Nile crocodile (*Crocodylus niloticus*) genetic diversity and population structure, within the lower Kunene and Okavango Rivers of northern Namibia

by

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Declaration

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Abstract

The Nile crocodile has experienced numerous stages of illegal hunting pressures in the mid-20th-century across most of the species' distribution. The reduced Nile crocodile populations have shown partial recovery and it is currently considered as a "lower risk" / "least concern" species on the Red List of International Union for Conservation of Nature. In Namibia, however, the Nile crocodile is recognised as a protected game species under the Nature Conservation Ordinance No 4 of 1975, allowing trophy hunting of the species only with the issuing of a hunting licence. Census and genetic data of the Nile crocodile is limited or non-existing in Namibia and the country has recently developed a species management plan to conserve the wild populations. During 2012 an aerial survey was conducted along the Lower Kunene River to estimate the abundance and distribution of the Nile crocodile population, by the use of a recently developed N-mixture model. Within the Lower Kunene River system a direct count revealed 562 crocodiles regardless of size, and an estimated population size of 806 individuals, after bias correction. The analyses suggested the class-structured model produced unbiased estimates of the Nile crocodile population in the Lower Kunene River system. To contribute to the conservation efforts of the Nile crocodile in the Lower Kunene River, the study also assessed the genetic diversity and structure within the Kunene and Okavango River system in comparison to neighbouring river basins. This study aimed to develop molecular markers, to assess the patterns of genetic diversity and population structure generated from 11 Short Tandem Repeats and the mitochondrial DNA, control region. The Lower Kunene and Okavango populations indicated a recent divergence with a single haplotype shared among the 64 samples sequenced and interestingly the haplotype was shared with populations in Gabon and Uganda. Moreover, there was no sharing of haplotypes found between the Lower Kunene and Okavango and the Lower Shire River system. Estimated for pairwise population differentiation, F-statistics, AMOVA and factorial correspondence analysis (FCA), based on Short Tandem Repeats, indicated significant structuring among the populations. Additionally, Bayesian clustering analyses detected three putative ancestral gene pools, of which two were present in the Okavango River population, supporting the findings of the Nile crocodile to be structure according to river basin formation. Despite no expansion or population bottleneck detected in the Nile crocodile populations, a contemporary genetic bottleneck may have gone undetected due to the crocodile's long-life span and breeding between overlapping generations. The contemporary restriction of gene flow and historical river topography are the most likely cause of genetic structure in the Nile crocodile populations of today. Even though the Kunene and Okavango Nile crocodile populations are experiencing different environmental and evolutionary pressures, the genetic data suggest a single evolutionary significant unit, with two management units. The Okavango River had a broad sampling range for the study, however both river populations will require more samples to validate fine-scale genetic structure.

Opsomming

Die Nyl krokodil het talle fases van onwettige jag ervaar in die middel van die 20ste eeu oor meeste van die spesie se verspreidingsgebied. Die verminderde Nyl krokodil bevolking het gedeeltelike herstel en word tans beskou as 'n laer risiko / minste kommer spesie op die rooi lys van die Internasionale Unie vir die Bewaring van die Natuur. In Namibië, is die Nyl krokodil erken as 'n beskermde wildsoort onder die Natuurbewaring Ordonnansie Nr. 4 van 1975, sodat trofeejag van die spesie net met die uitreiking van 'n jaglisensie uitgevoer mag word. Sensus en genetiese data vir die Nyl krokodil is beperk of nie-bestaande in Namibië en die land het onlangs 'n spesie bestuursplan, om die wilde bevolkings bewaar, ontwikkel. Gedurende 2012 is 'n lug telling opname langs die ‘Lower’ Kunene Rivier gedoen om die digtheid en verspreiding van die Nyl krokodil bevolking te skat, deur die gebruik van 'n onlangs ontwikkelde ‘N-mixture’ model. Die ‘Lower’ Kunene Rivier direkte telling, skat ongeveer 562 krokodille ongeag die grootte, en 'n geskatte bevolkingsgrootte van 806 individue na sydigheidskorreksie. Die analise blyk dat die klas gestruktureerde model kan onbevooroordeelde ramings van die Nyl krokodil bevolking in die ‘Lower’ Kunene Rivier stelsel produseer. Om verder by te dra tot die bewaringspogings van die Nyl krokodil in die ‘Lower’ Kunene Rivier, poog die studie om die genetiese diversiteit en -struktuur binne die Kunene- en Okavango Rivier stelsel, in vergelyking met die naburige rivierbekkens, te ondersoek . Elf mikrosatelliet merkers en mitochondriale DNA is vir die doel aangewend. Die ‘Lower’ Kunene en Okavango bevolkings dui op 'n onlangse divergensie met 'n enkele haplotipe wat gedeel word tussen die 64 monsters. Die haplotipe word interessantlik gedeel met bevolkings in Gabon en Uganda. Verder was daar twee takke van haplotiepes gevind tussen die ‘Lower’ Kunene en Okavango en die ‘Lower’ Shire-rivier stelsel. Na raming van paarsgewyse bevolking differensiasie, F-statistieke, AMOVA en faktoriaal korrespondensie analise (‘FCA’), gebaseer op mikrosatelliete, is daar beduidende genetiese strukturering tussen verskeie bevolkings. Daarbenewens, bevind ‘Bayesian’ groeperingsanalise dat daar drie vermeende voorvaderlike geen poele bestaan, waarvan twee teenwoordig was in die Okavango Rivier bevolking. Hierdie data ondersteun vorige waarneming dat Nyl krokodil populasiestruktuur beïnvloed word deur rivier vorming. Ten spyte van die beraamde afwesigheid van bevolkingsuitbreiding of bevolkingsbottelnekke in die Nyl krokodil, kan 'n kontemporêre genetiese bottelnek ongemerk gegaan het as gevolg van die krokodil se lang lewensduur en teling in oorvleueling generasies. Die kontemporêre beperking van genevloei en historiese rivier topografie is die mees waarskynlike oorsaak van die beskryfde genetiese struktuur in die Nyl krokodil bevolking. Selfs ervaar die Kunene en Okavango Nyl krokodil bevolkings verskillende omgewings- en evolusionêre druk, is daar slegs ’n enkele evolusionêre beduidende eenheid, met moontlik twee bestuurseenhede. Die Okavango Rivier het 'n breë monsterneming reeks vir die studie; Maar beide rivier bevolkings sal meer monsters benodig om fyn skaal genetiese struktuur te ondersoek.

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List of Abbreviations

λ	Mean abundance
µL	Microlitre
µM	Micromole
π	Nucleotide diversity
CI	Confidence interval
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTAB	Cetyltrimethylammonium Bromide [((C16H33)N(CH3)3Br]
DNA	Deoxyribonucleic Acid
DSS	Division Support System
ESU	Evolutionary Significant Units
FAM	5-carboyfluirescein (ABI-fluorescent label)
FCA	Factorial Correspondence Analysis
F_{ct}	Derivative of Wright's Fixation Index adapted for hierarchical AMOVA (group of populations relative to the total population)
F_{is}	Wright's Fixation Index (individual relative to the sub-population, equal to the inbreeding coefficient - f)
F_{sc}	Derivative of Wright's Fixation Index adapted for hierarchical AMOVA (sub-population relative to the group of populations)
F_{st}	Wright's Fixation Index (subpopulation relative to the total population)
GPS	Global Positioning System
H	Number of haplotypes
h	Haplotype diversity
H_e	Expected Heterozygosity
H_o	Observed Heterozygosity
HWE	Hardy-Weinberg Equilibrium
IAM	Infinite Allele Model
IUCN	International Union for Conservation of Nature and Natural Resources
k	Mean number of nucleotide differences between haplotypes
km	Kilometre
LD	Linkage Disequilibrium
MCMC	Markov Chain Monte Carlo
MET	Ministry of Environment and Tourism
mtDNA	Mitochondrial Deoxyribonucleic Acid
MU	Management Units
MUSCLE	Multiple Sequence Comparison by Log-Expectation
mya	Million years ago
N	Realised abundance
N_e	Effective population size
PIC	Polymorphic Information Content
Rand	Random Survey effect
R_s	Allelic Richness
S#	Session Number

SD	Standard Deviation
sec	Second
SMM	Stepwise Mutation Model
Spp.	Several Species
STRs	Short Tandem Repeats
SVL	Snout Vent Length
T_a	Annealing Temperatures
™	Trademark
TPM	Two-Phased Model

Chapter 1

Introduction: Literature Review, Research Aims and Objectives

1.1 Introduction to the study animal

The crocodile family, *Crocodylidae,* is considered part of the class *Reptilia* and consists of three genera, *Crocodylus*, *Ostelaemus* and *Tomistoma* (Britton 1995). The *Crocodylus* genus consists of 11 different crocodilian species including, but not limited to the Nile crocodile (*C. niloticus*), Morelet's crocodile (*C. moreletii*) (Platt *et al*. 2010) and Saltwater crocodile (*C. porosus*) (Webb *et al*. 2010). The *Crocodylidae* family is distributed worldwide with six genera residing in Asia and four in America (Britton & Ferioli 2012). Three distinct crocodilian species populate the fresh water river, lakes and/or swamps of Africa, however the status of the populations have only been reported in a few incidents (Graham 1968; Parker 1970; Hutton 1989; Leslie 1997; Platt & Thorbjarnarson 2000; Bourquin 2007; Chase 2009; Ferreira & Pienaar 2011; Wallace et al. 2013; Combrink 2014). The crocodile family feature as an apex predator in its natural environment and has been deemed important for conservation intentions as a keystone species (Musambachime 1987; Bourquin 2007; Aust 2009; Ashton 2010).

The limited research on crocodilians, in Africa, has highlighted the need to investigate the population dynamics of these species. The three crocodilian species are the Nile crocodile (*C. niloticus*) (Schmitz *et al*. 2003; Fergusson 2010), Dwarf crocodile (*Ostelaemus tetraspis*) (Schmitz *et al*. 2003; Eaton 2010) and Slender-snouted crocodile (*Mecistops cataphractus*) (Meredith *et al.* 2011; Shirley 2010). The distributions and greater localization of the species are found, but not limited to central and western Africa and in addition the Nile crocodile is widely dispersed over sub-Saharan Africa (Cites 1999 Figure 1.1). The Nile crocodile is easily identifiable by several exterior markers, namely two nuchals smaller than the other four, one row of post-occipitals and protuberance behind each of the eyes (Cites 1999; Figure 1.1).

The Nile crocodile has experienced numerous stages of illegal hunting pressures in the mid-20th-century across most of the species' distribution (Ross 1998). The reduction of the Nile crocodile in the meantime has shown partial recovery and it is currently considered a lower Risk / least Concern on the Red List of International Union for Conservation of Nature. Notwithstanding the difficulty of evaluating the total wild population size, rough estimates have been provided for 250 000 to 500 000 individuals (IUCN 2014). It has been noted that

wild Nile crocodile populations have shown partial recovery, but are currently still under threat due to habitat loss caused by anthropogenic actions. As such, crocodiles are considered peripherally endangered in developing countries, including Namibia (Griffin 2003). In order to aid the future survival of the affected populations, conservation efforts are required to identify different managements units of the Nile crocodile populations, making use of behavioural, biological and genetic analyses.

The Nile crocodile is regarded as a long lived species; reaching an average age of 45 years in the wild. Moreover, age estimates of the Nile crocodile in the wild are difficult to determine accurately and thus it is considered a function of total animal length (with the average length to being five meters) (Groombridge 1987).

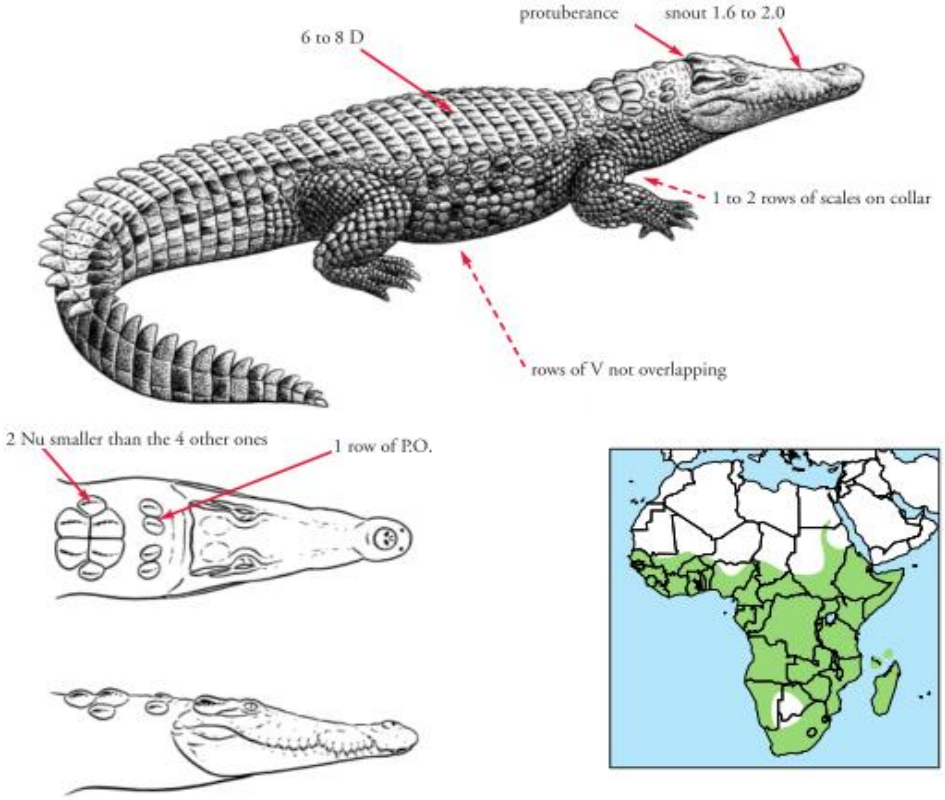


Figure 1.1 Exterior marker identification of the Nile crocodile (*C. niloticus*) for their identification in the wild and the distribution in Africa and its surrounding islands. Modified from CITES (1999).

Recent work conducted on crocodiles have brought about significant molecular and morphometric evidence, endorsing two lineages in the Slender-snouted crocodile (Shirley *et al.* 2014). Schmitz *et al.* (2003) reported that the Nile crocodile similarly shows a molecular lineage separation between the Eastern and Western Nile crocodile, namely paraphyletic East (Madagascan populations) and monophyletic West (Central African population) clades. A

study by Hekkala *et al.* (2010) supported these findings with further evidence for genetic divergence between the Eastern and Western African clades. Additionally, Nile crocodile karyotype differences exist between the two clades and current emerging evidence suggests the western clade is a historically extinct *Crocodylus* species, *C. suchus* (Figure 1.2). These findings provide important information regarding the conservation of the wild Nile crocodile populations. Nevertheless, the Crocodile Specialist Group currently recognizes the Nile crocodile as a single species (Crocodie Specialist Group 2012).

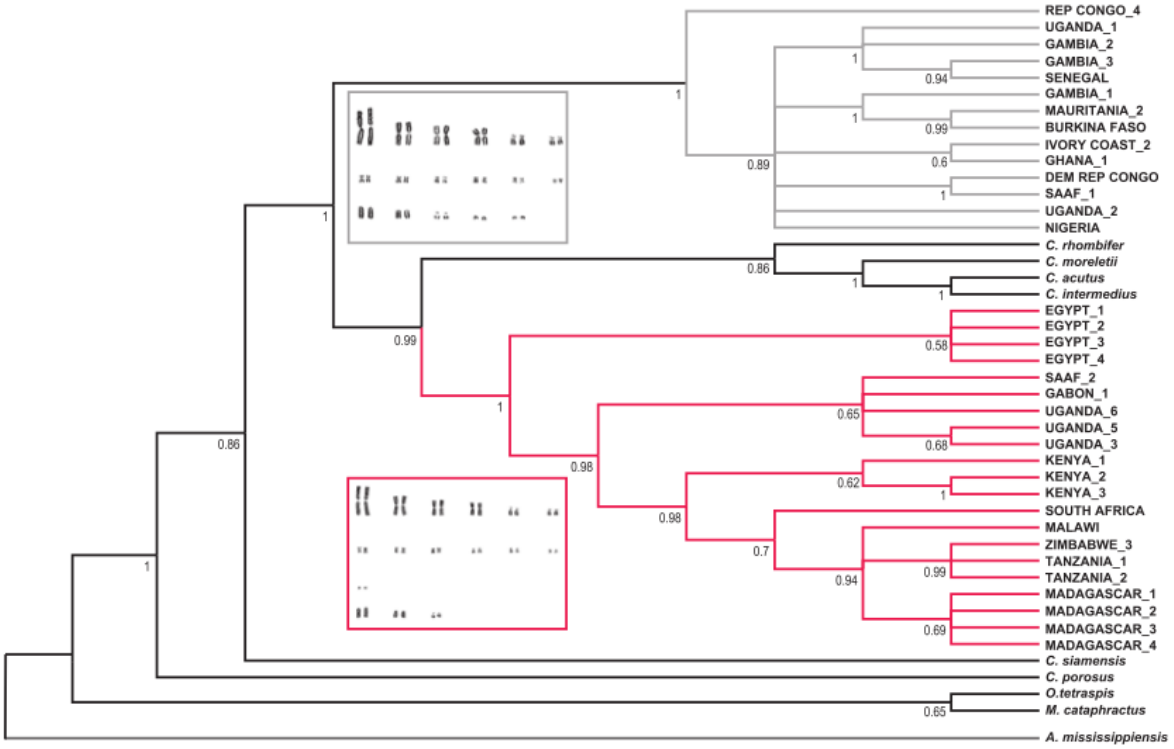


Figure 1. 2 Phylogenetic tree with karyotype insert to illustrate the lineage separation of the western (light grey) and eastern (red) Nile crocodile populations and the difference in chromosome pairs. The phylogenetic tree and karyotype analyses support a paraphyletic Nile crocodile with the predominantly western clade (light grey) as sister to a monophyletic New World and eastern Nile crocodile clade. Posterior probability (PP) are indicated above the branches with significant support indicated by PP > 0.90. Karyotype inserts displayed are those of individuals SAAF_1, SAAF_P (western) and SAAF_2 (eastern). Drafted from Hekkala *et al.* (2011)

1.2.1 Crocodilian fossil evidence and origin of the species

The origin of *Crocodylus* is unclear, however, the genus is thought to have originated in Africa during the Cretaceous period 65 million years ago (mya) (Sill 1968), owing their global distribution to continental drift (Brooks & O'Grady 1989). The oldest fossils recovered for the *Crocodylus* genus, *C. palaeindicus*, was found on the Indian subcontinent (South East Asia) dated to have lived during the late Miocene period (11 – 5.2 mya) (Brochu 2000). Additional *Crocodylus* fossils were discovered and dated in Australia (4.5-4 mya) (Willis 1997), Neotropics (Central-America) (+/- 4mya) (Miller 1980) and Africa (3-2 mya)

(Tchernov 1986). Fossil evidence suggests that continental separation and long distance travel may explain the current global species distribution of crocodilians. Long distance traveling has been reported for crocodile populations over a large geographical range. Evidence has been supportive of *Crocodylus* utilizing ocean currents for long distance travel. For example, *C. porosus* have been found to make use of ocean currents (Campbell *et al.* 2010) and reportedly travelled 800km (Bustard & Choudhury 1982) and 1360 km (Allen 1974) from land. The long distance migration of crocodiles allowed for the emergence of a new hypothesis to be considered for their global distribution.

Oaks (2011) formulated a hypothesis based on the origin and global distribution of the *Crocodylus* genus, with the genus nested with a common ancestor in the Tortonian Indo-Pacific 13.6-8.3 mya (Mekosuchinae, a sub-family of crocodiles), which shows the distribution relative to a time of mass extinction. If there is merit in the evidence presented by Oaks (2011) and the origin of *Crocodylus* as per the fossil evidence, then this can be used in support of *Crocodylus* and its movement into Africa during the post extinction period.

The location of *Crocodylus* entry into Africa has not been defined, however the Congo Basin has been considered as a point likely of origin (Hekkala *et al*. 2011). The probability of the Congo Basin origin is plausible, as several fish species have also been described as having originated from the Basin (Skelton 1975). The separation of riverine species in Africa has been considered relative to rift valley formation and topographic changes (Skelton 1975; Bell-Cross 1968; Salzburger *et al.* 2005; Eaton et al. 2009; Hekkala *et al.* 2011). The separation of the various isolated species are required to be evaluated for conservation management, as different conservation plans are required for diverged populations. The structuring of these populations are required and has previously been found for the Nile crocodile to be structured according to river basin formation (Hekkala *et al.* 2010).

1.2.2 Crocodilian conservation ecology

Census estimates of *Crocodylus* species have been conducted on various river systems (Graham 1968; Parker 1970; Hutton 1989; Platt & Thorbjarnarson 2000; Bourquin 2007; Chase 2009; Ferreira & Pienaar 2011; Wallace et al. 2013; Combrink 2014). Evidently, it is difficult to compare the various river system population sizes to one another due to different habitat types. Common methods of crocodile population estimations are achieved via spotlight boat, or helicopter surveys. Furthermore, these means of prediction provide good estimates for crocodile abundance, however each is not without its own bias (Bayliss 1987;

Pollock & Kendall 1987; Cherkiss *et al*. 2006; Bourquin 2007). Bias factors are considered different for boat surveys and aerial surveys.

Boat surveys are affected by the limited access to parts of a river, observer skill, boat speed (Cherkiss *et al.*, 2006), water level, water temperature, time of day and crocodile behaviour (Hutton & Woolhouse, 1989). Aerial surveys allow for a greater field of view, however is affected by dense vegetation, bad weather conditions and observer fatigue (Bayliss, 1987; Pollock & Kendall, 1987; Bourquin, 2007). Models have been proposed to limit and correct these bias factors for each of the counting methods. Even though aerial surveys have shown a lower detection rate for crocodilian counts compared to boat surveys (Woodward *et al*., 1996; Stirrat, *et al.*, 2001), the acknowledgement of statistical bias correction has shown, for both methods, to estimate similar results (Woodward *et al*. 1996; Stirrat *et al*. 2001; Ferreira & Pienaar 2011).

The predictive data generated from the various abundance estimates may be beneficial towards conservation efforts for establishing more effective management plans. Furthermore, comparison of census data within river systems over time requires surveys to be consistent every year. For example, conducting a census estimate during the rainy season (high water levels) during a single year should be repeated the following year during the same seasonal time. In support of the previous, encounter rates of crocodiles have been reported to differ between seasons (Woodward & Marion 1978; Messel 1979; Webb *et al*. 1990; Ron *et al*. 1998; Bourquin 2007). Comparisons of crocodile populations between river systems are problematic due to different habitats, therefore genetic variation can be considered for additional comparisons between populations (Hare et al. 2011) and in the same way genetic variation has been correlated to population size (Reed & Frankham 2003).

An estimate of genetic variation within a population is expressed as a function of effective population size (N_e) (Franklin 1980; Waples & Do 2010; Hare *et al*. 2011). The N_e provides an estimate for the potential numbers of breeders, which contribute to the following generation and in addition the contribution of genetic variation between generations (Waples & Do 2010; Hare *et al*. 2011). The influence of genetic drift on a small population is considered to be greater in comparison to a larger population (Franklin 1980). Effective population size indicates the loss of genetic variation between populations as the result of genetic drift over time, whereas the census size provides an estimate for the total population only.

The use of N_e has been considered a viable option for the monitoring of wild populations (Rieman & Allendorf 2001; Wang 2005; Palstra & Ruzzante 2008; Harmon &

Braude 2010). Long-lived species tend to have a delayed onset of sexual maturity and an overlap of individual mating between several generations, which allows for population recovery and relative maintenance of genetic diversity of the historical population. Effective population size estimates in the Okavango Delta (Bishop et al., 2009) supported populations to be considered under no threat of extinction, $N_e > 50$ (Franklin 1980), however populations may have been reduced by five-fold since the historical exploitation of the population in the mid-20th century. Further, the N_e of the Okavango Delta population has decreased, with genetic diversity remaining at moderate levels. In addition, the population sizes in West Africa have also decreased, with genetic diversity maintained among the populations. The above contrasting examples could be indicative that the loss of genetic diversity may not be exclusively dependant on N_e.

