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Orinoco crocodile
Crocodylus intermedius

Mating systems and the potential for genetic monitoring and paternity testing

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Abstract

The Orinoco crocodile (*Crocodylus intermedius*) is today one of the most critically endangered crocodile species in the world, with only two sustainable natural populations left in the wild. As a means to protect and reintroduce the species in Venezuela, breeding and reintroduction programs have been initiated, and relevant questions on the behavior and genetic status of the remaining populations have been raised. In this study the issue of mating systems and the potential for paternal testing and genetic monitoring of Orinoco crocodile populations and the potential of using microsatellite markers to address this subject has been investigated. The study showed that the obtained markers - originally developed for crocodile species closely related to the Orinoco crocodile - do not contain enough variation to support the marker resolution required for paternal testing or genetic monitoring in a population of low genetic diversity equal to that of the studied sample population. However, despite low resolution on the markers, proof of a multiple paternity mating system was discovered, as well as indications of only a few males being responsible for the offspring of several nests, suggesting that dominant males may gain more offspring than their subordinates. This will have implications on the outcome of any reintroduction program undertaken in Venezuela. To further study the subject, additional variable microsatellite markers will have to be developed.

I. Introduction

I.1. History & conservation of crocodilians

Crocodilian species were already present on earth when the dinosaurs first appeared, and have remained more or less unchanged for approximately 240 million years. There are today 23 species of crocodilians in the world, including the Alligatoridae, Crocodylidae, and Gavialidae families. Out of these, 11 species are considered vulnerable or endangered according to the IUCN red list of threatened species, and as many as 4 of them are considered critically endangered. Only one of the Alligatoridae species (*Alligator sinensis*) are critically endangered, while the single Gavialidae species (*Gavialis gangeticus*) is considered endangered. This means that looking at the Crocodylidae family, things look even worse as 9 out of 14 species are threatened, whereof 3 are red listed as critically endangered (2004 IUCN Red List of threatened Species database, *http://www.iucnredlist.org/*).

Crocodilians exist in most of the southern hemisphere, with most of the species located in tropical areas (Fig 1). South America has the highest amount of crocodilians in the world, with as many as 5 different species (*Paleosuchus palpebrosus*, *P. trigonatus*, *Caiman crocodilus*, *Crocodylus acutus*, and *C. intermedius*) within the Venezuelan wetlands. (Ramo et al, 1992).

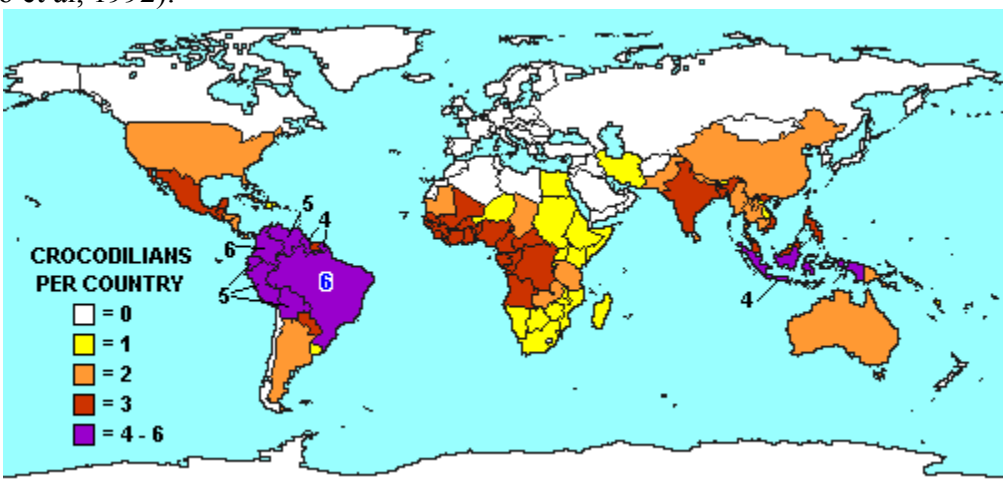

Fig 1: Distribution of crocodilian species in the world. From: The Crocodile Specialist Group homepage (http://www.flmnh.ufl.edu/natsci/herpetology/crocs.htm)

The IUCN/SSC crocodile specialist group, which includes crocodile specialists from around the world focusing their efforts on the conservation of crocodiles, completed the first world wide conservation plan for crocodile species in 1990. Since then, the knowledge and methods have been rapidly improving, adding to the survival rate of some species, while others remain highly threatened. Today, the conservation of 8 species is prioritized (Table 1), and the IUCN has compiled a list of species and countries that should remain the focus of conservation efforts, including "basic surveys, identification of key habitats and populations, protection of habitats and species, enhancement of national management and conservation capacity, captive breeding and restocking, and development of incentive for crocodile and habitat conservation" (Ross, 1998).

Table 1: Crocodile species and countries that should remain the focus of conservation efforts, according to the IUCN conservation plan from 1998 (Ross, 1998). The species are listed in priority order.

Priority	Species	Common name	Country
1	*Alligator sinensis*	Chinese alligator	China
2	*Crocodylus mindorensis*	Philippine crocodile	Philippines
3	*Tomistoma schlegelli*	False gharial	Indonesia, Malaysia
4	*Crocodylus siamensis*	Siamese crocodile	Cambodia, Vietnam, Laos, Thailand, Indonesia
5	*Crocodylus intermedius*	Orinoco crocodile	Colombia, Venezuela
6	*Crocodylus rhombifer*	Cuban crocodile	Cuba
7	*Gavialis gangeticus*	Gharial	India, Nepal, Pakistan, Bangladesh, Bhutan
8	*Crocodylus cataphractus*	Slender-snouted crocodile	Central & West Africa

The conservation of crocodiles is associated with a lot of problems. These species are the largest predators in their habitats, and often a direct threat to humans and livestock. Therefore, they are often hunted or have their eggs collected or destroyed by local communities. This fact makes it a challenge to gain the local support needed for conservation efforts in important areas. Most endangered species are also being exploited commercially due to their valuable skins, supporting trade worth half a billion dollars each year. Another important factor is the constant loss and degradation of habitats suitable for sustaining crocodile populations (Ross, 1998).

An important area for crocodile conservation is ex-situ breeding for future reintroduction into their natural habitats. This has for example been done successfully in India, where ten years of management resulted in 2364 crocodiles of three different species being released into the wild after successful captive breeding (Ramo et al, 1992). The method has proven effective mainly because natural death rates most often the highest among young hatchlings as a result of predation. Crocodiles also tend to grow slowly, reaching sexual maturity at a late age, which makes the populations sensitive to hunting and other activities that tend to focus on the adult population. The biological characteristics of many crocodile species makes many populations very resilient and able to cope with and recover from population depletion and high harvest rates, giving hope to the conservation work. As with all animal species however, there is a limit, and the hunting of adults together with habitat destruction might very well result in the extinction of crocodile populations (Ross, 1998). Building on the mentioned resilience of the species, limited harvesting of wild and ranched crocodiles, making the species economically important and thereby improving on to the good will of local communities and authorities have also been a way of adding to the protection of the different crocodile species.

In addition to the protection and breeding of crocodiles, gaining an understanding of the ecology and social behavior of these are important to make any efforts when dealing with crocodile conservation more effective. Ecological studies of crocodiles have often been difficult to carry out, not only because of the danger associated with working with large, aggressive predators in remote locations, but also as a result of the crocodiles spending a lot of their time in or below water. In later years, new ways of doing ecological studies have been made possible with the advance of different DNA techniques to study genetic

variation and relatedness issues. Very little has been done so far, but Fitzsimmons et al (2000) mention a few areas that need to be investigated more thoroughly. For example, genetic diversity within and between populations needs to be examined for use in restocking and reintroduction programs for Orinoco crocodile (*Crocodylus intermedius*), hybridization issues in Cuban (*Crocodylus rhombifer*), Siamese (*Crocodylus siamensis*) and American crocodile (*Crocodylus acutus*) need to be studied, gene flow between crocodile populations should be investigated, and genetic tools would also be a good method to study of the complex behavior of crocodile species, including mating systems and reproductive success.

I.2. Status and history of the Orinoco crocodile

The Orinoco crocodile *(Crocodylus intermedius)* is among the most critically endangered species of crocodiles in the world. It is red listed by the IUCN, and included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since the 1970s (Seijas and Chávez, 2000). The species was once widely distributed along the major river systems of the Llanos savannah of Colombia and Venezuela, and along many smaller rivers into the surrounding foothill areas of the Peruvian Andes. However, the commercial overexploitation of the species between the 1920s and the 1960s resulted in the Orinoco crocodile populations being almost fully depleted, and on the verge of extinction, and today its distribution is limited to the Orinoco basin, from the Guaviare river in Colombia to the Amacuro delta in Venezuela. During the peak of skin hunting in 1933-1935, a reported 900 000 crocodile skins were exported from Venezuela, although this figure probably includes skins from both Orinoco and American crocodiles (*Crocodylus acutus*), and at least 250 400 individuals were captured in Colombia (Medem, 1981, 1983). The city of San Fernando, Venezuela, functioned as the main center of skin trade, and 3000 to 4000 skins were sold on a daily basis during this peak period. In the 1960s, over-exploitation resulted in the demise of commercial hunting of skins in most areas, and the Orinoco crocodile finally gained legal protection from the Venezuelan and Colombian authorities in the 1970s, although this has had little effect on hunting in Colombia, and only little or no population recovery has taken place in both Colombia and Venezuela over the last 30 years (Thorbjarnarson and Hernández 1992). Due to the difficulties of doing research in Colombia, very little is known about the status of the Orinoco crocodile in the country, though a few populations are known to exist, and some surveys have been carried out on the initiative of the Estación de Biología Tropical and the Ministry of Environment, and a survey was also done in the early 1970s in connection to Dr. Federico Medem's work on South American crocodile species (Medem 1981, 1983). Due to the lack of information, the IUCN has defined the search for viable populations as a highly prioritized part of the Orinoco crocodile conservation work in Colombia. Other prioritized projects mentioned in the 1998 revised conservation plan, is the monitoring of populations of released captive-bred crocodiles in Venezuela, further surveying the periphery of the species range in Venezuela (some survey projects have already been completed since then, including Seijas and Chavez, 2000), analysis of genetic diversity within and among populations, and identification of areas suitable for reintroduction of captive-bred crocodiles in Colombia (Ross, 1998).

Fig 2: Present day distribution of Orinoco crocodile in South America. (From: The Crocodile Specialist Group homepage, http://www.flmnh.ufl.edu/natsci/herpetology/crocs.htm)

The range of the Orinoco crocodile populations in Venezuela is today quite well known, as surveys have been carried out since the 1970s. This work was accelerated in 1986 when a comprehensive conservation plan was elaborated, which included both up-to-date inventories of the rivers known to contain populations of Orinoco crocodile, and the initiation of ecological studies on several of the wild populations (Seijas and Chávez, 2000). This work was mainly coordinated and funded by the Venezuelan *Fundación para la Defensa de la Naturaleza*, and the Venezuelan ministry of the environment and renewable natural resources (*Ministerio del Ambiente y Recursos Naturales Renovables*), although several NGOs and environmental organizations are also involved (Thorbjarnarson and Hernández 1992). The most important, and only viable populations of Orinoco crocodile known to exist in Venezuela are located in the Capanaparo river system in the Apure region, and in the narrow river systems of the Cojedes region in the states of Cojedes and Portuguesa (Seijas and Chávez, 2000). Both of the areas are part of the Venezuelan Llanos and the Orinoco river basin. The Capanaparo river is located in an area consisting of high quality habitats, where the Orinoco crocodile populations historically reached their highest densities. In addition, most of the area has remained isolated from urban and industrial centers through modern history, and most of the river is included in the Santos Luzardo National Park, giving the crocodiles much needed protection from further disruption of their habitats. The Cojedes region however, is located in a region very close to some of the most important agricultural, urban, and industrial centers in Venezuela and no formal protection of the area exists. 547 non-hatchling Orinoco crocodiles were found in the river system in a 1999 survey making it the largest known population in Venezuela (Seijas and Chávez, 2000). Smaller populations can also be found in several other rivers in the Llanos, including in the Arauca, the Cinaruco, the Caño El Caballo, and scattered throughout parts of southern Venezuela, outside the Llanos, but conservation work in Venezuela is mainly focusing on the viability of the two larger populations. There are today still many factors that contribute to the present endangered status of the Orinoco crocodile, and the holding back

of any population recovery. These factors mainly include anthropogenic influence in the form of deliberate killing of crocodiles as vermin or for the valuable skin, the collecting of eggs for food (a traditional part of some Indian communities), and the capture of hatchlings for sale as pets. Another factor is accidental killing by drowning as crocodiles get caught in fishing nets. Degeneration and destruction of habitats, mainly through deforestation, draining of wetlands, and the pollution of water is an important factor in the decline of suitable crocodile habitats, and finally, competition from the widespread Caiman (*Caiman crocodilus*) populations, have proven to make the situation even worse for the Orinoco crocodiles, as these tend to increase both the competition for food and the predation on young hatchlings. In general, different factors are of varying importance in different areas. For example, the collecting of eggs for food and hatchlings for sale to tourists have had the most detrimental effect on the Capanaparo population, while 10 years ago, the Cojedes population was mainly affected by the habitat destruction that followed from the Venezuelan government irrigational projects (Thorbjarnarson and Hernández, 1992).

Four protected areas that include the range of the Orinoco crocodile populations exist in Venezuela, namely the Parque Nacional Aguaro-Guariquito in Guarico state, the Parque Nacional Cinaruco-Capanaparo in the Apure state, the Refugio de Fauna Estero de Chiriguare in the Portuguesa state, and the Refugio de Fauna Caño Guaritico in the Apure state. However, the present main method of conservation when dealing with the remaining Orinoco crocodile populations is today captive breeding, restocking of the present populations, and reintroduction of crocodiles into suitable habitats. Orinoco crocodiles are successfully being bred in captivity at three different field stations in Venezuela: At the Universidad Nacional Experimental de los Llanos Occidentales Ezequiel Zamora in the Portuguesa state, at the Hato Masagüeral in the Guarico state, and at the Estación Biológica El Frío in the Apure state (Ramo et al, 1992).

Fig 3: A Llanos wetland landscape during wet season. The whole area is flooded, and the grass is growing from beneath the water. Note the Orinoco crocodile female lying guard next to her nest at the sand bank.

I.3. The breeding and reintroduction program at Estación Biológica El Frío

El Frío field station is located in the National Park of Cinaruco-Capanaparo in the Apure state, Venezuela, and is among the oldest of the biological field stations responsible for the breeding and reintroduction of Orinoco crocodile in Venezuela. The area, which is part of the Llanos savannah is very rich in biological diversity, and in addition to external funding, the field station receives an income from the eco tourism that it arranges.

As part of the conservation project, the field staff of the El Frío field station constructs artificial sand nests along the wetland shore lines of the area, suitable for crocodile females to lay their eggs in. The eggs from each nest are then moved to incubation tanks at the field station, and incubated until they hatch. Upon hatching, the baby crocodiles are tagged and, as of lately, scale samples are collected and sent to Uppsala University for genetic analysis, sometimes accompanied with a sample from the egg shell. The crocodiles are bred at the field station until of sufficient size to avoid predators in the wild when they are about one year old, and then released back into nature. The population in the area does not belong to any of the previously mentioned wild populations of Orinoco crocodile in Venezuela, but is instead derived from reintroductions of captured individuals from both the nearby Capanaparo and the Cojedes Orinoco crocodile populations, as well individuals from lately extinct populations, and from captivity.

