sufficient for the selection of the best breeding pairs (Tzika *et al.,* 2008). Additionally, founders are assumed to be unrelated (founder assumption), but this is not always true (e.g., individuals born of the same brood) and thus may lead to an underestimation of relatedness resulting in incorrect management actions (Russello & Amato, 2004). To solve this, genetic information can guide the choice of individuals with the lowest mean kinship to be parents of subsequent generations to maximize the retention of genetic variation in the offspring, since it reduces the overall level of relatedness, maximizes founder representation, and minimizes the expression of deleterious alleles in inbred animals (Montgomery *et al.,* 1997).

Knowing that the EBTRF counts with a high genetic potential for the recovery of wild populations of *C. intermedius* but such genetic diversity is unevenly distributed in the population, two management guidelines need to be considered to produce neonates that maximize genetic variability: first, maintenance of high levels of heterozygosity by combining unrelated genetically variable adult individuals, and second prioritization of combinations with individuals that have rare alleles in order to not lose them (Chapter 1). Therefore, in this study, we combine information from studbook data and molecular genetic analyses to guide the combinations of individuals that maximize the genetic diversity of the captive breeding program of the endangered *C. intermedius* in Colombia. To ascertain relationships among

the founders and living crocodiles, we genotyped a big part of the five subpopulations from the breeding project using 16 polymorphic microsatellite loci. Based on this data, we analyzed their relationship and developed recommendations for the combination of breeding groups.

2.3 Methods

2.3.1 Sampling

Between 2004 and 2020, scales and/or muscle samples were taken from 73% of the crocodiles belonging to the captive breeding program coordinated by the EBTRF. Of the available samples, we processed 453 individuals. The missing samples are from the two largest subpopulations: 95 individuals from the EBTRF and 40 from Wisirare; the subpopulations of Merecure, Ocarros, and Piscilago were genotyped entirely.

From the processed samples, 15 corresponded to dead individuals: one crocodile from Piscilago, two females from Wisirare, three wild individuals (among which we found the crocodiles called Pancho and Dabeiba), eight individuals seized from the Rango Rudd hatchery, and a seized crocodile without provenance. The 438 remaining samples corresponded to the F0, F1, and F2 living crocodiles: 19 located in Merecure, 278 in the EBTRF, five in Ocarros, four in Piscilago, and 127 in Wisirare. Scales and muscle samples were preserved in 96% ethanol and kept at -20°C until processing.

2.3.2 Laboratory procedures and genotyping

Genomic DNA was extracted from preserved tissues using the same laboratory procedures described in Chapter 1. We amplified 16 polymorphic loci of microsatellite tested for cross-amplification by Laferriere *et al.,* (2016); for that four PCRs multiplex were performed (Multiplex 1: CpP302, CpP305, CpP314, CpP1409, CpP3216; Multiplex 2: Cj16, Cj122, Cu5123; Multiplex3: Cj18, Cj109, C391, CUJ131 and Multiplex 4: Cj101, Cj127, Cp801, CpDi13), using the Multiplex PCR kit MyTaq™ HS Mix (Bioline, USA). Reactions and thermocycling conditions were used as described in Chapter 1. Fragment lengths were determined using an ABI 3500 Genetic Analyzer. The Gene-Mapper 3.7 (Applied Biosystems Foster City, CA) and Osiris 2.13.1 (NCBI) software were used for scoring fragment lengths. Genetic laboratory work was conducted at the Molecular Ecology Laboratory of the Genetics Institute, National University of Colombia in Bogotá.

2.3.3 Data Analysis

- **Identification of key individuals with the presence of rare alleles**

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), allelic richness (AR), allelic frequencies, and inbreeding coefficient (F_{IS}) were calculated for each group using 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 & Rousset, 1995). Through this information, we identified genetically relevant individuals containing alleles with low frequencies.

- **Identification of kinship relationships within founding individuals and within living crocodiles**

Relationships among crocodiles were inferred using ML-RELATE (Kalinowski *et al.,* 2006), a Maximum Likelihood-based software that estimates relatedness coefficient (*r*) for each pair of individuals, providing a list of several possible relationships (i.e., Half-Sibling, Full-Sibling, Parents-Offspring and Unrelated). This index was combined with the Homozygosity by Loci index (see below) to propose the best combinations that maximize genetic diversity. Relatedness was analyzed within the five subpopulations to determine the degree of relationship of the crocodiles that have been reproducing. Finally, to test the "founder assumption", relatedness was also estimated among the five individuals coming from Cravo Norte river (one dead and four located in the EBTRF) and within the individuals seized from the Rangos Rudd hatchery (eight dead, two located in Piscilago, one in Ocarros and one in Wisirare).

- **Assessment of parental veracity**

To prove the provenance veracity of the captive-bred individuals registered in the archive 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) were compared to identify the most likely parents for each

offspring. Using the potential fathers of each of the groups, simulations of 10,000 offspring genotypes were run, 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. The eggs registered as coming from the Pancho - Dabeiba couple were evaluated with the genotypes of these two founders. The eggs registered as coming from Piscilago were evaluated considering as potential parentals the seven crocodiles that have been in Piscilago. The same was done for the eggs and individuals from Wisirare, Merecure, and Ocarros. The parent pairs analysis for the individuals from EBTRF eggs was not conducted since it is not certain that these eggs come from the Station and since there are many dead individuals who could be potential parents but were not genotyped. By not having the complete data set, even if supported kinship relationships are established, these can probably be wrong.

- **Identification of individuals with high genetic diversity**

For the formulation of guidelines and recommendations to preserve current genetic diversity as much as possible, we estimated inbreeding coefficients at the individual level for each of the living crocodiles using the GENHET 2.3 R script (Coulon, 2010). We estimate the Homozygosity by Loci (HL) which is a homozygosity index that weights the contribution of each locus depending on their allelic variability (Aparicio *et al.,* 2006). Hereby, each crocodile is assigned a value ranging from 0 (all loci are heterozygous) to 1 (all loci are homozygous), allowing us to identify the most genetically diverse individuals.

- **Management formulations**

To guide the choice of reproductive pairs that will produce neonates with high genetic diversity, the *r* and the HL indexes were combined with additional information from every single crocodile (i.e., size, age, sex, origin, current location) in a dynamic table. Using this tool, we proposed several options of viable crosses in the five subpopulations already established and in the reproductive nucleus that will soon be established in Unillanos University in Villavicencio, Meta department. The combinations were formulated considering the number of tanks currently available and those to be built in the future.

2.4 Results

The 16 microsatellite loci were successfully amplified for 450 of the 453 processed samples. For the missing three samples, between one and six loci were not obtained but were included in the analyzes. Our data set represents 73% of the living crocodiles of the Station. A total of 64 alleles were identified in the sampled individuals but the number differed between the five subpopulations (Table 1). As expected, the largest subpopulations (EBTRF and Wisirare) showed the highest number of alleles (61 and 56 alleles, respectively), while the population with the lowest number of alleles was Piscilago (43 alleles).

In turn, Wisirare and EBTRF subpopulations were the only ones to present unique alleles (one and four, respectively). However, although the other populations did not present unique alleles, they presented alleles at very low frequencies (Table 2). Allele 203 (Cj18) was present in five individuals, allele 193 (Cj131) in three individuals, 157 (Cj391) in 13 individuals, 151 (Cj391) in six individuals, 171 (Cj391) in three individuals, 173 (Cj391) in nine individuals, 179 (Cj391) in nine individuals, 354 (CCj101) in nine individuals, 358 (CCj101) in seven individuals, 341 (Cj127) in four individuals, 166 (Cpp801) in two individuals and 174 (CpP801) in two individuals. We identified and prioritized in the management guidelines the 60 reproductive individuals that contained alleles at low frequencies distributed in the five subpopulations (Table 3, Table S1). Despite being a priority, individual 181 was not considered since it has the penis partially amputated and cannot reproduce. The rest of the alleles were found in at least 35 living crocodiles. 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% of individuals had an index lower than 0.6 and most are grouped between 0.2 and 0.5 (Figure 1).

The results below are presented by subpopulation, evaluating the set of indices in each case. All the parental combinations were assembled by using the developed dynamic tool (Table S2) that combines the *r* and the HL indexes with complementary important information regarding every single crocodile (i.e., size, age, sex, origin, and current location). Using this tool, several options for logistical viable crosses were proposed considering the priority crocodiles identified with the allele frequencies, combining them with unrelated crocodiles in reproductive age that showed the lower HL. According to the specific requirements and necessities of each of the six subpopulations, we established options of combinations that guarantee the recovery of rare alleles and minimize mean kinship.

2.4.1 Ocarros subpopulation

The Ocarros subpopulation is made up of five adult individuals (three F0 males and two females) which are around 30 years old. Despite being the population with the lowest number of individuals along with Piscilago, this subpopulation is the third most diverse in number of alleles, containing 81% of the alleles present throughout the Program (Table 1). Nevertheless, according to the archive records and the information provided by the station officials, Ocarros contributed the least to the growth of the *C. intermedius ex-situ* population. According to the archive records, only two crocodiles currently in the EBTRF come from Ocarros eggs, but when performing the parental pairs analysis, potential parents from Ocarros were not identified (Table 5).

Since the five crocodiles of Ocarros are in the same tank, to avoid competition from the males and generate a productive reproduction, Ocarros has requested the exchange of two males for two reproductive females. The three males found in Ocarros are priority crocodiles since they have scarce alleles, but two of them have a relatedness coefficient greater than 0 with the females (Table 3a). Therefore, we suggest leaving male 156 and complete the nucleus with two females from the EBTRF. Based on the dynamic table (Table S2) and according to the parameters of priority females that are in reproductive age, an $r = 0$ with the male and an HL as low as possible, we choose females 172 and 272 to complete the reproductive nucleus (Table 3a).

2.4.2 Unillanos subpopulation

In accordance with the agreement made between the National University of Colombia and the Universidad de los Llanos, two tanks are being built to host two reproductive nuclei, each one composed of one male and three females. We propose to leave within each nucleus the priority males that are going to be exchanged in Ocarros, mentioned above. Since male 154 has an HL of 0.199, we select six priorities breeding females that have a higher HL coefficient than the male (between 0.309 and 0.363, Table 3b). On the other hand, since that male 157 has a high HL coefficient (0.614), we selected females with an $r = 0$ with the male and an HL as low as possible, even if they were not priority individuals (Table 3b).

2.4.3 Piscilago subpopulation

The Piscilago subpopulation is made up of four individuals (two F0 males and two females) that are around 30 years old. Compared to Ocarros that have a similar number of crocodiles, Piscilago has the lowest number of alleles (43), containing 67% of the alleles present throughout the Program. Nevertheless, according to the archive records and the information provided by the station officials, together with Wisirare, Pisilago is the population that has contributed the most to the growth of the *C. intermedius ex-situ* population. According to the archive records, 68 crocodiles currently in the EBTRF, came from Piscilago eggs. However, when performing the parental pairs analysis, 42 were confirmed as offspring of Piscilago crocodiles (Table 5).

Currently, Piscilago has two tanks but only one has a reproductive nucleus with the females 115-118 and the male 214, while male 213 is in an isolated tank only for exhibition (Table 3c). In accordance with the agreements made between the National University of Colombia and Piscilago, this subpopulation can receive an additional female for the tank containing the reproductive nucleus already established, and an additional nucleus composed of two females and one male in a new tank that they are currently adapting. Considering that the two males and the female 118 are priority crocodiles and that the relationship of this female with both males is greater than 0 (a degree of relationship of Half and Full Sibling), we proposed to leave both males in the nuclei and bring the female 118 to EBTRF for the inclusion in another reproductive nucleus (Table 3f). According to the parameters of priority females (if possible), that are in reproductive age, an r = 0 with the male involved and a HL as low as possible, we choose females 197 and 204 to complete the reproductive nucleus already established in tank 1, and females 238 and 456 to establish the new reproductive nucleus with male 213 in tank 3 (Table 3c). Finally, we propose to transfer to the exhibition tank male 181 housed in the EBTRF, since it has an amputated penis being unable to contribute to the breeding program but being appropriated to be exhibited.