1.3. Population dynamics

Estimating genetic diversity parameters have been considered valuable for the management of species. Several different techniques exist to monitor the historic and contemporary diversity and structure among populations namely, mainly through using different genetic markers, such as mitochondrial DNA and Short Tandem Repeats.

1.3.1 Historical population dynamics

Mitochondrial DNA (mtDNA) is a double stranded molecule and inherited maternally (Avise *et al*. 1987). Haplotypes may differ between individuals of the same species, although sharing of a haplotype is considered when individuals share a common ancestor. The differences seen among haplotypes are due to mutations within the various mtDNA regions. The rates of mutation have been shown to vary between different species of vertebrates and each mtDNA region. For instance, the mtDNA control region have shown a high frequency of polymorphisms (Quinn 1992; Stewart & Baker 1994; Baker & Marshall 1997) and little to no variation (Baker *et al*. 1994; Walker & Avice 1998) between different species. Mitochondrial DNA has been used to evaluate historical information of species and elucidate their geographical distribution (phylogeography) (Ciofi 2005; Luzhang *et al*. 2010; Valtonen *et al*. 2014; Velo-Antón *et al.* 2014). The historical evaluation of Galapagos tortoises (Ciofi 2005), Himalayen snowcock (Luzhang *et al.* 2010), fresh water seal (Valtonen *et al*. 2014) and Nile crocodile (Hekkala *et al*. 2011; Velo-Antón *et al.* 2014), demonstrates separate lineages between populations across a large areas and their most likely means of distribution.

The changes of environmental condition that effect crocodiles are the consequence of landscape changes by the formation of rift valleys or the aridification of the landscape. Landscape changes in Africa are those of the East African Rift Valley formation spanning from Mozambique into Asia (Wichura *et al*. 2011) and in addition the lesser known aridification of the Cuvelai basin in Northern Namibia (Hipondoka 2005; Hipondoka et al. 2006; Mendelsohn *et al*. 2013; Miller et al. 2010; Pickford 2013). The changes in the landscape formation have indeed diverged populations of fauna (de Menocal 2004; Moodley & Bruford 2007), including fish (Ribbink 1988) and crocodiles (Hekkala *et al*. 2011). The divergence among the populations requires conservation efforts to maintain their respective diversities and adaptability.

To combat and effectively assist the conservation crisis present with segregated populations, species specific management plans have been proposed by Moritz (1994), of which Evolutionary Significant Units (ESU) and Management Units (MU) have both been regarded as long- and short-term conservation solutions. Classification of the ESU relies on significant divergence of a species based on historically geographical isolated populations with restricted gene flow. To demonstrate, Bowen *et al.,* (1992) evaluated the green turtle global distribution, based on mtDNA, and found two ESUs for the population, namely Indo-Pacific and Atlantic-Mediterranean. Even though two ESUs were found several MUs, based on nuclear loci, were visible within each of the ESUs. The management of each MU ensures the contemporary maintenance of diversity within the meta-population (Funk et al. 2012). Even though it is ideal to maintain ESUs separate based on historical isolation, it should be considered best to maintain adaptive diversity within a population (Crandall *et al*. 2000).

The incorrect management of populations have previously resulted in the extinction of many species or populations. (Wolf *et al*. 2001. Loss of genetic diversity, due to the introduction of farm bred individuals, and hybridization within populations have been observed in the brown hare, *Lepus europaeus* (Mamuris *et al.* 2001), the wild boar, *Sus scrofa,* (Vernesi et al. , 2003) and the red-legged partridge, *Alectoris rufa*, (Negro, Torres & Godoy, 2001).

1.3.2 Contemporary population dynamics

Short tandem repeats (STRs) are found to be evenly spread within the non-coding region of the nuclear genome (Guichoux *et al*. 2011). The markers are widely used for population diversity and -structure analyses, genetic mapping, individual identification and pedigree inference in wild species (Arif & Khan 2009). The selection of STR markers for

population genetics is commonly preferred, since they are considered to be highly polymorphic and have the ability to be easily amplified at a low-cost (Guichoux *et al*. 2011). Short Tandem Repeat markers are cross-species amplifiable (Rico *et al*. 1996), however the level of polymorphisms might be negatively impacted (Primmer *et al*. 1996; Hughes *et al.* 1998; Dallimer 1999; Galbusera 2000). Non-amplifications may also result from cross-species amplification due to polymorphisms (indels and SNPs) within the primer binding sites and enzyme slippage resulting in incorrect allele scoring. Comparisons of STRs between different laboratories are also problematic as no well-established evolutionary model is available for STR marker comparisons (Beaumont & Bruford 1999). Polymorphisms within a population has shown correspondence to population fitness and diversity (Reed & Frankham 2003).

The number of alleles within a population is a good indication of the genetic diversity present and may be maintained in a population with the presence of gene flow. In the same way, the presence of gene flow between populations have been associated, but not limited to migration (Slatkin 1985). Gene flow may be described by several proposed models, namely the island model (Wright 1931) , the isolation by distance model (Wright, 1943) which was later attributed by Slatkin (1993) and the Stepping-stone model (Kimura 1953). The maintenance of gene flow between populations could increase their chance of survival as single alleles can play a crucial role towards adaption in a changing environment (Allendorf 1986; Fuerst & Muruyama 1986; Spielman *et al*. 2004). In the case of restricted gene flow and migration, populations may experience a loss in diversity.

For riverine populations, restricted gene flow is influenced by topographic changes and seasonal isolation of species, thereby impacting upon genetic drift between isolated populations (McElroy *et al*. 2012; Willet *et al.* 2014). Equally important is the population size as the magnitude of genetic drift is considered greater among small isolated populations (Huettel *et al.* 1980; Falconer & Mackay 1996). The size of the population has been noted to be supportive of diversity in the populations (Reed & Frankham 2003). On conditions that populations have been isolated over an extensive period of time, specific allelic frequency may reduce due to the potential increased likelihood of inbreeding and drift (Frankham 2005). Inbreeding has been found to be most prominent in small isolated populations as the chance of consanguineous mating is more likely, evidently increasing the expression of deleterious recessive alleles (Charlesworth *et al.* 2009). Small isolated populations with restricted gene flow are able to survive in their newly environment, unless the founding population size is very small or the populations has experienced a large population decline.

The translocation of populations between groups have been considered (De Smet 1998) and in addition the new genetic material will reduce lethal recessive allele expressions (Slatkin 1985).

Furthermore, the absences of gene flow between populations provide genetic structure. The presence of private alleles within a sub-population may demonstrate genetic adaptability towards the environmental pressure and provide structure within the meta-population. Classifying structure among populations require a broad range of sampling across the various environments of a species to identify the presence of exclusive alleles. In brief, gene flow is limited within riverine populations due to topographic or seasonal changes and is responsible for restricting gene flow between populations (Giodarno et al. 2007; Valtonen et al. 2014). The evaluation of structure among populations may demonstrate the genetic contribution between neighbouring populations. In contrast, sampling seasons have been noted to impact the structure of fish species to be sampled during the spawning season as compared to the non-spawning season (Sanches et al. 2012). Furthermore, natal philopatry have impacted gene flow between independent structured populations (Hekkala *et al*. 2010). For conservation management it is important to maintain gene flow and determine structure among populations to maintain diversity.

1.4 Population genetics for crocodile conservation and study rationale

Mitochondrial DNA primers have previously been developed for the Nile crocodile (Ray & Densmore 2002; Velo-Antón *et al*. 2014). Various regions have determined separate lineage formation in Nile crocodile populations (Schmitz *et al.* 2003; Meredith *et al.* 2011; Hekkala *et al*. 2011; Velo-Antón *et al.* 2014) and the phylogenetic tree constructed by Hekkala *et al.* (2011) (Figure 1.2) is considered to be the most informative. The findings observed in the previous mentioned studies gave reason for evaluating the extent of the divergence within the Nile crocodile population. Eastern Africa crocodile populations, as examined by Hekkala *et al.* (2011) showed two lineages, which is of interest to this study. However, divergence of the lineages in the aforementioned study could be described with more merit if the sample set were larger. The lack of sampled individuals from Southern African countries leaves curiosity of the lineage separation proposed and room for more efficient means of interpreting data from larger sample sets.

Lineage structures have been observed for Crocodilian species in the Neo-tropics, making use of various mtDNA regions, and the evaluation of *C. acutus* in Columbia alone revealed two lineages within the population (Bloor *et al*. 2015). Similarly, *Caiman*

crocodylus was also found to consist of significant genetic structuring over small geographic regions in Central America (Venegas-Anaya *et al.* 2008).

Short Tandem Repeat (STR) markers have not been designed for the Nile crocodile specifically, however success has been shown for cross-species amplification in other studies (Bishop *et al*. 2009; Hekkala *et al*. 2010; Velo-Antón *et al*. 2014).

Extensive genetic work has been conducted for various species in parts of southern Africa; but there is still a great lack of information concerning the Nile crocodile. Even though the estimates of diversity have shown to be informative (Bishop *et al*. 2009; Hekkala *et al*. 2010; Velo-Antón *et al*. 2014) there is still room for improvement for developing species specific STR markers.

In order to validate the separation of the lineages, further empirical evidence needs to be collected to support the trends observed amongst the Nile crocodile, with respect to its genetic diversity. As discussed earlier, sequencing data derived from mtDNA control regions as well as STR would be of greater significance should this analysis have access to a larger sample set. Additionally, publically available sequence may add value by their contribution toward a haplotype network, having taken into account the environmental impact factors proposed on the likelihood of dispersal in southern Africa, and more specifically Namibia.

Within northern Namibia the Cuvelai basin has been noted to house several wetland species (Hipondoka *et al*. 2006; Pickford 2013). The evaluation of sediment analyses in the Cuvelai basin demonstrated the Upper Kunene River to have been a contributory inflow. The Lower Kunene River corroded inland from the Atlantic ocean and redirected the Upper Kunene River basin catchment (Hipondoka 2005; Mendelsohn *et al*. 2013). The consequence of the Upper Kunene water being redirected towards the Atlantic Ocean, changed the environmental conditions of the Cuvelai basin. Even though the Upper Kunene was pirated during the Pliocene/Pleistocene, a great Paleo-lake persisted in the basin until 35 000 years ago after which aridification followed (Hipondoka 2005) (Figure 1.3).

The aridification of the Cuvelai basin has separated the Kunene and Okavango River systems from one another. Equally important to the separation of the river systems is the separation of the same species inhabiting these environments (Curtis *et al.* 1998). Geographical restriction of gene flow among these separated populations will allow for the populations to adapt to their newly formed environments. Limited genetic studies have been published on the presence of population divergence for the Kunene and Okavango River, however restricted gene flow between the same species within the Lower Kunene and Okavango River systems could be hypothesised. In other words, the restriction of gene flow

may have led to population divergence over time (Slatkin 1985). It is important to estimate species diversity and extent of divergence to incorporate into local management plans.

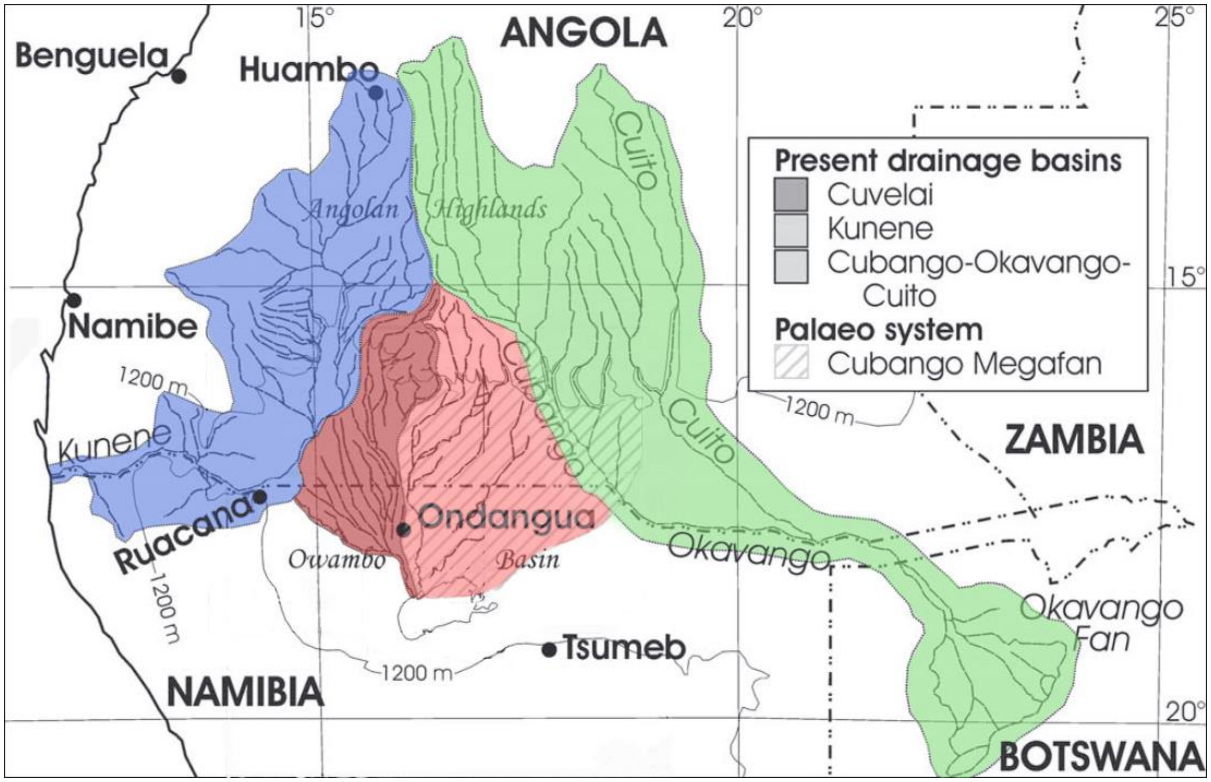


Figure 1. 3 The Northern river system basins of Namibia, originating in the Bie Highland of central Angola. Depicting the separation of the Kunene (Blue) and Okavango (Green) river systems by the Cuvelai basin (Red), historically considered a Paleo-lake system. Modified from Miller *et al*. (2010).

1.5 Aims and objectives

The Nile crocodile (*Crocodylus niloticus*) is understudied in terms of population genetic analyses and much work can still be done across the continent, by the use of STR and mtDNA to elucidate population structure and divergence. The study focuses on the genetic diversity and structure of the Lower Kunene, Okavango and Lower Shire River Nile crocodile populations. The populations within the previously mentioned river systems are compared to publically available data on Nile crocodile populations within the African continent.

The study objectives were to (1) estimate the total abundance of the Nile crocodile population within the Lower Kunene River system, (2) determine phylogeography of the Nile crocodile populations in selected Southern African river systems (including comparisons with to publically available data), (3) estimate the degree of polymorphisms of selected cross-species amplification STRs in the Nile crocodile and (4) estimate *C. niloticus* genetic

diversity, population structure, effective population size and historical bottleneck or population expansion within the Lower Kunene, Okavango and Lower Shire River systems.

In Chapter 2 a binomial mixture model and aerial count approaches were implemented to estimate the abundance and distribution of the Nile crocodile population in the Lower Kunene River, Namibia. For the binomial mixture model an *N*-mixture model was chosen as the most appropriate model as it can simultaneously estimate abundance and effective detection probability of animals. The Lower Kunene River system is considered a closed system, as it has no other permanent water bodies for crocodiles to migrate to. This data can be integrated to the current Namibian Nile crocodile management plan to assist with reducing human-crocodile conflict and the first Nile crocodile monitoring survey for the river system.

In Chapter 3 the aim was to evaluate the historical distribution of the Nile crocodile in the Lower Kunene, Okavango and Lower Shire sampling locations by assessing the control region and comparing to publically available sequences from the African continent, proving historical structure for the Nile crocodile and its population structure in Southern Africa. Furthermore, STR markers were evaluated for their effectiveness in cross species amplification in the Nile crocodile populations of the Kunene, Okavango and Lower Shire populations. The successful amplification of these markers will be recorded along with their degree of polymorphism. The use of the markers will evaluate the extent of gene flow between the populations to estimate their diversity and structure between the river systems. Moreover, the information gathered from the study will allow for identification of management units for short-term conservation efforts which can be considered for the Namibian Nile crocodile management plan.

Chapter 4 summarizes the findings in our study and the recommendations towards current management plans. Along with the short-coming experienced for the project and future recommendations.

Chapter 2

Nile crocodile population estimation in the Lower Kunene River, Namibia calculated using a binomial mixture model

Abstract

The Nile crocodile *Crocodylus niloticus* is found throughout sub-Saharan Africa, including in countries such as Namibia, Botswana and Angola. The species was transferred from CITES Appendix I to Appendix II in 2004, although it is recognised as peripherally endangered in Namibia due to diminishing habitat availability primarily from human encroachment. In 2013 a species management plan was approved in Namibia to assess the management of the Namibian Nile crocodile populations, as the species plays an important role within the environment and commercial industry. The Nile crocodile population in the Kunene River system needs to be re-assessed as there is very little data available, primarily due to the logistical difficulty in accessing large parts of the river system. During 2012 an aerial survey was conducted to provide an estimate of Nile crocodile population in the Lower Kunene River. A recently developed *N*-mixture model for estimation of abundance and spatial variation was used. Detection of crocodiles from the air can be difficult and is also dependant on their size; however an estimated 806 individuals were counted along the 352 km of the Kunene River system with a direct count estimate of 562 crocodiles regardless of size. The parameter estimates generated by the analysis suggested that the class-structured model can produce precise, unbiased estimates of total abundance and reliable estimates of local abundance for this population in the Kunene River system.

2.1 Introduction

The Nile crocodile, *Crocodylus niloticus,* is found throughout sub-Saharan Africa, including countries such as Angola, Botswana and Namibia (Aust., 2009; Fergusson, 2010; Leslie *et al.,* 2011) and is classified under Least Concern on the IUCN RED List of threatened species (IUCN, 2014). In 2004, Namibian authorities transferred the species from CITES Appendix I to Appendix II (CITES, 2004). This transfer was prompted in part due to diminishing habitat caused by human encroachment as settlements are found along the various river systems in Namibia (United States Fish and Wildlife Service, 1999; Griffin, 2003; Mendelsohn *et al.,* 2003), resulting in competition with crocodiles for both food and space (eg: basking and nesting areas). *Crocodylus niloticus* is recognised as a protected game species in Namibia under the Nature Conservation Ordinance No 4 of 1975, allowing trophy hunting of the species only with the issuing of a hunting licence. The quota for Namibia is assessed by the Ministry of Environment and Tourism (Ordinance No 4, 1975), with the current quota set at a total of 25 adult crocodiles per year, which was determined on 14 April 2014 (CITES, 2014). CITES Appendix II allows the trade of no more than 1600 skins of Nile crocodiles from Namibia originating from trophy hunting and ranched specimens combined (Act No 9, 2008; CITES, 2014). Crocodilians play an important role within the ecosystem (Mazzotti *et al.,* 2009) and they have been found to be economically beneficial towards tourism (Llewellyne, 2007) and trophy hunting (Lindsey *et al.,* 2007). A Namibian species management plan drafted in 2012 was approved in 2013 (Species Management Plan, 2012) and focuses on utilization of the crocodile species, by incorporating the economically beneficial factors whilst maintaining their contribution towards the ecosystem.

Human settlements are found along the Kunene River due to the limited availability of water in the Kunene area (Mendelsohn *et al.,* 2003), resulting in competition with crocodiles for both food and space (eg: basking and nesting areas). An ontogenetic shift in the Nile crocodile occurs between small mammals and fish in the upper end of the juvenile size class (SVL ≈ 40cm) based on stomach content (Wallace & Leslie, 2008) and replicated by scute keratin levels (Radloff et al., 2012). Sub-adult crocodilians consume mainly fish (Snout Vent Length (SVL) : >66.3 cm), until they exceed 119 cm SVL and undergo a second ontogenetic shift (Radloff et al., 2012), most likely for large terrestrial mammals (Cott, 1961). Local inhabitants have large herds of goats and cattle, which are considered to be a sign of wealth (Comaroff & Comaroff, 1990) and these animals forage along the banks of the Kunene River as the larger riverine trees provide good browsing for livestock during the dry

season (Irving & Ward, 1999). Livestock, along with local inhabitants who use the river for washing, swimming and water collection, form part of the prey base of larger crocodiles.

Census data of Nile crocodiles in Namibia are very limited as population counts have only been conducted in the eastern Namibian river systems (Okavango, Kwando, Mamili, Linyanti/Chobe and Zambezi Rivers) (Brown *et al.*, 2005; Chase, 2009). Reports of human crocodile conflict by Mr BM Siyanga (pers. comm.), a ranger for the Ministry of Environment and Tourism in Opuwo, stated that from January 2010 to March 2011 an estimated 44 animal deaths and one human death occurred in the Lower Kunene River system. The human/crocodile conflict reports only provide information on the number of livestock or individual human deaths and not on the exact number of crocodiles found at the incident site. The Lower Kunene River is situated in a very isolated area, making it difficult for locals to report on human/crocodile conflict and it is thus important to provide a solution to minimize such conflict. Establishing a crocodile management plan requires several parameters to be considered namely: conservation, egg harvesting and population control (Bayliss, 1987). These parameters will prevent over-exploitation and a possible population decline and additionally provide income to local communities.

Spotlight surveys by boat have been the most commonly used survey method to estimate crocodilian abundance in river systems (Letnic & Connors, 2006). Spotlight surveys are usually only conducted on a portion of the whole river and presented as a population index (Bayliss, 1987; Wallace *et al.*, 2011). These surveys are dependent on a number of environmental and physical factors, namely access to large parts of the river, observer skill, boat speed (Cherkiss *et al.*, 2006), water level, water temperature, time of day and crocodile behaviour (Hutton & Woolhouse, 1989). For the Lower Kunene River an aerial survey was considered the most viable option, as large areas of the river are inaccessible by car or boat. Aerial surveys have shown lower detection rates compared to boat surveys in Crocodylia (Woodward *et al.*, 1996; Stirrat, *et al.*, 2001), however helicopter use allows greater manoeuvrability, controlled speeds, wider fields of view and photographic data acquisition, to eliminate bias for submerged animals or dense vegetation areas effecting observer visibility (Bayliss, 1987; Pollock & Kendall, 1987; Bourquin, 2007).

In this study, an aerial survey was conducted to provide a population abundance estimate for Nile crocodiles in the Lower Kunene River to implement into a crocodile management plan for Namibia. The management plan will assist to mitigate human crocodile conflict and the utilization of crocodiles for tourism, commercial interests and trophy hunting.

One of primary objectives of this study was therefore to estimate distribution and abundance of the Nile crocodile across the study domain and understand patterns of variation in relation to environmental and anthropogenic factors. Detectability of individual animals is highly variable and nearly always < 1; and imperfect detection must be accounted for to reliably estimate population sizes and trends (Royle & Dorazio 2008). Due to expected heterogeneity in both local abundance and local detection probability in our case (due to environmental or sampling covariates), we considered the N-mixture model as the most appropriate model as it can simultaneously estimate abundance and effective detection probability of animals. The state process of the *N*-mixture model describes ecological mechanisms that generate spatial and temporal patterns in abundance, while the observation model accounts for the imperfect nature of counting individuals due to temporary immersion and false absences. This model also assumes sampling in a closed system, regarding mortality, recruitment immigration and emigration (Royle, 2004).

2.2 Materials and Methods

2.2.1 Study Area

The Kunene River is a fresh water perennial system and is fed from the natural springs in the Bie Highlands in Angola (Irving & Ward, 1999) and by a limited annual summer rainfall. (October-March) (Hay *et al.*, 1997),which ranges from 50 mm in the Namib desert, to 1500 mm in the highlands of Angola (Hay *et al.*, 1997). There is a high level of endemic fauna and flora in the central and eastern Lower Kunene, including: trumpet thorn (*Cataphractes alexandri),* gum myrrh (Commiphora *spp.*) (Irving & Ward, 1999), black faced impala (*Aepyceros melampus petersi*) and mountain zebra (*Equus zebra hartmannae*) to name a few (Kunene River Awareness Kit, 2014). Several communal conservancies are situated in the Kunene area with some bordering on the Lower Kunene River, namely Marienfluss, Uunolonkadhi-Ruacana and the Kunene conservancy (NACSO, 2009). The Skeleton Coast National Park is situated on the north-western Namibian shore, through which the Kunene River flows; the region consists of desert vegetation with no local tribal inhabitants. The estimated human population of the Kunene province was 88 300 in 2011 and approximately 18 000 (20.4%) of these individuals live in the Epupa constituency bordering the Lower Kunene River (Namibian Census, 2011).