Photos: Carles Vilá

Fig 4: Left: Artificial sand nest, constructed next to a shoreline
Middle: Incubation boxes for crocodile eggs
Right:A tagged Orinoco crocodile hatchling

I.4. Orinoco crocodile basic morphology, behavior and reproductive ecology

The Orinoco crocodile is one of the largest crocodilian species in the world, and the biggest predator in South America. Historical records report animals reaching sizes of 6-7 meters in length, though today the largest specimen reach only a maximum of 5 meters, mainly as a result of low survival rates among older crocodiles due to illegal hunting. Crocodilians of all species are able to grow very old, reaching ages of more than a century, and keep growing throughout their lives. The Orinoco crocodile comes in three color variations, including the most common color of amarillo (light, tan body with scattered dark areas), mariposo (grayish green body and dark dorsal patches), and negro

(dark grey). The crocodiles may also to some degree change color with time. The latin species name (*intermedius*) refers to the long and narrow snout which is shaped as something between that of the V-shape of most *Crocodylus* species, and the parallel-sided *Gavialis*. While the juveniles of the species live mainly on fish and invertebrates, larger animals usually dine on most vertebrates unfortunate to be at the wrong place at the wrong time, including capybaras, fish, birds, and even humans have been recorded to be preyed on. As hunters they are efficient on both land and in water, using their tail to propel them through the water when swimming. The structure of the head and nostrils, also allows for the Orinoco crocodile to remain almost fully submerged under water. Older animals are usually shyer than the younger specimens, and will most often choose to submerge below water if anything disturbs them (Medem, 1983).

Crocodilians share a well developed behavioral repertoire, and a very complex social behavior. Only a few species have been thoroughly studied previously, suggesting that the diversity in behavior between species may be even higher than recognized. A few studies have been made on Orinoco crocodile, beginning with the work on crocodile ecology by Federico Medem in the early 1980s (Medem, 1981, 1983), and also by Thorbjarnarson and Hernández (1993a, 1993b). Orinoco crocodiles communicate through the use of a diverse selection of visual, vocal, olfactory, and acoustic signals. The signaling behaviors are similar to that of other crocodile species, but combined in a way specific for the Orinoco crocodile. The main social display is the acoustic headslap, common for all crocodilian species, combined with a low frequency roar. It has also been shown that the roars involve the mandibular glands, suggesting that olfactory signals may be present in this basic assertion behavior as well. The assertion display is part of the hierarchical system of Orinoco crocodile, including the maintaining of territories and courtship. Mainly males display this behavior, but females have been known to headslap as well in situations of aggression, for example when defending a nest. It is believed that the assertion display communicates information on sex, location, size and individual identity. The Orinoco crocodile courtship period starts when the dry season sets in with dropping water levers. There is an increase in headslap and roaring behavior during this period, as the crocodiles establish mating territories and dominance hierarchies, and start to attract mates. During the few months of courting, it is not only the males that display territorial behavior, but the females will be protecting their territorial rights as well. The start of the dry season will result in the crocodiles being concentrated to the few remaining water concentrations, mainly including the major river channels. The higher the population densities, the stronger the dominance hierarchies will be, with only a few males mating at higher densities, but with distinct monogamous groups of crocodiles forming at lower densities. The wild populations of today usually contain a mix of both polygamy and monogamy. At the end of the year, mating takes place, with the crocodile display of assertion behavior growing more frequent and reaching its peak. The Orinoco crocodile is a hole nesting species. 4 weeks prior to nesting, the female will start making nocturnal visits, including trial excavations to a suitable nesting site of her choice. Usually, she will choose a sandbar next to the water, an island beach, or a mid-channel sandbar, preferably close to a deep pool, where she can lie protected, submerged under water while still being able to guard the eggs. In lack of good nesting sites, she may however choose other less suitable locations, and one crocodile female has even been

recorded to build her own nest from vegetation in an area where no sand beaches exist. Females will, however, always choose elevated areas to avoid flooding of the eggs. In general, suitable nest sites will usually be found where the river has eroded into the mixed savanna and forest habitat of the Llanos, with the nesting site consisting of high, well drained areas of sand deposition. Upon laying her eggs, the female will dig a hole in the sand, and once the eggs are put in it, she will cover them with sand, which is afterwards compacted as the mother tramples the nest site. The same nest is often used several seasons in a row. Eggs will hatch just before the beginning of the wet season (average incubation time is 70-95 days), and the female will arrive to dig up the hatchlings at late evening, moving them with her mouth from the nesting site to a nearby shoreline area with lots of protective vegetation. The offspring will keep together in a pod, with the female attending to them at any time, keeping males and predators away with her aggressive behavior. Despite the motherly protection, some eggs are still at risk due to vultures and tegu lizards, though other species usually prefer to prey on nearby turtle nests. After 4 weeks, the offspring will start dispersing to different locations at night, only to return to the protection of pod at morning. When having reached mature age (1-3 years), the crocodiles will disperse from the safety of the pod, though Orinoco crocodiles do not tend to move any greater distances. In fact, Muños and Thorbjarnarson (2000) showed that captive-bred crocodiles will disperse a maximum of about 12 kilometers and in general only a few kilometers from the release site. Clutch sizes vary between around 30 to 45 eggs, and the mean egg viability in wild population can be as high as 90%. Captive populations however, tend to have lower egg viability (most often less than 50%). Female fecundity and the associated egg size and mass are often connected to the size of the crocodile, with fecundity growing higher as they age. At a certain age however, the crocodiles will start loosing in fecundity as they grow older (Thorbjarnarson and Hernández, 1993b, Medem, 1983). Orinoco crocodiles reach sexual maturity at a late age, making it very important for the crocodiles to survive the early years for any reintroduced population to produce new breeding members.

I. 5. The multiple paternity mating system

Multiple paternity is a mating system in which a female mates with several males in a short time span, and then has clutches of offspring including the genes of several of them. This allows for the female to distribute her mating effort across several mates, and thereby increasing her chance to give birth to at least a few genetically fit individuals. Multiple mating also gives other benefits in the form of being able to counter skewed population sex ratios, and the female choice of mate is not as critical as she can always find a better one later. It also helps in avoiding genetic incompatibility, and allows for the selection of competitive sperm (Moore and Ball, 2002). Multiple paternity is a common mating strategy throughout nature, and is employed by such diverse species as cats, birds, turtles, lizards, alligators, and insects. Myers and Zamudio (2004) proposed that species involved in aggregate breeding should most often develop a multiple paternity system as well, as both mating systems support a higher degree of genetic mixing during short spans of time, and therefore tend to co evolve (both of the mating systems share the same evolutionary benefits and reasons for being evolved). Though *Crocodylus* species make use of an aggregate breeding system, it has been debated whether the species employ a multiple paternity mating strategy, and if such is the case, to what degree females choose

to mate with several males. Genetic tests have already shown that American alligator (*Alligator mississippienis*), do make use of a multiple paternity mating system (Davis et al, 2000), which should indicate that there is a great chance that any *Crocodylus* species share this trait.

For any reintroduced population to be viable, enough genetic diversity is needed for it to be able to adapt to future environmental changes. It is therefore very important to understand the mating patterns of the Orinoco crocodile, as any reintroduction effort will have to include the development of a breeding strategy that will allow for as many individuals as possible to breed, and to maximize the number and genetic variation of the produced offspring during the first years. In addition, as the reintroduced population of Orinoco crocodile at the El Frío field station consist of individuals from populations of varying mating seasons and social behavior, it is also important to take the risk of outbreeding depression into account when developing these breeding strategies.

The use of genetic markers when studying the mating behavior in animal populations, has become increasingly popular. The method can often yield information difficult or impossible to obtain through direct observations (Goodnight and Queller, 1999). This is especially evident in the case of multiple paternity, which can be very difficult to distinguish through observation only. Most of the work on the issue have been conducted on mammal and bird species, but the studies done on reptiles have been increasing lately (Laloi et al, 2004), partly as a result of the development of genetic methods.

I.6. Objectives of the study

It is not known if, or to what degree, dominant males monopolize offspring production in Orinoco crocodile populations. If one dominant male contributes more than the other males to the production of offspring, it is expected that this should show up as the same male being responsible for the offspring of several nests. This statement assumes that each female will lay her eggs in only one nest, which is very reasonable, as it is impossible for any female to excavate a nesting site without destroying the eggs already present.

Another question to address is the issue of multiple paternity. If multiple paternity is prevalent in the Orinoco crocodile population, this should show up as several fathers being responsible for the offspring of a single nest.

For the study to be successful, it is expected that the genetic tools available, including the control region mtDNA and microsatellite markers obtained from literature on previous genetic studies of crocodilian species, are variable enough to estimate relatedness within and between clutches of offspring.

II. Material and methods

II.1. The samples

The samples collected for DNA extraction and analysis originate from the reintroduced population of Orinoco crocodile of the El Frío field station in Venezuela. Ten scale samples used for the first PCR optimization tryouts came from one-year old crocodiles that had been bred at the El Frío biological station for future reintroduction into their natural habitat. The scale samples were cut from the dorsal side of the tail of each individual, and kept dry until used for lab analysis. The samples were kept dry in a freezer at Uppsala University between DNA extractions. The genetic markers were then typed on scale samples cut from newly born individuals, hatched and tagged at the field station. These samples were kept in eppendorf tubes, filled with 95% ethanol while being transferred to Uppsala University, and then kept in the same ethanol filled tubes at 8ºC between DNA extractions. For some of these individuals, egg shell samples from the eggs that they had been hatched from, were obtained in the same eppendorf tubes as those of the corresponding scale samples. Tag name and the nest of origin for each individual crocodile hatchling were recorded.

Samples were collected from eight different nests, including 133 different individuals. The sample size of each nest varied from 9 to 31 individuals. In nest VI though, only one individual had survived hatching as the mother had refused to put her eggs in the artificial nest supplied by the El Frío staff, but instead chose another site not as suitable for incubation. Due to the high humidity in this nesting site, almost all the eggs were rotten on excavation and only one hatched.

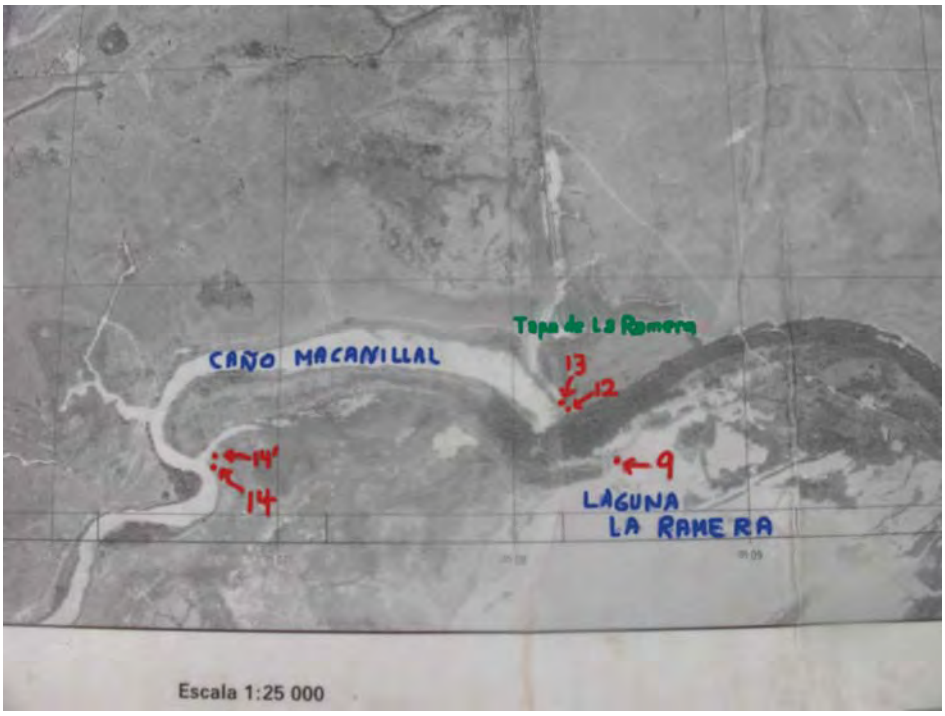

Fig 5: Location of the nests from where the samples were collected. Nest I, II, II, and V correspond to location 14', nest IV to location 14, nest VI to location 9, nest VII to location 13, and nest VIII to location 12. The map is in a scale of 1:25000

Table 2: UTM coordinates for the Orinoco crocodile nests included in the study. Eggs were collected at each nest and incubated at El Frío field station. Adjacent nests are represented by a single map location. The sample size of each nest is displayed, as well as the total number of scale samples obtained.

Nest	Map location	UTM coordinates	Sample size
I	14'	06706;66260	16
II	14'	06706;66260	22
III	14'	06706;66260	22
IV	14	06700;66243	19
V	14'	06706;66260	9
VI	9	08462;66256	1
VII	13	08207;66449	13
VIII	12	08219;66440	31
		Total sample size:	133

II.2. Extracting DNA, and measuring concentration

DNA extractions of the collected crocodile scale samples were carried out according to the conventional phenol-chloroform extraction protocols used at the Department of Evolutionary Biology, Uppsala University (Johansson, 2005), with some minor changes including the addition of dithiothreitol (DTT). Using clean scalpels, each of the scale samples was cut to suitable sizes. For the samples cut from the ten older crocodiles, a piece of approximately 0,5 * 0,5 cm in size were used, while in the scales from newly hatched crocodiles (which were smaller, but provided higher quality DNA due to the larger number of live cells) only a small piece of approximately 0,3 * 0,3 cm in size were used. The cut scale samples were put in tubes containing a mixture of 750µl of Laird's buffer (Tris, EDTA-Na_2, NaCl, SDS, ddH_2O, pH = 8,5), 20µl proteinase K (20mg/ml), and 10µl DTT. The samples were digested over night at 37-39ºC. For the extraction of DNA, 750µl of Phenol-Chloroform-Isoamyl-Alcohol (PCI) were then added to each tube to remove proteins. This step was usually repeated twice to make sure that all proteins were removed in the process. Chloroform was then added once to remove the remnants of PCI. To precipitate the DNA, 3M NaAc was added, and cold 96% ethanol (EtOH) was then added before incubating the samples in a freezer over night. Using cold 70% EtOH, the pellet containing the DNA was washed from salts, and left to dry after most of the alcohol had been removed using a pipette. The pellet was then dissolved in water.

DNA concentration was measured using a Nanodrop 2.5.3 and then diluted to a concentration of 10 ng/µl. If a concentration proved to be 10 ng/µl or less to start with, it was diluted no further, but used as it was.

II.3. Extraction of crocodile maternal DNA from egg shells

Following Strausberger and Ashley's (2001) extraction of maternal DNA from bird egg shells, an attempt to extract maternal DNA from pieces of the crocodile egg shells sampled with each hatched individual at the El Frío field station was carried out. The idea was to be able to amplify the alleles of both the mother and offspring and, comparing the observed alleles with those in the offspring, reconstruct the genotype of the mother. The extraction was done in the same way as for the scale samples (see section 3.2).

II.4. Choosing markers and designing primers

No genetic studies had been carried out on the Orinoco crocodile previous to this one. However, since many genetic studies - involving both sequencing of control region mitochondrial DNA (Ray and Densmore, 2002) and analysis of microsatellite variation (Fitszimmons et al, 2000) - had been done on various close related species, markers used on these could be expected to work on Orinoco crocodile as well.