2.4.4 Merecure subpopulation

Merecure subpopulation is made up of 19 individuals (eight females and 11 males) born between 1991 and 1994, that contain 75% of the alleles found in the captive population. Merecure has contributed considerably to the growth of the *ex-situ* population: of the 54 individuals registered as originating from Merecure eggs, with the parental pairs analysis 47 were confirmed (Table 5).

Currently, all the crocodiles of Merecure are in the same tank. However, to avoid competition between males and generate productive nuclei, it is intended to leave two males with six females in the tank. One female and three males were identified as priority crocodiles (Table 3d and Table S1). However, considering that males 141 and 145 present the same priority allele and that 145 has a greater HL than 141, males 138 and 141 were chosen to configure the two reproductive nuclei. According to the parameters of priority females (if possible), that are in reproductive age, an $r = 0$ with both males and the HL as low as possible, we choose females 134, 152, 376, 165, 520, and 170 to establish the reproductive nucleus (Table 3d).

2.4.5 Wisirare subpopulation

The Wisirare subpopulation is made up of 167 individuals of which 160 are juveniles, containing 88% of the alleles found in the *ex-situ* population. We genotyped the seven crocodiles that make up the living brood foot which are around 30 years old and 120 of the juveniles that supposedly come from these parental crocodiles. We found that juveniles 1147, 1162, 1163, 1166, 1169, 1176, 1177, 1184, 1188, 1202, 1204, 1228, 1230 are the only representatives of allele 354 (in locus CCj101) in the *C. intermedius ex-situ* population.

Unlike all the other subpopulations, Wisirare presents deviations of the F_{IS} coefficient in 12 of the 16 evaluated loci due to heterozygotes excess (Table 1). Of the 115 crocodiles recorded as originating from Wisirare eggs, 80 found potential parents in this subpopulation. Since we found an unrelated kinship level and a low HL in the individuals that make up the brood foot (Table 3f) and considering that the transport to Wisirare is the most complicated, for management guidelines we suggest leaving the pairs as they are, keeping two nuclei together or apart.

2.4.6 EBTRF subpopulation

The EBTRF represents the largest of the *C. intermedius* subpopulations. It is made up of 361 crocodiles, of which we genotyped 278: 90 juveniles, 185 adults (born between 1996 and 2010), and three remain to be confirmed. It contains 95% of the alleles from the entire captivity program and four unique alleles.

Considering priority individuals that have not been assigned to any reproductive nucleus, we proposed five reproductive nuclei for the Station. They were established by minimizing the number of individuals involved and combining priority and non-priority individuals (Table

3f). In these, we found female 118 that would be taken from Piscilago and the highest number of priority crocodiles that remained in the EBTRF (Table S1).

2.4.7 Founder assumption

When testing the relationships between the founding crocodiles that came from the same sites, we found that several are related (Table 4). Although the six individuals from Cravo Norte showed unrelatedness in some cases, most are related as Half and Full-Sibling (Table 4a). The same happened in the seized crocodiles from the Rango Rudd hatchery, where we found Unrelated individuals, but most are related as Half and Full-Sibling and even as Parental-Offspring.

2.5 Discussion

The breeding program for *Crocodylus intermedius* in Colombia aims at preserving and increasing as much as possible the current genetic diversity and at producing neonates with the highest genetic diversity possible to support management actions. To achieve this goal, we proposed a powerful system of 16 microsatellite loci to estimate the relationship and the individual diversity of the living crocodiles, that combined with information of age, size, sex, and location, also allowed us to design combinations to plan future breeding groups in each subpopulation. This innovative tool enables to simultaneously maximize genetic diversity combining diverse individuals and achieve a genetic gain by minimizing the relationships between the individuals combined.

Our work is novel and necessary since most of the 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 (Spitzweg *et al.,* 2018). Furthermore, it is the first study to combine relatedness information with the Homozygosity by Loci, which can be very useful when the number of individuals involved is large and discriminating only with the *r* index may not be enough. In addition, if the first proposed combinations cannot be achieved (e.g., if crocodiles do not have an adequate state of health, if they do not adapt well to the reproductive nuclei, or if they die) the tool allows to easily develop alternative crosses that fulfill the same purposes.

Our results are promising since despite the living crocodiles retained approximately 92% of the genetic diversity of the wild-caught founder individuals (Chapter 1), the presence of five

unique alleles and 12 rare alleles (Table 2), the difference in the number of alleles and the allele frequencies among the five subpopulations, showed that the diversity is unevenly distributed between groups. If no action is taken to balance this, the loss of genetic diversity in the next few generations can be very 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 provided here and not relying solely on the latter, since as we found when performing the parent pairs analysis, a large part of the archive file is wrong in determining the origin of the individuals. In addition, we make a strong call to be more rigorous when recording this information and to make it clear if it is not certain. Finally, we explicitly recommend implementing conservation genetic assessments for other captive breeding projects to preserve the maximum genetic diversity and to avoid inbreeding depression (as also recommended by Xu *et al.,* 2005; Tzika *et al.,* 2008; Spitzweg *et al.,* 2018).

We recommended completing the dataset with the missing crocodile samples to include them in the management guidelines since some of these crocodiles can be very relevant (e.g., five individuals from Cravo Norte River that have not been genotyped). In turn, it is necessary to genotype the crocodiles that are going to be born to have a complete genetic profile of the program and to evaluate future trends in allele frequencies and restructure combinations if necessary.

We suggest bringing to the *ex-situ* program new crocodiles from wild populations to refresh the genetic diversity and avoid future inbreeding (Chapter 1). These individuals must be genotyped to determine the presence of rare alleles, the individual genetic diversity, and their level 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). Fortunately, in the case of the Cravo Norte and Rango Rudd crocodiles, all of them were males, so there was not option to combine them. In the case of the Cravo Norte crocodiles, according to the information provided by the station officials, there is a high probability that these individuals came from the same clutch. With this, the differences in the kinship relationships found between these individuals could be indicating multiple paternity, a phenomenon already identified for the species in Venezuela (Laferriere *et al.,* 2016).

Based on our data, we suggest that selective breeding should be implemented, and some mating combinations must be avoided. Of the 60 crocodiles identified as a priority, 48 were assigned to a reproductive nucleus. Of the 13 missing, nine do not have reproductive size, two have a high Homozygosity by Loci (0.808 and 0.947) and the last one is located in Merecure and it is not recommended to transport it elsewhere. Nevertheless, we recommend considering them for future management guidelines.

Finally, in Merecure, Wisirare, and EBTRF we proposed the establishment of reproductive nuclei with more than one male. If reproduction is possible with the established ones (e.g., in case that coexistence between males and females allows it), this may be an option to further maximize diversity, since multiple paternity would allow obtaining clutches from both males.

2.5.1 Wisirare subpopulation

The Wisirare subpopulation is the largest contributor to the *ex-situ* population growth of *C. intermedius*. Between 2005 and 2011 the eggs spawned by Wisirare females were transferred to the EBTRF facilities in Villavicencio, in part because there was not an incubation infrastructure. However, after 2011 the entire egg incubation and rearing process could be completed in Wisirare. As a result, in Wisirare we found only one priority crocodile, which together with the management dynamics and allelic frequencies suggest that the current diversity of the station may reflect the great over-representation that Wisirare has generated, overshadowing the contributions of the other subpopulations.

The deviation of 12 of the 16 loci due to excess heterozygotes in Wisirare may be the result of always reproducing the same individuals that have a low HL, generating a group of individuals without generational turnover with many heterozygotes out of Hardy-Weinberg proportions. This should be a factor to consider when releasing these crocodiles since according to the genetic situation of the wild populations, depression could be generated by outbreaking (Banes *et al.,* 2016)

Finally, in Wisirare we found 13 juvenile individuals that are the only ones containing allele 354 (locus CCj101) in the *ex-situ* population. Since this allele is not found in any of the dead founders (Chapter 1) or in Wisirare's brood foot, these individuals likely come from a wild origin. This hypothesis is supported by the fact that in Wisirare the collection of wild eggs from Cravo Norte has been carried out, for their incubation and subsequent release.

Therefore, it is necessary to wait for these individuals (or part of them) to grow, to be sexed, and included in the reproductive nucleus to add this allele to the *ex-situ* breeding program. Also, we suggested adding individual 1192 to the reproductive nucleus when it grows up since it has an allele at low frequencies (193, locus Cj131).

2.5.2 EBTRF subpopulation

The EBTRF contains the largest subpopulation (about 370 individuals), the largest number of tanks available, and a high genetic diversity involving four 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 Merecure began to be carried to the Station. Considering that the EBTRF subpopulation has the highest number of adult crocodiles with unique diversity, it is necessary to re-implement the brood foot with these individuals. It is urgent to maintain a balance in the proportion of incubations of eggs according to the place where they come from, and the number of parents that produce them.

In the EBTRF we found juvenile individuals that we considered in priority because they contain alleles at low frequencies. However, these individuals are not yet at the reproductive age, so we recommend keeping them until they grow to be included in the reproductive nuclei.

With the implementation of the crosses proposed here, the program will ensure obtaining a highly genetically variable offspring preserving all 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, which, according to the requirements of natural populations, would be able to be released into the wild (Chapter 3).

2.6 References

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2.7 Tables and figures

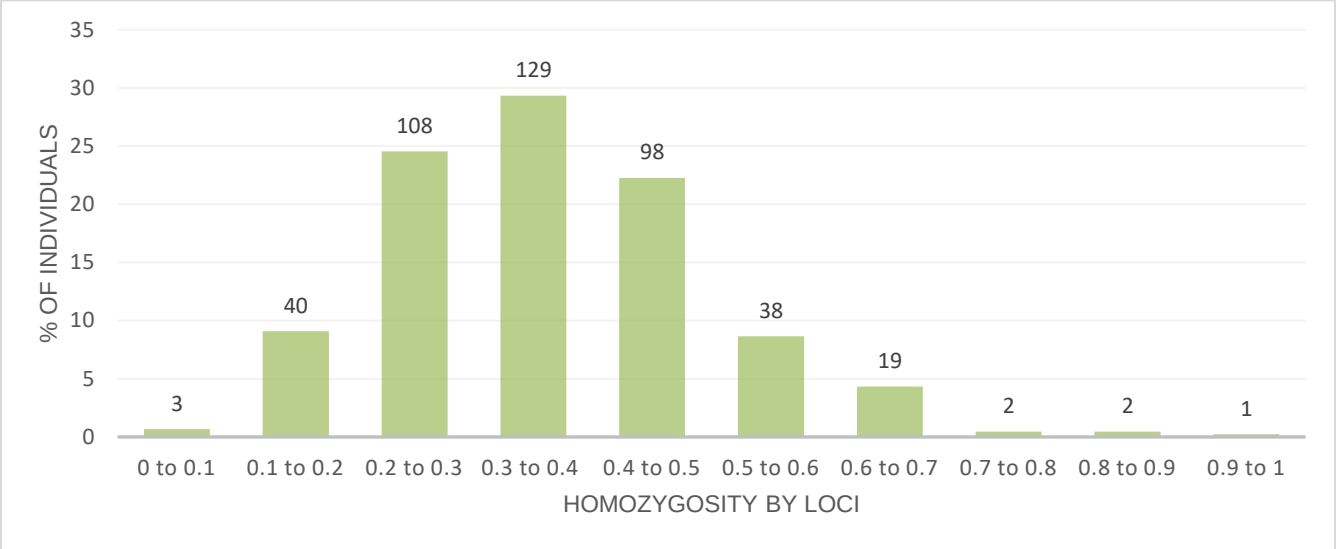

Figure 1. Distribution of individual diversity (homozygosity by loci, HL) of the living crocodiles that make up the *ex-situ* population managed by the Roberto Franco Tropical Station. The values above the columns indicate the number of individuals in each category.

Table 1. Genetic diversity of the current five *ex-situ* subpopulations of *Crocodylus intermedius* managed by Roberto Franco Tropical Biology Station (EBTRF). N – sample size, nA – alleles per locus, AR – allelic richness, F_{IS} - inbreeding coefficient.