The Kunene River system covers a total area of 110 200 km^2 (with an upper, middle and lower area) of which the lower Kunene forms the border between Namibia and Angola (14 900 km^2 (13.3%) (INBO, 2007). The portion considered for the aerial survey covered 352

km of the Kunene River from its mouth (km 0, altitude 0 meter above sea level, latitude -17.249515 and longitude 11.752746) to Ruacana falls (km 352, altitude 775 m above sea level, latitude -17.403902 and longitude 14.216841) (lower Kunene, 13.3%) (Fig. 2.1). The aerial survey was conducted under a Ministry of Environment and Tourism Division Support System (DSS) approved work activity permit (Permit number: 2003/2015).

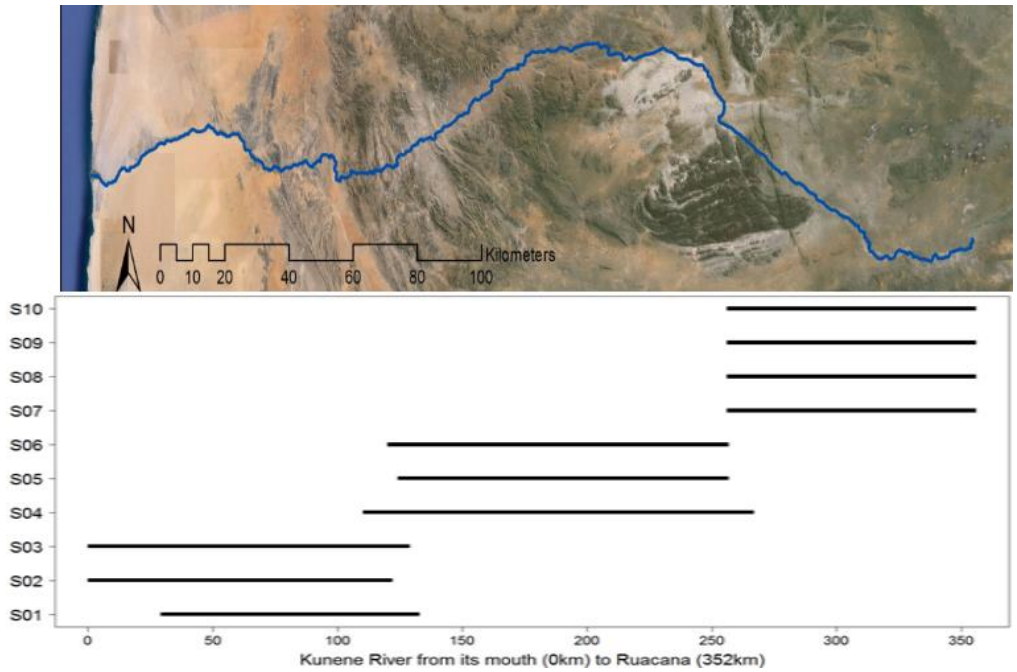


Figure 2.1 A map showing the lower Kunene River surveyed during the study. The Kunene river mouth is situated at the left/west (0km) and Ruacana dam at the right/east (352km) (See Appendix A1 Table S2.1).

2.2.2 Survey design and effort

The Lower Kunene River area surveyed in 2012 was separated into two parts, namely: east and west. The helicopter (Bell Jet Ranger B206 and a Bell Long Ranger B206L) had a pilot, two to three observers (two most experienced observers with the third interchangeable less experienced) and a data recorder and was flown at an altitude of 24 – 27 feet at 110-130 km/h. The western part of the river was surveyed from 24th - 28th April 2012 (early dry season) and the eastern part from 9th – 12th August 2012 (late dry season). Only a segment of each river part could be covered within a single day and flights were flown during the late morning or early afternoon, as the majority of crocodiles would be basking on the banks. The river segments were covered in 10 sessions (S#) (Fig. 2.1 and Appendix A1, Table S2.1).

For the statistical analyses, every session as described above was considered to be a different sampling occasion. The river was divided into segments of equal length, with every segment being considered as independent sampling unit referred to as a *site* in the rest of the

paper. At the *site* level, one aerial sampling occasion consisted of one flight over the river segment. Since segments are adjacent and since crocodiles could freely move in and out of the segments during the repeated surveys, the independence of each segment and the closure assumption are clearly violated. However, one could assume that the longer the segment and the shorter the survey, the less the impact of non-closure would be as a lesser proportion of animals are expected to move between sites. In addition, if movements are random, one could also expect that, on average, temporarily emigration equals temporary immigration. The size of the *site* (i.e., length of the river segment) was chosen to make sure it was large enough (see below) to reduce movement of crocodiles between sites over the duration of the survey and therefore, be as close as possible to the population closure assumption (Williams *et al.*, 2002) required for the statistical analysis. The *sites* of the river consisted of four days of flying for the western part and five days for the eastern part. As no GPS tracking data were available for the Kunene River crocodiles, we used Okavango River crocodile movement data collected by the Ministry of Environment and Tourism, on five adults from August 2011 to April 2013, assuming that animal movement patterns were similar in the two river systems (African Wildlife Tracking SAT collars, Iridium system). Previous studies have found that female crocodiles (>2.8 m) and male crocodiles (>3.2 m) tend to settle on a distinct home range (Modha, 1967; Hutton, 1989). The GPS data indicated that more than 90 % of the movements were shorter than 5 km over a five day period, with crocodiles seldom moving further than 8 km. We therefore considered an 8-km segment to be large enough to consider the local populations as close. The 352 km of river was divided into 44 consecutive, non-overlapping 8-km segments, each segment being considered as an independent *site* unit. The statistical method used to analyse the data required that all sites were surveyed at least once and a subsample of the sites were to be surveyed several times. In the study, two sites were surveyed twice, 21 sites three times, and sites 17 and 19 four times each.

2.2.3 Data Recording Survey

Data was logged as follows: each observation of a crocodile was recorded with its corresponding geographic coordinates (latitude and longitude), time of sighting and size class. The size class of crocodiles was based on its estimated length (Class 1 = 1-2m, Class 2 = 2.1-3m, Class 3 = 3.1-4m and Class 4 = <4.1m), estimated visually. Fig. 2.2 indicates the distribution of all observations along the river. Every observation was assigned to the nearest *site* (shortest perpendicular distance between the observation and the river segment) using ArcGIS software (ESRI, 2008). Hence the number of crocodiles observed at site *i* on session *j*

in size class *g*, is noted as n_{ijg}. Appendix A1, Table S2.2 shows an example of the data recorded on site #71. For this site $n_{71.Occ8.G2} = 2$.

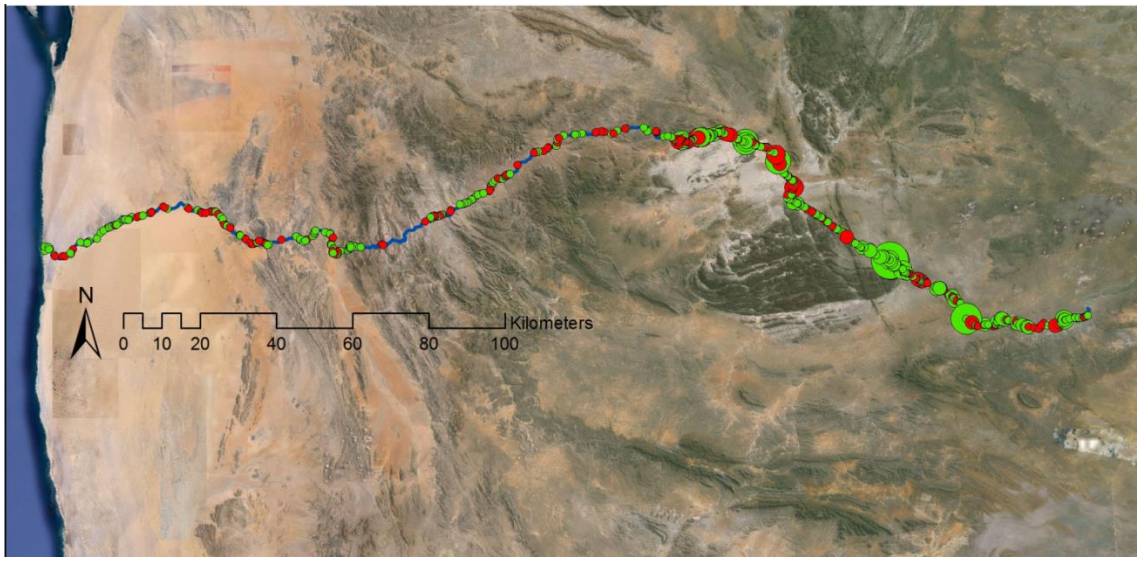


Figure 2.2 Observations of Nile crocodiles on the Lower Kunene River during the 2012 aerial survey. Green dots indicate animals between 1 and 3 meters in length, red dots indicate animals greater than 3 meters. The size of the circle is proportional to the number of individuals observed at the location (see Fig. 2.1.)

2.2.4 Site and sampling covariates

In the analyses, the first flight over a segment of the river was considered to be an exploratory flight (S01, S04 and S07). When the same portion of a river was flown over in a single day, S01; S02 and S05; S06, we modelled separately for each flight path. This was to account for any possible disturbance caused by the first flight, which would have made the crocodiles less detectable during the second flight. S07 and S08 were flown on different days. A subset of six predictor variables was chosen which were believed to be a potential contribution to the driving forces for abundance of species at the scale of this study (Jablonicky, 2013). Crocodiles (>3 meters) will be expected to show a preference for river width as they occupy large water bodies and shore steepness for crossing animals forming part of their diet. Crocodiles (<3 meters) are expected to correlate to number of channels as they seek shelter on the islands and possible nesting sites. Crocodiles feed on domestic animals around villages, with density of humans expected to have higher number of livestock. However, crocodiles are also under hunting pressure from human inhabitants. The predictor variables were derived from physical characteristics of the Lower Kunene River explained every km. Making use of high-resolution satellite imagery available on Google Earth, a 30 meter resolution digital elevation model based on ASTER satellite imagery, and

data from the Namibian Atlas (Mendelsohn *et al.*, 2003). This fine description of the river was therefore averaged along the 8-km segment (*site* unit) to build the set of *site* covariates to be used in the statistical analysis. To limit the co-linearity within factors, we used a principal components analysis and selected a subset of non-co-linear variables. The selected predictors and their respective sources are shown in Table 2.1 and effects in Fig. 2.3 and Fig. 2.4.

Table 2.1 Description of the environmental factors used as covariates in the statistical analysis. See also Appendix A2, Figure S2.1, S2.2 and S2.3.

Factor name	Description of the factor	Source	Data type and Unit
width	River width. Measured manually at every kilometre on Google earth and corresponds to the length of the perpendicular section of the river from one shore to the other after ground areas are excluded.	Google earth	Continuous, meter
shore	Shore steepness. Assessed visually every kilometre using Google earth pro software 3D imagery and Play tour mode to fly along the Lower Kunene River. Proxy for the accessibility to the river by large prey species.	Google earth	Categorical, index between 0 and 5, 0 corresponding to a flat shore.
channel	Index of river complexity. The number of channels was assessed visually at every 1-kilometer segment on Google earth software. Proxy for basking and nesting site availability.	Google earth	1, 2, 3, 4, and 5+ channels.
dis.V	Distance to the nearest village. Measured at every 1-kilometer segment using ArcGIS software. Proxy for environmental disturbance and hunting pressure.	Atlas of Namibia	Continuous, kilometer
den.H	Index of human population density. Assessed on an 8x10 km strip centred on the river course using ArcGIS software. Proxy for environmental disturbance and hunting pressure.	Atlas of Namibia	Continuous, inhabitants per square kilometre

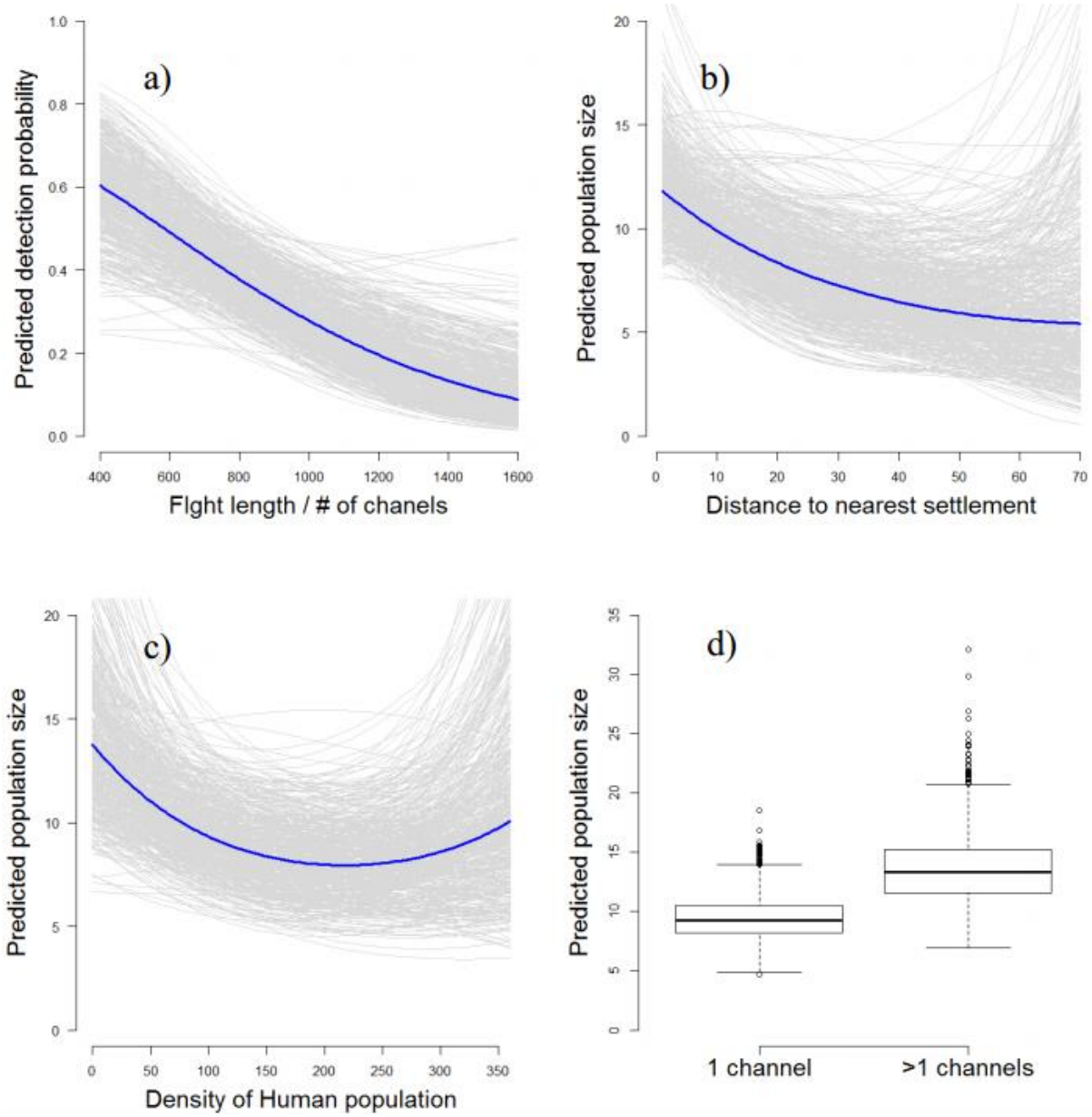


Figure 2.3 Predictions of the covariate relationships that account for estimation uncertainty, 1-3 meters crocodile class (group 1). (a) relationship between covariate and the detection probability. (b), (c) and (d) relationship between site covariates and predicted population size. The blue line shows the posterior mean, and grey lines show the relationships based on a random sample of 500 to visualize estimation uncertainty.

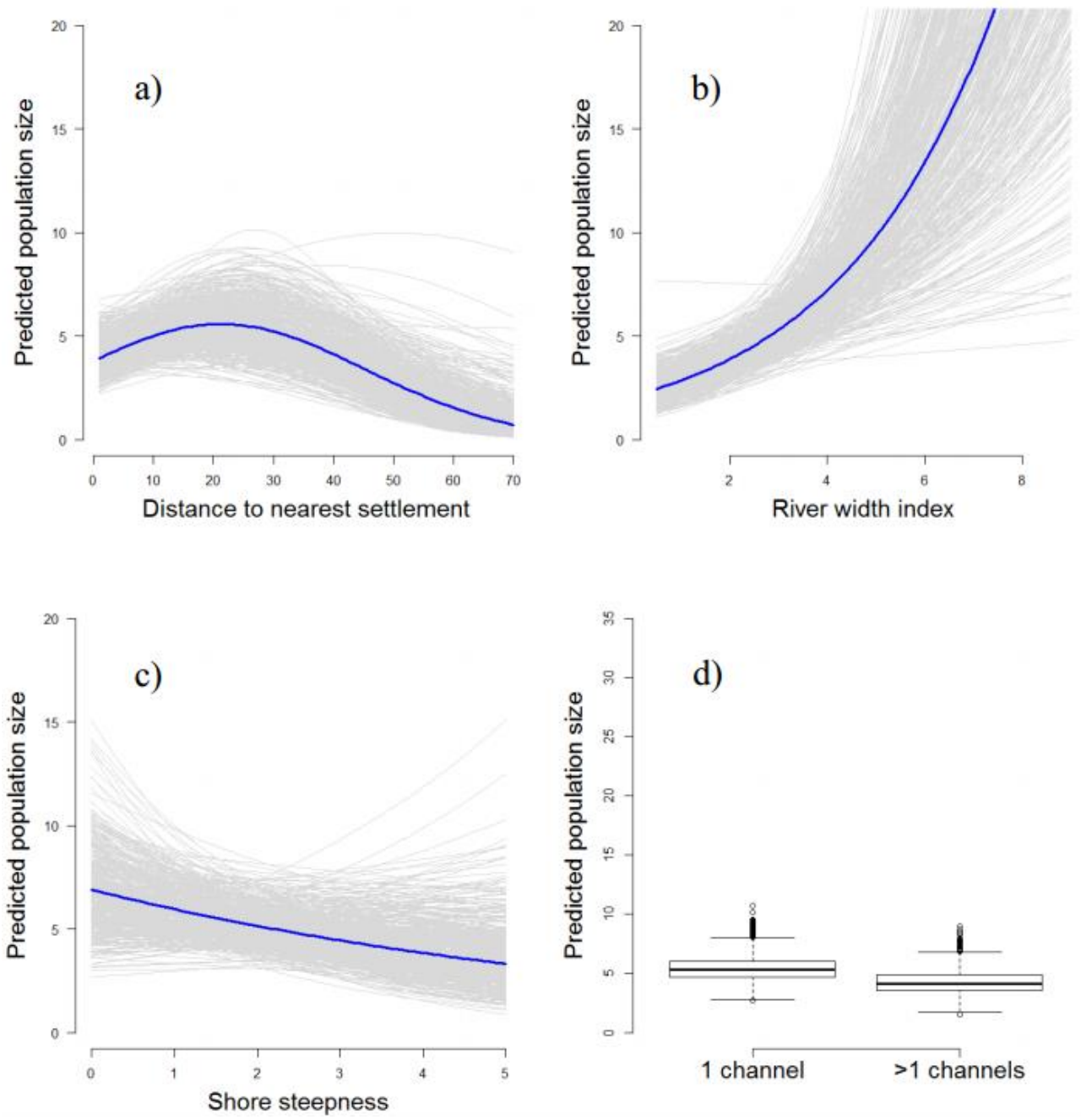


Figure 2.4 Predictions of the covariate relationships that account for estimation uncertainty, 3+ meters crocodile class (group 2). (a), (b), (c) and (d) relationship between the site covariates and the population size. Blue line shows the posterior mean, and grey lines show the relationships based on a random sample of 500 to visualize estimation uncertainty.

In addition, we also considered four independent sampling covariates that could affect the probability of detecting crocodiles at a *site*. We first considered two factors related to the intensity of a survey effort at a site. Factor one is described by length of the helicopter GPS track log divided by number of channels and factor two describes number of observers in the aircraft (Table 2.2 and 2.3). We expect the increase of flight length to indicate an increase in crocodile counts and number of observers to show a positive effect toward the survey method. All flights had two observers, except for flights S09 and S10 which had three observers aboard, in an attempt to increase the probability of detection of animals. Factors three and four were discovery and return flights respectively (Table 2.2 and 2.3).

Table 2.2 Summary of the N-mixture analysis for crocodiles in group 1 (crocodile size from 1.0-3.0 m). The table shows the Bayesian posterior mean, standard deviation and 95% credibility interval for each parameter included in the model as described in the text. Rhat < 1.05 indicates that the chains have converged.

Parameter		mean	sd	2.50%	97.50%	Rhat
α	Origin	2.227	0.187	1.863	2.595	1.001
α1	River width	0.033	0.131	-0.227	0.284	1.001
α2	Shore steepness	-0.048	0.123	-0.284	0.195	1.001
α3	Channels	0.363	0.184	-0.002	0.726	1.001
α4	Distance to village	-0.299	0.176	-0.652	0.040	1.001
α44	Distance to village (quadratic)	0.041	0.093	-0.140	0.227	1.001
α5	Density human population	-0.310	0.154	-0.610	-0.010	1.002
α55	Density human population (quadratic)	0.155	0.099	-0.035	0.356	1.001
β	Origin	-0.678	0.284	-1.241	-0.128	1.002
β1	Flight length	-0.477	0.173	-0.820	-0.147	1.001
β2	Discovery	-1.107	0.278	-1.662	-0.585	1.001
β3	Return	-0.990	0.333	-1.668	-0.354	1.001
β4	# of observers	1.444	0.378	0.749	2.217	1.001
sd.p	Random effect	0.964	0.138	0.710	1.251	1.001

Table 2.3 Summary of the N-mixture analysis for crocodiles in group 2 (> 3 meters in size). The table shows the Bayesian posterior mean, standard deviation and 95% credibility interval for each parameter included in the model as described in the text. Rhat < 1.05 indicates that the chains have converged.

Parameter		mean	sd	2.50%	97.50%	Rhat
α	Origin	1.669	0.188	1.290	2.037	1.001
α1	River width	0.491	0.153	0.190	0.802	1.001
α2	Shore	-0.157	0.124	-0.402	0.079	1.002
α3	Channels	-0.246	0.255	-0.743	0.256	1.002
α4	Distance to village	0.225	0.200	-0.171	0.618	1.001
α44	Distance to village (quadratic)	-0.256	0.110	-0.475	-0.041	1.001
α5	Density human population	-0.051	0.174	-0.388	0.286	1.001
α55	Density human population (quadratic)	0.110	0.107	-0.100	0.311	1.001
β	Origin	0.302	0.299	-0.283	0.873	1.001
β1	Flight length	0.132	0.269	-0.403	0.643	1.001
β2	Discovery	-0.387	0.323	-1.045	0.228	1.001
β3	Return	-0.514	0.410	-1.335	0.283	1.001
β4	# of observers	0.628	0.362	-0.043	1.403	1.001
sd.p	Random effect	0.964	0.138	0.710	1.251	1.001

2.2.5 Description of the model

Recently developed *N*-mixture models allow for the estimation of abundance and spatial variation in abundance from count data alone for closed (Royle, 2004) and open (Dail & Madsen, 2011) populations. *N*-mixture models are a class of state-space models in which the 'true' state of the system (abundance) is observed imperfectly. The 'true' abundance here "*is the (unobserved) abundance […] of individual on the spatial sample unit*" (Royle & Dorazio, 2008) or can be defined as well as the abundance corrected for imperfect detection (Kéry, 2010). Unlike classical state-space models used in ecology (e.g.: de Valpine & Hastings, 2002, Staples *et al.*, 2004), *N*-mixture models do not make unrealistic assumptions about the Gaussian process and sampling errors and instead assume that abundance is a discrete random variable (Buckland *et al.*, 2004). Similarly, *N*-mixture models attribute observation error to a specific phenomenon, such as the inability to detect all individuals that are available during sampling and are referred to as imperfect detection. The *N*-mixture model for a closed population (Royle, 2004) was considered, as surveys were only conducted in a single year. Animals ranging in size class from 1.0-3.0 m (henceforth referred to as group 1) and animals larger than three meters (henceforth referred to as group 2) were modelled separately. We used the respective frequencies of every group to estimate the total number of animals in each size class. A model accounting for covariate effects on abundance, both covariates effects and extra Poisson dispersion (extra heterogeneity) was used for detection probability. However, the introduction of random effects into linear predictors can be seen as

an over dispersion correction and it increases the uncertainty in the estimates. The total population size and its credibility interval over the 352 km river was computed directly in JAGS, by summing the segment-level abundance estimates (see Appendix A3, Line 828).