Two control region mtDNA primers - CR2H and tPhe-L (Ray and Densmore, 2002) - were ordered together with 23 chosen microsatellite primer pairs and their respective tags. To avoid the unnecessary costs associated with using one different fluorescently labeled primer for each marker, a tail was added to one of the primers and a third fluorescently labeled primer (Tag) was used in the PCR amplification which was complementary to this tail (Boutin-Ganache et al, 2001). This meant that the original forward primers of each marker – derived from the above mentioned literature - had to be modified by adding a tail. See table 1 for more information on the sequences of the primers and tags that were evaluated in the study, including both the sequences of the original primers and the modified ones (after adding a tail).

Table 3: Tag and primer sequences for each microsatellite (Fitszimmons et al, 2000) and mtDNA control region marker (Ray and Densmore, 2002) that were tested and evaluated in the study of Orinoco crocodile
Original primers = primers as they were defined in previous studies
Modified primers = primers after modification for tags to be able to attach

Tag	Sequence 5´ to 3´
CAG-tag	CAGTCGGGCGTCATCA
CAG-2-tag	CAGTCGGGCGTCAT
M13-2-tag	GGAAACAGCTATGAC

Primer	*Original sequence 5´to 3´*	*Modified sequence 5´to 3´*
C391-F	ATGAGTCAGGTGGCAGGTTC	GGAAACAGCTATGACCGAGTCAGGTGGCAGGTTC
C391-R	CATAAATACACTTTTGAGCAGCAG	
Cj16-F	CATGCAGATTGTTATTCCTGATG	GAAACAGCTATGACATGCAGATTGTTATTCCTGATG
Cj16-R	TGTCATGGTGTCAATTAAACTC	
Cj18-F	ATCCAAATCCCATGAACCTGAGAG	CAGTCGGGCGTCATCCAAATCCCATGAACCTGAGAG
Cj18-R	CCGAGTGCTTACAAGAGGCTGG	
Cj20-F	ACAATGGGGATCAGTGCAGAG	GGAAACAGCTATGACCAATGGGGATCAGTGCAGAG
Cj20-R	GTTTCAAATCCACAGTCATATAGTCC	
Cj35-F	GTTTAGAAGTCTCCAAGCCTCTCAG	GGAAACAGCTATGACTAGAAGTCTCCAAGCCTCTCAG
Cj35-R	CTGGGGCAAGGATTTAACTCTC	
Cj101-F	ACAGGAGGAATGTCGCATAATTG	CAGTCGGGCGTCATAGGAGGAATGTCGCATAATTG
Cj101-R	GTTTATACCGTGCCATCCAAGTTAG	
Cj104-F	TCCTTCCATGCATGCACGTGTG	CAGTCGGGCGTCATCTTCCATGCATGCACGTGTG
Cj104-R	GTTTCAGTGTCTGGTATTGGAGAAGG	
Cj105-F	CAACAGAAAGTGCCACCTCAAG	GGAAACAGCTATGACCACAGAAAGTGCCACCTCAAG
Cj105-R	GTTTGATTATGAGACACCGCCACC	
Cj107-F	ACCCCGCATTCTGCCAAGGTG	CAGTCGGGCGTCATCGCATTCTGCCAAGGTG
Cj107-R	GTTTATTGCCATCCCCACTGTGTC	

Cj109-F	GTATTGTCAACCCCACCGTGTC	CAGTCGGGCGTCATATTGTCAACCCCACCGTGTC
Cj109-R	GTTTCCCCTCCACAGATTTACTTGC	
Cj119-F	GTTTGCTGTGGAATGTTTCTAC	CAGTCGGGCGTCATCAGTTTGCTGTGGAATGTTTCTAC
Cj119-R	CGCTATATGAAACGGTGGCTG	
Cj122-F	GTTTCATGCTGACTGTTTCTAATCACC	AGTCGGGCGTCATCATGCTGACTGTTTCTAATCACC
Cj122-R	GGAACTACAATTGGTCAACCTCAC	
Cj127-F	CCCATAGTTTCCTGTTACCTG	AGTCGGGCGTCATCATAGTTTCCTGTTACCTG
Cj127-R	GTTTCCCTCTCTGACTTCAGTGTTG	
Cj128-F	ATTGGGGCAGATAAGTGGACTC	GGAAACAGCTATGACCTGGGGCAGATAAGTGGACTC
Cj128-R	GTTTCTTGCTTCTCTTCCCTACCTGG	
Cj131-F	GTTTGTCTTCTTCCTCCTGTCCCTC	GAAACAGCTATGACTGTCTTCTTCCTCCTGTCCCTC
Cj131-R	AAATGCTGACTCCTACGGATGG	
Cp10-F	GATTAGTTTTACGTGACATGCA	CAGTCGGGCGTCATTTAGTTTTACGTGACATGC
Cp10-R	ACATCAAGTCATGGCAGGTGAG	
CU4-121-F	GGTCAGCTAGCAGGGTG	CAGTCGGGCGTCATCAGGTCAGCTAGCAGGGTG
CU4-121-R	TGGGGAAATGATTATTGTAA	
CU5-123-F	GGGAAGATGACTGGAAT	GGAAACAGCTATGACAGATGACTGGAAT
CU5-123-R	AAGTGATTAACTAAGCGAGAC	
CUD68-F	GCTTCAGCAGGGGCTACC	CAGTCGGGCGTCATCACTTCAGCAGGGGCTACC
CUD68-R	TGGGGAAACTGCACTTTAGG	
CUI99.2-F	CACTGTGGGGGCCTCAATCTG	GGAAACAGCTATGACGTGGGGGCCTCAATCTG
CUI99.2-R	AGGCAGGTGGTAGGACCCTAGCAAT	
CUJ-131-F	GTCCCTTCCAGCCCAAATG	GGAAACAGCTATGACCCCTTCCAGCCCAAATG
CUJ-131-R	CGTCTGGCCAGAAAACCTGT	
CUJ-B131-F	CCTGCCCAAGCCCATCAAT	GGAAACAGCTATGACTGCCCAAGCCCATCAAT
CUJ-B131-R	CCCTTTTGGCATGGCACAGT	
Cr52-F	GATCAAAATGAAACACCACA	GGAAACAGCTATGACTCAAAATGAAACACCACA
Cr52-R	CATAAATACACTTTTGAGCAGCAG	
Control region		
tPhe-L	GAACCAAATCAGTCATCGTAGCTTAAC	
CR2H	GGGGCCACTAAAAACTGGGGG	

II.5. DNA amplification

The polymerase-chain-reaction (PCR) method was used to amplify the extracted DNA, though instead of using the standard fluorescently labeled primer pair, a fluorescently labeled tag (a third primer, see previous section) was added, which was designed to attach to a modified forward primer, and replicate the DNA in each PCR (see picture 1):

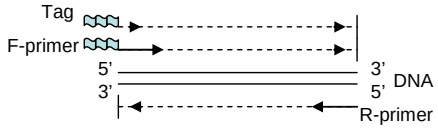

Fig 6: In addition to the standard two primer PCR settings, a third primer was used in the micro satellite study of Orinoco crocodile. The fluorescently labeled tag (3rd primer) attaches to the tail of the forward primer, and copies the DNA

The original PCR optimization for the use of microsatellite markers was done on DNA extracted from the ten first scale samples corresponding to older individuals (see above). As these samples proved to contain lower quality DNA than in the samples obtained from younger individuals, the PCR had to be reoptimized for higher quality DNA later on, to avoid the genotyping problems associated with too much PCR product, and to reduce the potential of primer dimers (the primers attach to each other, and copy themselves, adding to the amount of unspecific PCR product). As these PCR amplifications didn't work well with the lower quality DNA, often resulting in no product at all, these ten lower quality DNA samples have been excluded from the microsatellite analyses in this study.

The PCR of the microsatellite markers was carried out in 14 µl reactions, containing 2 µl DNA (10 ng/µl), 1,4 µl Qiagen 10x PCR buffer (Hot Star), 0,14 µl 20 mM dNTP mix, 0,04 µl 10 mM forward primer, 0,25 µl 10 mM reverse primer, 0,20 µl 10 mM tag, 0,06 µl Hot Star TAQ polymerase (Qiagen), and 9,91 µl double-distilled water. The reactions were done in an Eppendorf Mastercycler thermocycler, using the following conditions: 95 ºC in 15 min, 37 cycles of: 95 ºC for 45 s, annealing temperature (T_a) for 45 s, and 72 ºC for 1:30 min. Thereafter: T_a ºC for 1 min, 72 ºC for 15 min, and finally 4 ºC for 1 min. T_a varied between 51 and 60 ºC depending on the marker being used. See appendix C for detailed information

For the PCR optimization of the mtDNA control region, the lower quality DNA of the older scales was used successfully. The PCRs were carried out in 25 µl reactions, containing 2,5 µl DNA (10 ng/µl), 2,5 µl Qiagen 10x PCR buffer (HotStar), 0,25 µl 20 mM dNTP mix, 0,75 µl 10 mM forward primer, 0,75 µl 10 mM reverse primer, 0,125 µl Hot Star TAQ polymerase, and 18,125 µl double-distilled water. The reactions were carried out in a MJ research PTC-0225 DNA Engine Tetrad thermocycler, using the following conditions: 95 ºC for 15 min, 37 cycles of: 95 ºC for 45 s, 56 ºC for 45 s, and 72 ºC for 1:30 min. Thereafter: 56 ºC for 1 min, 72 ºC for 10 min, and finally 4 ºC for 1 min.

II.6. Genotyping and sequencing

PCR products of the control region mtDNA marker reactions were cleaned and the sequence reaction carried out, following protocols commonly used in the department (Kärf 2004). The samples were then sequenced on a Megabace 1000 capillary sequencer (Amersham), following the manufacturer's protocols, and the SEQUENCHER (Gene Codes Corporation) software was used for the subsequent analysis of electropherograms.

For the electrophoresis of the microsatellite markers, 1,3 µl of each PCR product were diluted with 10 µl of water. 1,3 µl of this was then added to a 10 µl water solution containing 0,25 µl ETRox 400 Size marker (Amersham). Genotyping was carried out on the Megabace sequencing instrument mentioned above, and scoring was done using the GENETIC PROFILER 2.2 (Amersham) software.

Each microsatellite marker was genotyped for at least 5 nests among the 133 individuals of higher quality DNA samples, and some markers were also genotyped using the ten individuals of lower quality DNA. For markers showing signs of variability during initial

scoring, all available samples were genotyped. Replicates of a randomly chosen set of samples for each marker were done as well, and for markers that proved to be difficult to analyze, 1-2 replicates were made of each genotype

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II.7. Data analysis

Sequences were aligned and compared with each other using the software Sequencer mentioned in section 3.5. Apart from the information obtained through direct observation by the use of this software, no further analysis was carried out with regard to the mtDNA control region due to its low variability (see results section).

The scored data of the genotyped microsatellite markers were compiled in the *MICROSATELLITE TOOLKIT* software and an initial analysis was carried out with respect to allele counts and frequencies, and the detection of matching samples. In addition, direct observation was used in the initial analysis of the data while trying to estimate the likely genotypes of each parent, and to detect nests where the distribution of alleles in the offspring could not be explained by only two parents. For example: if one nest (holding eggs from only one female) would contain more than 4 different alleles at one locus, the distribution of alleles could not be explained by only one father, but through the introduction of several fathers.

A combined F_{st} value (using the Weir & Cockerham 1984 approximation) for all the loci included in the study was calculated using the GENETIX (Belkhir et al, 1996-2004) software. This is a basic measure of genetic differentiation. The GENETIX software was also used to construct an AFC (factional analysis of correspondence) graph. An AFC graph provides a visualization of the degree of genetic differentiation between the samples. If a nest includes offspring from more than two males, individuals of the same nest are expected to show up in a number of different clusters in the AFC graph. In addition, if one male is responsible for the offspring of several nests, clusters of individuals are likely to overlap between nests.

To further study the obtained data with regard to the possibility of detecting a multiple paternity mating system and to investigate if one dominant male is mating with several of the females, the KINSHIP 1.3.1 (Goodnight) software for performing likelihood tests of pedigree relationships was used. This was used to calculate the pairwise relatedness between individuals. Histograms representing the distribution of relatedness values within each nest and for all the nests together were then constructed using the Minitab software, and an Anderson-Darling test for normality were carried out to determine if the values follows a normal distribution.

The KINSHIP software was also used to evaluate the power of the markers to differentiate between relationships. The program uses allele frequencies for all the markers in the population to simulate genotypes and to estimate the expected relatedness between unrelated, full sibling and half sibling individuals. For each of the three simulation settings, the relatedness values of the lower (<0.025) and higher (>0.975) percentile where then used to define a 95% confidence interval. Finally, the distribution of relatedness values obtained for the three simulation settings were compared to

determine to what degree the values were overlapping. The degree of overlap would thus indicate the resolution of the microsatellite markers to estimate relatedness between individuals in the study population.

As the sampled population consisted mostly of related individuals, population allele frequencies ought to be estimated from a separate source to avoid the risk of strong biases. To correct for this bias, population allele frequencies were calculated using the reconstructed parental genotypes obtained through the direct observation of offspring genotypes, before being added to the Kinship analysis. Nests where multiple paternity had been detected using direct observation, where however excluded from the population allele frequency calculations, as there was no reliable method to determine the exact genotype of the three (or more) parents. The genotype of the single individual in nest VI was also included in the calculations as it was expected not to have any relationship with any other offspring. To adjust for alleles present in the sampled population, but not represented among the allele frequencies estimated from the reconstructed genotypes (for example, alleles that were only found in nests with evidence of multiple paternity), these were included with an allele count of 1. This means that these alleles were treated as if they were present in heterozygous state in just one individual. Any risk of bias due to the same father being responsible for the offspring in several nests was not taken into account as it this method was the best available during the given circumstances.

III. Results

III.1. Evaluation and selection of microsatellite markers

Ten of the 23 tested microsatellite markers were clearly polymorphic, with a number of alleles ranging from 2 to 7. Due to lack of time and as a result of sudden PCR problems, the polymorphic marker CUJ-B131 had to be excluded from the study as it had not yet been tested thoroughly enough. In addition, the marker Cj119, with 3 defined alleles, proved to be too unreliable for use in the study as it was often difficult to distinguish the different alleles from each other, and further replicates would have to be obtained to confirm the results. Some of the other markers also posed genotyping problems. C391 proved to be an excellent marker, but low annealing temperatures (51°C as used in the study) often resulted in the presence of false bands, while higher temperatures (>51°C) often resulted in the applied M13-2 tag not being able to bind. Also, some of the lately acquired microsatellite primers were never optimized well enough to be genotyped (see table 2), due to the lack of time. In general, 133 different individuals in 8 nests were successfully genotyped with the 8 polymorphic microsatellite markers remaining. Three of the selected markers proved to be highly polymorphic with an observed number of 7 alleles, while 4 of the markers contained between 3-5 alleles, and two markers included only 2 alleles each. For the marker Cj127, only one nest proved to be polymorphic.