* Significance for heterozygous defect

** Significance for heterozygous excess

	Merecure (total alleles=48)					EBTRF (total alleles=61)					Ocarros (total alleles=52)					Piscilago (total alleles=43)					Wisirare (total alleles=56)				
Locus	N	nA	PA	AR	Fis	N	nA	PA	AR	Fis	N	nA	PA	AR	Fis	N	nA	PA	AR	Fis	N	nA	PA	AR	FIS
CpP3216	19	2	-	1.976	-0.478	278	2	-	1.971	-0.096	5	2	-	1.800	0.000	4	2	-	2	-0.500	127	2	-	1.973	-0.388**
CpP305	19	2	-	1.995	0.350	278	3	-	2.386	0.132	5	3	-	2.600	-0.067	4	3	-	3	-0.600	127	3	-	2.726	0.034
CpP1409	19	3	-	2.666	-0.094	278	3	-	2.508	-0.092	5	3	-	2.778	-0.200	4	2	-	2	-0.200	127	3	-	2.783	-0.331**
CpP302	19	4	-	3.323	-0.050	278	4	-	3.391	-0.024	5	3	-	2.778	-0.200	4	3	-	3	-0.091	127	4	-	3.400	-0.187**
CpP314	19	3	-	2.755	-0.109	278	3	-	2.845	0.063	5	3	-	3	0.200	4	3	-	3	0.000	127	3	-	2.830	-0.119
Cj16	19	3	-	2.806	-0.537**	278	4	-	2.688	-0.087	5	3	-	2.778	-0.200	4	3	-	3	-0.286	127	4	-	2.639	-0.120**
CU5123	19	4	-	3.668	-0.097	278	4	-	3.408	-0.065	5	4	-	3.578	0.077	4	3	-	3	-0.412	127	4	-	2.971	-0.125**
Cj122	18	4	-	3.268	0.053	278	5	-	3.899	-0.022	5	5	-	4.556	0.030	4	5	-	5	-0.200	127	5	-	3.657	-0.219**
Cj18	19	3	-	2.206	-0.320	278	5	2	2.994	-0.007	5	2	-	2.000	-0.600	4	2	-	2	-0.200	126	4	-	3.197	-0.150**
CUJ131	19	3	-	2.120	0.309	278	2	1	1.989	-0.094	5	3	-	2.978	1.000	4	2	-	2	0.143	126	3	-	1.990	-0.371**
Cj109	19	4	-	3.569	0.031	278	4	-	3.037	-0.073	5	4	-	3.600	-0.103	4	3	-	3	-0.125	126	4	-	3.452	-0.221**
Cj391	19	3	-	2.149	0.092	278	7	-	2.889	-0.078	5	6	-	5.356	0.111	4	2	-	2	0.143	126	5	-	2.180	-0.246**
CCj101	19	2	-	1.807	-0.172	278	3	-	2.091	-0.157	5	2	-	2.000	-0.600	4	2	-	2	0.571	127	4	1	2.261	-0.454**
CpDi13	19	3	-	2.755	-0.495**	278	3	-	2.263	0.135*	5	3	-	2.800	-0.391	4	3	-	3	-0.286	127	3	-	2.059	-0.085
Cj127	19	3	-	2.258	-0.234	278	3	-	1.819	-0.103	5	1	-	1	NA	4	2	-	2	0.000	127	2	-	1.808	-0.221
CpP801	19	2	-	1.897	-0.259	278	6	1	3.053	-0.106	5	5	-	4.400	0.000	4	3		3	0.500	127	3	-	2.531	-0.181**
Mean		3		2.576	-0.126		3.813		2.702	-0.042		3.250		3.000	-0.063		2.688		2.688	-0.096		3.500		2.654	-0.212
SD		0.730		0.617	0.262		1.377		0.595	0.085		1.291		1.108	0.379		0.793		0.793	0.322		0.894		0.578	0.126

Table 2. Allelic frequencies of 16 polymorphic microsatellite loci in the current five *ex-situ* subpopulations of *Crocodylus intermedius* in charge of the Roberto Franco Tropical Biology Station (EBTRF). N – sample size. [a] Private allele, [b] Alleles with very low frequencies.

Locus	Allele	Merecure (N=18)	EBTRF (N=278)	Ocarros (N=5)	Piscilago (N=4)	Wisirare (N=126)
CpP3216	137	0.667	0.644	0.900	0.625	0.641
	141	0.333	0.356	0.100	0.375	0.359
CpP305	176	0.000	0.061	0.100	0.375	0.168
	192	0.444	0.493	0.800	0.500	0.313
	196	0.556	0.446	0.100	0.125	0.520
CpP1409	245	0.250	0.295	0.200	0.750	0.246
	249	0.611	0.606	0.700	0.250	0.520
	253	0.139	0.099	0.100	0.000	0.234
CpP302	194	0.472	0.424	0.700	0.750	0.426
	196	0.194	0.171	0.100	0.000	0.203
	200	0.083	0.142	0.200	0.125	0.129
	208	0.250	0.263	0.000	0.125	0.242
CpP314	254	0.278	0.415	0.400	0.375	0.219
	258	0.556	0.344	0.300	0.250	0.391
	262	0.167	0.241	0.300	0.375	0.391
Cj16	141	0.194	0.067	0.200	0.250	0.004
	167	0.528	0.588	0.700	0.625	0.582
	171	0.278	0.299	0.100	0.125	0.285
	173	0.000	0.047	0.000	0.000	0.129
CU5123	202	0.333	0.255	0.200	0.375	0.203
	214	0.222	0.128	0.100	0.000	0.047
	216	0.222	0.209	0.600	0.375	0.184
	220	0.222	0.408	0.100	0.250	0.566
Cj122	378	0.056	0.169	0.200	0.375	0.121
	380	0.278	0.268	0.200	0.125	0.406
	386	0.417	0.225	0.400	0.250	0.063
	390	0.000	0.063	0.100	0.125	0.133
	392	0.250	0.275	0.100	0.125	0.277
Cj18	203	0.000	**0.009** [a,b]	0.000	0.000	0.000
	207	0.361	0.221	0.400	0.250	0.125
	209	0.028	0.149	0.000	0.000	0.227
	211	0.611	0.572	0.600	0.750	0.523
	213	0.000	0.049	0.000	0.000	0.125
CUJ131	185	0.222	0.439	0.400	0.500	0.672
	191	0.750	0.561	0.400	0.500	0.324
	193	**0.028** [b]	0.000	**0.200** [b]	0.000	**0.004** [b]

Cj109	372	0.361	0.363	0.400	0.375	0.305
	374	0.139	0.027	0.100	0.000	0.129
	382	0.222	0.239	0.100	0.125	0.215
	384	0.278	0.371	0.400	0.500	0.352
Cj391	153	0.722	0.622	0.300	0.500	0.730
	157	**0.028** [b]	**0.020** [b]	0.000	0.000	**0.004** [b]
	161	0.000	0.000	**0.100** [b]	0.000	**0.020** [b]
	169	0.250	0.138	0.100	0.000	0.016
	171	0.000	**0.005** [a, b]	0.000	0.000	0.000
	173	0.000	**0.011** [b]	**0.100** [b]	**0.500** [b]	0.000
	175	0.000	0.191	0.200	0.000	0.230
	179	0.000	**0.013** [b]	**0.200** [b]	0.000	0.000
CCj101	354	0.000	0.000	0.000	0.000	**0.035** [a, b]
	356	0.833	0.635	0.600	0.375	0.586
	358	0.000	**0.016** [b]	0.000	0.000	**0.004** [b]
	360	0.167	0.349	0.400	0.625	0.375
CpDi13	358	0.278	0.041	0.100	0.125	0.012
	360	0.556	0.590	0.600	0.625	0.645
	362	0.167	0.369	0.300	0.250	0.344
Cj127	337	0.750	0.820	1.000	0.875	0.816
	341	**0.056** [b]	**0.004** [b]	0.000	0.000	0.000
	343	0.194	0.176	0.000	0.125	0.184
CpP801	166	0.000	**0.002** [b]	**0.100** [b]	0.000	0.000
	170	0.222	0.196	0.100	0.000	0.152
	174	0.000	**0.002** [a, b]	0.000	0.000	0.000
	178	0.000	0.164	0.300	0.125	0.176
	182	0.778	0.568	0.400	0.750	0.672
	186	0.000	0.068	0.100	0.125	0.000

Table 3. Suggested parental combinations for the six proposed *ex-situ* subpopulations of *Crocodylus intermedius* in Colombia. a. Unillanos b. Ocarros c. Piscilago d. Merecure e. Wisirare f. EBTRF. The values in parentheses represent the Homozygosity by Loci for each individual. The values within the table represent the relatedness coefficient between both individuals compared. Females in the rows, males in the columns. Individuals in bold represent priority crocodiles. Relationships: ^{U} Unrelated; ^{HS} Half sibling; ^{FS} Full sibling; ^{PO} Parental offspring.

a. Ocarros

Current situation

F/M	**154 (0.199)**	**156 (0.485)**	**157 (0.614)**
155 (0.448)	0 ^{U}	0 ^{U}	0 ^{U}
158 (0.227)	0.124 ^{U}	0 ^{U}	0.115 ^{HS}

Suggested combinations

F/M	**156 (0.485)**
155 (0.448)	0 ^{U}
158 (0.227)	0 ^{U}
172 (0.249)	0 ^{U}
272 (0.292)	0 ^{U}

b. Unillanos

Suggested combinations

Tank 1

F/M	**154 (0.199)**
126 (0.363)	0 ^{U}
171 (0.309)	0 ^{U}
168 (0.334)	0 ^{U}

Tank 2

F/M	**157 (0.614)**
240 (0.170)	0 ^{U}
256 (0.137)	0 ^{U}
235 (0.213)	0 ^{U}

c. Piscilago

Current situation

	Tank 1	Tank 2
F/M	**214 (0.459)**	**213 (0.351)**
115 (0.227)	0 ^{U}	0 ^{U}
118 (0.352)	0.350	1

Suggested combinations

	Tank 1	Tank 2	Tank 3	
F/M	**214 (0.459)**	**181** (Amputated male)	F/M	**213 (0.352)**
115 (0.227)	0		**238 (0.330)**	0
258 (0.211)	0		239 (0.326)	0
345 (0.307)	0			

d. Merecure

Current combinations

F/M	131 (0.377)	132 (0.316)	135 (0.468)	136 (0.247)	137 (0.403)	**138 (0.214)**	139 (0.417)	**141 (0.454)**	142 (0.509)	143 (0.391)	**145 (0.707)**
130 (0.317)	0.180 HS	0.418 PO	0.176 HS	0.264 HS	0.500 PO	0.007 U	0 U	0.079 U	0.259 HS	0 U	0 U
133 (0.498)	0 U	0.244 HS	0 U	0.178 U	0.195 HS	0 U	0.129 U	0.130 HS	0.210 HS	0 U	0.009 U
134 (0.377)	0.202 HS	0.067 U	0.127 HS	0 U	0.272 HS	0.014 U	0.290 FS	0 U	0 U	0.078 U	0 U
147 (0.405)	0.467 PO	0.628 FS	0.138 HS	0.133 HS	0.555 PO	0 U	0.392 FS	0.070 U	0.351 HS	0.029 U	0 U
148 (0.170)	0.468 PO	0 U	0.414 FS	0.486 PO	0.446 PO	0.300 FS	0.258 HS	0.038 U	0.511 PO	0.216 U	0.136 HS
150 (0.264)	0 U	0.333 HS	0.102 U	0.136 HS	0.182 HS	0.522 FS	0.416 PO	0.236 HS	0.500 PO	0.098 U	0 U
151 (0.425)	0.087 U	0.500 PO	0.038 U	0.230 U	0.583 FS	0.270 HS	0.710 FS	0.096 U	0.451 FS	0.243 U	0 U
152 (0.283)	0.375 FS	0 U	0.462 FS	0.632 FS	0.250 HS	0 U	0 U	0 U	0.071 U	0.507 PO	0.014 U