The hierarchical model is described below (Refer to Table 2.1 for complete description of covariates' abbreviations).

Level 1

The realized abundance of animals for size group *g* at site *i* is:

$N_{i,g} \sim \text{Poisson}(\lambda_{i,g})$

GLM for level 1:

The mean abundance ($\lambda_{i,g}$) at site i for group g is described by the following relation

$\text{Log}(\lambda_{i,g}) = \alpha_g + \alpha_{1,g} * \text{width}_i + \alpha_{2,g} * \text{shore}_i + \alpha_{3,g} * \text{channel}_i + \alpha_{4g} + \text{dis.V}_i + \alpha_{44,g} * \text{dis.V}_i^2 + \alpha_{5,g} * \text{den.H}_i + \alpha_{55,g} * \text{den.H}_i^2$

Level 2

The observed count for group g at site i and on survey j is:

$C_{i,j,g} | N_{i,g} \sim \text{Binomial}(N_{i,g}, P_{i,j,g})$

GLM for level 2:

The detection probability at a site i for group g and survey j is described by the following relation

$\text{Logit}(P_{i,j,g}) = \beta_g + \beta_{1,g} * \text{flight}_{i,j} + \beta_{2,g} * \text{discov}_{i,j} + \beta_{3,g} * \text{return}_{i,j} + \beta_{4,g} * \text{observ}_{i,j} + \text{rand}_{i,j,g}$

Level 2b (random survey effect):

$\text{Rand}_{i,j,g} \sim \text{Normal}(0,\sigma)$

A Bayesian approach to estimate the model parameters was used as this provides a computationally tractable method to integrate across unobserved states and quantifies the uncertainty of the estimates. A Bayesian analysis requires specification of prior distributions for parameters. We assumed vague priors in all analyses presented in this paper. We ran three chains of the model, each for 2,200,000 iterations after a burn-in of 200,000 and thinned by 2000. We implemented our analyses with the program R (R Core Team, 2012) using the software program JAGS (Plummer, 2003) to use Markov chain Monte Carlo (MCMC) to approximate posterior distributions for each of the parameters. The model code for the analysis can be found in Appendix A3.

2.3 Results

2.3.1 Model fit and performance

Visual inspection of the MCMC and Rhat values, all smaller than 1.05, indicated that chains of all parameters have mixed properly and converged (Gelman and Rubin 1992) (Table 2.2 and Table 2.3). In addition, the comparison of the discrepancy between the observed and the simulated data (Fig. 2.5) shows that they correlated, suggesting that the model is adequate for the data set. This is supported by a Bayesian posterior predictive *p*-value of 0.52 (Fig.2.5). This *p*-value quantifies the discrepancies between the data and the model, and a *p*-value near 0 or 1 indicates a lack of fit of the model (see Gelman et al., 2004).

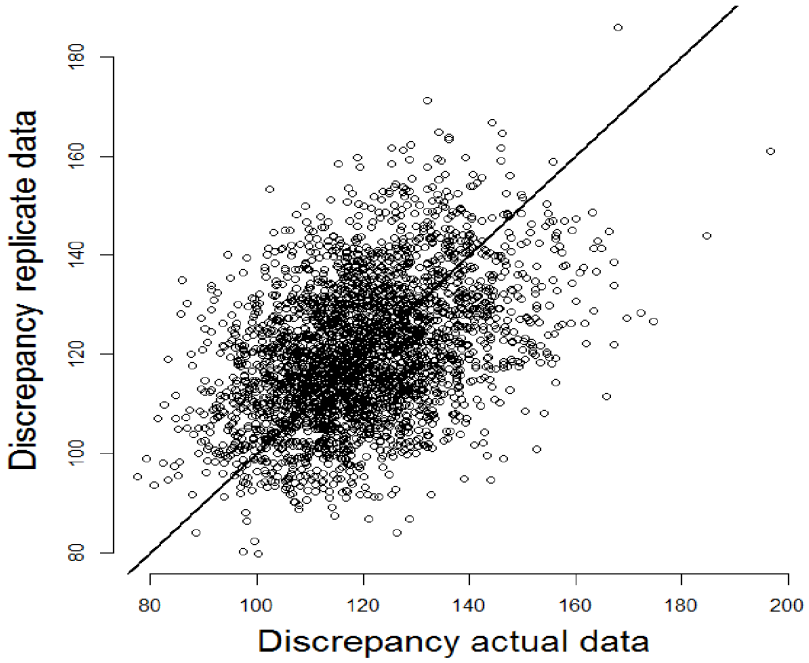


Figure 2.5 Posterior predictive check of model fit by a scatter plot of the discrepancy measure for replicate (simulated) versus actual (observed) data in an N-mixture model. The Bayesian p-value is the proportion of points above the 1:1 line.

The parameter estimates generated by the analysis have demonstrated that the class-structured model can produce precise estimates of total abundance and reliable estimates of local abundance for the Lower Kunene River population of crocodiles as shown in Fig. 2.5.

2.3.2 Mean detection probability and total population size

Mean detection probability was significantly higher for group 2 (mean: 0.55, 95% CI: 0.454-0.626) than for group 1 (mean: 0.35, 95% CI: 0.273-0.418) and the difference can be considered significant as the CIs of the two estimates do not overlap. Along the 352 km stretch of the Lower Kunene River, the total population of crocodiles was estimated at 806 individuals (95% CI: 674-1015) (Table 2.4). For the different size-classes the model estimated 239 (189-320), 340 (268-455), 149 (131-180), and 78 (68-94) individuals for class 1, 2, 3 and 4, respectively (Table 2.4). These values are to be compared with the naïve population estimate which is given by the sum of the maximum number of individuals observed at every site on a single sampling occasion. In this survey, the naïve estimates in each size class, from the smallest to the largest, were 154, 199, 131 and 78 individuals with a total of 562 crocodiles of all sizes.

Table 2.4 Total population size and number of crocodiles in each size-class.

	Mean	SD	Bayesian Credibility Interval 95%	
			Low	High
# of crocodiles [1-2m]	238.98	35.12	189	320
# of crocodiles [2.1-3m]	340.26	50.00	268	455
# of crocodiles [3.1-4m]	149.01	12.65	131	180
# of crocodiles [>4.1 m]	78.12	6.63	68	94
Total # of crocodiles	806.36	91.03	674	1015

2.3.3 Covariate effects on detection probability and local abundance

Covariate effects on both detection and local abundance were considered significant when the credibility interval did not contain the zero value. The covariates that were tested in the model showed very different responses between the two groups. None of the covariates had a significant effect on the detection probability of crocodiles from group 2, while all were significant for detection probability of group 1. The estimates and credibility intervals of the parameters are shown in Tables 2.2 and 2.3. Results indicated that the length of the flight path for the discovery and return flight mode had a negative effect on detection probability, while the number of observers participating in the aerial survey had a positive effect on the

probability of detecting a crocodile at a *site*. The variance for the random effect σ was estimated at 0.964 (0.710-1.251 95% CI), which represents the part of the variance in the detection probability that is not explained by the covariates. Local abundance of crocodiles was highly variable among sites for both size classes (Fig. 2.4), and usually higher for group 1. The upper part of the river (segment #27 - #32) had a much higher density of crocodiles from group 2 than the rest of the river, while there was no such clear pattern for group 1 crocodiles. It is also worth noting that the precision of the estimate is much higher for group 2. Local abundance ranged from 8.30 (2-19 95% CI) individuals on segment #5 to 21.28 (15-32 95% CI) on segment #17 for group 1, and from 0.17 (0-1 95% CI) on segment #2 to 18.46 (14-27 95% CI) on segment #27 for group 2. The results in Tables 2.2 and 2.3 also show that the local abundance for the two different size classes is explained by different covariates namely, channels and flight length.

2.4 Discussion

The survey conducted along the Lower Kunene River, provided valuable insight into the abundance and distribution of the Nile crocodile. Furthermore, the accuracy of the N-mixture model was evaluated to provide an indication of accuracy within the study and the possibility of using the model for future studies.

2.4.1 Total abundance

Along the Lower Kunene River, Nile crocodiles were estimated at a naive abundance of 562 total individuals (1.60 crocodiles per km). Final population abundance estimated at a total of 806 individuals after considering observer and environmental bias (2.29 crocodiles per km). An estimate of 2.29 crocodiles per km can be considered plentiful compared to other African river populations (Bourquin, 2007). This could be the result of limited poaching in the area in the past and very few tribal home settlements situated on the river. That result in fewer disturbances on the crocodile natural habitat in the Kunene River system. Ignoring detection probabilities of crocodilians clearly leads to an underestimate of the population size, in particular for animals less than 3 meters in length (Fujisaki *et al.,* 2011). The underestimation for the naïve abundance could be the result of inexperienced observers, crocodile submergence and/or the distance from the area/transects being surveyed (Bayliss 1987; Hutton &Woolhouse 1989; Jablonicky, 2013), as shown in previous studies (Shirley et al., 2012; Jablonicky, 2013). This supports the need for detection probability in crocodilian counts to prevent underestimation of populations. Naïve estimates of large crocodiles can be

considered accurate if the river is surveyed multiple times as in this study (3.4 times on average per session) (see above: Survey design and effort).

2.4.2 Local abundance and covariates effects

Estimates of local abundance of crocodiles along the Lower Kunene River are highly variable for both size classes (Fig. 2.4) specifically for group 1. Detection probability is higher for larger sized crocodiles (>3 m in length) when compared to smaller crocodiles, similar to a study by Fujisaki et al. (2011) on *Alligator mississippiensis*. Local abundance of crocodiles from group 1 and 2 in this study were influenced by different environmental factors namely, number of channels and human settlements respectively. Abundance of group 1 animals seems primarily correlated to the number of channels (Table 2.2, Fig. 2.3d) indicating complexity of the river system. Channel shape effect seems logical, as the islands that separate the channels are ideal for crocodile nesting (Leslie & Spotila, 2001; Aust, 2009). Vegetation and water depth in these areas further provide more shelter for animals of small to medium size (Group 1). This was corroborated in a study by Hutton (1989) who showed that Nile crocodiles smaller than 2.2 meters were restricted to nesting areas in the Ngezi River, with larger crocodiles occupying the lake system. Abundance of group 1 crocodiles was found to be greatest nearest to human settlements (monotonous negative trend; see Table 2.2, Fig. 2.3b) and where human density was slightly higher (positive quadratic effect; see Table 2.2, Fig. 2.3c), but the uncertainty around the effects of human density is high. There was a possible impact from human population density and distance to nearest settlement, but the indication was low and would thus require more data for confirmation. This could be the result of combining two size classes and each size class responding differently to each of these factors. Splitting the group into two sub groups was not possible in the analysis and would require a larger dataset to be explored thoroughly. This is in contrast to a study along the lower Zambezi Valley with Nile crocodiles occurring in abundance in protected areas (3.1 crocodiles per kilometre), when compared to human inhabited areas (1.4 crocodiles per km) (Wallace *et al.*, 2011), which corresponds for group 2 crocodiles (Table 2.3, Fig. 2.4a).

Within our study the distance for group 2 crocodile abundance increases and decreases again after 20 km from a village. A village can produce a positive effect on crocodile abundance due to the presence of large prey (cattle) farmed by tribal inhabitants. However, crocodiles occupying space close to villages are negatively affected due to habitat disturbance and hunting pressures (Musambachime, 1987; McGregor, 2005). The conservancies situated adjacent to the Lower Kunene River allow for trophy hunting and

hunting of so called problem animals (Ordinance No 4, 1975). The main environmental driving force for group 2 animals has been shown to be river width (monotonic positive trend; see Table 2.3, Fig. 2.4b), indicating that crocodiles 3 meters and above show preference for exploiting larger bodies of water (Fig. 2.4d), corresponding to previous studies for larger crocodilians (for eg: Aust, 2009). Group 2 crocodile abundance was also found to decrease with steepness of the bank (negative monotonous trend; see, Table 2.3, Fig. 2.4c). This effect may be related to the availability of large prey mammals in these areas which prefer accessing and crossing the river at low to mild steepness of the bank (Jarman, 1972).

2.4.3 Detection probability

As expected, the model showed that the detection of crocodiles is imperfect and animal size dependant. The probability of detection was slightly lower during the first flight when compared to the return flight. This could be the result of observer effectiveness and fatigue. During aerial surveys it is impossible to change observer's mid-flight or on 30 min intervals as during boat survey studies (Bourquin, 2007). The loud noise from the helicopter also results in animals seeking shelter during the return flight. It would be recommended to have a 2 hour break before the return flight around midday rather than a 30 min break midday to refuel the helicopter as during this survey. The 2 hour break will alleviate observer fatigue and reduce animal disturbance for the sites counted. Surprisingly, the detection probability of crocodiles from group 1 decreased proportionally with the length of the flight (Fig. 2.3a), while it was expected to increase. The observed increase in flight length is primarily due to extra flight loops conducted when the course of the river was more complex due to numerous channels, swampy areas and more dense vegetation. These riverine areas offer more shelter and decrease the detection of small crocodiles when compared to more open portions of the river, having a negative effect on flight length. Crocodiles are able to hide under the shrubs and in the swampy areas to reduce chances of mortality (Woodward *et al*. 1987). Group 2 has shown to be only affected by the covariates as they are easier to detect and not covariate dependant. Detection probability in our case is clearly imperfect and correlates to animal size and environmental covariates. Therefore this parameter should not be ignored and needs to be modelled accordingly to obtain unbiased estimates of population size.

2.5 Conclusion

The parameter estimates generated by this analysis have demonstrated that a class-structured model can produce precise, unbiased estimates of total abundance and reliable estimates of local abundance for this population of crocodiles. Covering long river segments in short survey sessions, preventing crocodile movement between sights. This study represents a good benchmark for the monitoring of the population in the future. The recent development of open population models based on animal counts (Dail & Medsen, 2011; Zipkin *et al.*, 2014), as conducted in our study, indicated sufficient information to monitor the trend of a population over time and perhaps estimate other demographic parameters required to effectively manage a population in the future.

The abundance of Nile crocodiles in the Lower Kunene River was estimated at 806 individuals after considering observer and environmental bias (2.29 crocodiles per km) and is considered plentiful, compared to other African River populations (Bourquin, 2007). Due to the Lower Kunene River being a mostly protected and uninhabited area, it was difficult to compare the distribution of the Nile crocodile to other studies (e.g. Bourquin *et al*. 2011; Wallace *et al*. 2013). The slight comparisons that were possible are those of adult crocodiles to prefer occupying areas not inhabited by human occupants. The effectiveness of the plan will also require the implementation of current on-going studies and updated aerial surveys of the Okavango, Kwando, Linyanti, Chobe and Zambezi River systems. By estimating the total size of the Namibian crocodile populations, we are now able to effectively implement the National crocodile species management plan, together with the assessment of genetic diversity, determination of population structure, dietary habits and identification of locating nesting sites.

Chapter 3

Genetic diversity and population genetic structure in the Lower Kunene, Okavango and Lower Shire River system Nile crocodile (*Crocodylus niloticus*) populations in Southern Africa.

Abstract

With the distribution of a species over a large area, geographical barriers are commonly found to separate individual geographic groups into sub-populations. Such isolated populations are particularly subjected to random genetic drift that may lead to random allele fixation or loss within populations, reducing diversity within each sub-population. Within Namibia, the Kunene and Okavango Rivers harbour Nile crocodiles and little is known about the relation between the species in the river systems and their diversity. The Kunene and Okavango Nile crocodile populations are evaluated based on their mitochondrial control region and Short Tandem Repeat markers. The mtDNA sequences are compared to publically available sequences to evaluate their phylogeography and separation. The Lower Kunene and Okavango River systems showed low haplotype diversity with a single haplotype observed in a total of 64 individuals collected from four locations and no haplotype diversity observed among the two rivers (h=0; π=0). The single haplotype shared between the Kunene and Okavango populations indicate recent divergence between the populations. Short Tandem Repeat diversity was comparable to neighbouring river systems and effective population size estimates were high for each river system population. The Nile crocodile in the Kunene and Okavango Rivers share a single haplotype among all the sequences samples and can be considered a single ESU. However, with the present separation of the two river systems resulted in two Management Units.

3.1 Introduction

Fresh-water species are abundant in lakes, rivers and swamps and are normally distributed over large areas that coincide with these water bodies. With the distribution of a species over a large area, geographical barriers are commonly found to separate individual geographic groups into sub-populations. These geographical barriers are generally topographic features of the landscape, such as the result of formation of rift valleys or river piracy (Wichura *et al*. 2011; Mendelsohn *et al*. 2013).

Sub-populations are of interest to various studies, because limited migration creates isolated populations with independent evolutionary trajectories (Waples & Gaggiotti 2006). Such isolated populations are particularly subjected to random genetic drift that may lead to random allele fixation or loss within populations, reducing diversity within each sub-population. Although loss of diversity within long lived species are often not detected, due to overlapping generations, the breeding population might still have decreased. This seems to be the case in crocodile, where numbers were reduced in the Okavango Delta and Western Africa Nile crocodile; however the genetic diversity within the populations are still maintained (Bishop et al. 2009; Brito et al. 2011; Velo-Antón et al. 2014).

Even though these decreased populations have shown relatively high diversity, several crocodilian species are still poorly understood, especially with regards to population genetic dynamics. The few studies, which are available are those for *C. acutus* and *C. moreletii* (Dever *et al*. 2002; Ray *et al*. 2004). In these studies, the populations were isolated from one another; however genetic connectivity was detectable between the populations, despite the large distance separation between the populations. It is, for example, known that *Crocodylus acutus* can tolerate salt water and move large distances. Even though no genetic structure studies have been published for *C. porosus* it may be assumed that no structure is present amongst regional populations, due to their large migration patterns (Campbell *et al*. 2010; Campbell *et al*. 2013). Unlike the previously mentioned species, the Nile crocodile (*Crocodylus niloticus*) is dispersed over Sub-Saharan Africa and large geographical barriers exist between regional populations.

The empirical data derived from genetic studies, is supportive and pertinent for more efficient conservation methods and management plans of the Nile crocodile (Bishop *et al*. 2009; Hekkala *et al*. 2010; Hekkala *et al*. 2011). The Nile crocodile being a least concern species, as described by the IUCN, raises concerns especially in developing countries where crocodiles suffer endangerment due to habitat loss as a result of human infringement. For

instance, in Namibia, Griffin (2003) documented habitat loss of the Nile crocodile to be of concern.

It has been hypothesised that the Nile crocodile originated from the Congo basin (Hekkala *et al.* 2011), from where it spread, inhabiting various habitats throughout sub-Saharan Africa. Among various factors, riverine basin topography played a crucial role in the distribution of the Nile crocodile as it separated the populations into an eastern and a western clade (Schmitz *et al*. 2003). Moreover, these river systems constitute a variety of environments, such as desert, semi-arid, grassland, woodland and tropical, however they all house fresh water rivers, lakes and / or swamps. The aforementioned, suggest genetic variation within the Nile crocodile population as a result of demographic and biogeographic influences (Hekkala *et al*. 2010), which may require independent management plans for conserving the various populations depending if separate lineages exist.

To effectively assist the conservation of diverged crocodile populations, specific management plans have been proposed by Moritz (1994), of which Management Units (MU) and Evolutionary Significant Units (ESU) have both been regarded as short and long term conservation solutions. The classification of ESUs relies on significant divergence of populations within a species based on historically geographical isolation due to restricted gene flow; whereas MU considers the contemporary population dynamics (Crandall *et al*. 2000).

The current study aims to determine the phylogeography and diversity of the Nile crocodile in the Lower Kunene and Okavango sampling sites. The Lower Kunene and Okavango Nile crocodile populations are evaluated based on their mitochondrial (mtDNA) control region and Short Tandem Repeat markers to evaluate their historical and temporal relation to contribute to the identification of management units in the species. The mtDNA sequences are compared to publically available sequences to evaluate the phylogeography of the Nile crocodiles in Southern African river systems (Lower Kunene, Okavango and Lower Shire River). It is hypothesised that a single lineage within the Lower Kunene and Okavango River system; however two separate populations exist between the two previously mentioned river systems. For southern Africa it is hypothesised that two lineages of the Nile crocodile exists along with two separate populations present.

3.2 Material and Methods

3.2.1 Sample collection and DNA extraction

A total of 139 Nile crocodile samples were collected from wild and wild-caught, ranch held individuals in four different countries and river systems respectively; Botswana (Okavango Delta, n= 29), Malawi (Lower Shire River, n=52), Namibia (Lower Kunene, n=12; Okavango n= 20; Otjiwarongo Crocodile Ranch n= 13) and South Africa (Izintaba crocodile farm, n=13) (Table S3.1 (See Appendix B: Fig S3.1a-3.1c,). The Okavango River was subdivided into three sampling populations: Bwabwatwa National Park (Namibia, n=20), Okavango Delta (Botswana, n=29) and Otjiwarongo Crocodile Farm (Botswana, n=13). Crocodiles from the Otjiwarongo Crocodile Farm were considered a true representation of a wild population, as the farm has maintained the same breeding pairs since their removal (from the wild) in 1986 from the Okavango Delta. Additionally, two populations were considered in the Lower Shire River system (Malawi) using the Nchalo Sugar Estate as a landmark: northwards to Kapichira Falls (n=27) and southwards to the Zambezi Confluence (n=25). Importantly, the Lower Kunene River (n=12) samples were comparatively small and considered a single population, although they originate from two sampling sites (Appendix B: Fig S3.1a-S3.1c,). Izintaba crocodile farm (n=13) from South Africa were considered a single population due to the small samples set available.

Blood samples were collected from the ventral caudal tail vein and stored in K_2EDTA vacutubes. Tissue samples were also collected, by scute removal (1-2 scutes) in a unique pattern for future identification of the individual Nile crocodile (Leslie 1997, Appendix B: Fig S3.2). All samples were stored at -20°C until DNA extraction. Total DNA was extracted using a CTAB protocol (Saghai-Maroof *et al*. 1984) and stored at -20°C until use. All samples were collected under the appropriate CITES Scientific Authority and collection permits for each country. Ethical clearance for this study was received from Stellenbosch University Ethics committee (SU-ACUD15-00007).

3.2.2 MtDNA sequences

Primers were manually designed using publically available sequences of the *C. niloticus* mtDNA control region (Appendix B: Table S3.2) and aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar 2004), implemented in Geneious software v7.1 (Kearse *et al*. 2012). The 514-bp fragment of the mtDNA control region was sequenced for 112 individuals using primers CnP1F (5'-AGTCATCGTAGCTTAACTCACA-3') and CnP1R (5'-TGTATAACGAGCATTAAA

TATTTATG-3'). All amplifications were performed in a total volume of 10 µl containing: KAPA Taq ReadyMix (KAPA Biosystems, Cape Town, SA), 0.8 µM forward- and reverse primers each and DNA. As follows: initial denaturation at 95 °C for 5 min, 35 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 80 sec and a final extension at 72°C for 5 min. Negative controls were included in all DNA extractions and amplifications. Sequencing reactions were performed in the forward direction using the BigDye® Terminator v3.1 sequencing kit (Applied Biosystems) as per manufacture's specifications and capillary electrophoresis was performed on an ABI3730xl sequencer (Applied Biosystems).

DNA sequences were visually inspected for ambiguities in nucleotide base assignment and manually corrected using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, http://www.geospiza.com). Sequences were aligned using the MUSCLE algorithm implemented in Geneious software v7.1. For the purpose of phylogeography, publicly available mtDNA sequences from previous studies and their geographical collection sites were retrieved from GenBank and datadryad (Meredith *et al*. 2011; Hekkala *et al*. 2011) (Appendix B: Table S3.2).