Table 4: Overview of microsatellites tested in the Orinoco crocodile study with respect to the applied tag, annealing temperature (Ta), the number of typed individuals (and the number of nests represented among these), the number of alleles and the range in size of the alleles found

Marker	TAG	Ta (ºC)	# of genotyped individuals (# nests in parenthesis)	# Alleles	Range in fragment size (bp)
C391	M13-2-tag	51	132 (8)	7	173-204
Cj16	M13-2-tag	56	133 (8)	5	160-190
Cj18	CAG-2-tag	56	133(8)	4(5)**	224-230 (232)**
Cj20	M13-2-tag	56	57 (6)	1	184
Cj35	M13-2-tag	56	69 (8)	1	163
Cj101	CAG-2-tag	56	132 (8)	3	370-378
Cj104	CAG-2-tag	56	75 (8)	1	224
Cj105	M13-2-tag	56	10 (5)	1	376
Cj107	CAG-2-tag	60	52(8)	1	220
Cj109	CAG-2-tag	56	133 (8)	7	382-407
Cj119	CAG-tag	56	133 (8)	3	188-190
Cj122	CAG-tag	60	132 (8)	4	394-406
Cj127	CAG-tag	60	133 (8)	2	352-356
Cj128	M13-2-tag	56	44 (5)	1	238
Cj131	M13-2-tag	56	9 (5)	1	230
Cp10	CAG-2-tag	56	116 (8)	1	207
CU4-121	CAG-tag	56	131 (8)	1	186
CU5-123	M13-2-tag	*			
CUD68	CAG-tag	56	90 (5)	1	127
CUI99.2	M13-2-tag	*			
CUJ-131	M13-2-tag	56	133 (8)	2	200-206

CUJ-B131	M13-2-tag	56	***	2-3	178-180
Cr52	M13-2-tag	*			

** optimization failed*
*** allele 232 was only present in a sample of 10 scales from older crocodiles not included in the analysis*
**** variation detected, but further typing needed for the marker to be included in any analysis*

III.2. The mtDNA control region

The sequencing of the mitochondrial control region in samples of ten older individuals from five different nests was successful, but no variation was detected. Therefor, no further sequencing was done in the remaining crocodile hatchlings, since this marker was unlikely to provide useful information within the population. The sequence obtained was approximately 500 - 635 base pairs depending on the success and quality of the sequencing.

III.3. Expected parental genotypes, and derived population allele frequencies

The efforts to extract maternal DNA from egg shells failed, as typing of extracted samples show that only offspring DNA was obtained from the extractions. Therefore, further analyses had to be carried out without knowledge of the mothers' genotype.

In a majority of the nests (5 out of 8) it was possible to assign parental genotypes from the offspring allele distributions, using direct observation. The exceptions were nest I and nest III as these nests showed signs of multiple paternity (the distribution of alleles in the nest could not be explained by the genotypes of only two parents). In nest I however, it was assumed that by removing the single individual with a genotype different from the more frequent genotypes, it should be possible to obtain the genotypes of the mother and the father with the assumed higher amount of offspring. This could not be done in nest III, as it proved impossible to tell exactly what, or even how many, parental genotypes were present in the nest, and as these were more evenly distributed than in nest I. In addition, as nest VI contained only the genotype of one individual, the parental genotypes could not be reconstructed, but instead the single offspring genotype was used. A more detailed summary on the reconstruction of the parental genotypes can be seen in table 5.

The parental genotypes were reconstructed on a locus-by-locus basis as it is not possible to reconstruct multi-locus genotypes. However, the same multi-locus genotype could be reconstructed for one of the parents for nests I, IV, VII and VIII, suggesting that all of them could be offspring of the same male. The same happens for nests II and V. Alleles present in the only individual sampled from nest VI show that it could not have derived from any of those two males (locus Cj109 excluded the possibility of having the same father than II and V; multiple loci excluded the possibility of having the same father as nests I, IV, VII and VIII). Since it was impossible to tell what the parental genotypes for nest III were, it was not possible to tell which could be the potential males either. The fact that the same father could be represented by several of the studied nests implies that the allele frequencies derived for the population could still be biased by the large contribution of some males.

Table 5: Estimated parental genotypes (A &B) for each of the included nests, and the derived observed heterozygosity, in the Orinoco crocodile study.

Marker↓	# alleles ↓	Nest → /Ind ↓	I	II	III	IV	V	VI*	VII	VIII	Marker H_0 ↓
C391	7	A	177, 190	188, 190	**	188,190	177, 190	190, X	173, 173	173, 190	
		B	188, 200	188, 200	**	188, 200	188, 190	190, X	188, 200	188, 200	0,77
Cj16	5	A	186, 188	160, 170	**	186, 170	160, 170	170, X	170, 186	160, 186	
		B	186, 190	170, 186	**	186, 190	170, 186	170, X	186, 190	186, 190	0,92
Cj18	5	A	(226, 228)	224, 228	226, 226	226, 226	226, 226	226, X	226, 226	226, 228	
		B	(226, 228)	226, 226	226, 228	226, 228	228, 228	230, X	226, 228	228, 230	0,62
Cj101	3	A	374, 374	374, 378	**	374, 378	370, 374	374, X	374, 378	374, 378	
		B	378, 378	374, 378	**	378, 378	374, 378	378, X	378, 378	378, 378	0,62
Cj109	7	A	382, 398	382, 390	**	382, 390	390, 390	388, X	382, 390	388, 390	
		B	388, 390	405, 407	**	388, 390	405, 407	400, X	388, 390	390, 390	0,85
Cj122	4	A	(400, 404)	394, 406	**	394, 406	394, 406	394, X	394, 394	400, 404	
		B	(400, 404)	406, 406	**	400, 404	406, 406	406, X	400, 404	400, 406	0,77
Cj127	2	A	352, 352	352, 352	352, 352	352, 352	352, 352	352, X	352, 352	352, 352	
		B	352, 352	352, 352	352, 352	352, 352	352, 352	352, X	352, 356	352, 352	0,08
CUJ-131	2	A	200, 206	200, 206	200, 206	206, 206	200, 206	206, X	200, 206	206, 206	
		B	206, 206	200, 206	206, 206	206, 206	200, 206	206, X	206, 206	206, 206	0,46
Evidence of polygamy in nest?			(yes) ***	no	yes	no	no	no	no	no	**Mean H_0↓**
Nest H_0			0,69	0,75	-	0,63	0,63	0,5	0,56	0,63	**0,64**

** The nest includes only one individual. When calculating H_0, the genotype of this single individual is used instead of the parents*
*** The nest and marker have an allele distribution that indicate multiple paternities*
**** The evidence of multiple paternity in this nest is limited to one individual (ID 10), which has alleles at loci Cj18 and Cj122 which exclude the possibility of having one unique father for the entire clutch*

The mean observed heterozygosity in the reconstructed parental population was 0,64. This is slightly higher than in the sampled population, which had an observed heterozygosity of 0,58. The derived allele frequencies used for the kinship analyses are indicated in table 6.

Table 6: Allele frequencies for the reconstructed parental Orinoco crocodile population. Individuals of nest III were not included in the construction of the expected allele frequencies table. Alleles found in the natural sampled population, but not in the expected parental genotypes after nest III had been removed, were added with an allele count of 1. The genotype of the single individual of nest VI was included in the calculations as well

Locus	*Allele*	*Frequency*	*Locus*	*Allele*	*Frequency*
C391	173	0,107	Cj109	382	0,154
	177	0,071		388	0,192
	188	0,286		390	0,423
	190	0,286		398	0,038
	196	0,036		400	0,038
	200	0,179		405	0,077
	204	0,036		407	0,077
Cj16	160	0,385	Cj122	394	0,231
	170	0,038		400	0,231
	186	0,154		404	0,192
	188	0,115		406	0,346
	190	0,308			
			Cj127	352	0,962
Cj18	224	0,037		356	0,038
	226	0,519			
	228	0,333	CUJ-131	200	0,231
	230	0,074		206	0,769
	232	0,037			
Cj101	370	0,038			
	374	0,385			
	378	0,577			

III.4. Multiple paternity and alpha male dominance mating systems

As mentioned in section III.2 direct observation of genotypes among the offspring in nest III reveals that the distribution of alleles in this nest can not be explained in terms of only two parents. This could also be the case in nest I, though only one individual (individual 10) does not fit with the rest of the rest of the clutch.

As predicted, the Genetix FCA representations showed all the members of the same clutch close to each other (fig 6), demonstrating that there is a differentiation of genotypes between nests. This result is also supported by the high value observed for the multilocus F_{st} estimate (Weir & Cockerham 1984) between nests of 0.192, a highly significant value. The FCA graphs provide further information. Individuals from nest III

seem to be present in two separate groups, supporting the idea of multiple paternity in this nest.

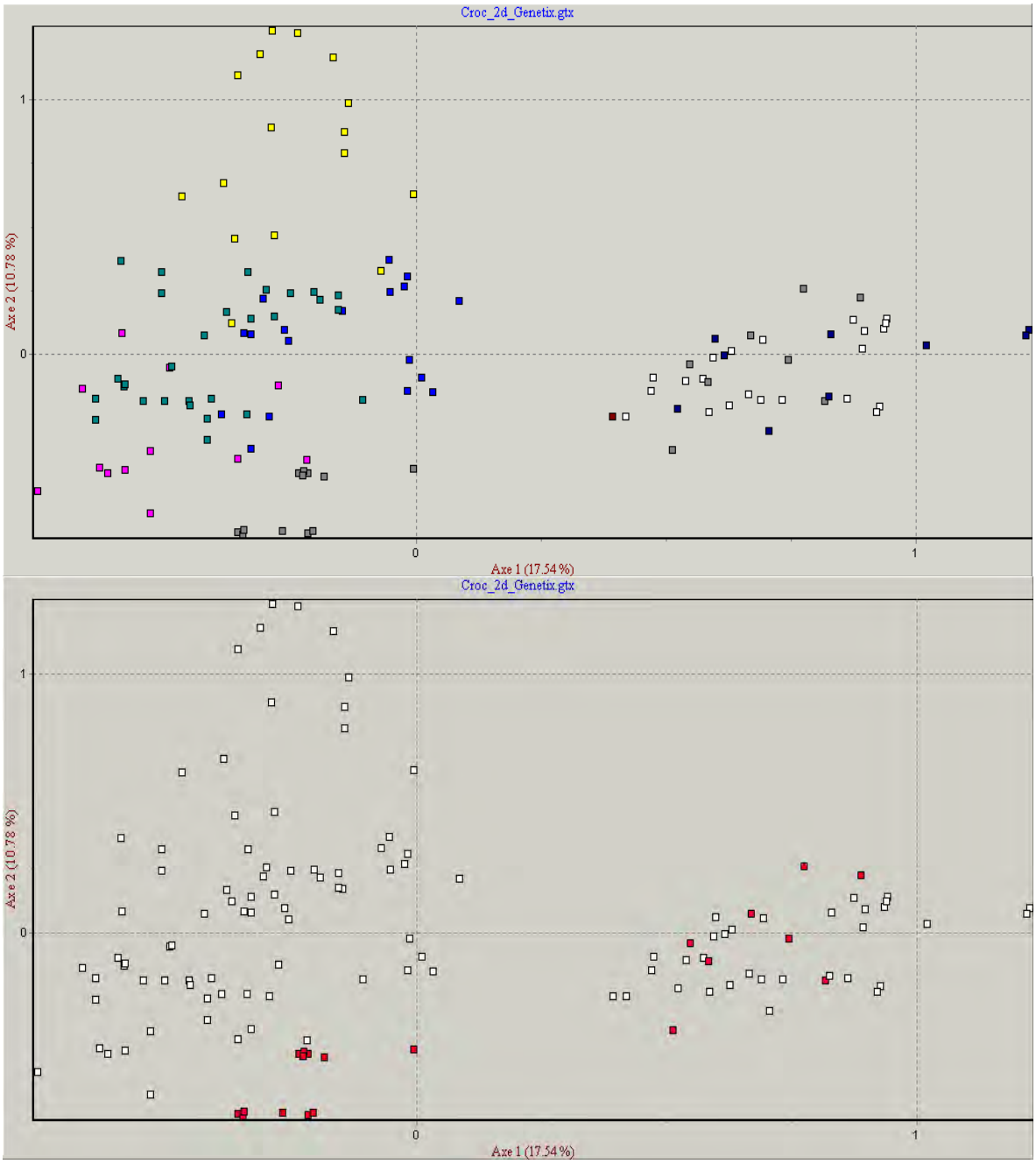


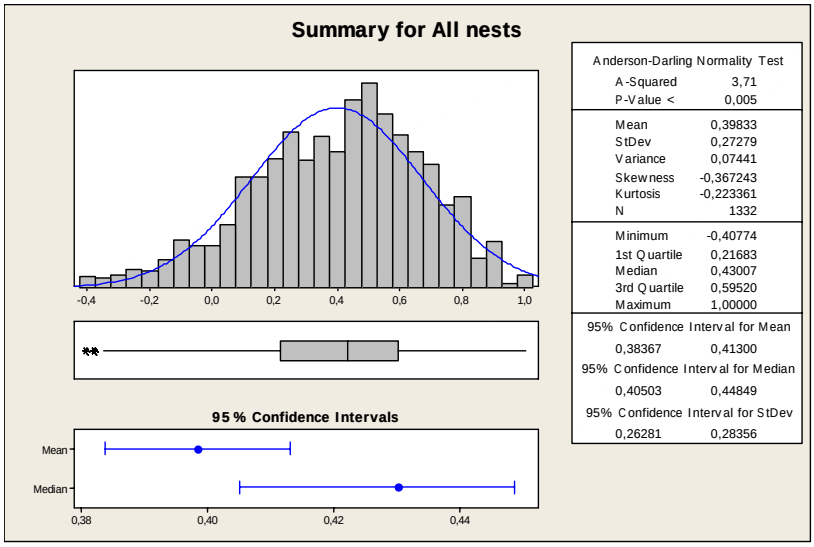
Fig 6: FCA representations for all included individuals in the Orinoco crocodile study. Individuals of the same nest are represented by a common color. Note that the left side blue (blue) and the right side blue (marine) are different. The percentage values of the X- and Y-axes indicate the proportion of the total variance represented by the axes. Individuals with similar genotypes occupy neighboring positions in the graph. Upper figure: Yellow = nest I, white = nest II, gray = nest III, blue = nest IV, marine = nest V, red = nest VI, cerise = nest VII, green = nest VIII. Lower figure: Red = nest III, white = any individual, not included in nest III.

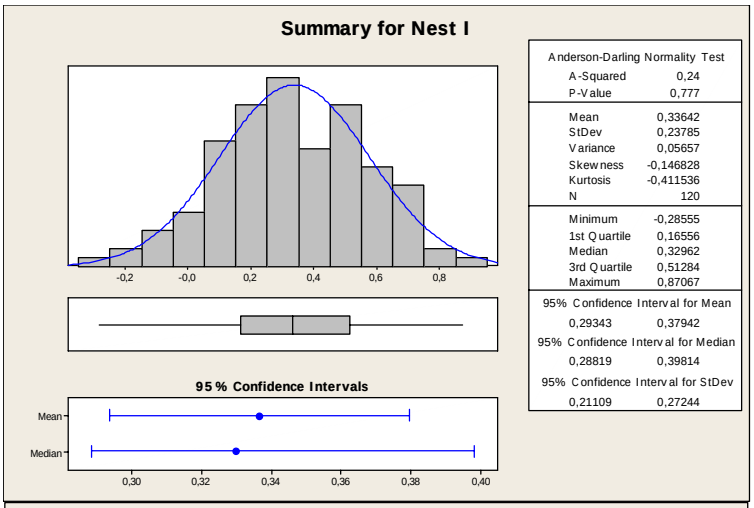
In addition, there appear to be two larger clusters of individuals. This supports the notion that only a few dominant fathers are responsible for the offspring of several nests. One of

the groups includes all individuals from nest II and V, while the other one includes all individuals in nest I, IV, VII and VIII, suggesting that the reconstructed genotypes from section IV.3 do show that those nests share the same father. Interestingly, the locations of individuals from nest III suggest that those two males could be the two fathers involved in this nest.