Suggested combinations

F/M	**138 (0.214)**	**141 (0.454)**
134 (0.377)	0 U	0 U
152 (0.283)	0 U	0 U
376 (0.207)	0 U	0 U
165 (0.438)	0 U	0 U
520 (0.532)	0 U	0 U
170 (0.534)	0 U	0 U

e. Wisirare

Current and suggested combinations

F/M	385 (0.466)	389 (0.195)
384 (0.433)	0 U	0 U
387 (0.309)	0 U	0 U
388 (0.288)	0 U	0 U
391 (0.170)	0 U	0.209 U
392 (0.260)	0 U	0 U

f. EBTRF

Suggested combinations

Tank 1

F/M	**579 (0.304)**	**593 (0.334)**
221 (0.490)	0 U	0 U
306 (0.245)	0 U	0 U
450 (0.217)	0 U	0 U
453 (0.319)	0 U	0 U

Tank 4

F/M	519 (0.250)	206 (0.486)
118 (0.351)	0 U	0 U
164 (0.310)	0 U	0 U
232 (0.589)	0 U	0 U
378 (0.470)	0 U	0 U

Tank 2

F/M	**179 (0.525)**	**183 (0.540)**
288 (0.199)	0 U	0 U
220 (0.261)	0 U	0 U
275 (0.338)	0 U	0 U
274 (0.266)	0 U	0 U
291 (0.225)	0 U	0 U
286 (0.358)	0 U	0 U

Tank 5

F/M	**197(0.265)**	**204 (0.278)**
231(0.264)	0 U	0 U
295(0.261)	0 U	0 U
290 (0.347)	0 U	0 U
255 (0.350)	0 U	0 U
583 (0.372)	0 U	0 U
365 (0.385)	0 U	0 U

Tank 3

F/M	**592 (0.289)**
233 (0.292)	0 U
590 (0.288)	0 U
270 (0.296)	0 U
364 (0.302)	0 U

Table 4. Relatedness coefficient and possible relationship within the founder crocodiles from Cravo Norte River (a) and Rango Rudd. hatchery (b).

Relationship: ^{U} Unrelated; ^{HS} Half sibling; ^{FS} Full sibling; ^{PO} Parental offspring.

a.	575	579	581	584	592	593
575	-					
579	0.247 ^{HS}	-				
581	0.102 ^{U}	0.618 ^{FS}	-			
584	0.466 ^{FS}	0 ^{U}	0 ^{U}	-		
592	0.360 ^{HS}	0.634 ^{FS}	0.781 ^{FS}	0 ^{U}	-	
593	0.232 ^{HS}	0.441 ^{FS}	0.482 ^{FS}	0.006 ^{U}	0.498 ^{FS}	-

b.	105	106	122	127	128	156	162	163	213	214	215	385
105	-											
106	0.545 ^{FS}	-										
122	0.500 ^{PO}	0.198 ^{HS}	-									
127	0 ^{U}	0.066 ^{HS}	0.500 ^{PO}	-								
128	0.410 ^{FS}	0.231 ^{HS}	0.267 ^{HS}	0.345 ^{FS}	-							
156	0.122 ^{HS}	0.145 ^{HS}	0.415 ^{FS}	0.500 ^{PO}	0 ^{U}	-						
162	0.260 ^{HS}	0.227 ^{HS}	0.828 ^{FS}	0.370 ^{HS}	0.163 ^{HS}	0.307 ^{HS}	-					
163	0 ^{U}	0 ^{U}	0.500 ^{PO}	0.567 ^{PO}	0.130 ^{U}	0.282 ^{HS}	0.421 ^{FS}	-				
213	0.500 ^{PO}	0.352 ^{FS}	0.259 ^{HS}	0.200 ^{U}	0 ^{U}	0.302 ^{HS}	0.252 ^{HS}	0 ^{U}	-			
214	0.168 ^{HS}	0.133 ^{HS}	0.588 ^{PO}	0.346 ^{HS}	0 ^{U}	0.670 ^{PO}	0.671 ^{FS}	0.292 ^{HS}	0.350 ^{HS}	-		
215	0 ^{U}	0 ^{U}	0.435 ^{FS}	0.362 ^{HS}	0 ^{U}	0.752 ^{FS}	0.340 ^{HS}	0.259 ^{HS}	0.122 ^{U}	0.756 ^{FS}	-	
385	0 ^{U}	0 ^{U}	0.436 ^{FS}	0.351 ^{HS}	0.591 ^{FS}	0.009 ^{HS}	0.344 ^{HS}	0.539 ^{FS}	0.003 ^{U}	0.120 ^{U}	0.009 ^{U}	-

Table 5. 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=68)	Wisirare (N=115)
Number of individuals with potential parents	7	0	47	46	80
Number of individuals without potential parents	0	2	7	22	35

2.8 Annexes

Table S1. Information of priority crocodiles presenting alleles at low frequencies. HL- Homozygosity by loci.

Annexed table in Excel format.

Table S2. Dynamic table generated to guide management guidelines comparing all living individuals of the program. HL- Homozygosity by loci. r- relatedness coefficient

Annexed table in Excel format.

Chapter 3

Behind the release of *Crocodylus intermedius'* individuals from a captive-breeding program in Colombia: the genetic approach.

3.1 Abstract

Translocation of captive breed individuals is a major tool in the management of threatened species. In Colombia, the critically endangered *Crocodylus intermedius* became locally extinct in great part of its original distribution and showed negative trends on populations recovery. Thus, efforts to rescue wild populations have been mainly focused on the reintroduction of individuals from a captive breeding program; to date, 240 crocodiles have been reintroduced. However, such translocations did neither consider the genetic component of the released individuals, nor the genetic profile of the intervened populations. To provide insights on the genetic component of the released individuals and to inform future movements, we used 16 polymorphic microsatellite loci to genotype and analyze: 1. Fifty-three crocodiles already moved into Guayabero / Losada, Guarrojo, Manacacias, and Tomo Rivers (four river nuclei with different conservation scenarios) and 2. Fifty-nine individuals that will be released in the Tomo River, contributing to the reinforcement of a *de novo* population. To enhance the population's long-term survival, it is necessary to include crocodiles with genetic diversity as high and different as possible from the already incorporated. Individuals released in Guayabero / Losada Rivers represent the only intervention to a possibly stable remaining population, while individuals released in Manacacias and Guarrojo Rivers represent interventions in non-cohesive populations. In both cases, crocodiles translocated presented kinship relationships and diversity indices that can be improved by considering the genetic profiles before liberations. We propose that in the short-term, reintroductions should only be carried out in places where it is certain that the populations have become extinct (e.g., Tomo or Bita Rivers in Tuparro National Natural Park). In case the species is still present, it is necessary to accurately estimate the genetic profile (i.e., diversity, population size and structure, inbreeding) before implementing reintroduction and other management actions.

Key words: microsatellites, population structure, *de novo* population, genetic potential, genetic diversity.

3.2 Introduction

Reintroductions from captive breeding programs are important tools for recovering endangered species, allowing the re-establishment of a population at a site where it has become extinct or currently exists in small numbers (Seddon *et al*., 2012). However, the aim of reintroductions goes beyond simply increasing the number of individuals but should also target genetic aspects that may be critical to the successful establishment of reintroduced populations (Drauch & Rhodes, 2007; Casena *et al.,* 2016). Genetic variation is the basis

for a species' evolutionary flexibility and responsiveness to environmental change in that it provides the raw material for future adaptations (Keller *et al.,* 2012). Nevertheless, genetic assessments tend to have a lower priority because of the long-time frame over which genetic factors act, relative to other agents of decline such as habitat loss (Jamieson *et al*., 2008; Jamieson & Lacy, 2012).

When reintroduction is based on individuals raised in captivity, the amount of genetic diversity available for translocation will be governed by the genetic constitution of the source population, which is heavily influenced by the species' population history and the bottlenecks experimented during the creation of the captive programs (Groombridge *et al.,* 2012). The first bottleneck occurs before conservation measures when populations become endangered and small (Keller *et al.*, 2012). The second bottleneck occurs when the captive breeding population is founded with a few wild-caught individuals, which will be the only source of genetic material for the growth of the population (Keller *et al.,* 2012). If genetic rules such as equalizing founder representation or avoiding close inbreeding are not considered, the population may lose genetic diversity over the generations (see Chapter 1; Ballou & Lacy, 1995; Ballou *et al*., 2010; Jamieson & Lacy, 2012). Finally, if we do not consider genetic profiles, when animals are released back into their former range, the population may experience the third bottleneck. Taking those factors into account, to provide the best possible start (Lacy, 1994), apart from having genetic requirements at the time of establishing and manage captive breeding programs (Chapter 2), there must be a genetic consideration in the selection of the individuals to be released (Jamieson & Lacy, 2012).

Maximizing genetic diversity is not enough since historical population profiles and future translocation strategies are intrinsically linked (Groombridge *et al.,* 2012). In cases where isolated remnant populations have already lost considerable genetic diversity or experience high levels of inbreeding, introductions of new genetic variants can prevent the negative consequences of disrupted gene flow and isolation (Tallmon *et al.*, 2004; Hedrick & Fredrickson, 2010). Contrarily, deliberate out-crossing can lead to unintentional genetic consequences in the form of outbreeding depression and disruption of local gene adaptations (Edmands, 2007; Jamieson & Lacy, 2012). When these genetic and population structure considerations are considered, targeted reintroductions have shown high-efficiency recovery even when species might be beyond hope genetically (e.g., *Falco punctatus*, Nicoll *et al.,* 2004; *Acrocephalus sechellensis*, Richardson *et al.,* 2006).

Reintroduction has become an important management tool for the conservation of the critically endangered *Crocodylus intermedius* (Graves, 1891) (see Lafferier *et al.,* 2016) that was historically distributed along the Orinoco River basin (Castro *et al.,* 2012). In Colombia, the commercial hunting of the last century caused the decline of its natural populations, which today is reflected in three different situations. Firstly, the species became totally extinct in several localities such as the Tomo, Bita, Ariporo, Cravo Sur, La Hermosa, or Picapico Rivers (Lugo, 1997; Castro *et al.,* 2012; Balaguera-Reina *et al.,* 2017; Parra *et al.*, 2020). Secondly, some populations became locally extinct but with remaining individuals such as in the middle course of the Meta River or in the Vichada River (Castro *et al.,* 2012). Thirdly, some populations decreased in size but remained stable, for instance, the Cravo Norte River (Castro *et al.,* 2012) and probably Guayabero River (Balaguera-Reina *et al.,* 2017). Thus, each of these sites has different population status and conservation necessities. For example, the largest population restricted to Ele, Lipa, and Cravo Norte Rivers in the Arauca department, seems to maintain its viability despite the killing of adults and the harvest of nests, while for other populations the natural recovery is almost impossible (e.g., Vichada subpopulation Castro *et al.,* 2012).

To assist in the recovery of the species in Colombia, a captive breeding program for its conservation was established in 1971 in the Roberto Franco Tropical Station (EBTRF) in Villavicencio, Meta department. Currently, the *ex-situ* population maintains more than 600 crocodiles distributed in five subpopulations, from which the 240 crocodiles released into the wild came. To date, the following crocodiles have been released: 71 in the Tomo River in El Tuparro National Park (Vichada); 32 in the La Aurora Civil Society Nature Reserve (CSNR) (Casanare); 29 on the Cravo Norte River (Arauca); 20 in the CSNR Corozito (Casanare); 25 in the CSNR Palmarito (Casanare), 40 in the CSNR Hato Venecia (Casanare); 15 in the Manacacias River (Meta); four in the Guayabero / Losada Rivers in La Macarena (Meta); and four in the Guarrojo River (Meta).