3.2.3 MtDNA sequence analysis

The following sequence diversity measures were estimated for each population: number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π) and average number of pairwise nucleotide differences (k), using Arlequin v3.5 (Excoffier & Lischer 2010). Genetic differentiation among populations was estimated using pairwise Phi-st (Φ-st) values (with significance determined using 1000 bootstrapped replicates), and population structure was further evaluated using AMOVA (significance estimated using 10,000 iterations) based on a distance matrix of pairwise differences calculated in Arlequin v3.5 (Excoffier & Lischer 2010). Populations were grouped for western (Lower Kunene and Okavango) vs. eastern (Lower Shire and South Africa) for the first round of AMOVA, following this was a second round of analyses only considering the Namibian populations (Lower Kunene vs. Okavango). A median-joining haplotype network was constructed to illustrate the evolutionary relationship among haplotypes with default parameters using Network software v4.6.3 (Bandelt *et al.* 1999) (For phylogenetic tree, see Appendix B: Fig 3.3).

3.2.4 STR selection, multiplexing and genotyping

Twelve loci were selected from previous publications, of *C. porosus* and *C. johnstonii* tested in *C. niloticus*, for cross-species amplification in the Nile crocodile, based on number of alleles (A_n>6) and observed heterozygosity (H_o>0.300) (Bishop *et al*. 2009; Miles *et al.* 2009b), including tetra and dinucleotide markers. Six samples (two from each river system) were used for initial singleplex gradient PCR tests to assess optimal annealing temperatures (T_a) and polymorphism of loci. Locus CpP305 (Miles *et al.* 2009a) was included in the preliminary tests, however it was removed due to the the inability to score the marker.

Three multiplex PCRs were considered based on T_a, expected allele range and fluorescent labels (Appendix B: Table S3.3). Due to T_a and fluorescent label constrictions, locus C391 was poolplexed with Multiplex 2. Multiplex amplifications were performed in 10 µl total volume containing KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems, Cape Town, SA) 0.8 µM of each primer and DNA, as follows: initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 15 sec, T_a for 30 sec, 72°C for 50 sec, and a final extension at 72°C for 80 sec. Negative controls were included in all DNA extractions and amplifications. Singleplex and multiplex PCR products were run on an ABI3730xl Genetic Analyser™ (Applied Biosystems) using capillary electrophoresis with GeneScan™ 600 LIZ® (Applied Biosystems, Foster City, CA, USA) as internal standard. Genotypes were scored using GeneMapper® v4.1 (Applied Biosystems). The presence of null alleles (Brookfield 1996) and scoring errors, due to stuttering, were tested for each locus using Micro-checker v2.2.3 (Van Oosterhout *et al.* 2004).

3.2.5 STR population genetic analyses

Departure from Hardy Weinberg equilibrium (HWE) (exact probability test, 500 batches, 10,000 iterations), number of alleles (A_n), expected - (H_e) and observed heterozygosity (H_o) was calculated in Arlequin v3.5 (Excoffier & Lischer 2010), integrated over all STR loci and all STR loci per population, corrected for multiple testing (Bonferroni correction). Furthermore, allelic richness (R_s) and inbreeding coefficient (F_{is}) were estimated between populations in FSTAT v2.9.3.2 (Goudet 1995). Polymorphic information content (PIC) was calculated using Microsatellite Tools v3.1 (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/index.ph). Two neutrality tests were also conducted: Evens-Watterson test (Slatkin's exact test derivative, in Arlequin) and an F_{st}-outlier test as implemented in Lositan v1.44 (10,000 permutations assuming the Stepwise mutation model) (Antao *et al*. 2008).

Pairwise F_{st} and Analysis of Molecular Variance (AMOVA, 10,000 permutations) were performed on a locus-by-locus basis, integrated over all loci calculated in Arlequin. Populations were grouped for western (Lower Kunene and Okavango) vs. eastern (Lower Shire and South Africa) for the first round of AMOVA, following this was a second round of analyses only considering the Namibian sampling sites only (Lower Kunene vs. Okavango). To visualise population distinctness, a factorial correspondence analysis plot was drawn in Genetix v4.05.2 (Belkhir *et al.* 2000). Ancestral population structure of *C. niloticus* was inferred using Structure software v2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2007; Hubisz *et al.* 2009). An initial analysis was conducted for *K*-values between 1 and 6, using the 'admixture model' with independent allele frequencies. A second round of analyses was conducted to assess structure within the major clusters recovered from the first analyses. For this purpose, the total data set was divided into groups comprising the individuals assigned to each of the clusters retrieved in the first analysis. The *K*-values tested ranged from 1 to the number of different sampling sites in each subset (10 replicates for each *K*, 15,000 steps of burn-in period followed by 35,000 steps of MCMC). The estimated log probabilities for each *K* value were calculated using the rate of change in the log probability values and plotted on a graph representing the uppermost level of structure in Structure Harvester software v0.6.93 (Earl & VonHoldt 2012).

Contemporary effective population sizes (N_e) were estimated by the linkage disequilibrium- (LD) (0.02 critical value and the Jack-knife [95% CI]) and heterozygosity excess methods for each population, as implemented in NEESTIMATOR v2.01 (Do *et al.* 2014). The temporal method was not considered for estimating N_e as the individuals were sampled around the same time. Wild populations of the Okavango and Lower Shire River systems were grouped as single populations, respectively, to prevent ambiguity N_e for the river population. This was considered as populations sampled within the river system are likely of migrating between sites.

Testing for recent bottlenecks or radial expansion was evaluated using the Wilcoxon signed rank test for significant deviation from heterozygosity excess and deficiency under the Infinite Allele Model (IAM), Single Mutation Model (SMM) and the two-phased model (TPM) implemented in BOTTLENECK v1.2.02 (Piry *et al.* 1999). Analyses in BOTTLENECK were performed using 1 000 replications at the 5% nominal level and a TPM composed of 70% SMM and 30% IAM (Piry *et al.* 1999). The TPM has been considered to be the best fit model for STR data analyses for recent bottleneck identification (Piry *et al.* 1999).

3.3 Results

3.3.1 Mitochondrial Analysis

The 514 bp mitochondrial control region was amplified and sequences for 112 Nile crocodile individuals. In the Lower Kunene, Okavango and Lower Shire populations, five haplotypes were observed, of which the Lower Kunene, Okavango populations clustered separately from the Lower Shire River populations. The consideration of publically available sequences (Meredith *et al*. 2011; Hekkala *et al*. 2011) (Appendix B: Table S3.2) indicated two clusters representing the two Nile crocodile eastern and western clades, which were previously described across Africa (Schmitz *et al.* 2003; Hekkala *et al*. 2011). The populations from the Lower Kunene, Okavango and Lower Shire River system clustered in the Eastern clade, which was separated from the Western clade by 15 mutational steps. The total dataset showed 12 haplotypes defined by 12 variable sites (all of which consisted of transitions) among 146 individuals (Fig. 3.1a).

The Lower Shire River system (Malawi) showed the highest haplotype diversity among the three Rivers (h=0.332±0.083; π=0.015±0.008) with four haplotypes observed in a total of 47 individuals (Appendix B: Table S3.4). Two of the haplotypes seem to have evolved recently and was exclusive to Malawi, both at a frequency of 0.04 and one mutational step derived from previously described haplotypes. Both of the other two haplotypes (frequency=0.83) was found to be shared with Madagascar, and the other (frequency=0.09) with Madagascar, Tanzania, South Africa and Zimbabwe sequences reported in a previous study (Hekkala *et al.* 2011).

The Lower Kunene and Okavango River systems showed no haplotype diversity with a single haplotype observed in a total of 64 individuals collected from four locations and no haplotype diversity observed among the two Rivers (h=0; π=0). Pairwise PHI-st values indicated significant differentiation between the Lower Shire populations in comparison to Lower Kunene (mean Φ_{st} = 0.940, P<0.05) and the three Okavango populations (mean Φ_{st} = 0.932, 0.947, 0.919, P<0.05) (Table 3.1). Population differentiation among the Lower Kunene, Okavango vs Lower Shire River systems was also supported by AMOVA analysis, with only significant differentiation observed within populations (F_{st}=0.800; 20%; P<0.05) (Table 3.2). No significant population differentiation was observed between the Lower Kunene and Okavango populations.

Table 3.2 Genetic divergence among populations of the Nile crocodiles in Kunene, Okavango (Bwabwatwa National Park, Okavango Delta and Otjiwarongo Crocodile Farm), Lower Shire populations (Lower Shire (North) and Lower Shire (South)) populations and South Africa commercial population. Pairwise F_{st}-values using STRs below diagonal line and pairwise Φ_{st}-values using mtDNA above diagonal line. N/A = No amplification.

	Kunene	Bwabwatwa National Park	Okavango Delta	Otjiwarongo Crocodile Farm	Lower Shire (North)	Lower Shire (South)	South Africa Comm.
Kunene	-	-0.012	-0.034	0.075	0.902*	0.977*	N/A
Bwabwatwa National Park	0.138*	-	-0.046	-0.044	0.896*	0.967*	N/A
Okavango Delta	0.116*	0.005	-	0.034	0.920*	0.975*	N/A
Otjiwarongo Crocodile Farm	0.138*	0.055*	0.07275*	-	0.882*	0.955*	N/A
Lower Shire (North)	0.222*	0.175*	0.171*	0.174*	-	0.008	N/A
Lower Shire (South)	0.200*	0.159*	0.158*	0.160*	0.003	-	N/A
South Africa Comm.	0.162*	0.119*	0.112*	0.197*	0.083*	0.074*	-

*Values that indicate significant differentiation, P<0.05.

Table 3.3 AMOVA results for standard computations (haplotype format) of the control region, excluding South African samples. Two separate analyses were conducted, namely populations clustered in two groups. Group 1: West (Lower Kunene and Okavango) vs east (Lower Shire), Group 2: Lower Kunene vs Okavango populations.

West and east, southern Africa			
Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	21.602	0.39427	80.050
Among populations within groups	0.377	-0.00024	-0.050
Within populations	10.441	0.09850	20.000
Total	32.420	0.49252	
F_{ST}: 0.80002	P: 0.000*		
F_{SC}: -0.00245	P: 0.320		
F_{CT}: 0.80051	P: 0.066		
Kunene vs. Okavango			
Source of variation	Sum of squares	Variance components	Percentage variation
Among groups	0.056	-0.00012	-0.20
Among populations within groups	0.116	-0.00005	-0.09
Within populations	3.582	0.05873	100.29
Total	3.754	0.05856	
F_{ST}: -0.003	P: 0.585		
F_{SC}: -0.001	P: 0.684		
F_{CT}: -0.002	P: 0.499		

* Statistical significance at the 1% nominal level

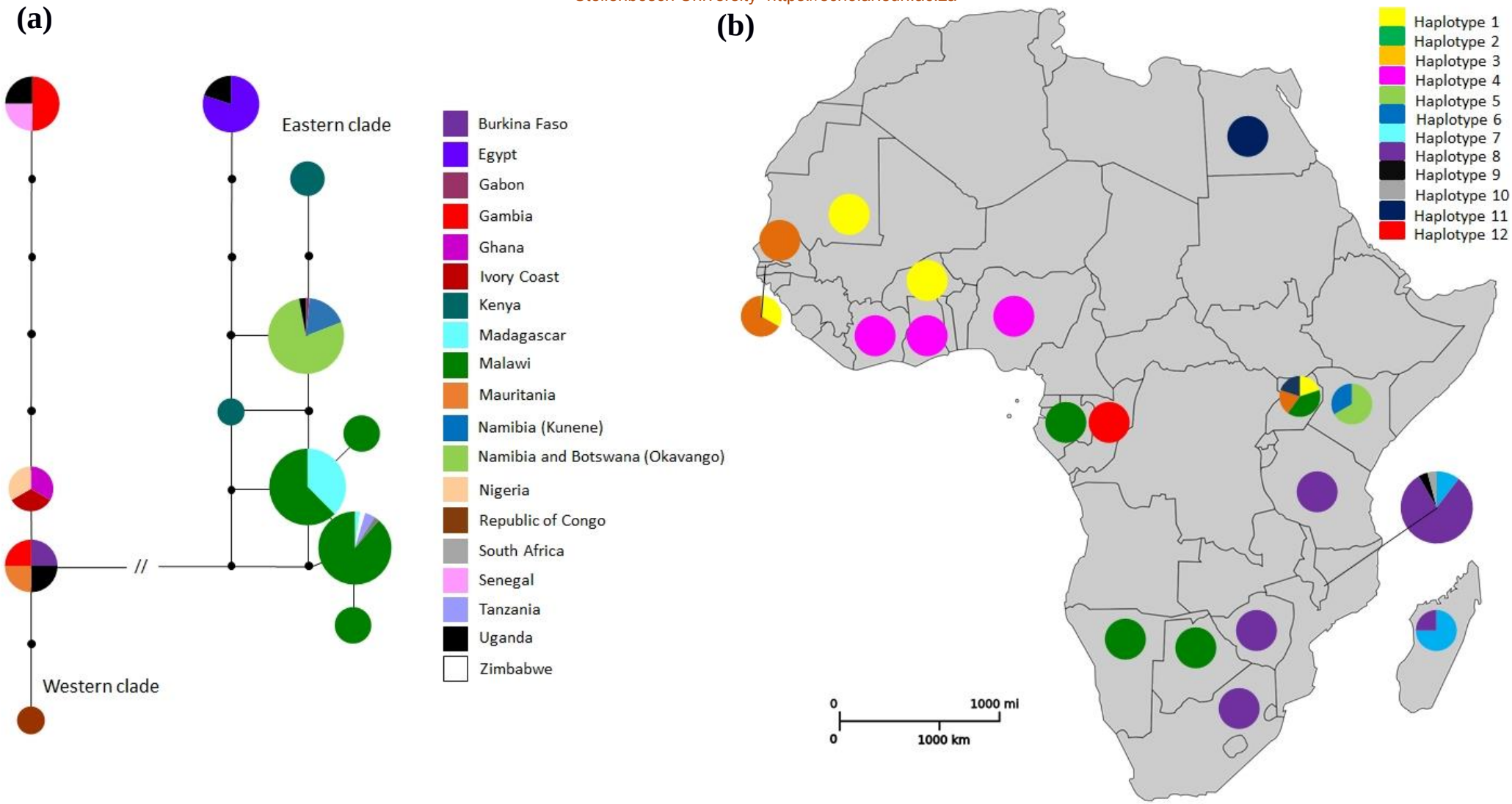


Figure 3.1 **(**a) The Medium-Joining haplotype Network depicting two groups of haplotypes, namely the Western and Eastern clades. Haplotype colours correspond to the countries where the samples were collected. Circles represent mtDNA haplotypes, lines connecting haplotypes represent a single substitution step, and black dots represent hypothetical haplotypes. // represents 15 mutational steps. (b), indication of the 12 haplotypes found within the Nile crocodile portrayed for each country of origin in Africa. Then samples within the study from the Lower Kunene and Okavango share the same haplotype with Gabon and Uganda. Furthermore, the populations of Southern Africa show two different haplotypes among each other separating those of Lower Kunene and Okavango from the Lower Shire population, which shares haplotypes with the surrounding countries.

3.3.2 Genetic diversity based on STR analysis

A total of 122 alleles were observed across all loci, with the number of alleles per locus variying between four (C391 and CpP309) and 23 (CpP307) (Appendix B: Table S3.5). All markers amplified in more than 95% of the samples, and only CpP307 and Cj18 failed in 12% and 14% of the samples, respectively, probably due to intra-specific sequence polymorphisms. Fixation indices (F_{IS}) showed significant heterozygous deficiency (thus deviation from HWE) when considering all populations as a single population group, with values ranging from 0.048-0.263. No large allele dropout was detected, but loci C391 and CpP2504 showed signs of stuttering. No evidence of selection based on F_{st} outlier and Ewan-Watterson tests were found for any of the 11 STR loci (Fig. 3.2 and Appendix B: Table S3.5). Nine loci were moderately informative (PIC>0.44) and two were highly informative (PIC>0.77). Except for Cj18 and CpP309, all loci showed evidence of null alleles (0.0183-0.1213) and this most likely explain the significant deviation from Hardy-Weinberg Expectation at each locus.

Departure from Hardy-Weinberg Expectation was non-significant when considering each population separately; however Hardy-Weinberg Expectation indicated departures at one locus (CpP307) in both Lower Shire populations and another (CpP1409) in the Botswana population after Bonferroni correction for multiple tests. All loci showed moderate values of H_e (≥0.551) and H_o was lower than H_e for most loci. The two Lower Shire River populations were the most diverse groups (H_e=0.67, H_o=0.63), and R_s=5.53 (averaged across the two groups) compared to the Lower Kunene (He=0.58, H_o=0.50, and R_s=4.10) and the Okavango populations (He=0.59, H_o=0.58 and R_s=4.46, averaged across the three groups) (Table 3.3). Fixation indices F_{is} indicated an excess of homozygotes in the Lower Kunene (F_{is}=0.15) and Lower Shire (F_{is}=0.10 and 0.07) populations, compared to the Okavango River populations (F_{is}=-0.01, 0.01 and 0.04).

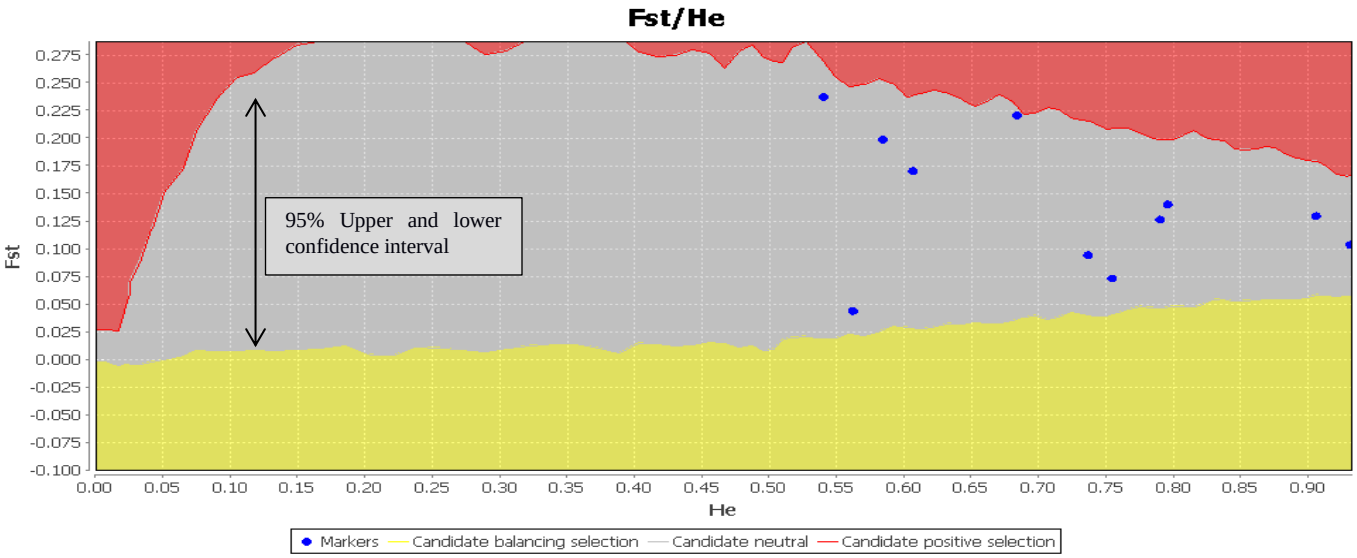


Figure 3.2 LOSITAN results indicating outlier loci as candidate loci under positive (red) and balancing (yellow) selection. All loci (indicated in blue dots) were considered to be neutral.

Table 3.4 Genetic diversity in the Nile crocodile populations genotyped in this study for mean values of Panmixia of Southern Africa populations, Lower Kunene population, Okavango populations (Bwabwatwa National Park, Okavango Delta and Otjiwarongo Crocodile Farm), Lower Shire populations (Lower Shire (North) and Lower Shire (South) and South African commercial population. For complete table refer to Appendix B: Table S3.4. N - number of individuals, An - number of alleles, He - expected heterozygosity, Ho - observed heterozygosity, HWE - Hardy Weinberg Equilibrium test (*P*-value), Rs - mean allelic richness, Fis - mean frequency of inbreeding coefficient.

Primer	N	An	He	Ho	HWE (P)	Rs	Fis
Panmixia	139	11.1	0.712	0.607	0.003	10.848	0.144
Lower Kunene	12	4.2	0.583	0.495	0.587	4.097	0.149
Bwabwatwa National Park	20	5.3	0.599	0.605	0.616	4.481	-0.009
Okavango Delta	29	3.0	0.880	0.750	0.613	4.749	0.150
Otjiwarongo Crocodile Farm	13	4.3	0.562	0.535	0.537	4.137	0.043
Okavango	62	5.2	0.595	0.584	0.531	4.456	0.015
Lower Shire (North)	27	6.9	0.664	0.617	0.337	5.519	0.098
Lower Shire (South)	25	6.9	0.684	0.625	0.367	5.540	0.071
Shire	52	6.9	0.674	0.621	0.352	5.529	0.085
South Africa comm.	12	3.8	0.519	0.586	0.273	3.734	-0.104

3.3.3 Contemporary genetic connectivity and genetic structure

Genetic distance, based on STR data, (F_{st} = 0.05-0.15, $p < 0.001$) was observed between the Kunene and all three Okavango River populations and great genetic distance (F_{st} = 0.15-0.25, $p<0.001$) between the Kunene, Okavango and Shire populations (Table 3.1). Genetic differentiation was supported by F_{st} between the Lower Shire River population in comparison to the Lower Kunene (F_{st} =0.222 and 0.200, $P<0.05$) and three Okavango

populations (F_{st} =0.175 and 0.159, 0.171 and 0.158, 0.634 and 0.784, p<0.001) (Table 3.1). The sampled populations distributed between the Eastern (Lower Shire River) and Western (Lower Kunene-Okavango River) regions of southern Africa detected two populations clusters (K=2), using the Bayesian structure analyses assuming no prior assumption of population structure, (Fig. 3.3) as the log probability only slightly increased for K values greater than 2 (Appendix B: Fig. S3.3).

The two separate clusters were further supported by significant differentiation amongst groups (F_{ct}=0.133; 13%), within groups (F_{sc}=0.053; 5%) and over all groups and populations (F_{st}=0.179; 82%), determined by AMOVA (Figure 3.4) and depicted by two separate clusters in a factorial correspondence plot (Fig. 3.5).

Additional analyses between the Lower Kunene and the Okavango River populations revealed further cluster sub-structure between the river systems; interestingly two sub-structured populations were present in the Okavango Delta (Appendix B: Fig. S3.4). Clustering of the Lower Kunene and Okavango River were supported by AMOVA amongst groups (F_{ct}=0.069; 14%), within groups (F_{sc}=0.050; 3%) and over all groups and populations (F_{st}=0.115; 83%) (Fig. 3.4) and depicted by two separate clusters in a factorial correspondence plot (Fig. 3.5). The signal for two populations present in the Okavango was very weak and not displayed in the factorial correspondence plot.