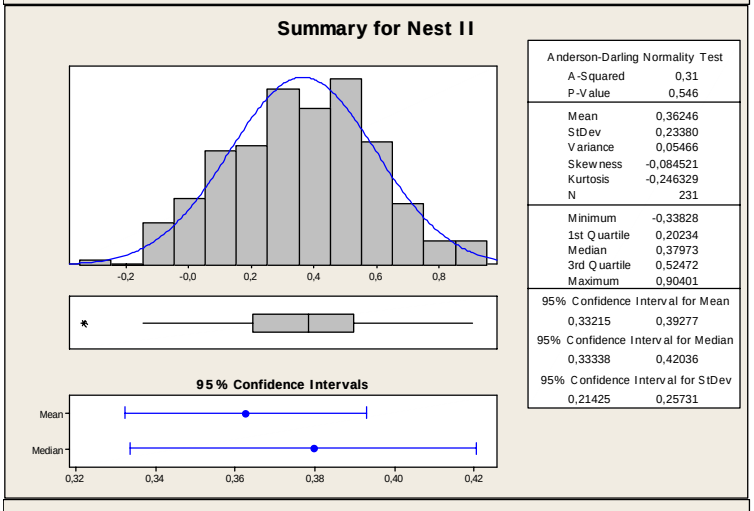
Individual number 10, in comparison with the rest of the individuals of nest I, do not seem to share the pattern of separate clutches observed in nest III. Also, nest VI does not appear separated from the rest despite the earlier stated indications on it being derived from a different father. This could be due to the fact that the nest is only represented by one offspring and that the AFC representation optimizes the separation of the majority of samples, so that the deviations in these two have a relatively small weight on the overall representation.

Histograms based on relatedness values of each nest show that the values for most of the nests are not significantly different from a normal distribution (Nest I, P = 0,777; Nest II, P = 0,546; Nest IV, P = 0,053; Nest V, P = 0,872; Nest VIII, P = 0,208; fig. 7b,c,e,f,h). However, significant deviations were observed for two nests (Nest III, P < 0,005; Nest VII, P < 0,005; fig. 7d,g). Joining all the nests together in one histogram, the Anderson-Darling test show that the relatedness values do not follow a normal distribution (P< 0,005; fig 7a), which could be expected since each nest derives from a different combination of parents. For nest III, the distribution of values can be explained by the presence of multiple paternity in this nest. No previous observation can explain the low P-value in nest VII, though the lack of normally distributed relatedness values could perhaps be explained by multiple paternity as well.

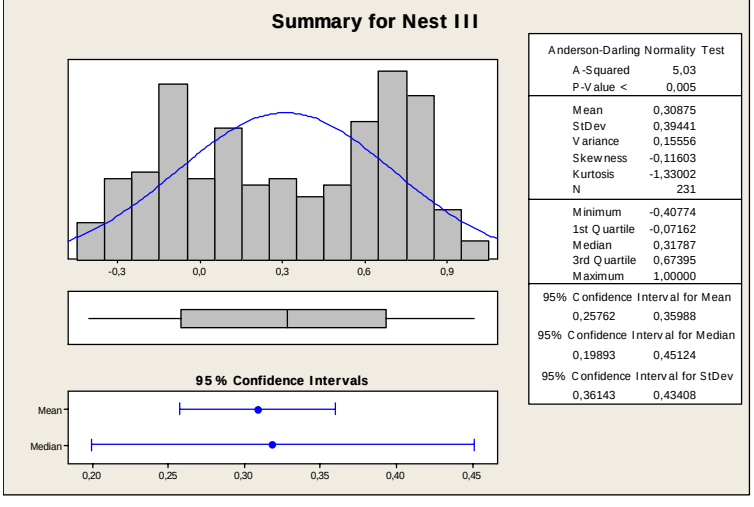




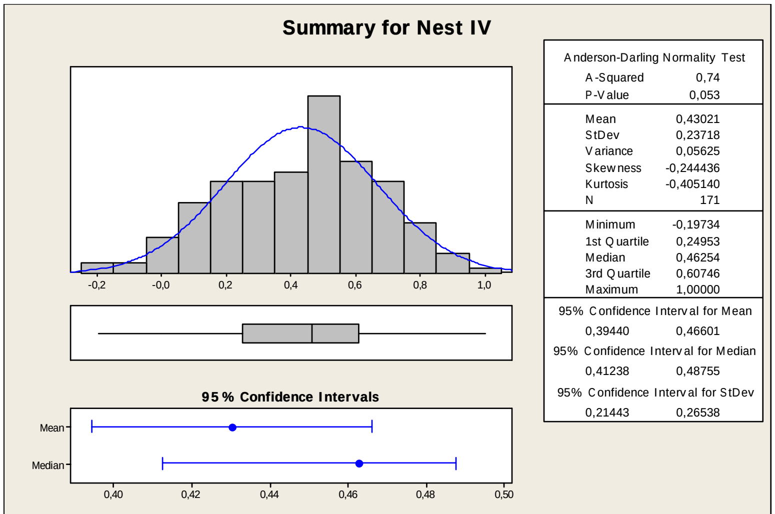
Anderson-Darling Normality Test	
A-Squared	0,24
P-Value	0,777
Mean	0,33642
StDev	0,23785
Variance	0,05657
Skewness	-0,146828
Kurtosis	-0,411536
N	120
Minimum	-0,28555
1st Quartile	0,16556
Median	0,32962
3rd Quartile	0,51284
Maximum	0,87067
95% Confidence Interval for Mean	
0,29343	0,37942
95% Confidence Interval for Median	
0,28819	0,39814
95% Confidence Interval for StDev	
0,21109	0,27244

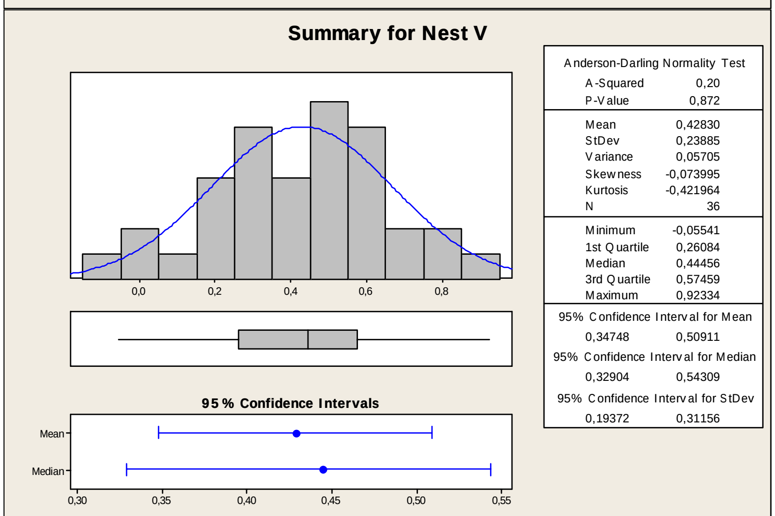


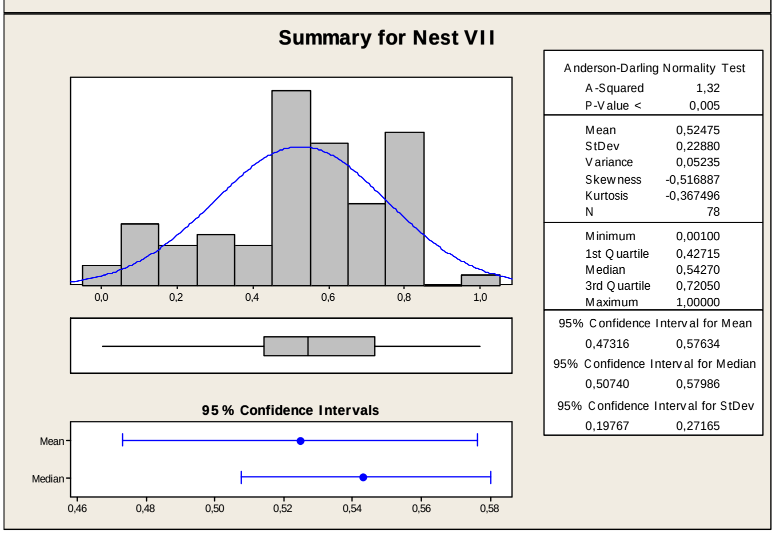
Anderson-Darling Normality Test	
A-Squared	0,31
P-Value	0,546
Mean	0,36246
StDev	0,23380
Variance	0,05466
Skewness	-0,084521
Kurtosis	-0,246329
N	231
Minimum	-0,33828
1st Quartile	0,20234
Median	0,37973
3rd Quartile	0,52472
Maximum	0,90401
95% Confidence Interval for Mean	
0,33215	0,39277
95% Confidence Interval for Median	
0,33338	0,42036
95% Confidence Interval for StDev	
0,21425	0,25731



Anderson-Darling Normality Test	
A-Squared	5,03
P-Value <	0,005
Mean	0,30875
StDev	0,39441
Variance	0,15556
Skewness	-0,11603
Kurtosis	-1,33002
N	231
Minimum	-0,40774
1st Quartile	-0,07162
Median	0,31787
3rd Quartile	0,67395
Maximum	1,00000
95% Confidence Interval for Mean	
0,25762	0,35988
95% Confidence Interval for Median	
0,19893	0,45124
95% Confidence Interval for StDev	
0,36143	0,43408


Summary for Nest IV
Anderson-Darling Normality Test
A-Squared 0,74
P-Value 0,053
Mean 0,43021
StDev 0,23718
Variance 0,05625
Skewness -0,244436
Kurtosis -0,405140
N 171
Minimum -0,19734
1st Quartile 0,24953
Median 0,46254
3rd Quartile 0,60746
Maximum 1,00000
95% Confidence Interval for Mean
0,39440 0,46601
95% Confidence Interval for Median
0,41238 0,48755
95% Confidence Interval for StDev
0,21443 0,26538
-0,2 -0,0 0,2 0,4 0,6 0,8 1,0
95% Confidence Intervals
Mean
Median
0,40 0,42 0,44 0,46 0,48 0,50


Summary for Nest V
Anderson-Darling Normality Test
A-Squared 0,20
P-Value 0,872
Mean 0,42830
StDev 0,23885
Variance 0,05705
Skewness -0,073995
Kurtosis -0,421964
N 36
Minimum -0,05541
1st Quartile 0,26084
Median 0,44456
3rd Quartile 0,57459
Maximum 0,92334
95% Confidence Interval for Mean
0,34748 0,50911
95% Confidence Interval for Median
0,32904 0,54309
95% Confidence Interval for StDev
0,19372 0,31156
0,0 0,2 0,4 0,6 0,8
95% Confidence Intervals
Mean
Median
0,30 0,35 0,40 0,45 0,50 0,55


Summary for Nest VII
Anderson-Darling Normality Test
A-Squared 1,32
P-Value < 0,005
Mean 0,52475
StDev 0,22880
Variance 0,05235
Skewness -0,516887
Kurtosis -0,367496
N 78
Minimum 0,00100
1st Quartile 0,42715
Median 0,54270
3rd Quartile 0,72050
Maximum 1,00000
95% Confidence Interval for Mean
0,47316 0,57634
95% Confidence Interval for Median
0,50740 0,57986
95% Confidence Interval for StDev
0,19767 0,27165
0,0 0,2 0,4 0,6 0,8 1,0
95% Confidence Intervals
Mean
Median
0,46 0,48 0,50 0,52 0,54 0,56 0,58

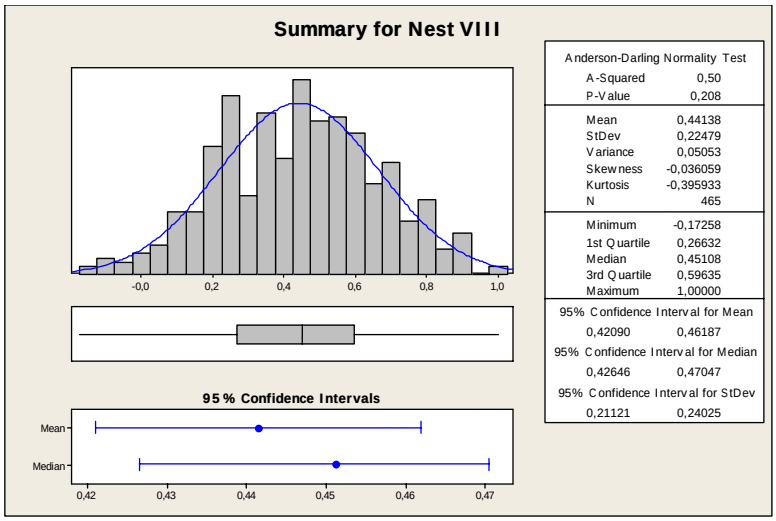


Fig 7a-h: Right: Summary statistics for histograms constructed using pairwise relatedness values of the Orinoco crocodile individuals included in each nest. An Anderson-Darling test has been carried out to test if the values follow a normal distribution. The histograms display the distribution of values, as well as the normal distribution curve. Mean and median confidence intervals are also represented. A significant P-value (P< 0.05) for the Anderson-Darling normality test indicates that the values do not follow a normal distribution

III.5. Power of the microsatellite markers

Out of 133 individuals, 121 individuals carried unique genotypes, demonstrating a high resolution power of the microsatellite markers. However, 6 pairs shared an identical genotype across the 8 loci included in the study. The individuals of identical genotype were always from the same nest (in nest III, IV, and VIII).

The simulation of relatedness values showed a high degree of overlap between the values expected for half sibling, full sibling, and unrelated individuals (fig 8). In theory, full siblings should have a relatedness value of 0,5, and half siblings 0,25. Unrelated individuals should have a relatedness value of 0. Due to low marker resolution, these values vary in such high degree that it is very difficult to distinguish between half and full sibling individuals.

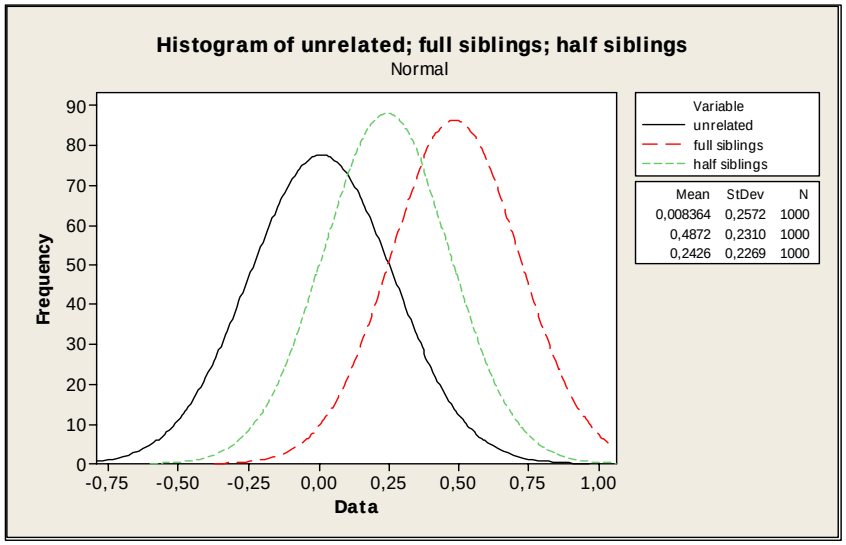


Fig 8: Distribution of relatedness values for unrelated, half sibling and full sibling individuals in the studied Orinoco crocodile population. The values are overlapping to a very high degree. The simulations were based on allele frequency data obtained from the reconstruction of parental genotypes.