However, those reintroductions did neither contemplate the genetic component of individuals, nor the genetic profile of the intervened populations. Considering that genetic management must be a part of any translocation strategy to ensure the success of reintroduction programs (Groombridge et al., 2012), in this study we aimed at: 1. providing and evaluating the genetic characterization of four already performed releases and 2. providing relevant suggestions for future interventions. For this, we used 16 polymorphic

microsatellite loci to genotype and analyze 53 individuals released in the Guayabero/Losada, Manacacias, Guarrojo, and Tomo Rivers and 59 individuals to be released in a coming event. The analysis of this genetic information is crucial for: 1. the genetic understanding of the interventions already performed, 2. the recognition of the gene pool of the crocodiles intended to be introduced, and 3. informing coming translocations.

3.3 Methods

3.3.1 Sampling

Tissue samples were taken from individuals of *C. intermedius* released in four distinct nuclei of the natural distribution of the species (Figure 1). The first release comprised four crocodiles (two females and two males) from the EBTRF subpopulation, introduced into the Guayabero/Losada Rivers near to La Macarena municipality in the Meta department in 2015. The Guayabero/Losada Rivers apparently host a stable natural population (Figure 1A). The second release comprised 14 individuals (11 females and three males) from the EBTRF subpopulation, introduced into the Manacacias River, Puerto Gaitán municipality, Meta department in 2017. The Manacacias River apparently hosts seldom vagrant individuals and does not constitute a cohesive population (Figure 1B). The third release comprised four females from the EBTRF subpopulation, introduced into the Guarrojo River, Puerto Gaitán municipality, Meta department in 2018. Guarrojo River apparently hosts seldom vagrant individuals and does not constitute a cohesive population (Figure 1C). The fourth release comprised 31 individuals from the Wisirare subpopulation, introduced in the Tomo River, in the Tuparro National Natural Park in 2019. The Tomo River is part of the historical distribution of the species, but its population was extirpated (Figure 1D).

Additionally, tissue samples were taken from 59 of the 80 juvenile individuals that we consider potential to be released because: 1. they are not part of the reproductive nuclei (established in Chapter 2), 2. they have a size between 180 and 240 cm (Figure 2), and 3. they are healthy and have had a normal growth according to the growth model estimated in the EBTRF (María del Pilar Venegas and Germán Preciado, EBTRF officials, pers. comm.). Scales and muscle samples were preserved in pure ethanol and kept at -20°C until processing. All the translocated and to be released individuals were marked with microchips that allow their identification, except from the Tomo River's crocodiles.

3.3.2 Laboratory procedures and genotyping

Genomic DNA was extracted from the 115 preserved tissues using the same laboratory procedures described in Chapter 1. We amplified 16 polymorphic loci of microsatellite tested for cross-amplification by Laferriere *et al.,* (2016); four PCRs multiplex were performed (Mix1: CpP302, CpP305, CpP314, CpP1409, CpP3216; Mix2, Cj16, Cj122, Cu5123; Mix3: Cj18, Cj109, C391, CUJ131; Mix4: Cj101, Cj127, Cp801, CpDi13) using the Multiplex PCR kit MyTaq™ HS Mix (Bioline, USA). Reactions and thermocycling conditions were used as described in Chapter 1. Fragment lengths were determined using an ABI 3500 Genetic Analyzer. The Gene-Mapper 3.7 (Applied Biosystems Foster City, CA) and Osiris 2.13.1 (NCBI) software were used for scoring fragment lengths. Genetic laboratory work was conducted at the Molecular Ecology Laboratory of the Genetics Institute, National University of Colombia in Bogotá.

3.3.3 Data Analysis

- **Identification of genetic diversity**

In all crocodiles (i.e., the four groups of released individuals and the group of individuals to be released), number of alleles per locus and allelic frequencies were calculated in FSTAT 2.9.3.2 (Goudet, 2001). Considering that the liberation carried out in the Tomo River is the only one that contributes to the creation of a population *de novo*, to have a starting point we estimate inbreeding coefficient (F_{IS}) in FSTAT 2.9.3.2 (Goudet, 2001), expected heterozygosities (He) and observed heterozygosities (Ho) in ARLEQUIN 3.5.1.2 (Excoffier *et al.* 2005). The same software was used to test for Hardy Weinberg equilibrium (HWE) and linkage equilibrium; Bonferroni corrections were applied for both calculations. F_{IS} significances for excess and defect of heterozygous were evaluated in Genepop 4.7.5 (p-value < 0.005, Raymond & Rousset, 1995).

- **Identification of kinship relationships**

Relationships between the crocodiles released at each site and between the crocodiles to be released were inferred using ML-RELATE (Kalinowski *et al.,* 2006), a Maximum Likelihood-based software that estimates relatedness coefficient (*r*) for each pair of individuals and provides a list of several possible relationships (Half-Sibling, Full-Sibling,

Parental-Offspring and Unrelated). Considering that the program does not consider size and age factors and that the relationship indexes between Full Siblings and Parents/ Offspring are similar, in cases where we find Parental/Offspring relationships, we consider them as Full Sibling relationships since the crocodiles are in the same size and age ranges and it is improbable a Parent-Offspring relationship.

- **Determination of individual diversity**

To determine the level of diversity of each analyzed individual, we estimated inbreeding coefficients at the individual level using the GENHET 2.3 R script (Coulon, 2010). We estimated the Homozygosity by Loci (HL), which is a homozygosity index that weights the contribution of each locus depending on their allelic variability (Aparicio *et al.,* 2006). Consequently, each crocodile is assigned a value ranging from 0 (all loci are heterozygous) to 1 (all loci are homozygous), allowing us to identify the individual diversity of each crocodile. To identify crocodiles to be released, we considered individuals that had an HL <0.6, which is the condition of 95% of the live crocodiles that we find in the Program (Chapter 2).

3.4 Results

The 16 polymorphic microsatellite loci were successfully amplified for the 112 processed samples. In the released individuals we found 54 alleles, but the number varied between each group (Table 1). In the four individuals released into the Guayabero/Losada Rivers we found 42 alleles (on average 2,6 alleles per locus), in the 15 individuals of the Manacacias River we found 53 alleles (on average 3,3 alleles per locus), in the 4 individuals of the Guarrojo River we found 54 alleles (on average 3,4 alleles per locus) and in the 31 individuals of the Tomo River, we found 49 alleles (on average 3,1 alleles per locus). In the 59 potential crocodiles to be released, we found 56 alleles (on average 3,5 alleles per locus).

The observed heterozygosity (Ho) and expected heterozygosity (He) in the Tomo River group were 0.637 and 0.547, respectively and the inbreeding coefficient F_{IS} was -0.164 (Table 1). Of the 16 evaluated loci, locus CpP801 was not found in Hardy-Weinberg equilibrium, and loci Cj391 and CpP801 presented statistically significant deviations in the inbreeding coefficient F_{IS} due to excess of heterozygotes.

Allele frequencies varied between groups. While we found alleles that maintained an equal frequency in the four liberations, even when comparing with potential crocodiles to be released (e.g., alleles 200 locus CpP302, allele 167 locus Cj16, alleles 337 and 343 locus Cj127, Table 2), other alleles presented different frequencies in each group (e.g., allele 386 locus Cj122, allele 185 and 191 locus CUJ131, allele 372 locus Cj109, allele 182 locus Cpp801, Table 2). Additionally, some alleles were not found in all groups. For example, allele 141 locus Cj16 was found in individuals released in the Manacacias and Guayabero/Losada Rivers, but it was not found in the other liberations or potential crocodiles to be released. Furthermore, allele 157 and 173 locus Cj391 were only found at very low frequencies in crocodiles to be released and in individuals released in the Manacacias River. Alleles 358 locus CCj101, 358 locus CpDi13 and 166 locus CpP801 were only found in individuals to be released (Table 2).

The individual diversity (HL) of the four Guarrojo River individuals varied between 0.287 and 0.429 (Figure 3, Table 3 a). Individuals 599 and 44 had a Half Sibling relationship and individuals 603 and 599 had a Full Sibling relationship; the other crocodiles were not related (Table 3a). The individual diversity of the four Guayabero/Losada Rivers individuals was 0.458 and 0.597 for the two females and 0.447 and 0.578 for the two males (Figure 3 and Table 3b). Individuals 321 (female) and 201 (male) had a Full Sibling relationship, and individual 615 (female) had a Half Sibling relationship with individuals 208 (male) and 321 (female) (Table 3b). The individual diversity of the 14 Manacacias River individuals varied between 0.215 and 0.604 for the females and between 0.336 and 0.539 for the males (Figure 3 and Table 3c). When comparing the relationships between the 14 individuals, we found 10 Half Sibling relationships, 12 Full Sibling relationships, and 69 Unrelated relationships (Table 3c). Finally, the individual diversity of the Tomo River individuals varied between 0.03 and 0.581 (Figure 3 and Table 3d). Of the 465 relationships determined when comparing the 31 individuals, we found 278 Unrelated relationships, 100 Half Sibling relationships, and 87 Full Sibling relationships (Table 3d).

The individual diversity of the juveniles to be released varied between 0.136 and 0.801, and of the 59 individuals, three had a Homozygosity by Loci higher than 0.6 (individuals 584, 596, and 610, Table S2). Of the 1891 relationships determined when comparing the 59 individuals, we found 1352 Unrelated relationships, 282 Half Sibling relationships, and 257 Full Sibling relationships (Table 3d). With the kinship information provided (Table S1) and

the genotypes of these individuals (Table S2), it is possible to establish liberation groups that contain individuals with an HL <0.6 that are the least related possible, and that have specific allele frequencies.

3.5 Discussion

The current conservation of the Orinoco crocodile is critical (Castro *et al.*, 2012), and one of the most promising strategies to recovery is reintroduction. We presented the genetic profiles and kinship relationships of 53 individuals of *Crocodylus intermedius* released in four localities part of its historical distribution: Guayabero/Losada, Manacacias, Guarrojo, and Tomo Rivers. This baseline provides us with crucial information for planning and directing future interventions for the recovery of the species. We found a high release potential in captive juvenile individuals based on our evaluations of the genetic profile and kinship relationships which combined with health, sex, and location allow us to plan future actions. With the information provided, the forward steps include the selection of the most appropriate genetically diverse and less related crocodiles, according to logistic capacities, to contribute to natural population constitution or enrichment.

Considering that the long-term success of the reintroduction programs is influenced by key demographic and genetic components (Drauch & Rhodes, 2007), based on our genetic results, we discuss about specific reintroduction initiatives in the context of the natural populations intervened.

3.5.1 *De novo* population: the Tomo River

The reintroductions made in the Tomo River represent the only translocation to the wild where the species seems to be locally extinct without apparent remnant individuals, making it the only release that contributes to the creation of a *de novo* population. Although 71 individuals from the Wisirare subpopulation have been reintroduced in the Tuparro National Natural Park in Tomo River, only samples of 31 individuals were studied. When comparing the subpopulation of origin with the subsample of the released individuals, we found that while in Wisirare there were 56 alleles, in the Tomo River´s released individuals there were 49 alleles (Table 1). Alleles 141 (CJ16), 193 (CUJ131), 157/161 (Cj391), 354/358 (CCj101), and 358 (CpDi13) are absent in the released individuals but present in the Wisirare

subpopulation at very low frequencies (Chapter 2), making them prone to being lost and not being included in releases by chance.

When comparing the allele frequencies between both groups, we found that, although there are loci where the frequencies varied a little (e.g., allele 213 locus CJ18 or allele 374 locus Cj109), in general they were similar in both groups (Table 2). It would be expected that if we evaluate allele frequencies adding the 40 released non-genotyped crocodiles, the genetic frequencies will not vary much and should adjust to the frequencies of the Wisirare subpopulation.

Despite that the original –now extinct– population of Tomo River may have had a particular genetic fingerprint, one approach to enhance the long-term survival of these *de novo* populations is maximizing the genetic diversity introduced (Groombridge *et al.,* 2012). To reach that objective and considering that the *ex-situ* population of *C. intermedius* has a genetic diversity greater than that found in the Wisirare subpopulation (Chapter 2), it is necessary to complement the management with crocodiles coming from other subpopulations that have a genetic diversity as high and different as possible from the one already included. In addition, individuals released in Tuparro River come from a maximum of nine parents (Chapter 2), which is reflected in that approximately 40% of the evaluated relationships have a kinship level of Half or Full Sibling (Table 3d).