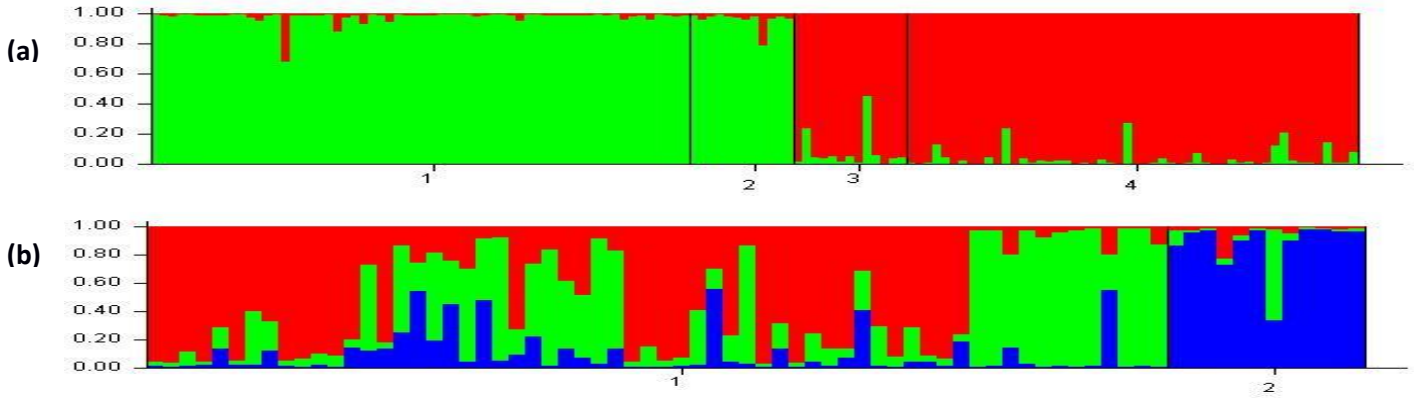
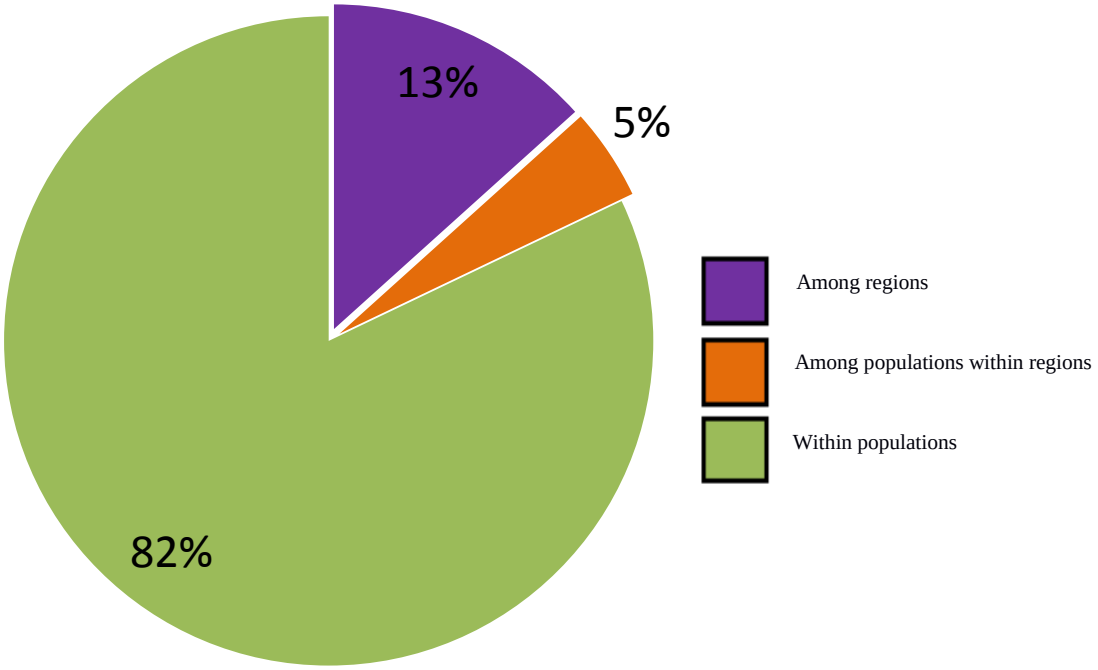


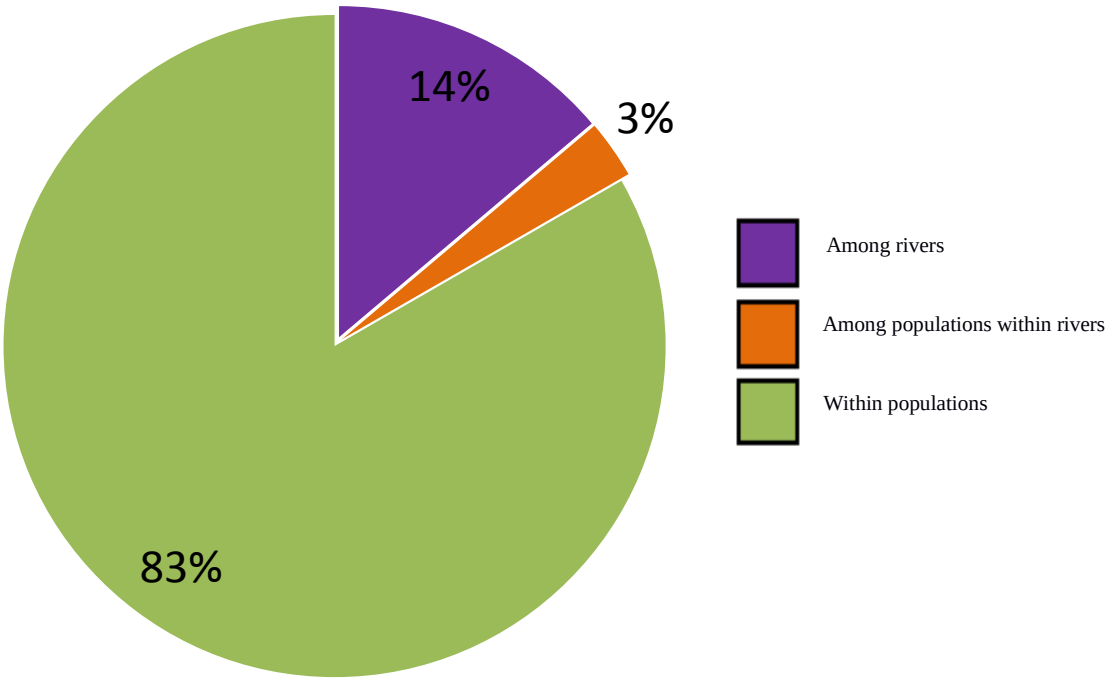
Figure 3.3 Genetic structure of Crocodylus niloticus populations based on Bayesian clustering analyses [Structure software v2.3.4 (Pritchard *et al.* 2000)] a) Genetic clusters in Southern Africa (complete dataset) , K = 2 and (b) Genetic clusters in the Kunene and the Okavango samples. Populations: (1) Okavango, (2) Kunene, (3) South Africa Commercial and (4) Lower Shire.

(a)



Fct = 0.133*; Fsc = 0.053*; Fst = 0.179*
*Statistical significance at the 0.01% nominal level

(b)



Fct = 0.069*; Fsc = 0.050*; Fst = 0.115*
*Statistical significance at the 0.01% nominal level

Figure 3.4 Locus by locus AMOVA results with populations clustered (a) in two geographical groups, Lower Kunene and Okavango river populations vs. Lower Shire River population and (b) two river groups, Lower Kunene river population vs. Okavango River population (*significance as the 0.01% nominal level).

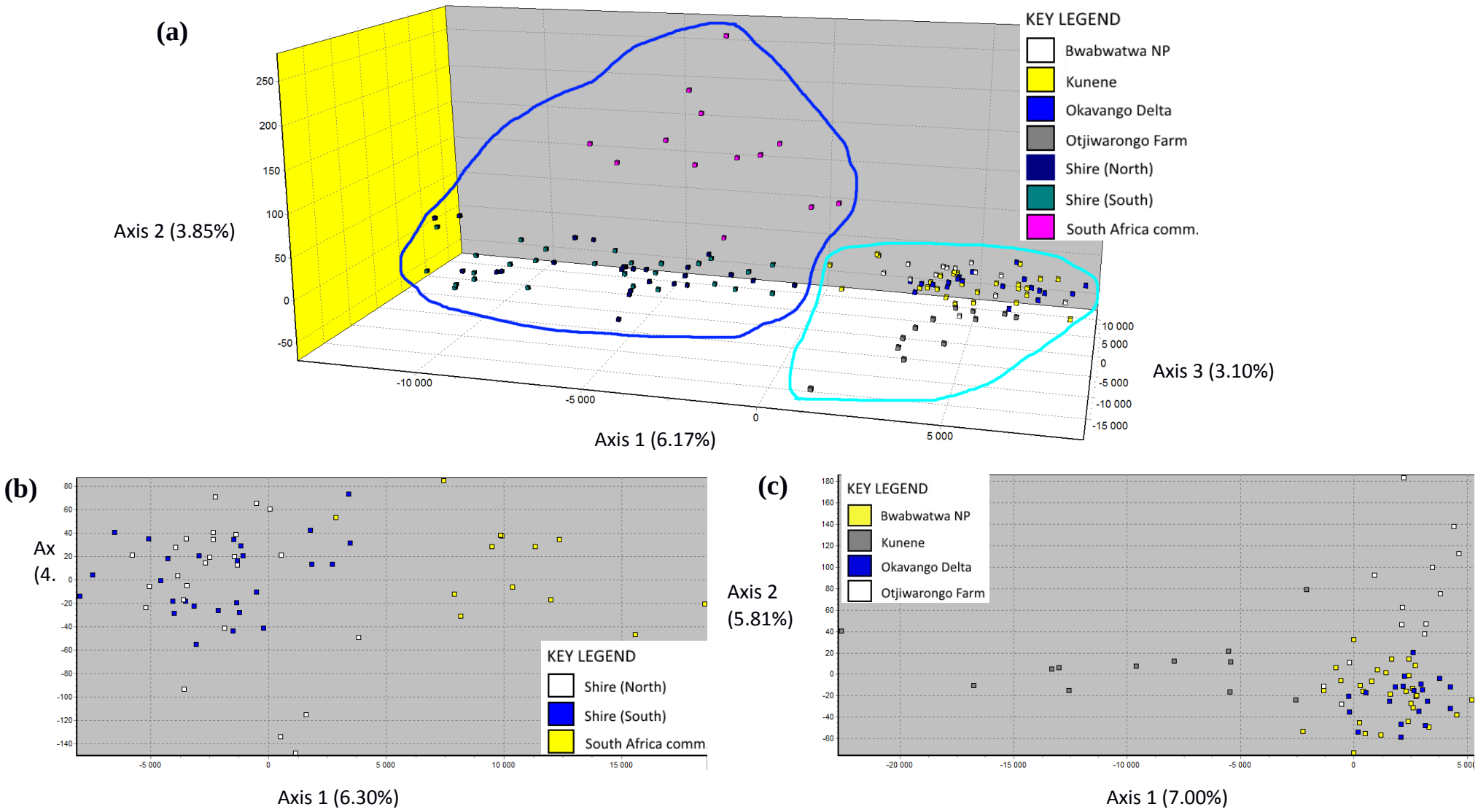


Figure 3.5 Factorial Correspondence Analyses plots. (a) Four *Crocodylus niloticus* populations grouped into their various river systems (Dark Blue indicates South Africa and Lower Shire populaitons and Light Blue indicates Lower Kunene and Okavango populations). Heterogeniety (b) within South Africa and the Lower Shire along factor 1 and 2, (c) within Lower Kunene and Okavango along factor 1 and 2.

3.3.4 Effective population size and potential population bottleneck

Effective population size, as estimated by the LD estimates were generally high; except for the Bwabwatwa National Park 38.6 (17.7-432.7) and Shire (South), 43.4 (29.4-75.2) (Table 3.4). Subsequently, due to the presence of gene flow within the Okavango and Shire river system, an overall estimate was determined for each river system, Okavango; 404.6 (107.3-∞) and Shire; 143.5 (74.5-743.8). There was no significant heterozygosity deficiency observed in any of the populations considering any of the three models (IAM, TPM, SSM: Wilcoxon signed-rank test, P>0.05), however heterozygosity excess was significant in the Shire (North) sampling site considering the IAM (P<0.05), however the combined Shire population considering the IAM indicated no significance (P>0.05) (Table 3.5).

Table 3.5 Estimates of contemporary N_e size based on the Linkage Disequilibrium method [95% CI], combined N_e of the Okavango and Lower Shire populations are in the shaded areas.

Populations	LD test for N_e [95% CI]		Heterozygosity excess test for Ne	
Lower Kunene	∞ (24.7-∞)		∞ (∞-∞)	
Bwabwatwa National Park	38.6 (17.7-432.7)		56.2 (10.1-∞)	
Okavango Delta	292.3 (44.7-∞)	404.6 (107.3-∞)	∞ (12.8-∞)	∞ (16.7-∞)
Otjiwarongo Crocodile Farm	62.04(18.1-∞)		∞ (13.1-∞)	
Lower Shire (North)	754.4 (54.1-∞)	143.5 (74.5-743.8)	∞ (∞-∞)	∞ (∞-∞)
Lower Shire (South)	43.4 (29.4-75.2)		∞ (∞-∞)	
South Africa Comm.	∞ (30.9-∞)		10.3 (3.5-∞)	

Table 3. 6 Results from the BOTTLENECK test of Short Tandem Repeats from the seven populations tested across the three different models Infinite Allele Model (IAM), Two-Phase Modeal (TPM) and Single Mutation Model (SMM). Combined test for all three models of the Okavango and Lower Shire populations are in the shaded areas.

Population	IAM				TPM				SMM			
	H def	H exc	H def	H exc	H def	H exc	H def	H exc	H def	H exc	H def	H exc
Lower Kunene	0.880	0.139			0.711	0.319			0.207	0.817		
Bwabwatwa National Park	0.880	0.139	0.926	0.087	0.517	0.517	0.618	0.416	0.120	0.897	0.120	0.897
Okavango Delta	0.913	0.103			0.650	0.382			0.120	0.897		
Otjiwarongo Crocodile Farm	0.768	0.260			0.382	0.650			0.120	0.897		
Lower Shire (North)	0.880	0.139	0.995	0.006*	0.517	0.517	0.650	0.382	0.139	0.880	0.087	0.926
Lower Shire (South)	0.998	0.002*			0.840	0.183			0.416	0.618		
South Africa Comm.	0.875	0.150			0.500	0.545			0.150	0.875		

H def = Heterozygosity deficiency, H exc = Heterozygosity excess, * Significant values, P<0.05.

3.4 Discussion

The empirical data derived from genetic studies, is supportive and a prerequisite toward more efficient conservation methods and management plans of the Nile crocodile. As previously found (Hekkala *et al*., 2010), the Nile crocodile populations in Namibia are structured relative to river basin formations. The importance of assessing genetic diversity is evident, especially in isolated populations which has been considered for the Nile crocodile populations per river system. It is proposed, from supporting evidence that the Nile crocodile originated from the Congo basin (Hekkala *et al.* 2011) and spread through sub-Saharan Africa.

3.4.1 Divergence in the southern Africa crocodilian population

The presence of two lineages of the Nile crocodile in southern Africa, indicated by the clustering of haplotypes in the Lower Shire (Malawi), separated by the single haplotype of the Lower Kunene and Okavango populations (Fig. 1a). Comparison to publically available sequences revealed two common haplotypes to be observed in Southern Africa, namely haplotype 2 and 8 (Fig. 1b). The aforementioned is further validated when pairwise Φ_{st} values between the Lower Shire populations are compared to the Lower Kunene and Okavango populations, indicating restriction of gene flow (Table 3.1). Further, should the Φ_{st} values not differ significantly from the null distribution of the variance of the population, the sub

populations would not be differentiated from the larger population. The study findings are in support of Hekkala *et al.* (2011) for a two lineage separation of the Nile crocodile in Southern Africa.

A historical migration, with the hypothesis of the Nile crocodile originating from within the Congo basin of Central Africa, may be indicated by geographical influences in southern Africa along with the assessment of mtDNA sequences into southern Africa. The dispersal of fresh water species into Southern Africa may be relative to the Kasais headwaters to be captured by the Upper Zambezi River systems, leading the way to the Okavango and Kunene River system respectively (Cotterill 2006). Observation of a single haplotype within the Kunene and the Okavango populations to be shared with an individual from Gabon and Uganda (Fig 1a and 1b) could be indicative of their possible dispersal from central Africa and a recent separation between the Kunene and Okavango River systems (Hipondoka 2005; Hipondoka *et al*. 2006; Mendelsohn *et al.* 2013). Furthermore, the lack of haplotype diversity could be indication of these Nile crocodile populations to be the most south-westerly populations in Africa. Considering diversity within a population to be greatest for populations in the abundant centre and decreasing due to genetic drift and isolation relative to the dispersal of the populations (Eckert *et al.* 2008).

3.4.2 Genetic diversity and contemporary population dynamics

Allelic diversity (H_e, H_o and A_n) and richness has been a key focus point on estimating diversity within populations (Allendorf 1986; Fuerst & Muruyama 1986; Spielman *et al.* 2004). Short Tandem Repeat markers used within the study indicated no impact of markers to be under selection within the population (Appendix B: Table S3.5) or between the populations (Fig. 3.2). Allelic diversity within the study respective to each river system (Table 3.3) was found to corroborate with river systems in Africa: Botswana (H_o = 0.51) (Bishop *et al*. 2009) South Africa (St Lucia) (H_o = 0.48), Tanzania (Ruaha River) (H_o = 0.66) and Zimbabwe (Zambezi River) (H_o = 0.54) populations (Hekkala *et al*. 2010). Even though crocodilian populations have previously experienced population declines due to over exploitation, reports were still supportive of moderate levels of genetic diversity within the various populations.

3.4.3 The Split of Namibian Nile crocodile populations

In the present study, STR analyses as an indicator of genetic differentiation was moderate (F_{st} = 0.05-0.15, $p < 0.001$) in the Lower Kunene as well as the Okavango River populations. Additionally, a great genetic differentiation (F_{st} = 0.15-0.25, $p<0.001$) was observed between the Lower Kunene, Okavango and Lower Shire populations (Table 3.1). The above indicates a significant measure of genetic variance contained within the respective sub populations, relative to the total genetic variance. This suggests a clear representation of sub population groups, which are historically related and independently diverse populations. The populations have limited genetic connectivity to one another and each river system has become its own independent population. Additionally, a large percentage of variation was seen within the populations depicted by an FCA separation (Fig. 3 FCA Single) by genetic distance and grouped the populations into two groups, namely West Southern Africa (Lower Kunene and Okavango) and East Southern Africa (Lower Shire).

Bayesian structure analyses within southern Africa further revealed the divergence of two populations (Fig. 5a) endorsing the divergence of two populations, whereas the presence of two populations within the Lower Kunene and Okavango River system contradict one another. However, several species have been separated within the Kunene and Okavango River systems with the aridification of the Cuvelai basin (Curtis *et al*. 1998).

Analyses of contemporary effective population size showed the Bwabwatwa and Lower Shire (South) populations to have a decrease in allelic diversity due to genetic drift ($N_e<50$) (Franklin 1980). However, the presence of genetic connectivity in the whole Okavango and Lower Shire River system required revaluation of the Ne, as several sampling populations were present within each of the river systems. The revaluation indicated no threat of genetic diversity loss within the Okavango and Lower Shire River. This was mirrored by the TPM Wilcoxon test to indicate no recent expansion or bottleneck event. Additionally, low levels of mtDNA structuring has been reported for long lived species (Glenn *et al.* 2002), to be partially explained by their low metabolic rates (Ray *et al.* 2004) influencing low mutation rates (Bromham 2002). Moreover, their slow ‘rate of evolution’ would have allowed them to recover from a historical bottleneck.

The comparison of mtDNA control region to that of the STR nuclear DNA results for the Lower Kunene and Okavango populations contradict each other. A single haplotype within the Lower Kunene and Okavango populations are not in agreement with STR results of two independent populations. The pattern of the single haplotype is possible due to the Cuvelai basin which connected the Kunene and Okavango River (Hipondoka 2005;

Hipondoka *et al*. 2006; Mendelsohn *et al.* 2013) with the water dependent species occupying the habitats. The presence of a single haplotype present indicates a recent separation and no time for lineage separation between the populations and the populations can be recognised under a single ESU.

The high mutation rate within STR display to separate populations with restricted gene flow between them, most likely caused by the aradification in the previously mentioned basin. With the current restriction of gene flow and possible migration between the two river populations, each river system would be recommended to have its own MU to maintain the diversity within the population.

3.5 Conclusion

The Nile crocodile in the Lower Kunene and Okavango River share a single haplotype among all the sequences samples and can be considered a single ESU. However, with the present separation of the two river systems resulted in two separate populations, MU, due to restricted gene flow. Mitochondrial DNA analysis has also confirmed that the Nile crocodile populations within the Lower Kunene and Okavango River systems form part of the Eastern Clade in Africa, as indicated by haplotype clustering. In the present study, STR analyses as an indicator of genetic differentiation was moderate in Lower Kunene as well as the Okavango River populations, indicated by significant F_{st} values. Additionally, a great genetic differentiation was observed between the Lower Kunene, Okavango and Lower Shire populations. This suggests the presence of two divergent lineages of the Nile crocodile in Southern Africa, which was also found for this study depicted by mtDNA and STR analyses.

The above suggests a significant measure of genetic variance contained within the respective sub populations, relative to the total genetic variance. It is therefore a clear representation of sub population groups, which are historically related and independently diverse populations as well. Converging lines of evidence point toward the necessity in monitoring *C. niloticus* genetic diversity and structure, as it may provide more efficient risk stratification tools in order to assist conservationists in developing future management plans.

Chapter 4
Concluding remarks, short comings and future recommendations

4.1 Overview of the study findings

The primary focus of this study was to investigate further the genetic diversity and structure of the Nile crocodile (*C. niloticus*), which inhabits various environments in sub-Saharan Africa. The separated geographical locations of the Nile crocodile across sub-Saharan Africa point toward a possible genetic influence as a result of topographical changes. Additionally the genetic variation also seen in the Nile crocodile could be intrinsically linked to the topography of the landscape. An aerial survey was conducted on the Nile crocodile along the Lower Kunene River system of Namibia and census size has been found to be supportive of genetic diversity.

In Chapter 2, results of an aerial survey conducted to estimate the abundance and distribution of the Nile crocodile in the Lower Kunene River system is reported. Problems which arise for surveys are generally factors influencing observer and habitat bias. The recent development of open population models based on animal counts (Dail & Medsen, 2011; Zipkin *et al*., 2014), as conducted in our study, provided sufficient information to monitor the trend of a population over time and perhaps estimate other demographic parameters required to effectively manage a population. Additionally, long river segments were covered in brief survey sessions, to allow for minimal crocodile migration between sites. The estimated abundance of Nile crocodiles in the Lower Kunene River was estimated at 806 individuals after considering observer and environmental bias (2.29 crocodiles per km) and this is considered plentiful, compared to other African River populations (Bourquin, 2007).

In Chapter 3, three populations from the Lower Kunene, Okavango and Lower Shire Rivers were assessed for their phylogeographic separation from the Congo basin, which has been considered the central origin of the Nile crocodile (Hekkala *et al.* 2011). Two primary haplotypes were found to be present within Southern Africa, justifying the two lineages of the Nile crocodile as was previously described (Hekkala *et al.* 2011). Additionally, the sharing of a single haplotype among the 64 samples of the Lower Kunene and Okavango populations indicate a historical joint population. The extend of the haplotype sharing towards central Southern African is yet to be determined as no samples were available from these river systems, namely Kwando, Mamili, Linyanti/Chobe and Upper Zambezi.

All three previous mentioned populations including a population from a South African commercial farm was evaluated for genetic diversity and structure. Nile crocodile populations were structured relative to river basin formation, which is the result of the change in topography and the presence of two separate populations in Southern Africa. Additional analyses for the Lower Kunene and Okavango River system indicated the same result, however gene flow was present within the Okavango River system and associated with nesting site locations. Genetic diversity was not found to be lacking as a result of genetic drift, thereby suggesting the absence of a bottle neck or expansion within the populations. However, long lived species could under represent the presence of genetic drift over time.

4.2 Contribution towards conservation efforts

Genetic conservation has been become more abundant in proposed management plans for species. The study has provided preliminary findings of the Nile crocodile in Namibia relative to neighbouring river systems to provide guidance for conservation management efforts, for the maintenance of diversity within Southern Africa, specifically the Lower Kunene, Okavango and Lower Shire River populations. Two divergent populations are present in southern Africa populations of the Nile crocodile, with mtDNA and STR analyses supporting this notion. Identification of the site of secondary contact between the two diverged lineages may be of importance to investigate population trends in central Southern Africa.

Within the two Namibian river systems, the Lower Kunene and Okavango populations, a single lineage was observed by mtDNA, indicative of a single ESU. However, STR analyses are suggestive of two MUs required for the populations within each river system. The data derived from the Lower Kunene River system is lacking, primarily due to the absence of individuals and therefore requires further investigation to ascertain stronger evidence with respect to their genetic structure. However, the estimated abundance of crocodiles and protected areas along the Lower Kunene River and the diversity within the populations are indicative of moderate population fitness.

Within the Okavango populations structure was observed for the presence of two populations and the presence of gene flow between these populations. The findings of Bishop *et al*. (2009) indicating a decreasing Nile crocodile population can now consider the individuals from Namibia to be included to the gene pool and a re-evaluation to determine the contribution of the Namibian population towards the Okavango Delta population. Especially since the Namibian populations are protected in Bwabwatwa and Mahango National Park.