IV. Discussion

No previous genetic studies had been made on the genetics of the Orinoco crocodile, making this a pilot project. It was therefore not known whether the use of microsatellite markers originally developed for other close related crocodile species, would be of any use in the study. However, some of the markers did prove to contain variability which was actually similar to that of other genetically studied crocodile species when taking into account only the number of identified alleles of each marker. Though the marker resolution is too weak at the moment for any thorough genetic monitoring or paternal testing to be carried out, the high quality of some of the markers leaves hope for this to be possible in the future, with only a little more effort needed for the development of additional microsatellite markers. However, the quality of the obtained microsatellite resolution was good enough to provide a fundamental understanding of the mating behavior in the studied population.

Another setback to the study was that it proved impossible to extract maternal DNA from offspring egg shells, which meant that the maternal genotype could not be identified, though it was usually possible to reconstruct the parental genotypes without defining which belongs to the mother and father respectively. For this to work in the future, egg shell samples shall either have to be collected from the nest during early nesting (although Strausberger and Ashley 2001 indicate that this does not guarantee any results either), or a sample will have to be taken from the mother herself while she is guarding the nest. In the latter case, this will have to be done so that there will be no mistake on which mother is responsible for which nest. However, there is a great difficulty (and danger) in handling these huge predators, making it necessary to develop safe methods for this in such a case. In addition, while it worked out fine to optimize the PCR for scale samples derived from young (hatchling) crocodile specimens, it proved very difficult to do the same for scales from older individuals. While some markers worked out all right on both younger and older scales, other markers would not work at all on the older ones, hence the decision not to include these in the final analysis. In the future, it might be wise to continue using only scales from young individuals, or start collecting the DNA from other sources (blood, et c).

Despite these obvious difficulties in the early part of the study, a few important discoveries have already been made. Firstly, there is clear evidence for a multiple paternity mating system in the studied population of Orinoco crocodiles. There are also indications that only a few males are breeding, but it is not possible to give an exact number, though a minimum of three males are required to explain the genotypes in the sampled population. It is clear that at least one nest (III) displays evidence for multiple paternity. Nest I might also show evidence on multiple fathers, but in this case there is only one hatchling that could derive from a secondary male. Such large differences in the number of offspring between males are possible, but the possibility that the mismatching juvenile could have accidentally arrived from another nest should be carefully evaluated. Multiple paternity would not be unlikely in this nest though, as it is often the case in multiple paternity mating systems that some males are often able to produce more offspring than others due to factors like for example higher quality sperm (*the intrinsic*

male quality hypothesis, which assumes that multiple mating enables sperm competition and cryptic females choice, Myers and Zamudio 2004), thereby resulting in a reproduction skew even within one nest with multiple paternity. It may also be the case (especially in nest VII) that some of the nests do display multiple paternity, but none that can be detected through direct observation, either because of low sample sizes or because of low genetic variation in the population. Adams et al (2005) suggest that it is often very difficult to distinguish multiple fathers of common or similar alleles using simple allele counts, as might be the case in some of the nests of this study.

In addition, the low resolution of the markers, combined with the low genetic variation in the sampled population will make it difficult to detect any multiple paternity unless observed directly as is the case in nest I and III. However, when the distribution of relatedness values where tested for normality in chapter III.4, nest VII (and perhaps in nest IV as the P value was very close to significant) showed a strange pattern and deviation from normality, which was otherwise only observed in nest III. The low marker resolution makes it difficult to tell if this deviation from normality is due to chance and perhaps low sampling sizes, or if it is evidence for multiple paternity in this nest as well. It should however be treated as indications of that multiple paternity may be even more prevalent in the population than can be detected through direct observation. There was no clear indication on multiple paternity in nest VII when taking the FCA chart into account. However, as in the case of nest I, the possibility exists that the distribution of clutches can be defined in such a way that nest VII shows signs of multiple paternity. The lower and upper part of the distribution of nest VII individuals in the chart may perhaps reflect the offspring of two different males.

Unfortunately, the low marker resolution also leaves the question on whether dominant males receive more offspring more or less unanswered. It is already known that there is a strong hierarchy among crocodile males during the mating season, and that the dominant male will try to monopolize the females (Thorbjarnarson and Hernández, 1993b), but it has also been shown in this study that the same female will also accept to breed with several males during one mating season, making it possible for the less dominant males to participate in the production of offspring as well. How will this then affect the overall genetic variation in the offspring? Will one dominant male still be responsible for a majority of the offspring, or will there be a higher amount of males involved in reproduction? There are indications in the results of this study that a few males were responsible for the offspring of several nests. This evidences that one dominant male is able to monopolize reproduction to some extent, and thereby also reduce the effect that any multiple paternity mating system will have on the number of males involved in reproduction. In addition, if only a few males can breed, the consequences for any reintroduction program will be quite bad. This would mean that genetic variation in the population may be lower than expected, and any introduction of new genetic material will be made more difficult when using male crocodiles.

Hopefully, the effect of a few dominant males monopolizing the females can be compensated through the presence of the multiple paternity mating system. To further study the effect and the co existence of multiple paternity and crocodile male breeding

hierarchies, an improved microsatellite marker resolution will be required, as this would allow for an improved estimate of the actual number of males involved in reproduction. As it is now, it is impossible to tell exactly how many males breed, though there are indications of that it might be less than one male per nest despite the presence of multiple paternity. This reasoning does not take into account the risk of some males breeding, but not being able to produce offspring fit enough to survive hatching. In addition, there is also the risk of some males and females trying to breed at the wrong season as they have been brought from populations with different seasonal variations.

The distribution of individuals in the FCA chart can perhaps be used to give a rough estimate on the number of breeding males, but this is likely to ignore males that have a very limited contribution. It should be possible to detect an approximate number of breeding males by comparing the FCA chart (Fig 6a) in chapter III.4. with the distribution of nests (table 2) in chapter III.1, assuming that the genetic variation between all the males is high enough that they will appear different at the FCA chart. Doing this gives a roughly estimated number of 3-4 breeding males (male 1 corresponds to the offspring of nest I, IV, VII and VIII, male 2 to nest II and V, male 3 to nest VI, and finally individual 10 might be from a fourth father). This procedure has however not been tested properly, and should be considered a hypothesis. There is also the possibility that the FCA chart represent only two breeding males, with one left side (male 1) and one right side (male 2) clutch.

In addition, the above hypothesis also allows for the conclusion to be drawn, that males mating at the western nesting sites (nesting site 14 and 14', including nest I, II, III, IV, and V), have no problem with being present to mate at the eastern nesting sites as well (9, 12, and 13, including nest VI, VII, and VIII), approximately 2.7 kilometers away. In the case of a few males monopolizing the females, the size of the area in which one dominant male can be sexually active during breeding season will be important as it will determine the distance required between artificially set nests to maximize the number of males breeding. If some nests were placed further away, a few more males would perhaps be allowed to breed.

There is also a slight chance that the breeding males do not represent a selection of dominant males among a larger amount of submissive individuals, but that there are actually only a few mature males available to breed in this area. If this is the case, the results of the study would not constitute any indication on male dominance. In addition, it might be that a few more males breed than recorded, but only hatchlings of some individuals survive.

Genetic variability in the population could not be estimated. However, based on the data obtained, a few basic conclusions can be drawn despite the high relatedness of the individuals included. It was expected that the population would show a very low genetic variation due to the recent bottleneck that occurred between the 1930s and the1960s. When looking at the control region mtDNA marker, this idea is supported since no variation between individuals was detected. Though as much as half of the tested microsatellite markers proved to be monomorphic, it is difficult to draw any conclusions

from this fact as the markers were not originally developed for the Orinoco crocodile, but for closely species related species. On the other hand, the markers have been tested in a variety of different crocodile species in which a much smaller degree of monomorphism was recorded. Note that this conclusion will be very biased as most of the studies conducted have probably removed loci that did not prove to be polymorphic. However, among the polymorphic microsatellite markers, the variability within each nest was comparable to that of other *Crocodylus* species. In several aspects, the variability was actually even higher. For example, while the polymorphic markers in three studies of *Crocodylus porosus* (Isberg et al, 2004), *Crocodylus moreleeti* (Dever and Densmore, 2001), and *Crocodylus siamensis* (Fitzsimmons et al, 2002) had a mean observed heterozygosity of 0,45 – 0,59, the sampled Orinoco crocodile population in this study showed a similar mean observed heterozygosity of 0,58 (see Appendix J). The mean observed heterozygosity of the reconstructed parental genotypes was as high as 0,64. However, as the El Frío Orinoco crocodile population has been reintroduced starting with several individuals from several different wild and captive populations in Venezuela, the study population is bound to have a greater genetic diversity than any of these natural populations. Conversely, other (wild) populations of Orinoco crocodile may be expected to contain a lower degree of genetic variation.

Due to the expected lower variability of the wild populations of Orinoco crocodile, an even greater marker resolution will be required to study these genetically and to establish relationships within a population. For genetic studies of differentiation between populations, the markers might however be much more useful, as genetic drift should produce a situation where the genetic variation within each separate population are lowered with time, while the differentiation between populations increase. This means that some of the markers that were monomorphic in the study population might be polymorphic in others. To ensure the development of good reintroduction strategies and to detect any risk of inbreeding depression in the wild populations, there is a critical need for genetic studies of Orinoco crocodile. Ecological aspects, such as the extent of multiple paternity, and to what degree dominant males are able to monopolize breeding should be further studied, and other basic genetic studies such as the differentiation and genetic variability within and among different populations of Orinoco crocodile should be initiated. Other genetic research projects of interest is the relationship between the Orinoco crocodile and the neighboring American crocodile (C*rocodylus acutus*), which lives at the coast of Venezuela and Colombia among other places. There is a great concern that these very similar crocodile species may hybridize, which will cause even greater problems for the conservation of Orinoco crocodile.

As mentioned in the introduction, crocodilian populations are usually very resilient and able to recover after serious population declines. Despite the current critical state of the Orinoco crocodile, if effective conservation efforts are taken and uphold, it should be possible to save this species from further population depletion and extinction. This means that an effort will be needed to spread acceptance of this dangerous predator among local communities within the species distribution range, as well as to understand the complex ecology and behavior of the species to make any reintroduction program successful. Genetic methods are a good way to study the species without much disturbance, and

hopefully future research will continue to increase its chances of survival through a better understanding of the needs of the species.

V. Acknowledgements

Firstly I'd like to thank Carles Vilà and Frank Hailer for their excellent supervising. Carles for his help with data analysis, collecting information, and for his comments on the report. Frank for his commitment to making this project work with respect to the PCR and genotyping problems, and everything else that just kept going wrong at the start of the project, as well as his help with the scoring of microsatellite typings. I'd also like to thank Carles and Santiago Castroviejo-Fisher for obtaining the samples. Jennifer Leonard earns a big thank you for teaching me the laboratory methods, and Eva Hedmark for helping me with the kinship analysis. Last but not least, big thanks go to the El Frío field station in Venezuela for supplying us with material to work with, and of course, a great deal of final thanks to all of the 143 crocodiles, especially those of nest III, for accepting to participate in the study!

Photo: Orinoco crocodile female watching her nest. Most of her time will be spent in the pool next to it.

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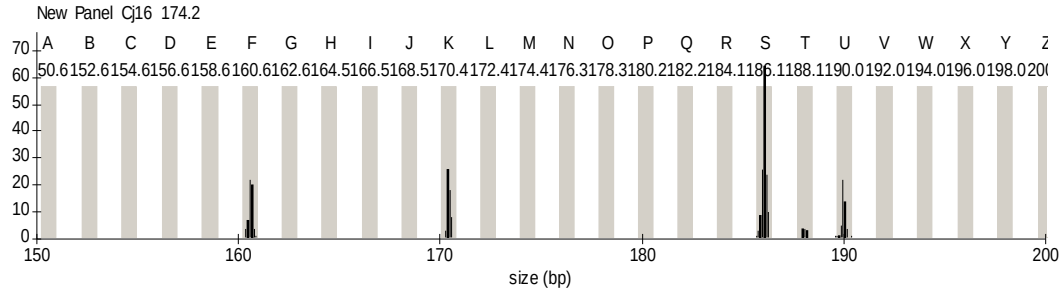
Appendixes

Appendix A: Overview of microsatellite marker Cj16

Forward primer: Cj16-F Reverse primer: Cj16-R TAG: M13-2-Tag

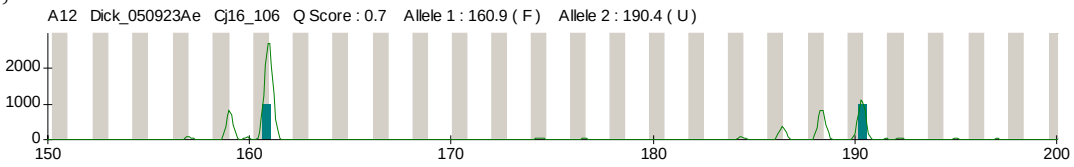
Allele distribution:



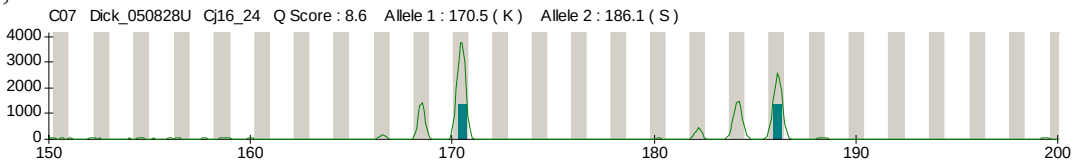
Known alleles: 160, 170, 186, 188, 190

Genotype definitions:

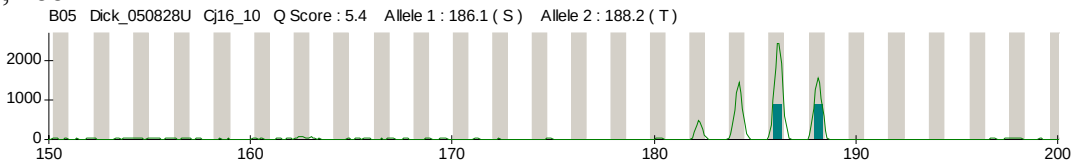
160, 190



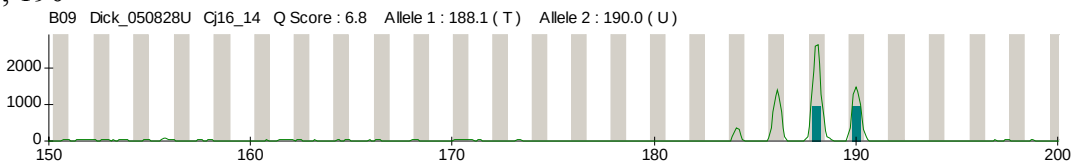
170, 186



186, 188



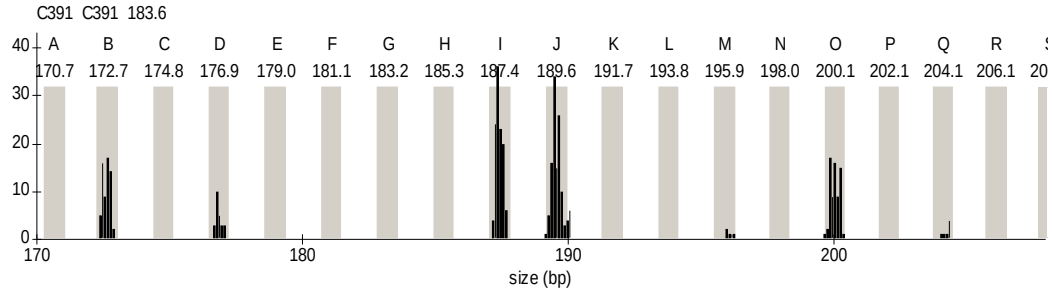
188, 190



Appendix B: Overview of microsatellite marker C391

Forward primer: C391-F Reverse primer: C391-R TAG: M13-2-Tag

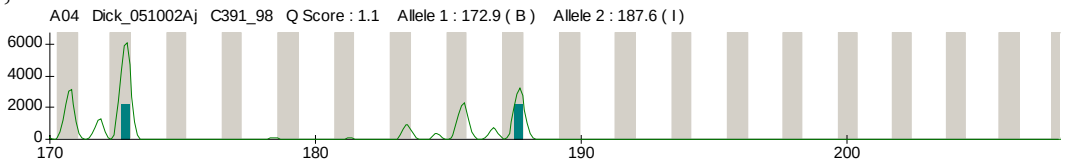
Allele distribution:



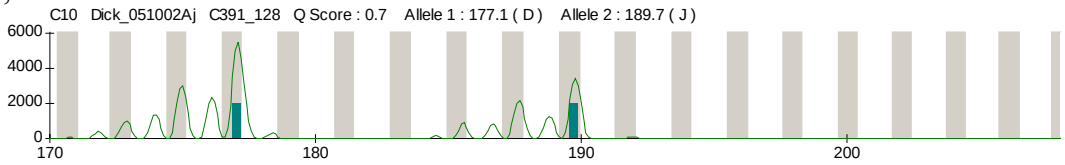
Known alleles: 173, 177, 188, 190, 196, 200, 204

Genotype definitions:

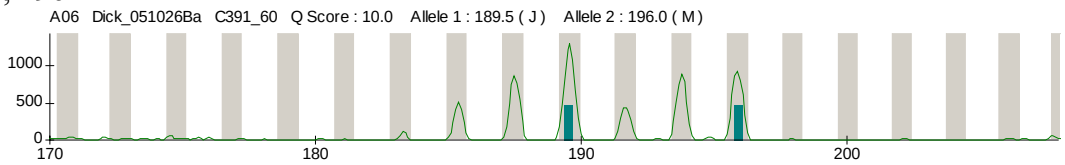
173, 188



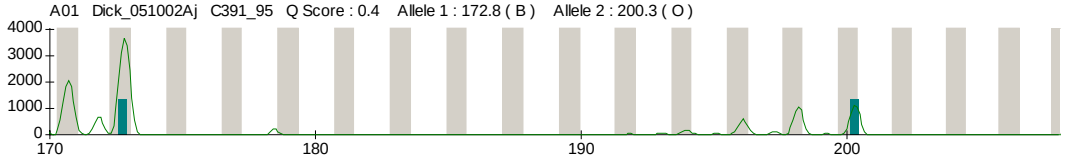
177, 190



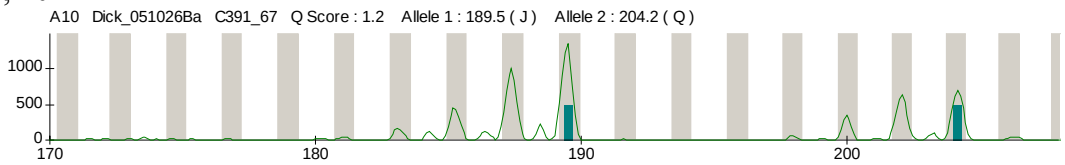
190, 196



173, 200



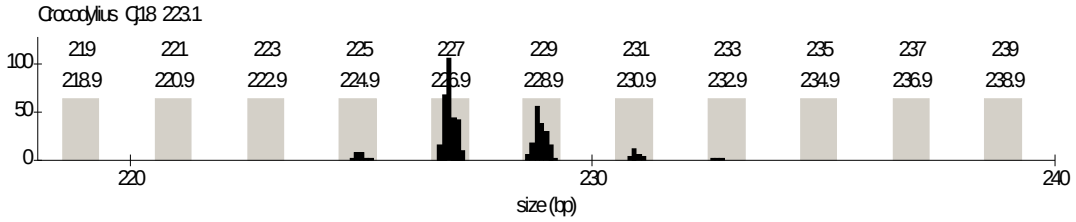
190, 204



Appendix C: Overview of microsatellite marker Cj18

Forward primer: Cj18-F Reverse primer: Cj18-R TAG: CAG-2-Tag

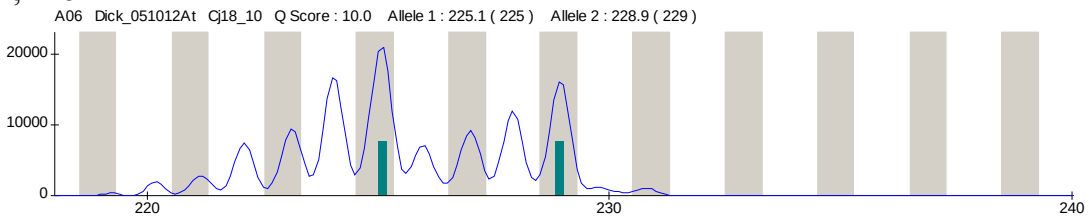
Allele distribution:



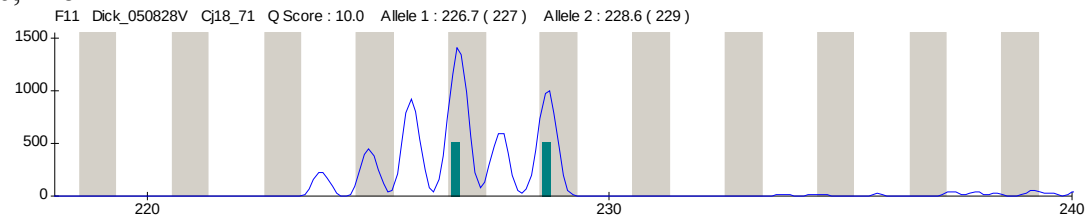
Known alleles: 224, 226, 228, 230, 232

Genotype definitions:

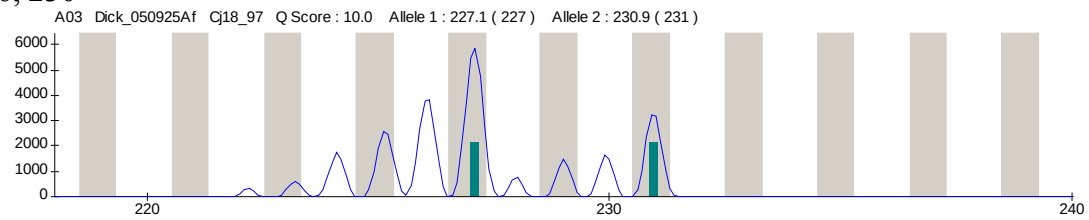
224, 228



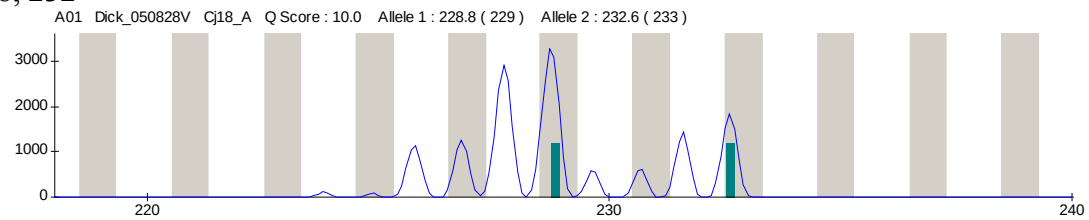
226, 228



226, 230



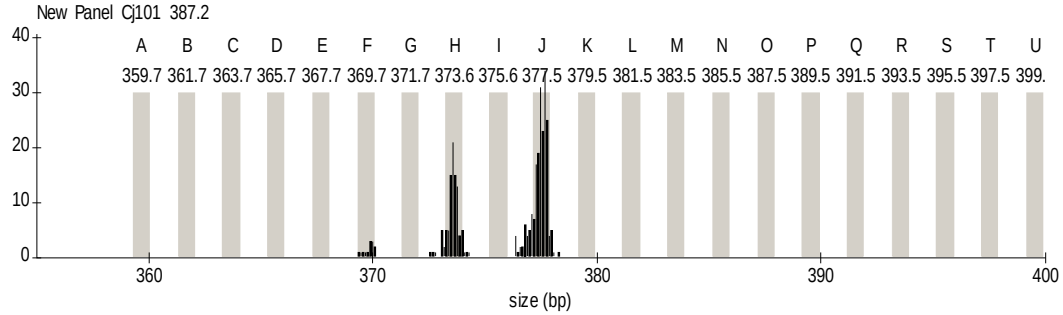
228, 232



Appendix D: Overview of microsatellite marker Cj101

Forward primer: Cj101-F Reverse primer: Cj101-R TAG: CAG-2-Tag

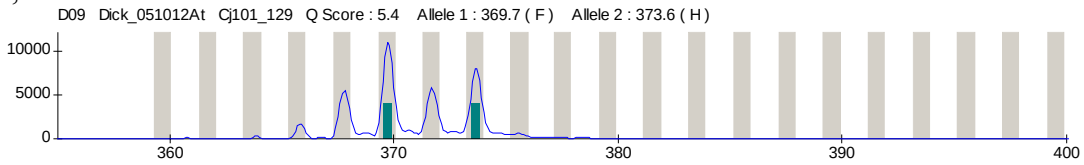
Allele distribution:



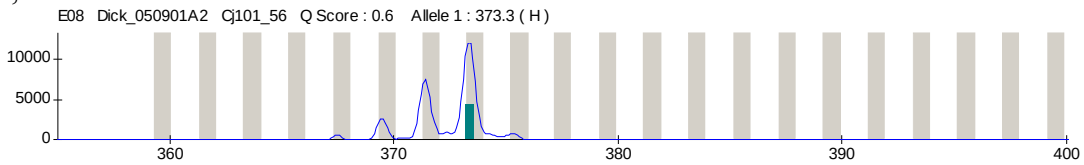
Known alleles: 370, 374, 378

Genotype definitions:

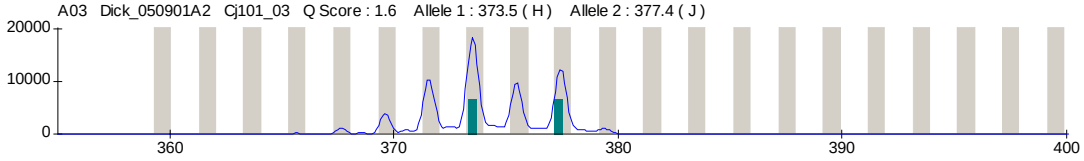
370, 374



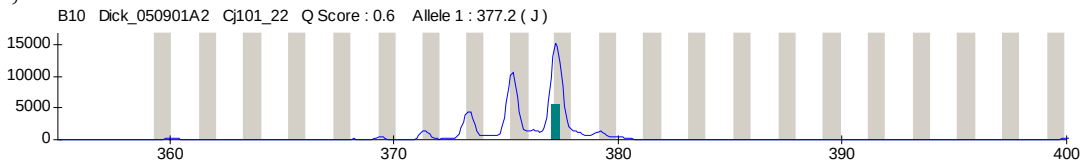
374, 374



374, 377



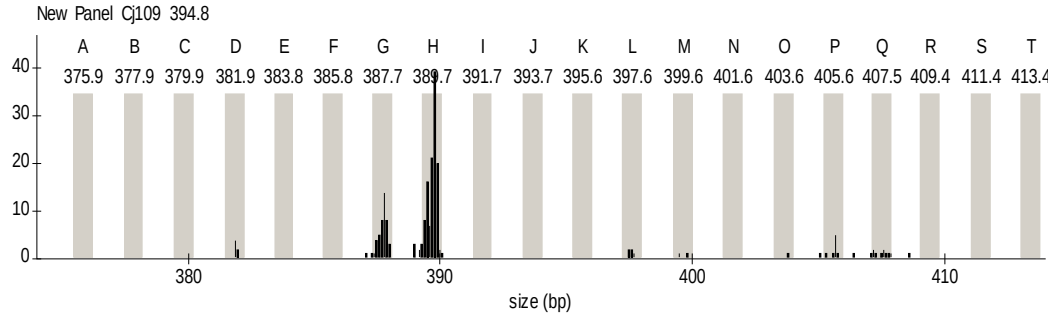
377, 377



Appendix E: Overview of microsatellite marker Cj109

Forward primer: Cj101-F Reverse primer: Cj101-R TAG: CAG-2-Tag

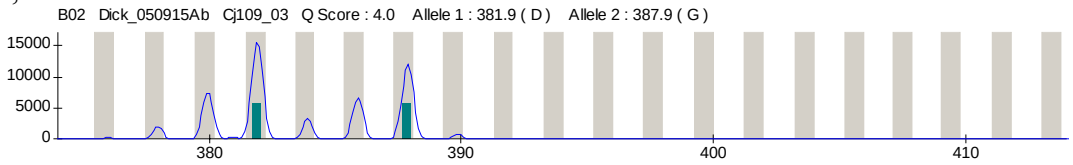
Allele distribution:



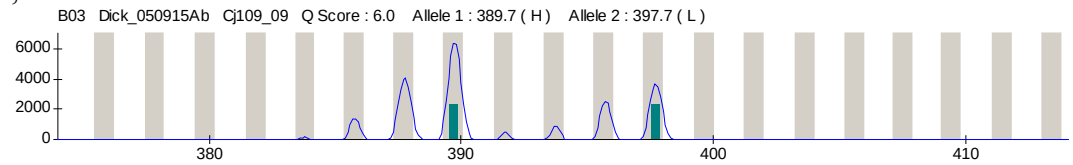
Known alleles: 382, 388, 390, 398, 400, 405, 407

Genotype definitions:

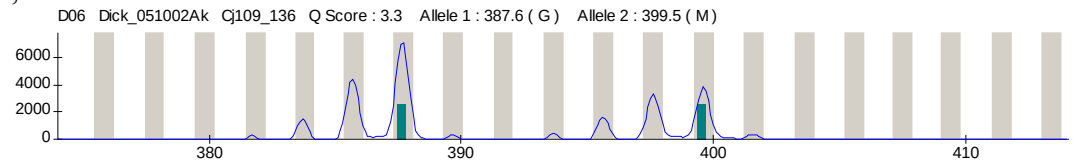
382, 388



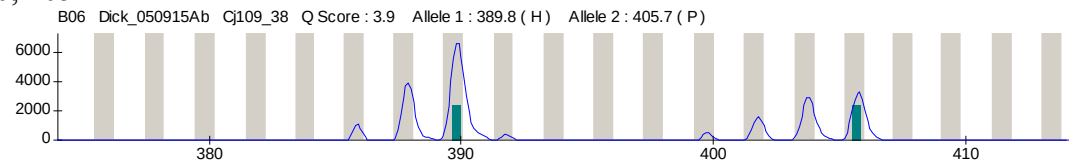
390, 398



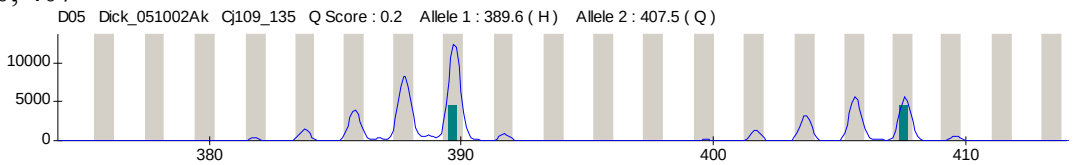
388, 400



390, 405



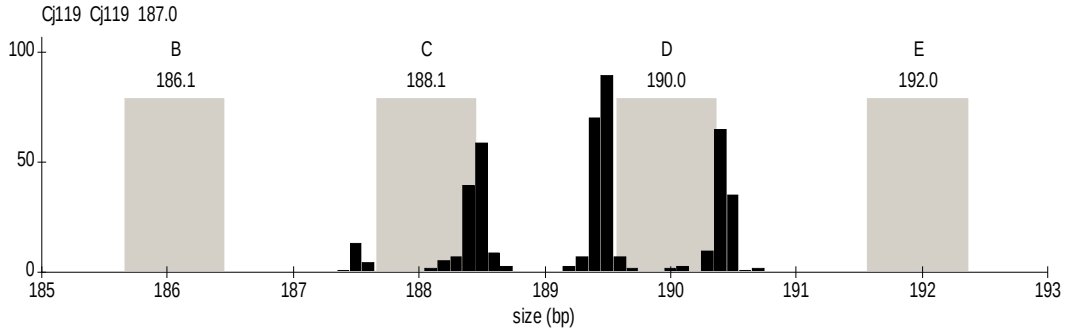
390, 407



Appendix F: Overview of microsatellite marker Cj119

Forward primer: Cj119-F Reverse primer: Cj119-R TAG: CAG-Tag

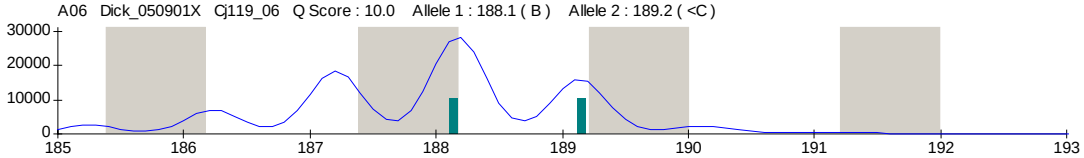
Allele distribution:



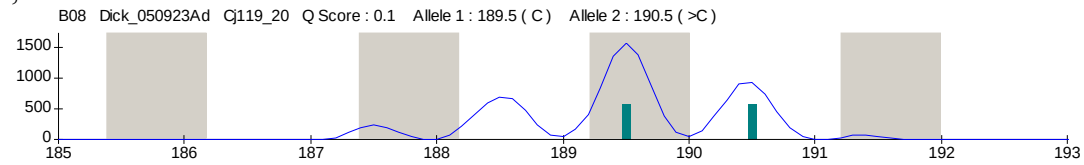
Known alleles: 188, 189, 190

Genotype definitions:

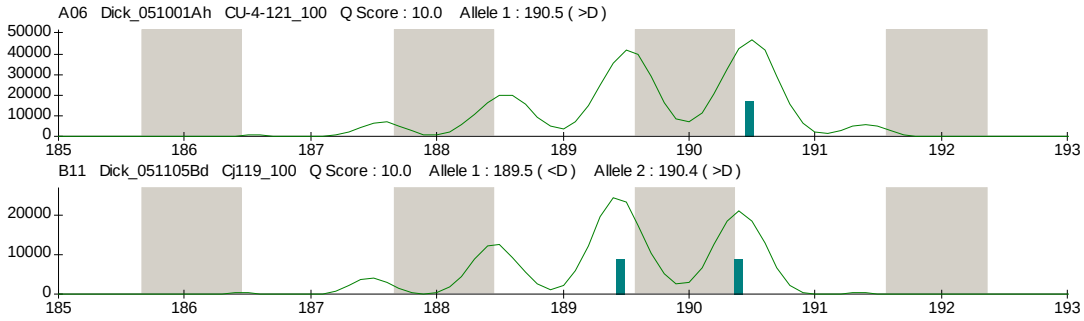
188, 190



189, 190



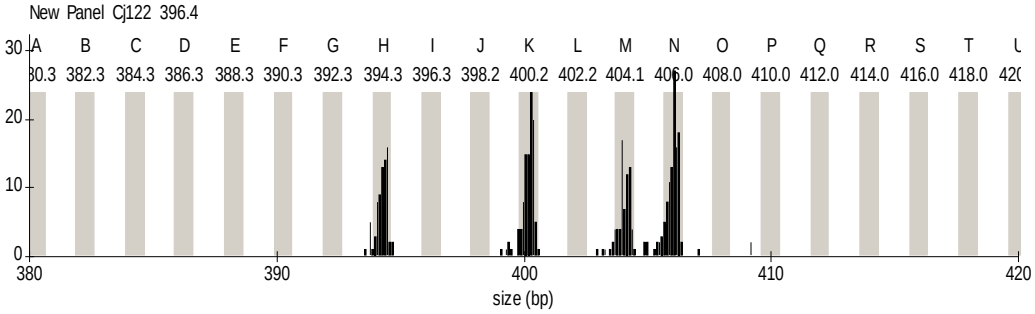
Scoring difficulties: The two different electropherograms below are of the same individual, but of different genotypings. Like in many other individuals when using the Cj119 marker, it is impossible to tell if the individual is a homo- or heterozygote. Note that the CU-4-121_100 should actually be named Cj119_100.



Appendix G: Overview of microsatellite marker Cj122

Forward primer: Cj122-F Reverse primer: Cj122-R TAG: CAG-Tag

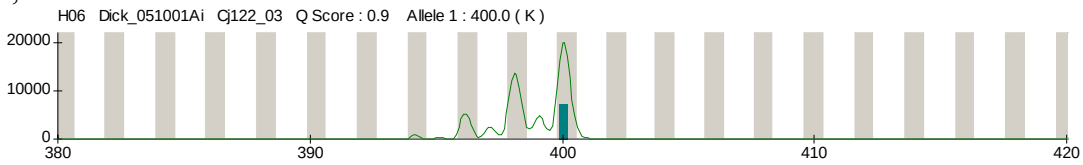
Allele distribution:



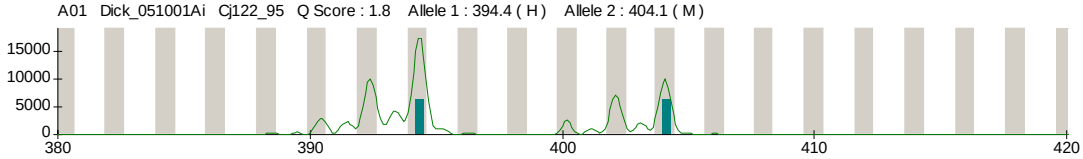
Known alleles: 394, 400, 404, 406

Genotype definitions:

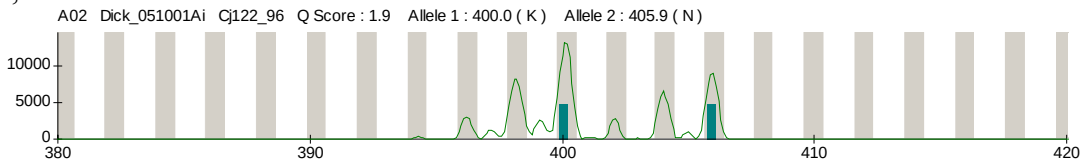
400, 400:



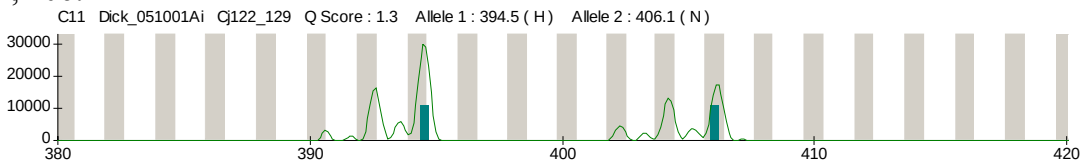
394, 404:



400, 406:



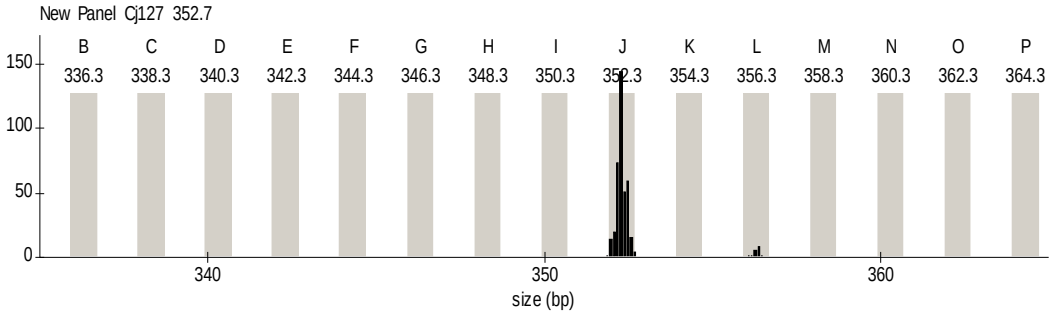
394, 406:



Appendix H: Overview of microsatellite marker Cj127

Forward primer: Cj127-F Reverse primer: Cj127-R TAG: CAG-Tag

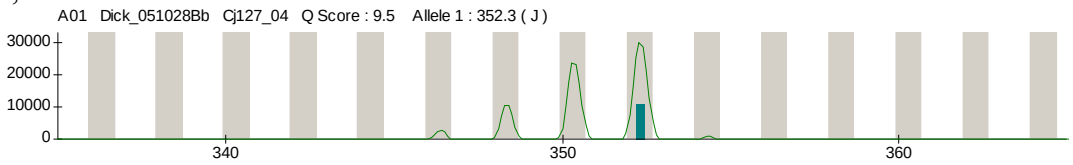
Allele distribution:



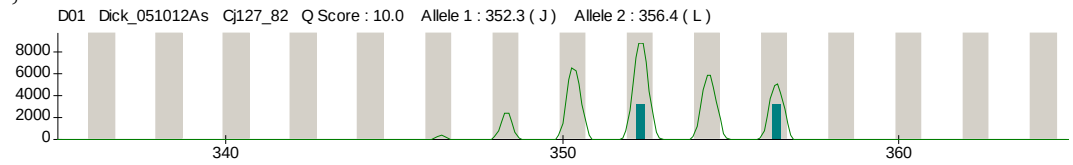
Known alleles: 352, 356

Genotype definitions:

352, 352



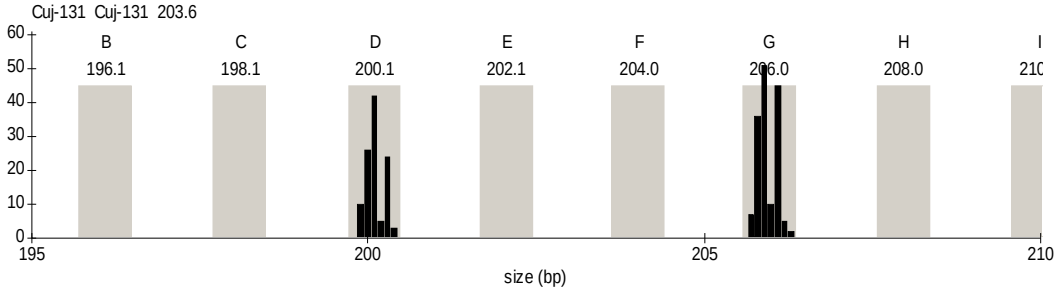
352, 356



Appendix I: Overview of microsatellite marker CUJ-131

Forward primer: CUJ-131-F Reverse primer: CUJ-131-R TAG: M13-2-Tag

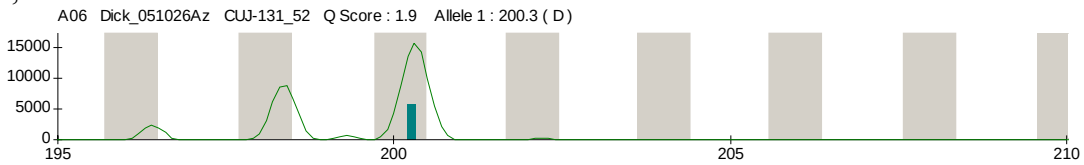
Allele distribution:



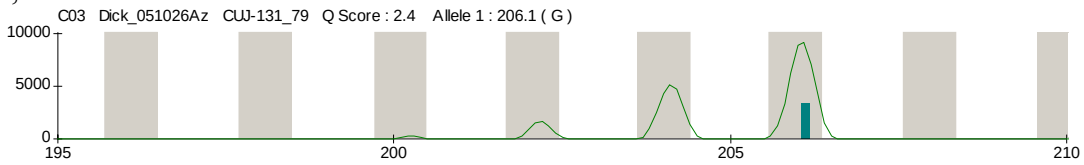
Known alleles: 200, 206

Genotype definitions:

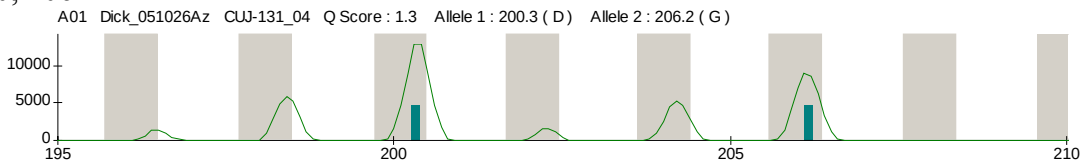
200, 200



206, 206



200, 206



Appendix J – Allele frequencies for each marker

Table: Allele frequencies for each marker in the sampled Orinoco crocodile population of 133 individuals from 8 nests

Locus	*Allele*	*Frequency*	*Locus*	*Allele*	*Frequency*
C391	173	0.1391	Cj109	382	0.0714
	177	0.0639		388	0.2481
	188	0.2932		390	0.4962
	190	0.3008		398	0.0376
	196	0.0188		400	0.0038
	200	0.1504		405	0.0752
	204	0.0338		407	0.0677
Cj16	160	0.1767	Cj122	394	0.1932
	170	0.1842		400	0.3030
	186	0.4286		404	0.2008
	188	0.0414		406	0.3030
	190	0.1692			
			Cj127	352	0.9624
Cj18	224	0.0376		356	0.0376
	226	0.5489			
	228	0.3421	CUJ-131	200	0.2368
	230	0.0714		206	0.7623
	232	0.0000			
Cj101	370	0.0341			
	374	0.3068			
	378	0.6591			