In the group of potential individuals to be released from the EBTRF subpopulation, we found that alleles 157/161 (Cj391), 358 (CCj101), (CpDi13), 179 (Cj391), and 166/186 (CpP801) are not present in the Tomo River´s released individuals (Table 2). To increase genetic variability, individuals to be released that have these alleles can be identified in Table S2 and should be prioritized in the next translocation event that will take place in the Tomo River. Considering the genetic profile of those individuals (Table S2) and the kinship relationships between them (Table S1), it is possible to assemble unrelated groups that contain the greatest amount of genetic diversity. The number of individuals per group will be determined by the number of logistically viable individuals to be moved.

The Tomo River is an ideal place to introduce crocodiles in the short term because it lies within a protected area and there is almost no human presence. This aspect is very important since the killing of adult specimens due to local inhabitants' fear could reduce the number of adult crocodiles in the wild (Castro *et al.,* 2012). For this reason, releases should

be made with a strong component of environmental education and in the meantime, areas without human presence can be prioritized and intervened with the individuals that have the genetic, size, health, and age requirements to be released (Table S2). Another place with similar conditions is the Bita River in the Tuparro National Natural Park, which is part of the historical distribution range of the species and where no relictual population or remnant individuals have been reported either (Parra *et al.*, 2020).

3.5.2 Stable relictual populations: Guayabero/Losada Rivers

Among the four release events evaluated, the liberation in the Guayabero/Losada Rivers was the only one performed in one of the four relict populations of *C. intermedius* in Colombia (Castro *et al.,* 2012). The two females and two males released had an individual diversity lower than that found in general in the *ex-situ* population (Chapter 2), and they showed a high degree of relationship. This reflects the need to implement genetic management when choosing the crocodiles, since the incorporation of individuals with low genetic diversity could have long-term repercussions (Jamieson & Lacy, 2012).

The Guayabero River complex in Colombia was defined as one of the areas with the most optimal conditions for long-term preservation and maintenance of *C. intermedius* populations (Balaguera-Reina *et al.,* 2017), but the last censuses were made more than 10 years ago and currently, we do not know the status of those populations (Castro *et al.,* 2012). Furthermore, in such places where it is believed that stable relictual populations remain, the hybridization and introgression caused by the reintroduction of different genetic lineages may have negative effects on the population's overall fitness. If two populations have been separated for a long time or if there are significant habitat differences, the populations are likely to show significant genetic divergence and possibly local adaptations (Frankham *et al.,* 2010; Frankham *et al.,* 2011; Banes *et al.,* 2016). Without considering the populations' genetic profile, reintroductions could generate homogenization, an effect already reported in other species (e. g. *Psittacula eques*, Groombridge *et al*., 2012).

For *C. intermedius*, Medem (1981) established that the Maipures and Atures streams in Vichada could represent natural geographical barriers for the movement of crocodiles and that before the populations decline of the XX century, differences (e.g., in population sizes) were evident when comparing populations located in the lower part of the streams (Guaviare River and tributaries) with those found in the upper portion of the streams (Meta River and

tributaries) (Medem, 1981). Although an approach to the genetic of natural populations found limited evidence of geographical structuring and suggested that the Orinoco Crocodile could be managed as a single genetic unit in Colombia (Posso-Peláez *et al*., 2018), the results are questionable due to the molecular marker used (Cytochrome b and Cytochrome c oxidase subunit 1; Posso-Peláez *et al*., 2018), the lack of inclusion of localities recognized as Regional Habitat Priority / Conservation Crocodilians Units (RHP / CCU) (Balaguera-Reina *et al.* 2017) and especially the lack of samples from the Guaviare River zone. Therefore, it is necessary to reevaluate the existence of a genetic structure in the populations of *C. intermedius* in Colombia with variable genetic markers that have been successful in inferring population structure in crocodilids, such as microsatellites and the control region of the mitochondria (Ray & Densmore, 2003; Lapbenjakul *et al.* 2017; Rossi *et al*. 2020; Vashistha *et al.* 2020). The presence of a genetic footprint in this isolated and relictual population is highly expected, therefore it should be assessed, recognized, and preserved.

While we do not know the presence of a structure and the current state of the population of the Guayabero River complex, we can neither know the effect that the four released individuals may have had in the population (if they survived and reproduced), nor can we plan future interventions. In these cases, a good approach for the conservation of the species is through the reintroduction of wild-caught rather than captive-reared crocodiles, considering that at least the survival probability increases by eliminating threats such as egg and neonate predation (e.g., see Barros *et al.,* 2010). These efforts have been carried out in Wisirare, where eggs from the Rivers Ele and Cravo Norte have been collected to incubate them and release the hatchlings. This strategy could also be implemented in other rivers.

3.5.3 Unstable remnant populations: Manacacias and Guarrojo Rivers

Aside from the two stable populations of *C. intermedius* in Colombia (Cravo Norte and Guayabero River complexes), two other non-stable relictual populations composed of some solitary individuals have been reported in the Vichada River and the middle course of the Meta River (Castro *et al.,* 2012). Currently, these unstable populations are considered the most threatened, and their natural recovery without human intervention is almost impossible (Castro *et al.,* 2012). However, despite the censuses carried out by Federico Medem in the 1970s, by the Ministry of the Environment in the 1990s, by the Chelonia Association and the

EBTRF in the past decade (Barahona & Bonilla, 1999; Lugo 1996; Castro *et al.,* 2012), it is probable that remnants of other populations persist but have not been identified yet, such as in the Guarrojo, Manacacías, Yucao, Arauca Rivers or in the section of the Meta River that borders Venezuela (Rafael Antelo, pers. comm; see Balaguera-Reina *et al.,* 2017).

Despite not knowing the situation and even the presence of the species in rivers such as the Manacacias and the Guarrojo, 14 and four individuals were released in 2017 and 2018, respectively. The individuals involved presented similar individual genetic diversity indices compared with those found in the captive breeding program (Chapter 2), and in both groups most individuals are not related. However, we consider that this diversity could be maximized, and relatedness minimized by knowing the genetic profiles before releasing.

In the case of the Manacacias River, although there are allele frequencies that are almost identical when compared with the frequencies of the EBTRF subpopulation (e.g., locus CpP3216, EBTRF subpopulation allele frequencies available in Chapter 2), there are other alleles that present very different frequencies (e.g., alleles at locus CUJ5123). Additionally, we found alleles in the EBTRF that are not present in the released individuals (alleles 390 locus Cj122, 207 locus Cj18, 171 locus Cj391, 358 locus CCj101, 358 locus CpPdi12, 341 locus Cj127,174 locus CpP801). This is because liberations considered *C. intermedius* populations as a single genetic unit (Posso-Peláez *et al*., 2018), did not contemplate genetic profiles and the sample is not large enough to capture a representative diversity of the EBTRF subpopulation. In the case of the Guarrojo River, since only four individuals were released, the allelic frequencies obtained are also the result of having chosen genotypes randomly. What is noteworthy is that this group of four individuals of the Guarrojo River has 12 more alleles than the four individuals released in Guayabero/Losada Rivers and one more allele than the 14 individuals released in Manacacias River.

As we have already mentioned, since we do not know the current state of the populations, we cannot infer about the effects that the inclusion of these individuals may cause (if they survived and reproduced). If it is determined that these populations are incapable of recovering naturally and if they are in a vortex of extinction due to inbreeding and small populations size, the introduction of novel genetic variants can augment genetic diversity, reversing indications of inbreeding depression and increasing population sizes (Banes *et al*., 2016). The benefits of 'genetic rescue' have been demonstrated in several conservation initiatives (e.g., *Puma concolor coryi* Pimm *et al.,* 2006; Ovis canadensis Hogg *et al.,* 2006;

Groombridge *et al.,* 2012), being effective even with the inclusion of a single migrant (e.g., *Canis lupus* Vila *et al.,* 2003), and have resulted not only in increasing the fitness but succeeded in restoring genetic diversity to ancestral levels (e.g., *Tympanuchus cupido* Bouzat *et al.,* 2009). Nevertheless, what is clear is that only by comparing levels of genetic variation before and after, we may be able to measure the need and the effect of genetic rescue (as suggested by Groombridge *et al.,* 2012). As long as we do not have this information, as in the case of stable populations, a measure of immediate conservation is through the incubation and subsequent release of wild individuals.

3.5.4 The future of releases

Long-term monitoring of genetic diversity and inbreeding in reintroduced populations needs to be incorporated into field programs, to provide the data and the statistical power to look for the consequent effects of the genetic interventions and to plan long-term actions such as later supplementary translocations (Groombridge *et al.,* 2012). Nevertheless, even introductions can be effective management strategies, recovery and long-term viability would not be realized unless there are complementary managements that ensure reproductive ecological and environmental conditions such as the presence of nesting beaches, preserved riparian landscape, or human coexistence and tolerance among others (Bouzat *et al.,* 2009; Jamieson & Lacy, 2012; Keller *et al.,* 2012).

It is necessary to evaluate the effectiveness of releases to determine whether it is possible to improve the choice of individuals to be released. For example, by modeling mark-recapture monitoring data, it is possible to adjust management decisions on the age of individuals to be released, which can vary even among sites (Casena *et al.,* 2016).

While neutral genetic markers can be employed to assess various parameters relevant to population genetics, they may not reflect variation at functional loci important to the fitness of the species in question (Reed & Frankham, 2001; van Tienderen *et al.,* 2002; Bekessy *et al.,* 2003). If the numbers of founders required to translocate variation from a population are determined by neutral variation, the numbers needed to retain the higher levels of functional variation could be underestimated (Groombridge *et al.,* 2012). Then, using a combination of genome-wide neutral markers and specific ‘critical’ loci (e.g., genes of the MHC; Hughes, 1991) may be the safest way to assess neutral and adaptive genetic diversity (Groombridge *et al*., 2012).

Studies like the presented here illustrate how genetic management may have an impact upon the reintroductions since translocations must be done based on the requirements of natural populations, which are only understood by studying them. By ignoring the genetic structure of populations and the genetic profile of the individuals' release, allelic frequencies and reintroduced genetic diversity are the result of chance, which in the end will not contribute to ensure the survival of the populations and, on the contrary, affect their dynamics and the genetic structure.

3.6 References

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3.7 Tables and figures

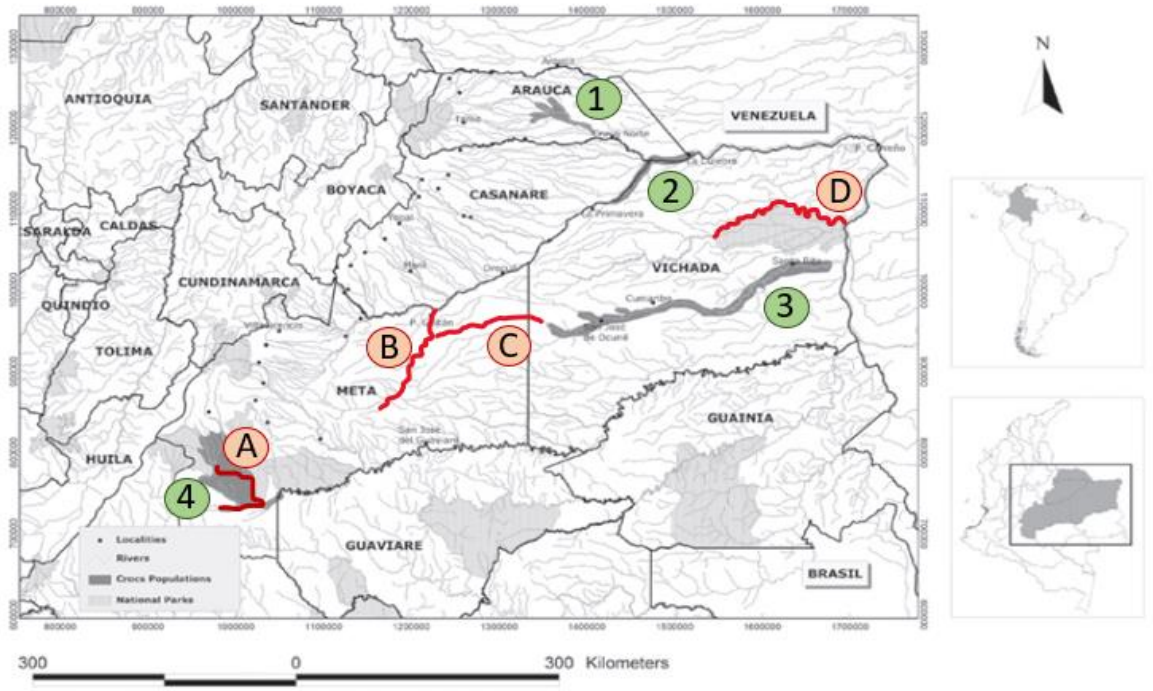

Figure 1. Green circles identify the location of the four relict populations of *C. intermedius* in Colombia (dark grey): (1) Cravo Norte River complex, (2) Middle curse of the Meta River, (3) Vichada River and (4) Guayabero River complex. Orange circles identify the four releases events (red lines): (A) Guayabero/Losada Rivers, (B) Manacacias River, (C) Guarrojo River and (D) Tomo River. Taken and modified from Castro *et al.* (2012).