4.3 Limitations and future research

The limitations encountered within the study are those biases which influence all wildlife sampling of predators in remote areas. Samples obtained from the Lower Shire and Okavango River systems can be considered good baseline data for the structure and diversity within the river systems. [As a sample set of more than 50 is recognised as an adequate number of samples to be representative of the populations (Ruzzanter 1998).] The collection of the samples were possible as past samples have been collected from the Okavango Delta and several Crocodile Ranches are located along the Lower Shire River systems. The Lower Kunene River is under representative as only 12 samples were collected from the system for the study and the large distance between the sampling locations will not provide as informative data as those of the Okavango and Lower Shire populations.

With the on-going collection of crocodile samples along the Namibian river systems, more fine scale structure may be determined within the respective river systems. The Kunene River has several waterfalls and rapids which may act as barriers and will be of interest to investigate to determine whether sub-structuring is present within the Kunene River populations. Communication with the professional hunters in the area may be helpful to collect tissue samples and during trawling times of the Namibian Inland Fisheries Institute as reports have been noted for several small crocodiles to be collected among the fish species.

Further, the Okavango River should be considered to be sampled outside of the National parks but this may prove difficult as equipment is not as secured as in the National Park. The presence of gene flow between the two Nile crocodile populations in the Okavango river system could be aided by environmental authorities collaborating from Botswana and Namibia. The additional use of GPS tagging methods currently in use along the Kunene and Okavango Rivers may validate the movement of the Nile crocodile within the river systems to establish new protected areas with the incorporation of genetic structure (Species Management Plan, 2012).

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Appendix A

Appendix A1

Table S2.1 The 10 sessions flown along the Kunene river system during the aerial survey. Shown for each day flown along the river and the distances covered on every single day. The river mouth was considered 0km and the Ruacana dam 352km.

Session	Date	Distant points covered
01	24-April-2012	from km 30 to km 132
02	24-April-2012	from km 0 to km 121
03	25-April-2012	from km 1 to km 128
04	27-April-2012	from km 111 to km 266
05	28-April-2012	from km 125 to km 256
06	28-April-2012	from km 121 to km 256
07	9-August-2012	from km 257 to km 352
08	10-August-2012	from km 257 to km 352
09	11-August-2012	from km 257 to km 352
10	12-August-2012	from km 257 to km 352

Table S2 2 Sample of the count data recorded on the Kunene River at site #71. Figures indicate the number of crocodiles observed at the site on a particular sampling occasion. NA indicates that this site was not surveyed on this particular occasion. Occ = occasion / session

Group	Occ1	…	Occ6	Occ7	Occ8	Occ9	Occ10	Total
Class 1	NA	…	NA	4	3	6	4	17
Class 2	NA	…	NA	2	2	3	3	10
Class 3	NA	…	NA	1	0	0	1	2
Class 4	NA	…	NA	3	1	1	1	6
Total	NA	…	NA	10	6	10	9	35

Appendix A2

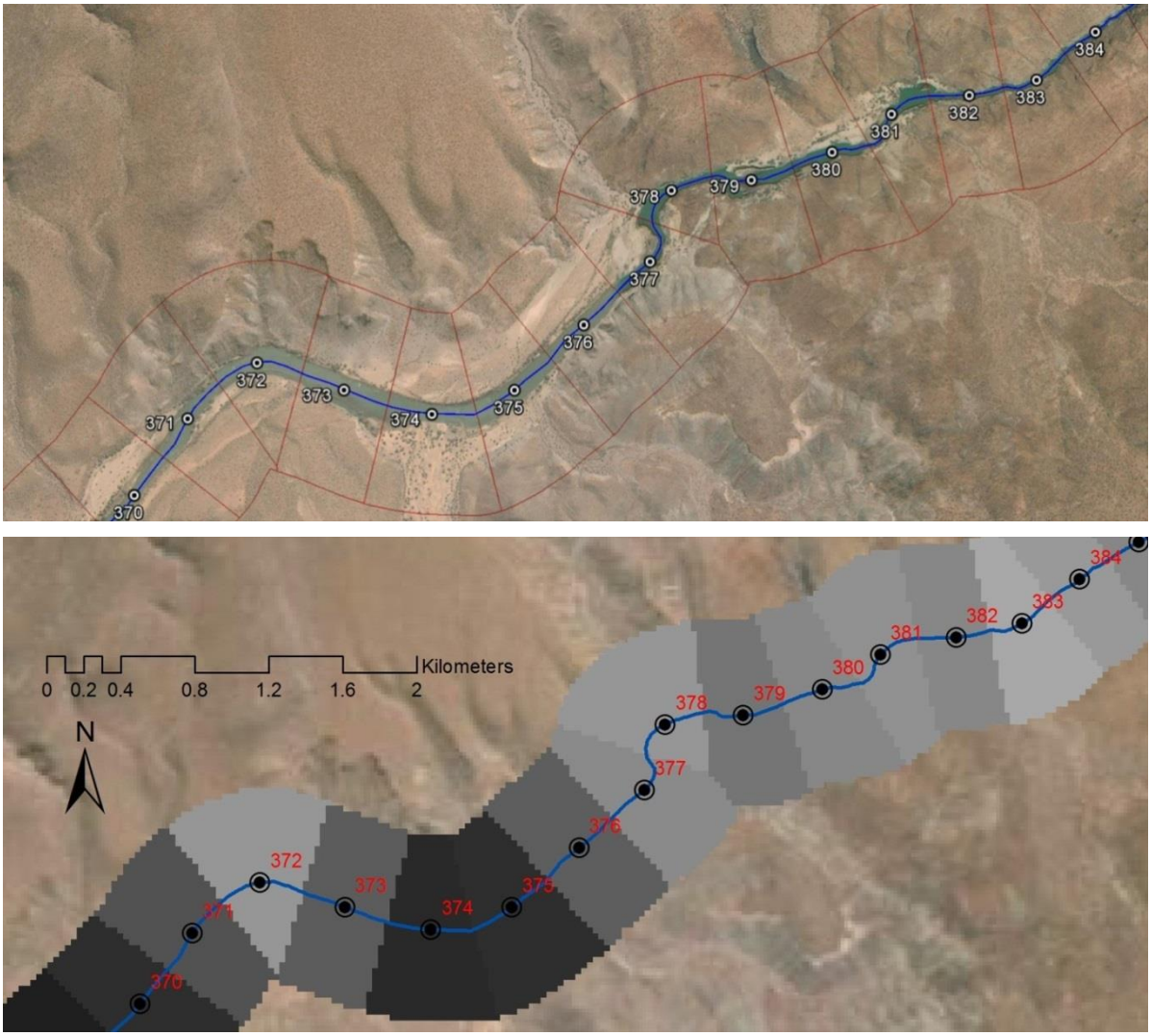

Figure S2. 1 Covariates description for river segments 371-380

#500m segment	371	372	373	374	375	376	377	378	379	380
River width	3	4	5	3	3	3	1	2	2	2
Alt variation	31.6	36.5	21.8	14.3	20.2	32.4	40.8	39.3	35.9	39.7

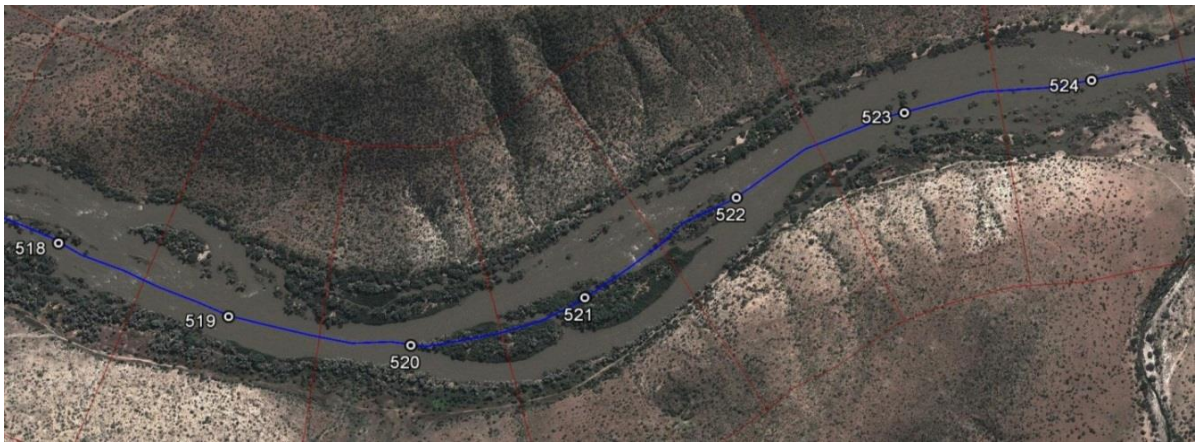

Figure S2. 2 Covariate description segments for river segments 518 to 524

#500m segment	518	519	520	521	522	523	524
# of channels	2	2	2	2	2	2	2

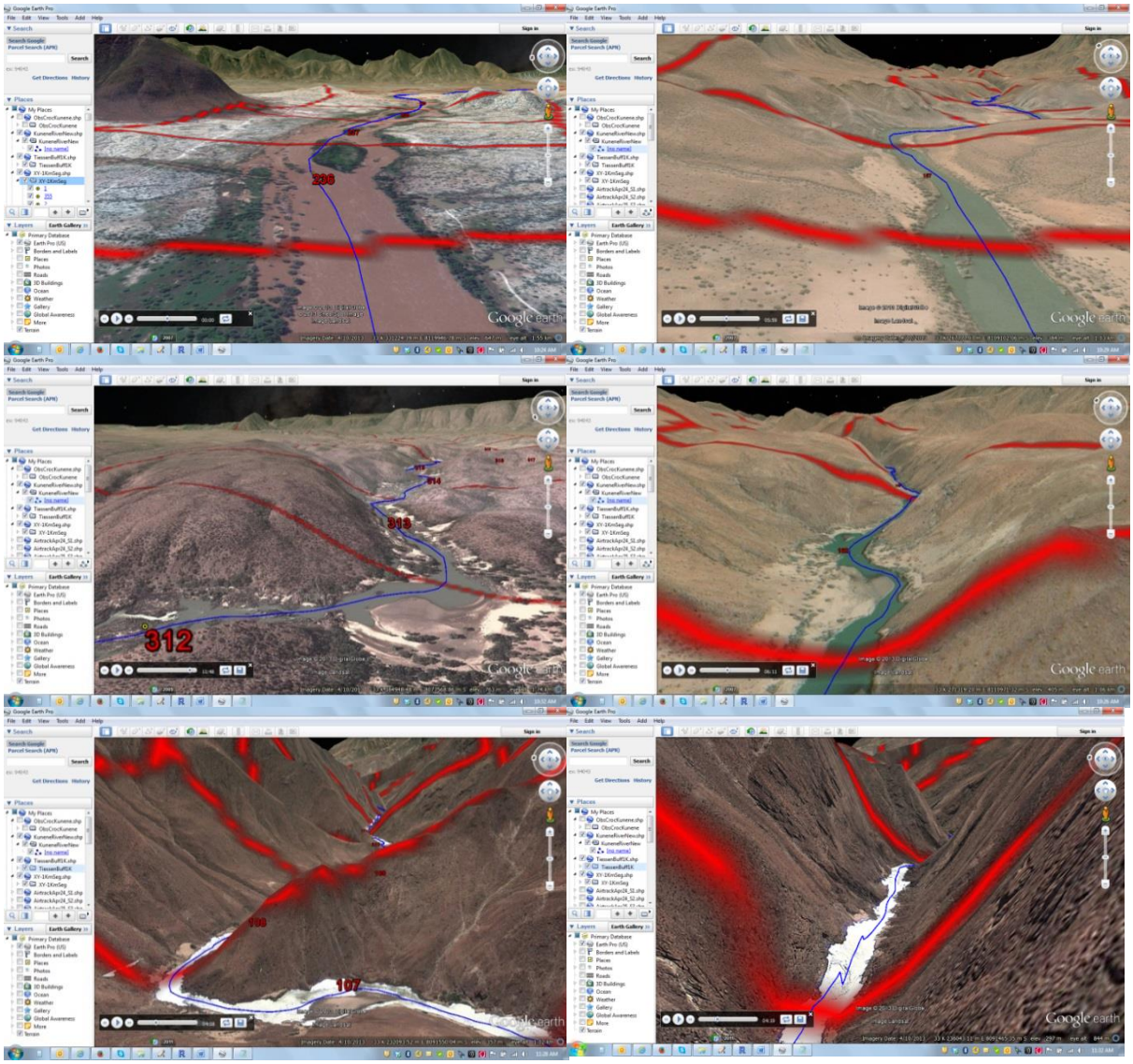

Figure S2.3 Covariate description

Appendix A3

JAGS code for the crocodile data analysis

The JAGS code that we used to estimate parameters for the crocodile population in Kunene River is presented below. We used vague priors for all parameters based on the data. We ran three chains of the model, each for 2200,000 iterations after a burn-in of 200,000 and thinned by 2000. We assessed model convergence by visually examining the chains and assuring that R-hat values were all less than 1.05.

```
# Priors
# Standard vague prior for lambda
for (g in 1:nGrp){
  alpha[g]  ~ dnorm(0, 0.1)
  alpha1[g]  ~ dunif(-10, 10)
  alpha2[g]  ~ dunif(-10, 10)
  alpha3[g]  ~ dunif(-10, 10)
  alpha4[g]  ~ dunif(-10, 10)
  alpha44[g]  ~ dunif(-10, 10)
  alpha5[g]  ~ dunif(-10, 10)
  alpha55[g]  ~ dunif(-10, 10)
  }
# Standard vague prior on detection
for (g in 1:nGrp){
  beta[g]  ~ dnorm(0, 0.1)
  beta1[g]   ~ dunif(-10, 10)
  beta2[g]   ~ dunif(-10, 10)
  beta3[g]   ~ dunif(-10, 10)
  beta4[g]   ~ dunif(-10, 10)
 }
# prior on for random effect
tau.p   <- 1 / (sd.p * sd.p)
sd.p     ~ dunif(0, 3)

# Likelihood
# Biological model for true abundance
for (i in 1:nSit) {
 for (g in 1:nGrp) {
    N[i,g] ~ dpois(lambda[i,g])            # Abundance  per size class
    log(lambda[i,g]) <- alpha[g] + alpha1[g] * sitcov1.N[i]
                        + alpha2[g] * sitcov2.N[i]
                        + alpha3[g] * sitcov3.N[i]
                        + alpha4[g] * sitcov4.N[i]
                        + alpha44[g] * pow(sitcov4.N[i],2)
                        + alpha5[g] * sitcov5.N[i]
                        + alpha55[g] * pow(sitcov5.N[i],2)
  } #g
  NSit[i] <- sum(N[i,])
} # i

# Observation model for replicated counts
for (g in 1:nGrp) {
 for (iv in 1:nSmp) {
```

```
            n[v[iv,1],v[iv,2],g] ~ dbin(p[v[iv,1],v[iv,2],g], N[v[iv,1],g])        # Detection with
site-by-day-by-group random effect
            p[v[iv,1],v[iv,2],g] <- (1/(1+exp(-lp[v[iv,1],v[iv,2],g])))
            lp[v[iv,1],v[iv,2],g] <- beta[g] + beta1[g] * obscov1[v[iv,1],v[iv,2]]
                        + beta2[g] * obscov2[v[iv,1],v[iv,2]]
                        + beta3[g] * obscov3[v[iv,1],v[iv,2]]
                        + beta4[g] * obscov4[v[iv,1],v[iv,2]]
                        + rand[v[iv,1],v[iv,2],g]
            rand[v[iv,1],v[iv,2],g] ~ dnorm(0, tau.p)
            p[v.2[iv,1],v.2[iv,2],g] <- 0       # p for all not surveyed occasion * sites

            # Compute fit statistic E for observed data
            eval[v[iv,1],v[iv,2],g] <- p[v[iv,1],v[iv,2],g] * N[v[iv,1],g]    # Expected values
            E[v[iv,1],v[iv,2],g]   <-   pow((n[v[iv,1],v[iv,2],g]   -   eval[v[iv,1],v[iv,2],g]),2)   /
(eval[v[iv,1],v[iv,2],g] + 0.5)
            # Generate replicate data and compute fit stats for them
            n.new[v[iv,1],v[iv,2],g] ~ dbin(p[v[iv,1],v[iv,2],g], N[v[iv,1],g])
            E.new[v[iv,1],v[iv,2],g]              <-              pow((n.new[v[iv,1],v[iv,2],g]              -
eval[v[iv,1],v[iv,2],g]),2) / (eval[v[iv,1],v[iv,2],g] + 0.5)
            p.list[iv,g] <- p[v[iv,1],v[iv,2],g]
          } # v
        } #g
        # Derived and other quantities for (g in 1:nGrp){    totalNGrp[g] <- sum(N[,g])
          mean.det[g]  <- mean(p.list[,g])
          #for (j in 1:nRep){
          #        jg.p[j,g] <- mean(p[,j,g])
          #}
        }
        totalN <- sum(N[,])
        N.Class[1] <- totalNGrp[1] * fq.n1
        N.Class[2] <- totalNGrp[1] * fq.n2
        N.Class[3] <- totalNGrp[2] * fq.n3
        N.Class[4] <- totalNGrp[2] * fq.n4

        for (g in 1:nGrp){
          for (iv in 1:nSmp) {
            fit.list[iv,g] <- E[v[iv,1],v[iv,2],g]
            fit.new.list[iv,g] <- E.new[v[iv,1],v[iv,2],g]
            }
        }

        for (g in 1:nGrp){
          for (iw in 1:10) {
            jg.p[iw,g] <- mean(p.list[w[iw]:w[iw+1],g])  #vector that tells the number of sites
with
          }
        }

        fit <- sum(fit.list[,])
        fit.new <- sum(fit.new.list[,])
```

Appendix B

Table S3.1 Origin of Nile crocodile individuals used within the study for phylogeographic analyses for comparison of Nile crocodile distribution in Africa, using mtDNA control region. Indicating country of origin, river system, latitude, longitude, sample type and accession number.

Country	River System Locality	Latitude	Longitude	Samples type
Botswana	Okavango Delta	-18.25759	21.54353	Blood
	Okavango Delta	-18.21855	21.51949	Blood
	Okavango Delta	-18.25133	21.54088	Blood
	Okavango Delta	-23.72061	21.85913	Blood
	Okavango Delta	-18.28806	21.83225	Blood
	Okavango Delta	-18.25339	21.78478	Blood
	Okavango Delta	-18.46762	22.07741	Blood
	Okavango Delta	-18.32437	21.83028	Blood
	Okavango Delta	-18.28844	21.82317	Blood
	Okavango Delta	-18.29211	21.81610	Blood
	Okavango Delta	-18.41975	21.97619	Blood
	Okavango Delta	-18.73840	22.25302	Blood
	Okavango Delta	-18.75076	22.24855	Blood
	Okavango Delta	-18.72373	22.24522	Blood
	Okavango Delta	-18.72066	22.23969	Blood
	Okavango Delta	-18.71440	22.21235	Blood
	Okavango Delta	-18.71241	22.18826	Blood
	Okavango Delta	-18.75176	22.26060	Blood
	Okavango Delta	-18.70344	22.18106	Blood
	Okavango Delta	-18.68061	22.18108	Blood
	Okavango Delta	-18.66118	22.18812	Blood
	Okavango Delta	-18.80586	22.36799	Blood
	Okavango Delta	-18.60625	22.11565	Blood
	Okavango Delta	-18.61554	22.10932	Blood
	Okavango Delta	-18.62895	22.16772	Blood
	Okavango Delta	-18.61554	22.10932	Blood
Namibia	Okavango	-18.17831	21.74166	Scute
	Okavango	-18.17831	21.74166	Scute
	Okavango	-18.17831	21.74166	Scute
	Okavango	-18.17337	21.74191	Scute
	Okavango	-18.24451	21.78623	Scute

	Okavango	-18.24451	21.78623	Scute
	Okavango	-18.24451	21.78623	Scute
	Okavango	-18.17458	21.69928	Scute
	Okavango	-18.17337	21.74191	Scute
	Okavango	-18.17458	21.69928	Scute
	Okavango	-18.17458	21.69928	Scute
	Okavango	-18.17458	21.69928	Scute
	Okavango	-18.18167	21.74532	Scute
Namibia	Kunene	-17.21149	12.20063	Scute
	Kunene	-17.25853	13.57198	Scute
	Kunene	-17.36022	13.88841	Scute
	Kunene	-17.25853	13.57198	Scute
	Kunene	-17.25853	13.57198	Scute
	Kunene	-17.25853	13.57198	Scute
	Kunene	-17.25853	13.57198	Scute
	Kunene	-17.36022	13.88841	Scute
	Kunene	-17.23711	12.24267	Scute
	Kunene	-17.23711	12.24267	Scute
	Kunene	-17.23689	12.24006	Scute
	Kunene	-17.24008	12.25133	Scute

Table S3.2 Additional samples of Nile crocodile mtDNA control regions of publically available sequences. Geographic location, River System Locality, N – Number of samples, Accession Number and Source.

Region and Geographic location	River System Locality	N	Accession number	Source
West Africa				
Burkina Faso	Unknown	1	None	(Hekkala *et al*. 2011)
Gambia	Kedougou, Gambia river	1	None	(Hekkala *et al*. 2011)
Gambia	River Gambia, NP	2	None	(Hekkala *et al*. 2011)
Gambia	Gambia River	1	JF502243	(Meredith *et al*. 2011)*
Ghana	Mole National Park	1	None	(Hekkala *et al*. 2011)
Ivory Coast	Go River	1	None	(Hekkala *et al*. 2011)
Mauritania	Aioun el-Atrouss	1	None	(Hekkala *et al*. 2011)
Mauritania	Guelta Linsherbe	1	JF502244	(Meredith *et al.* 2011)*
Nigeria	Escravos River, Niger Delta	1	None	(Hekkala *et al*. 2011)
Senegal	Casamance River	1	None	(Hekkala *et al*. 2011)
East Africa				
Botswana	Okavango Delta	29	(See Appendix B: Table 1)	This study
Egypt	Lake Nasser, near Aswan	4	None	(Hekkala *et al*. 2011)
Gabon	Petit Loango, Loango NP	1	None	(Hekkala *et al.* 2011)
Kenya	Tana river	3	None	(Hekkala *et al*. 2011)
Malawi	Lower Shire	27	(See Appendix B: Table 1)	This study
Malawi	Lower Shire	25	(See Appendix B: Table 1)	This study
Malawi	Salima Bay	1	None	(Hekkala *et al*. 2011)
Namibia	Okavango river	20	(See Appendix B: Table 1)	This study
Namibia	Kunene river, Hartmann Valley	5	(See Appendix B: Table 1)	This study
Namibia	Kunene river, Swartboois Drift	7	(See Appendix B: Table 1)	This study
Namibia	Otjiwarongo crocodile ranch	13	(See Appendix B: Table 1)	This study
Republic of Congo	Likouala aux herbes	1	None	(Hekkala *et al*. 2011)
South Africa	Lake St. Lucia	1	None	(Hekkala *et al*. 2011)

Tanzania	Lake Rukwa	1	None	(Hekkala *et al*. 2011)
Tanzania	Rufiji River	1	None	(Hekkala *et al*. 2011)
Uganda	Kidepo Valley, NP	2	None	(Hekkala *et al*. 2011)
Uganda	Victoria Nile, Murchison falls NP	1	None	(Hekkala *et al*. 2011)
Uganda	Semliki River, Semuliki NP	1	None	(Hekkala *et al*. 2011)
Uganda	Lake Mburo, Ruizi Drainage, Lake Mburo NP	1	None	(Hekkala *et al*. 2011)
Zimbabwe	Lake Kariba	1	None	(Hekkala *et al*. 2011)
Zimbabwe	Sengwa, Lake Kariba	1	JF502245	(Meredith *et al.* 2011)*
Madagascar				
Madagascar	Ankarana Caves	2	None	(Hekkala *et al*. 2011)
Madagascar	Betsiboko river	1	None	(Hekkala *et al*. 2011)
Madagascar	Estuary, Fort Dauphin	1	None	(Hekkala *et al*. 2011)
Madagascar	Near Fort Dauphine	1	JF502246	(Meredith *et al.* 2011)*

* = sequences were excluded from the analyses due to short fragment lengths

Table S3.3 Eleven STR marker panel optimised for Nile crocodile genotyping in three PCR multiplex reactions and a singleplex reaction with primer information, repeat motif, dye label, estimated allele ranges, T_a - primer annealing temperature and PCR conditions of primers used. Loci were selected from [1](Miles *et al*. 2009a) and [2] (Bishop *et al.* 2009).