Figure 2. Potential juvenile crocodiles to be released located at the Roberto Franco Tropical Biology Station (EBTRF) in Villavicencio, Meta.

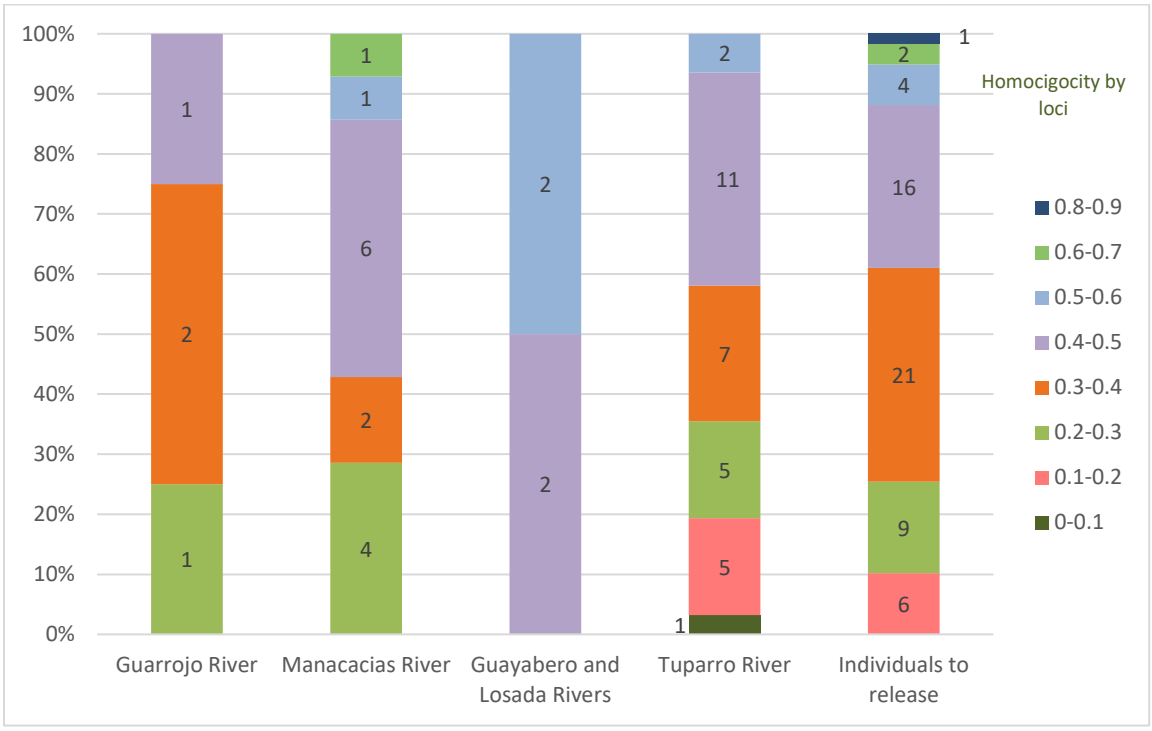

Figure 3. Distribution of individual diversity (homozygosity by loci, HL) of the individuals released in the four localities and in the group of potential individuals to be released. The values within the columns indicate the number of individuals in each category.

Table 1. Genetic diversity of the four groups of individuals released and potentials to be released of *Crocodylus intermedius* in charge of the Roberto Franco Tropical Biology Station.

N – sample size, nA – alleles per locus, Ho – observed heterozygosity, He – expected heterozygosity, HWE – Hardy-Weinberg equilibrium, F_{IS} - inbreeding coefficient.

	Juveniles' potential to be released (total # of alleles = 56)		Guayabero (total # of alleles = 42)		Manacacias (total # of alleles = 53)		Guarrojo (total # of alleles = 54)		Tomo (total # of alleles = 49)					
Locus	N	nA	N	nA	N	nA	N	nA	N	nA	Ho	He	HWE	F_{IS}
CpP3216	59	2	4	2	14	2	4	2	31	2	0.3548	0.4744	Yes	0.255
CpP305	59	3	4	2	14	3	4	3	31	3	0.4839	0.5923	Yes	0.186
CpP1409	59	3	4	3	14	3	4	3	31	3	0.7419	0.5653	Yes	-0.319
CpP302	59	4	4	4	14	4	4	4	31	4	0.6452	0.6626	Yes	0.027
CpP314	59	3	4	3	14	3	4	3	31	3	0.7742	0.6748	Yes	-0.15
Cj16	59	3	4	3	14	4	4	4	31	3	0.6774	0.5537	Yes	-0.228
CU5123	59	4	4	4	14	4	4	4	31	4	0.8065	0.6478	Yes	-0.25
Cj122	59	5	4	4	14	4	4	5	31	5	0.8065	0.7314	Yes	-0.105
Cj18	59	4	4	3	14	4	4	4	31	4	0.7097	0.5854	Yes	-0.217
CUJ131	59	2	4	2	14	2	4	2	31	2	0.4516	0.3554	Yes	-0.277
Cj109	59	4	4	2	14	4	4	4	31	4	0.8387	0.6446	Yes	-0.308
Cj391	59	6	4	2	14	6	4	6	31	3	0.8065	0.5484	Yes	-0.482 *
CCj101	59	3	4	2	14	2	4	2	31	2	0.5807	0.495	Yes	-0.176
CpDi13	59	3	4	2	14	2	4	2	31	2	0.4839	0.4744	Yes	-0.02
Cj127	59	2	4	2	14	2	4	2	31	2	0.1613	0.1507	Yes	-0.071
CpP801	59	5	4	2	14	4	4	4	31	3	0.871	0.5881	No	-0.493 *
Mean		3.5		2.625		3.313		3.375		3.063	0.637	0.547		-0.164
SD		1.155		0.806		1.138		1.204		0.929	0.201	0.140		0.208

* significance for heterozygous excess

Table 2. Allelic frequencies of 16 polymorphic microsatellite loci in the four groups of individuals released and potentials to be released of *Crocodylus intermedius* in charge of the Roberto Franco Tropical Biology Station.

Locus	Allele	Juveniles to be released (N=59)	Guayabero Losada (N=4)	Manacacias (N=14)	Guarrojo (N=4)	Tomo (N=31)
CpP3216	137	0.661	0.375	0.643	0.500	0.629
	141	0.339	0.625	0.357	0.500	0.371
CpP305	176	0.073	0	0.036	0.250	0.097
	192	0.468	0.375	0.357	0.125	0.452
	196	0.460	0.625	0.607	0.625	0.452
CpP1409	245	0.266	0.250	0.179	0.125	0.258
	249	0.637	0.500	0.643	0.750	0.597
	253	0.097	0.250	0.179	0.125	0.145
CpP302	194	0.476	0.25	0.679	0.625	0.516
	196	0.145	0.375	0.036	0	0.194
	200	0.177	0.125	0.179	0.250	0.113
	208	0.202	0.25	0.107	0.125	0.177
CpP314	254	0.411	0.125	0.571	0.750	0.290
	258	0.282	0.625	0.250	0	0.355
	262	0.306	0.250	0.179	0.250	0.355
Cj16	141	0	0.125	0.071	0	0
	167	0.677	0.750	0.607	0.750	0.613
	171	0.266	0.125	0.286	0.250	0.242
	173	0.056	0	0.036	0	0.145
CU5123	202	0.290	0.125	0.107	0.750	0.161
	214	0.056	0.250	0.071	0	0.097
	216	0.250	0.250	0.536	0	0.210
	220	0.403	0.375	0.286	0.250	0.532
Cj122	378	0.234	0	0.357	0.375	0.145
	380	0.347	0.125	0.393	0.500	0.419
	386	0.121	0.625	0.107	0	0.065
	390	0.065	0.125	0	0	0.113
	392	0.234	0.125	0.143	0.125	0.258
Cj18	207	0.169	0.125	0.143	0	0.048
	209	0.169	0	0.214	0.250	0.274
	211	0.605	0.750	0.571	0.500	0.581
	213	0.056	0.125	0.071	0.250	0.097
CUJ131	185	0.605	0.125	0.679	0.750	0.774
	191	0.395	0.875	0.321	0.250	0.226
Cj109	372	0.323	0.625	0.179	0.250	0.435
	374	0.040	0	0.179	0.125	0.016
	382	0.274	0	0.250	0.500	0.161
	384	0.363	0.375	0.393	0.125	0.387
Cj391	153	0.621	0.625	0.357	0.500	0.565
	157	0.040	0	0.071	0	0
	169	0.073	0.375	0.179	0.250	0.065
	173	0.040	0	0.036	0	0
	175	0.194	0	0.214	0.250	0.371
	179	0.032	0	0.143	0	0
CCj101	356	0.581	0.875	0.643	0.375	0.581
	358	0.048	0	0	0	0
	360	0.371	0.125	0.357	0.625	0.419
CpDi13	358	0.016	0	0	0	0
	360	0.653	0.875	0.714	0.625	0.629
	362	0.331	0.125	0.286	0.375	0.371
Cj127	337	0.895	0.875	0.821	0.875	0.919
	343	0.105	0.125	0.179	0.125	0.081
CpP801	166	0.008	0	0	0	0
	170	0.081	0.125	0.071	0.125	0.323
	178	0.202	0	0.179	0.250	0.129
	182	0.589	0.875	0.536	0.375	0.548
	186	0.121	0	0.214	0.250	0

Table 3. Relatedness coefficient and possible relationship within the crocodiles released in the Guarrojo River (a), Guayabero / Losada Rivers (b), Manacacias River (c) and Tomo River (d). The values in parentheses represent the Homozygosity per Loci for each individual.

Relationships: U Unrelated; HS Half sibling; FS Full sibling

a. Guarrojo River

	444 (0.305) ♀	473 (0.287) ♀	599 (0.429) ♀	603 (0.343) ♀
444 (0.305) ♀	-			
473 (0.287) ♀	0.012 U	-		
599 (0.429) ♀	0.281 HS	0 U	-	
603 (0.343) ♀	0 U	0.020 U	0.774 FS	-

b. Guayabero/Losada Rivers

	201 (0.578) ♂	208 (0.447) ♂	321 (0.597) ♀	615 (0.458) ♀
201 (0.578) ♂	-			
208 (0.447) ♂	0.005 U	-		
321 (0.597) ♀	0.374 FS	0.007 U	-	
615 (0.458) ♀	0 U	0.194 HS	0.141 HS	-

c. Manacacias River

	212 (0.475) ♂	237 (0.539) ♂	246 (0.255) ♀	501 (0.260) ♀	503 (0.335) ♂	547 (0.246) ♀	578 (0.478) ♀	588 (0.459) ♀	591 (0.604) ♀	594 (0.402) ♀	597 (0.284) ♀	605 (0.362) ♀	607 (0.417) ♀	611 (0.450) ♀
212 (0.475) ♂	-													
237 (0.539) ♂	0 U	-												
246 (0.255) ♀	0.020 U	0.114 U	-											
501 (0.260) ♀	0.011 U	0.055 U	0.259 U	-										
503 (0.335) ♂	0.173 HS	0.165 U	0.254 FS	0 U	-									
547 (0.246) ♀	0.237 U	0 U	0.345 HS	0 U	0.412 FS	-								
578 (0.498) ♀	0.083 U	0 U	0 U	0 U	0 U	0 U	-							
588 (0.459) ♀	0 U	0.263 U	0.073 U	0 U	0.007 U	0.5 FS	0.258 HS	-						
591 (0.604) ♀	0.118 U	0.117 U	0 U	0 U	0.244 HS	0.094 U	0.116 U	0.313 HS	-					
594 (0.402) ♀	0.225 HS	0.577 FS	0.097 U	0 U	0.123 U	0 U	0 U	0.067 U	0.032 U	-				
597 (0.284) ♀	0.005 U	0.500 FS	0.038 U	0.073 U	0.157 U	0.019 U	0 U	0 U	0.018 U	0.765 FS	-			
605 (0.362) ♀	0.068 U	0.453 FS	0 U	0 U	0.266 HS	0 U	0 U	0.033 U	0.091 U	0.582 FS	0.538 FS	-		
607 (0.417) ♀	0.123 U	0.501 FS	0.003 U	0 U	0.352 HS	0 U	0 U	0 U	0.126 U	0.652 FS	0.825 FS	0.344 HS	-	
611 (0.450) ♀	0.193 U	0 U	0 U	0 U	0 U	0 U	0.292 HS	0 U	0.069 U	0 U	0 U	0.083 U	0 U	-

d. Tomo River (Next page)

	T1 (0.453)	T10 (0.418)	T11 (0.415)	T12 (0.265)	T13 (0.337)	T14 (0.441)	T15 (0.445)	T16 (0.030)	T17 (0.477)	T18 (0.520)	T19 (0.362)	T2 (0.144)	T20 (0.300)	T21 (0.423)	T22 (0.581)	T23 (0.135)	T24 (0.466)	T25 (0.216)	T26 (0.331)	T27 (0.472)	T28 (0.325)	T29 (0.156)	T3 (0.104)	T30 (0.149)	T31 (0.326)	T4 (0.446)	T5 (0.215)	T6 (0.500)	T7 (0.339)	T8 (0.254)	T9 (0.280)
T1 (0.453)	-																														
T10 (0.418)	0 U	-																													
T11 (0.415)	0 U	0.020 U	-																												
T12 (0.265)	0.138 U	0 U	0.055 U	-																											
T13 (0.337)	0 U	0 U	0 U	0.340 FS	-																										
T14 (0.441)	0 U	0 U	0.363 HS	0.243 HS	0.184 HS	-																									
T15 (0.445)	0.328 HS	0.079 U	0.236 HS	0.023 U	0 U	0.073 U	-																								
T16 (0.030)	0 U	0 U	0.066 HS	0.445 FS	0.490 FS	0.584 FS	0 U	-																							
T17 (0.477)	0.227 HS	0 U	0.475 FS	0.176 HS	0 U	0 U	0.536 FS	0.118 U	-																						
T18 (0.520)	0.318 HS	0 U	0.571 FS	0 U	0 U	0.082 U	0.458 FS	0.034 U	0.686 FS	-																					
T19 (0.362)	0 U	0.332 HS	0 U	0.038 U	0 U	0 U	0 U	0 U	0.175 U	0.014 U	-																				
T2 (0.144)	0 U	0 U	0 U	0 U	0.550 FS	0.228 HS	0 U	0.705 FS	0.137 U	0 U	0 U	-																			
T20 (0.300)	0.658 FS	0.097 U	0 U	0.011 U	0 U	0 U	0.500 FS	0.017 U	0.500 FS	0.240 HS	0 U	0.137 HS	-																		
T21 (0.423)	0.144 U	0 U	0.316 HS	0.453 FS	0.249 U	0.225 HS	0.500 FS	0 U	0.400 FS	0.271 HS	0.179 HS	0.008 U	0.077 U	-																	
T22 (0.581)	0.116 U	0.218 HS	0.500 FS	0.034 U	0 U	0 U	0.290 HS	0 U	0.500 FS	0.668 FS	0.319 HS	0 U	0 U	0.186 HS	-																
T23 (0.135)	0 U	0 U	0 U	0.635 FS	0.697 FS	0.039 U	0 U	0.208 U	0.500 FS	0 U	0.244 HS	0.028 U	0 U	0.390 FS	0 U	-															
T24 (0.466)	0.761 FS	0 U	0.244 HS	0.118 U	0 U	0 U	0.073 U	0.031 U	0.361 HS	0.464 FS	0.030 U	0.087 U	0.534 FS	0.052 U	0.352 HS	0.050 U	-														
T25 (0.216)	0.273 HS	0 U	0.350 HS	0 U	0 U	0 U	0.336 HS	0.115 U	0.436 FS	0.500 FS	0.341 HS	0.033 U	0.425 FS	0.070 U	0.177 HS	0 U	0.500 FS	-													
T26 (0.331)	0 U	0.440 FS	0.302 HS	0 U	0.095 U	0.127 U	0 U	0.040 U	0.236 HS	0.223 HS	0.325 HS	0.168 HS	0.194 U	0.266 HS	0.414 FS	0.045 U	0.144 U	0.048 U	-												
T27 (0.472)	0 U	0.500 FS	0.473 FS	0.099 U	0 U	0.222 HS	0.253 HS	0 U	0.253 HS	0.278 HS	0.532 FS	0 U	0 U	0.503 FS	0.621 FS	0 U	0 U	0.039 U	0.500 FS	-											
T28 (0.325)	0 U	0 U	0.084 U	0 U	0.490 FS	0.455 FS	0 U	0.090 U	0 U	0 U	0.103 U	0.407 FS	0 U	0.160 U	0 U	0.105 U	0 U	0.036 U	0.269 HS	0 U	-										
T29 (0.156)	0.332 HS	0.111 U	0 U	0.337 HS	0.019 U	0 U	0 U	0.100 U	0.256 HS	0 U	0.314 HS	0.040 U	0.500 FS	0.138 HS	0.500 FS	0.500 FS	0.500 FS	0.082 U	0.270 HS	0.329 HS	0 U	-									
T3 (0.104)	0.074 U	0.001 U	0 U	0.136 U	0.298 U	0 U	0.082 U	0.363 FS	0.137 U	0.009 U	0 U	0.649 FS	0 U	0.054 U	0 U	0 U	0 U	0 U	0.188 HS	0.038 U	0.264 HS	0.062 U	-								
T30 (0.149)	0.125 U	0.040 U	0.220 HS	0.500 FS	0.216 HS	0.061 U	0 U	0.289 FS	0.217 HS	0 U	0 U	0.210 HS	0.251 HS	0.217 HS	0 U	0.500 FS	0.164 HS	0.034 U	0 U	0.146 U	0 U	0.1257 HS	0.151 HS	-							
T31 (0.326)	0.121 U	0.004 U	0.052 U	0.430 FS	0.313 HS	0.500 FS	0.211 HS	0.667 FS	0.500 FS	0.249 HS	0 U	0.354 FS	0.500 FS	0.091 U	0.209 HS	0.315 FS	0.500 FS	0.007 U	0.034 U	0.116 U	0.129 HS	0.500 FS	0.180 U	0.113 U	-						
T4 (0.446)	0.115 U	0 U	0.155 U	0.326 HS	0.059 U	0.078 U	0.226 HS	0.377 FS	0 U	0.010 U	0 U	0.058 U	0 U	0.219 HS	0.082 U	0 U	0 U	0.008 U	0 U	0 U	0.174 HS	0 U	0.319 HS	0 U	0 U	-					
T5 (0.215)	0.370 HS	0.123 U	0 U	0.171 HS	0 U	0.042 U	0 U	0 U	0.247 HS	0.280 HS	0.312 HS	0 U	0.125 U	0.423 FS	0.500 FS	0.163 HS	0.293 HS	0 U	0.500 FS	0.384 HS	0 U	0.565 FS	0.067 U	0 U	0.046 U	0.006 U	-				
T6 (0.500)	0.190 U	0.264 HS	0 U	0.032 U	0 U	0.263 HS	0 U	0 U	0.189 HS	0.075 U	0.366 HS	0 U	0 U	0.342 HS	0.396 FS	0 U	0.045 U	0 U	0.500 FS	0.498 FS	0 U	0.139 HS	0.083 U	0.150 U	0 U	0.121 U	0.312 HS	-			
T7 (0.339)	0 U	0.284 HS	0.551 FS	0.076 U	0 U	0.163 HS	0.234 HS	0.314 HS	0.135 HS	0.235 HS	0.255 HS	0.035 U	0 U	0.114 U	0.683 FS	0.067 U	0.002 U	0 U	0.419 FS	0.577 FS	0.029 U	0.500 FS	0.064 U	0 U	0.114 U	0.248 HS	0.500 FS	0.295 HS	-		
T8 (0.254)	0.500 FS	0 U	0 U	0.500 FS	0.160 HS	0 U	0.289 U	0.249 HS	0 U	0.120 U	0 U	0.248 U	0 U	0 U	0.090 U	0.093 U	0.530 FS	0 U	0 U	0 U	0.148 HS	0 U	0.343 FS	0 U	0.419 FS	0.500 FS	0.111 U	0.028 U	0 U	-	
T9 (0.280)	0 U	0 U	0.053 U	0.289 FS	0.395 FS	0.403 FS	0 U	0.077 U	0.077 U	0 U	0.108 U	0.483 FS	0 U	0.395 FS	0 U	0.130 U	0 U	0 U	0.2 U	0.116 U	0.450 FS	0 U	0.485 FS	0.269 HS	0 U	0.227 HS	0 U	0.137 HS	0.068 U	0.141 HS	-

3.8 Annexes

Table S1. Relatedness coefficient and possible relationship within the crocodile's potential to be released hosted in the Roberto Franco Tropical Biology Station (EBTRF). Relationships: U Unrelated, HS Half sibling, FS Full sibling.
The values in parentheses represent the homozygosity per loci of each individual.

Table S2. Genotypes of the 16 polymorphic microsatellite loci for the 59 juvenile crocodiles to be released.

HL: Homocigosity by loci.

Conclusions

Successful reproduction (Chapter 1 and 2) and reintroduction (Chapter 3) are necessary steps in the recovery of *Crocodylus intermedius*. For this, the *ex-situ* population of the Orinoco crocodile founded in Colombia 50 years ago and currently in charge of the Roberto Franco Tropical Biology Station presents a genetic reservoir for the species towards the recovery of wild populations.

Although from the foundation until today there was a loss of alleles due to an inadequate reproductive management in the rearing system that allowed the mating of a limited group of reproducers, the population maintains high levels of genetic diversity. Nevertheless, genetic variability is unevenly distributed in the population and therefore two management guidelines need to be considered: first, maintain high levels of heterozygosity by combining unrelated genetically variable adult individuals, and second prioritize the combinations with individuals that have rare alleles to not lose them.

With the introduction of a breeding strategy that considers the genetic profile of each individual and combines less related individuals, the percentage of genetic diversity can be significantly preserved and increased. Resulting offspring with high genetic diversity can be released into the wild according to the requirements of natural populations.

The EBTRF population covers a very restricted range of the historical natural distribution of the species in Colombia. Our results suggested 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 the wild populations, as well as to bring wild individuals from unsampled sites, to refresh the diversity of the Program and avoid future inbreeding.

We propose that in the short-term, reintroductions should only be carried out in places where it is certain that the populations have become completely extinct (e.g., Tomo or Bita Rivers in Tuparro National Natural Park). We especially recommend including crocodiles

with genetic diversity as high and different as possible from the already incorporated in Tomo River.

In case the species is present, it is necessary to accurately estimate the population size and assess its genetic profile before implementing reintroduction measures or any other management action. Developing activities considering the species as a single genetic unit could generate a homogenization of remaining populations, losing genetic diversity and evolutionary potential.

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