Primer	Primer sequence 5'-3'	Repeat motif	Dye label	Estimated allele range (bp)	T_a (°C)	PCR conditions
CpP1409[1]	F-GTTTATGCCCTACTGGTTATCTATC	$(AGAT)_n$	NED	250-302	55	MP1
	R-CAGTCGGGCGTCATCAGGGAAGGGGATTTAATAAT				55	
CpP2504[1]	F-CAGTCGGGCGTCATCACTCATATTTCCCAACTATCAC	$(AGAT)_n$	FAM	346-398	55	MP1
	R-GTTTCATTCCCACAATACACATAA				55	
CpP309[1]	F-GTTTAATACCTGGCATGTGTTCTTC	$(AAAC)_n$	PET	209-223	55	MP1
	R-CAGTCGGGCGTCATCACATCAGGTTGGCATTTCA				55	
CpP4311[1]	F-CAGTCGGGCGTCATCAGGCTGCTCTGTGTTTG	$(AGAT)_n$	FAM	194-222	55	MP1
	R-GTTTGGGTTTAGCATCATGT				55	
CpP218[1]	F-GTTTGGCATTTGAATTATTAACT	$(ACCC)_n$	PET	176-192	50	MP2
	R-CAGTCGGGCGTCATCACTGGCAAATCACTTCTG				50	
CpP307[1]	F-CAGTCGGGCGTCATCAGAAACCAGAGGCCAATA	$(ACAT)_n$	NED	338-398	50	MP2
	R-GTTTCTTGTCTTTGGCAGATT				50	
C391[2]	F-ATGAGTCAGGTGGCAGGTTC	$(CA)_n$	VIC	127-133	63	Singleplex
	R-CATAAATACACTTTTGAGCAGCAG				63	
Cj18[2]	F-ATCCAAATC CCATGAACCTGAGAG	$(CA)_n$	PET	202-218	63	MP3
	R-CCGAGTGCTTACAAGAGGCTGG				63	

CUD68[2]	F-GCTTCAGCAGGGGCTACC	$(CA)_n$	NED	112-128	63	MP3
	R-TGGGGAAACTGCACTTTAGG				63	
CJ119[2]	F-GTTTGCTGTGGAATGTTTCTAC	$(CA)_n$	FAM	160-190	63	MP3
	R-CGCTATATGAAACGGTGGCTG				63	
Cj35[2]	F-GTTTAGAAGTCTCCAAGCCTCTCAG	$(CT)_nTA(CA)_n(CT)_n$	VIC	156-166	63	MP3
	R-CTGGGGCAAGGATTTAACTCTC				63	

Table S3.4 Genetic diversity for the Nile crocodile, Crocodylus niloticus, integrated over all mtDNA control region haplotypes from each sampling location. N - number of samples, H - number of haplotypes (unique haplotypes), h - haplotype diversity, π - nucleotide diversity, k - mean number of nucleotide differences between haplotypes.

	N	*H*	h	*π*	*k*
Kunene	12	1 (1)	0	0	0
Okavango	52	1 (1)	0	0	0
Malawi	47	4 (4)	0.332±0.083	0.015±0.008	8.144

Table S3.5 Genetic diversity measures and Hardy-Weinberg Equilibrium test in Southern Africa Nile crocodile populations. a) over all populations, b) Kunene population, c) Okavango populations (Bwabwatwa National Park, Okavango Delta and Otjiwarongo Crocodile Farm), d) Shire populations (Shire (North) and Shire (South) and e) South Africa commercial samples. N - number of individuals, An - number of alleles, He - expected heterozygosity, Ho - observed heterozygosity, HWE - Hardy Weinberg Equilibrium test (P-value), Rs - mean allelic richness, Fis - mean frequency of inbreeding coefficient, Null Alleles – Brookfield 1, Ewens-Watterson homozygosity test Frequencies (P-value) and PIC - polymorphic information content.

Primer	N	A_n	H_e	H_o	HWE (*P*)	Rs	Fis	Null Alleles	EW test F (*P*)	PIC
a)										
CpP1409	135	28	0.931	0.807	0.000	26.960	0.133	0.0621	0.035 (0.041)	0.923
CpP2504	138	20	0.791	0.667	0.002	19.598	0.157	0.0681	0.821 (0.312)	0.771
CpP309	139	4	0.589	0.547	0.019	3.777	0.071	0.025	0.135 (0.346)	0.505
CpP4311	139	8	0.724	0.655	0.000	7.728	0.096	0.039	0.218 (0.180)	0.687
CpP218	137	5	0.557	0.482	0.010	5.000	0.135	0.0471	0.334 (0.089)	0.511
CpP307	110	23	0.887	0.655	0.000	22.927	0.263	0.1211	0.397 (0.325)	0.872
C391	137	4	0.560	0.489	0.000	4.000	0.127	0.044	0.188 (0.090)	0.496
Cj18	108	8	0.748	0.713	0.004	8.000	0.048	0.018	0.168 (0.079)	0.707
CUD68	134	6	0.706	0.582	0.000	5.956	0.177	0.0711	0.098 (0.211)	0.649
Cj119	136	10	0.784	0.640	0.002	9.546	0.184	0.0791	0.161 (0.320)	0.747
Cj35	130	6	0.553	0.446	0.000	5.831	0.194	0.0681	0.489 (0.382)	0.506
Mean		11.1	0.712	0.607	0.003	10.848	0.144	0.058	0.277 (0.216)	0.670
b) Kunene										
CpP1409	12	5	0.750	0.833	0.817	4.917	-0.117	-0.067	0.188 (0.182)	0.675
CpP2504	12	3	0.554	0.500	0.740	2.917	0.102	0.020	0.309 (0.469)	0.428
CpP309	12	3	0.562	0.500	1.000	2.917	0.114	0.025	0.258 (0.428)	0.432
CpP4311	12	4	0.659	0.333	0.042	3.917	0.506	0.183[1]	0.289 (0.378)	0.561
CpP218	12	4	0.576	0.583	0.678	3.993	-0.013	-0.020	0.549 (0.367)	0.506
CpP307	12	9	0.855	0.750	0.493	8.736	0.128	0.038	0.482 (0.393)	0.798
C391	11	2	0.455	0.455	1.000	2.000	0.000	-0.014	0.242 (0.242)	0.340
Cj18	11	4	0.710	0.545	0.521	4.000	0.241	0.079	0.141 (0.222)	0.615
CUD68	12	3	0.301	0.333	1.000	2.917	-0.114	-0.035	0.823 (0.823)	0.264
Cj119	12	5	0.435	0.250	0.053	4.750	0.436	0.118	0.962 (0.962)	0.393
Cj35	11	4	0.558	0.364	0.119	4.000	0.360	0.111	0.656 (0.599)	0.482
Mean		4	0.583	0.495	0.587	4.097	0.149	0.040	0.445 (0.460)	0.499
c) Okavango										
Bwabwatwa National Park										
CpP1409	19	9	0.784	0.789	0.106	7.076	-0.007	-0.015	0.648 (0.715)	0.734
CpP2504	20	10	0.606	0.600	0.281	7.333	0.011	-0.006	0.986 (0.960)	0.577
CpP309	20	2	0.513	0.500	1.000	2.000	0.026	0.000	0.012 (0.012)	0.375
CpP4311	20	4	0.568	0.650	0.824	3.797	-0.149	-0.062	0.431 (0.230)	0.509
CpP218	20	3	0.145	0.150	1.000	2.354	-0.036	-0.008	0.937 (0.937)	0.136

CpP307	20	10	0.823	0.800	0.516	7.629	0.029	0.001	0.531 (0.681)	0.778
C391	20	3	0.512	0.400	0.468	2.550	0.223	0.066	0.330 (0.525)	0.397
Cj18	15	5	0.678	0.667	0.877	4.666	0.018	-0.007	0.407 (0.387)	0.605
CUD68	18	3	0.514	0.556	0.844	2.997	-0.083	-0.037	0.326 (0.170)	0.449
Cj119	20	4	0.729	0.750	0.351	3.966	-0.029	-0.023	0.022 (0.28)	0.657
Cj35	19	5	0.721	0.789	0.505	4.919	-0.098	-0.051	0.172 (0.051)	0.665
Mean		5	0.599	0.605	0.616	4.481	-0.009	-0.013	0.437 (0.427)	0.535
Okavango Delta										
CpP1409	28	12	0.880	0.750	0.001*	8.891	0.150	0.061	0.126 (0.210)	0.850
CpP2504	29	13	0.685	0.724	0.510	8.126	-0.058	-0.030	0.981 (0.912)	0.660
CpP309	29	2	0.503	0.345	0.134	2.000	0.319	0.100	0.051 (0.051)	0.372
CpP4311	29	4	0.551	0.586	1.000	3.748	-0.065	-0.029	0.397 (0.182)	0.498
CpP218	28	3	0.229	0.250	1.000	2.563	-0.092	-0.020	0.729 (0.656)	0.211
CpP307	24	8	0.717	0.708	0.812	5.975	0.013	-0.004	0.659 (0.683)	0.656
C391	29	3	0.556	0.690	0.404	2.769	-0.246	-0.093	0.140 (0.231)	0.443
Cj18	28	5	0.738	0.679	0.157	4.844	0.082	0.027	0.063 (0.032)	0.680
CUD68	27	5	0.665	0.704	0.622	4.529	-0.059	-0.031	0.277 (0.153)	0.608
Cj119	27	5	0.624	0.481	0.059	4.180	0.232	0.081	0.409 (0.405)	0.553
Cj35	29	6	0.716	0.828	0.166	4.616	-0.160	-0.073	0.269 (0.435)	0.649
Mean		6.0	3.083	0.624	0.613	0.011	4.749	0.562	-0.001	0.442
Otjiwarongo Crocodile Farm										
CpP1409	13	6	0.778	0.923	0.721	5.692	-0.195	-0.100	0.252 (0.307)	0.711
CpP2504	13	6	0.745	0.769	0.697	5.825	-0.034	-0.031	0.444 (0.264)	0.682
CpP309	13	2	0.409	0.538	0.499	2.000	-0.333	-0.104	0.287 (0.287)	0.316
CpP4311	13	4	0.588	0.538	0.664	3.828	0.087	0.017	0.499 (0.569)	0.496
CpP218	13	2	0.148	0.154	1.000	1.982	-0.043	-0.010	0.725 (0.725)	0.132
CpP307	12	8	0.757	0.750	0.739	7.659	0.010	-0.014	0.850 (0.831)	0.697
C391	13	3	0.643	0.615	0.695	3.000	0.045	0.002	0.075 (0.071)	0.546
Cj18	12	3	0.507	0.500	0.0234	2.996	0.015	-0.009	0.443 (0.342)	0.424
CUD68	13	4	0.455	0.385	0.450	3.843	0.161	0.037	0.762 (0.666)	0.407
Cj119	13	5	0.785	0.462	0.0059	4.846	0.422	0.167[1]	0.048 (0.101)	0.712
Cj35	12	4	0.370	0.250	0.408	3.833	0.333	0.077	0.920 (0.920)	0.330
Mean		4	0.562	0.535	0.537	4.137	0.043	0.003	0.482 (0.416)	0.496
Total Mean		5.18	0.595	0.584	0.531	4.456	0.015	-0.004	0.431 (0.416)	0.531
d) Shire										
Shire (North)										
CpP1409	26	15	0.908	0.846	0.529	10.641	0.069	0.024	0.188 (0.227)	0.881
CpP2504	27	14	0.755	0.667	0.195	8.297	0.119	0.043	0.968 (0.976)	0.720
CpP309	27	3	0.611	0.630	0.102	2.997	-0.030	-0.018	0.067 (0.055)	0.531

CpP4311	27	6	0.825	0.667	0.024	5.769	0.194	0.079	0.001 (0.004)	0.781
CpP218	27	5	0.662	0.704	0.873	4.288	-0.065	-0.033	0.295 (0.278)	0.591
CpP307	20	8	0.721	0.450	0.003*	6.700	0.382	0.148[1]	0.727 (0.502)	0.677
C391	27	3	0.414	0.370	0.093	2.799	0.108	0.026	0.469 (0.334)	0.358
Cj18	18	7	0.840	0.889	0.931	6.768	-0.060	-0.040	0.028 (0.011)	0.793
CUD68	27	4	0.649	0.741	0.417	3.781	-0.144	-0.063	0.151 (0.212)	0.572
Cj119	27	8	0.756	0.741	0.420	6.360	0.021	0.001	0.445 (0.327)	0.715
Cj35	24	3	0.159	0.083	0.124	2.308	0.480	0.062	0.880 (0.880)	0.148
Mean		7	0.664	0.617	0.337	5.519	0.098	0.021	0.384 (0.338)	0.615
Shire (South)										
CpP1409	25	17	0.906	0.800	0.0095	11.200	0.119	0.047	0.551 (0.623)	0.879
CpP2504	25	11	0.793	0.600	0.010	7.404	0.248	0.100[1]	0.725 (0.836)	0.748
CpP309	25	3	0.624	0.560	0.047	2.999	0.104	0.032	0.062 (0.054)	0.541
CpP4311	25	7	0.810	0.840	0.951	6.201	-0.038	-0.026	0.058 (0.054)	0.764
CpP218	25	5	0.711	0.720	0.370	4.720	-0.013	-0.0137	0.151 (0.088)	0.647
CpP307	22	13	0.855	0.545	0.001*	9.609	0.368	0.158[1]	0.679 (0.547)	0.819
C391	25	3	0.438	0.400	0.112	2.832	0.089	0.021	0.450 (0.306)	0.375
Cj18	24	5	0.720	0.833	0.511	4.690	-0.162	-0.075	0.127 (0.091)	0.654
CUD68	25	4	0.610	0.440	0.058	3.603	0.283	0.099	0.266 (0.271)	0.519
Cj119	25	6	0.774	0.800	0.965	5.685	-0.034	-0.024	0.084 (0.031)	0.725
Cj35	24	2	0.284	0.333	1.000	1.996	-0.179	-0.044	0.376 (0.376)	0.239
Mean		7	0.684	0.625	0.367	5.540	0.071	0.025	0.321 (0.298)	0.628
Total Mean		6.9	0.674	0.621	0.352	5.529	0.085	0.023	0.352 (0.318)	0.622
e)										
South Africa										
CpP1409	12	6	0.801	0.750	0.222	3.993	-0.333	-0.0435	0.173 (0.080)	0.736
CpP2504	12	8	0.790	0.833	0.129	7.659	-0.058	-0.1733	0.707 (0.775)	0.723
CpP309	13	3	0.665	0.923	0.010	3.000	-0.412	-0.1954	0.042 (0.042)	0.566
CpP4311	13	4	0.566	0.846	0.092	3.692	-0.526	-0.1404	0.559 (0.741)	0.462
CpP218	12	4	0.634	0.833	0.577	3.000	0.452	0.000	0.356 (0.266)	0.547
CpP307	0	0	0.000	0.000	0.000	0.000	0.000	-0.077	0.000 (0.000)	0.000
C391	12	3	0.409	0.500	1.000	2.917	-0.234	0.000	0.649 (0.684)	0.341
Cj18	0	0	0.000	0.000	0.000	0.000	0.000	0.002	0.000 (0.000)	0.000
CUD68	12	5	0.728	0.833	0.041	4.996	0.048	-0.080	0.056 (0.039)	0.716
Cj119	12	6	0.786	0.750	0.648	5.826	-0.152	0.0978	0.546 (0.597)	0.649
Cj35	11	3	0.325	0.182	0.280	5.989	0.066	-0.055	0.806 (0.806)	0.282
Mean		4	0.519	0.586	0.273	3.734	-0.104	-0.0435	0.354 (0.366)	0.457

* = Hardy Weinberg Equilibrium significance after Bonferroni correction for multiple tests (p-level<0.0045).

[1] = Presence of Null alleles

Table S3.6 Nile crocodile populations (Lower Kunene, Bwabwatwa, Okavango Delta, Otjiwarongo Crocodile Ranch, Lower Shire (North), Lower Shire (South) and South Africa Commercial samples) allele frequencies for 11 STR loci (N = 139), including; $Allele_n$ = mean allele size and N = number of individuals.

Locus	$Allele_n$	Lower Kunene	Bwabwatwa	Okavango Delta	Otjiwarongo Crocodile Ranch	Lower Shire (North)	Lower Shire (South)	South Africa Commercial
C_391	**N**	11	20	29	13	27	25	12
	127	0,318	0,600	0,448	0,500	0,741	0,720	0,750
	129	-	0,025	0,052	0,308	-	-	-
	131	0,682	0,375	0,500	0,192	0,056	0,060	0,208
	133	-	-	-	-	0,204	0,220	0,042
Cj_119	**N**	12	20	27	13	27	25	12
	160	-	-	0,019	-	-	-	-
	166	0,042	0,375	0,537	0,038	0,111	0,100	0,417
	168	-	-	-	0,231	0,019	-	0,083
	178	-	0,100	0,074	0,154	0,130	0,060	-
	180	0,125	0,275	0,074	0,308	0,444	0,380	0,333
	182	0,750	0,250	0,296	0,269	0,111	0,240	0,042
	184	0,042	-	-	-	-	-	-
	186	-	-	-	-	0,037	-	-
	188	0,042	-	-	-	0,130	0,140	0,083
	190	-	-	-	-	0,019	0,080	0,042
Cj_18	**N**	11	15	28	12	18	24	0
	202	0,318	0,500	0,321	0,667	0,167	0,375	-
	204	-	-	-	-	-	0,063	-
	208	-	0,067	0,089	-	0,111	-	-
	210	0,045	0,133	0,107	0,250	0,167	0,146	-
	212	-	-	-	-	0,111	-	-
	214	-	-	-	-	0,083	0,063	-
	216	0,227	0,267	0,375	0,083	0,306	0,354	-
	218	0,409	0,033	0,107	-	0,056	-	-
Cj_35	**N**	11	19	29	12	24	24	11
	156	-	-	0,017	-	-	-	-
	158	-	0,079	0,034	-	-	-	-
	160	0,636	0,158	0,379	0,792	0,917	0,833	0,818
	162	0,091	0,474	0,328	0,042	0,063	0,167	0,136
	164	0,045	0,158	0,034	0,042	-	-	-
	166	0,227	0,132	0,207	0,125	0,021	-	0,045
CpP_1409	**N**	12	19	28	13	26	25	12
	250	-	-	-	-	0,038	-	-
	252	-	-	-	-	0,019	-	-
	256	-	-	-	-	-	0,020	-
	257	-	-	-	-	-	-	0,375
	258	-	0,395	0,214	0,231	0,115	0,040	-
	259	-	-	-	-	-	-	0,167
	260	0,167	0,026	0,196	-	0,115	-	-
	262	0,042	-	0,018	0,038	0,019	0,120	-
	264	-	-	0,018	-	0,038	0,020	-

	266	-	-	-	-	-	0,020	-
	268	-	0,026	-	0,385	-	0,020	-
	270	-	-	0,018	-	-	0,020	-
	272	-	0,079	0,054	-	0,019	0,120	-
	274	-	-	0,071	-	0,192	0,220	-
	276	-	0,026	-	-	-	0,020	-
	277	-	-	-	-	-	0,000	0,083
	278	0,250	-	-	0,038	0,096	0,140	-
	280	-	0,211	0,125	-	0,038	0,040	-
	281	-	-	-	-	-	-	0,083
	282	0,417	0,053	0,125	0,154	0,058	0,040	-
	284	-	-	-	-	0,058	0,060	-
	285	-	-	-	-	-	-	0,208
	286	0,125	-	-	-	0,019	0,020	-
	287	-	-	-	-	-	-	0,083
	288	-	0,158	0,054	-	-	-	-
	290	-	0,026	0,089	0,154	0,019	0,020	-
	292	-	-	0,018	-	-	-	-
	302	-	-	-	-	0,154	0,060	-
CpP_218	**N**	12	20	28	13	27	25	12
	176	0,208	-	-	-	0,481	0,420	0,292
	180	-	0,025	0,036	-	0,037	0,080	-
	184	0,083	-	-	0,077	0,130	0,120	0,083
	188	0,625	0,925	0,875	0,923	0,315	0,320	0,542
	192	0,083	0,050	0,089	-	0,037	0,060	0,083
CpP_2504	**N**	12	20	29	13	27	25	12
	346	-	0,050	0,052	0,115	0,037	-	-
	348	-	-	-	-	0,056	0,040	-
	360	0,542	0,075	0,052	0,115	0,037	0,060	0,042
	362	-	-	0,017	-	-	-	0,042
	364	-	-	0,017	-	0,463	0,280	0,083
	366	-	-	0,034	-	0,019	-	-
	368	0,417	0,625	0,552	0,462	0,167	0,340	0,333
	370	-	-	-	-	0,019	0,020	-
	372	-	0,050	0,052	0,038	0,093	0,140	0,083
	374	-	-	0,017	-	0,019	0,020	-
	376	0,042	-	-	0,077	-	-	-
	378	-	0,025	0,017	0,192	0,019	0,020	-
	380	-	0,050	-	-	-	-	-
	382	-	0,050	0,086	-	0,019	-	0,042
	384	-	-	-	-	0,019	0,040	0,042
	386	-	-	-	-	-	-	0,333
	388	-	0,025	0,052	-	-	0,020	-
	390	-	0,025	0,017	-	0,019	0,020	-
	392	-	0,025	0,034	-	-	-	-
	398	-	-	-	-	0,019	-	-
CpP_307	**N**	12	20	24	12	20	22	0
	340	0,250	-	-	-	0,100	0,045	-

	342	0,083	0,025	0,021	0,083	0,500	0,227	-
	344	0,042	0,150	0,083	-	-	0,045	-
	348	-	-	-	-	-	0,023	-
	350	-	-	-	-	0,150	0,295	-
	352	-	-	-	-	0,075	-	-
	356	-	0,025	-	0,042	-	-	-
	358	-	-	-	0,042	-	-	-
	360	0,083	0,025	-	-	-	0,023	-
	362	0,292	0,325	0,417	0,208	-	-	-
	366	-	-	-	-	0,025	0,068	-
	368	0,042	0,225	0,333	0,458	-	-	-
	370	0,042	-	-	-	-	0,045	-
	372	-	0,075	0,021	0,042	-	0,045	-
	374	-	-	-	0,042	0,075	0,091	-
	376	-	0,025	0,042	-	-	-	-
	378	-	-	-	-	0,050	0,023	-
	380	0,083	-	-	-	-	-	-
	382	-	-	-	-	-	0,023	-
	384	0,083	0,100	0,042	0,083	-	-	-
	386	-	0,025	-	-	-	-	-
	388	-	-	-	-	0,025	0,045	-
	398	-	-	0,042	-	-	-	-
CpP_309	**N**	12	20	29	13	27	25	13
	209	-	-	-	-	0,185	0,280	0,308
	213	0,500	0,500	0,552	0,731	0,537	0,520	0,231
	217	0,458	0,500	0,448	0,269	0,278	0,200	0,462
	223	0,042	-	-	-	-	-	-
CpP_4311	**N**	12	20	29	13	27	25	13
	194	-	-	-	-	-	0,040	-
	198	0,042	-	-	-	-	-	-
	202	0,125	0,175	0,138	0,077	0,148	0,300	0,346
	206	-	0,150	0,172	0,308	0,204	0,240	0,038
	210	0,458	0,625	0,638	0,577	0,241	0,180	0,577
	214	0,375	0,050	0,052	0,038	0,056	0,060	0,038
	218	-	-	-	-	0,222	0,040	-
	222	-	-	-	-	0,130	0,140	-
CUD_68	**N**	12	18	27	13	27	25	12
	112	-	-	-	-	-	-	0,083
	116	-	-	0,074	-	0,130	0,040	0,208
	118	0,042	0,167	0,148	0,115	0,333	0,380	0,167
	120	0,125	0,167	0,222	0,115	0,481	0,500	0,375
	122	0,833	0,667	0,519	0,731	0,056	0,080	0,167
	128	-	-	0,037	0,038	-	-	-

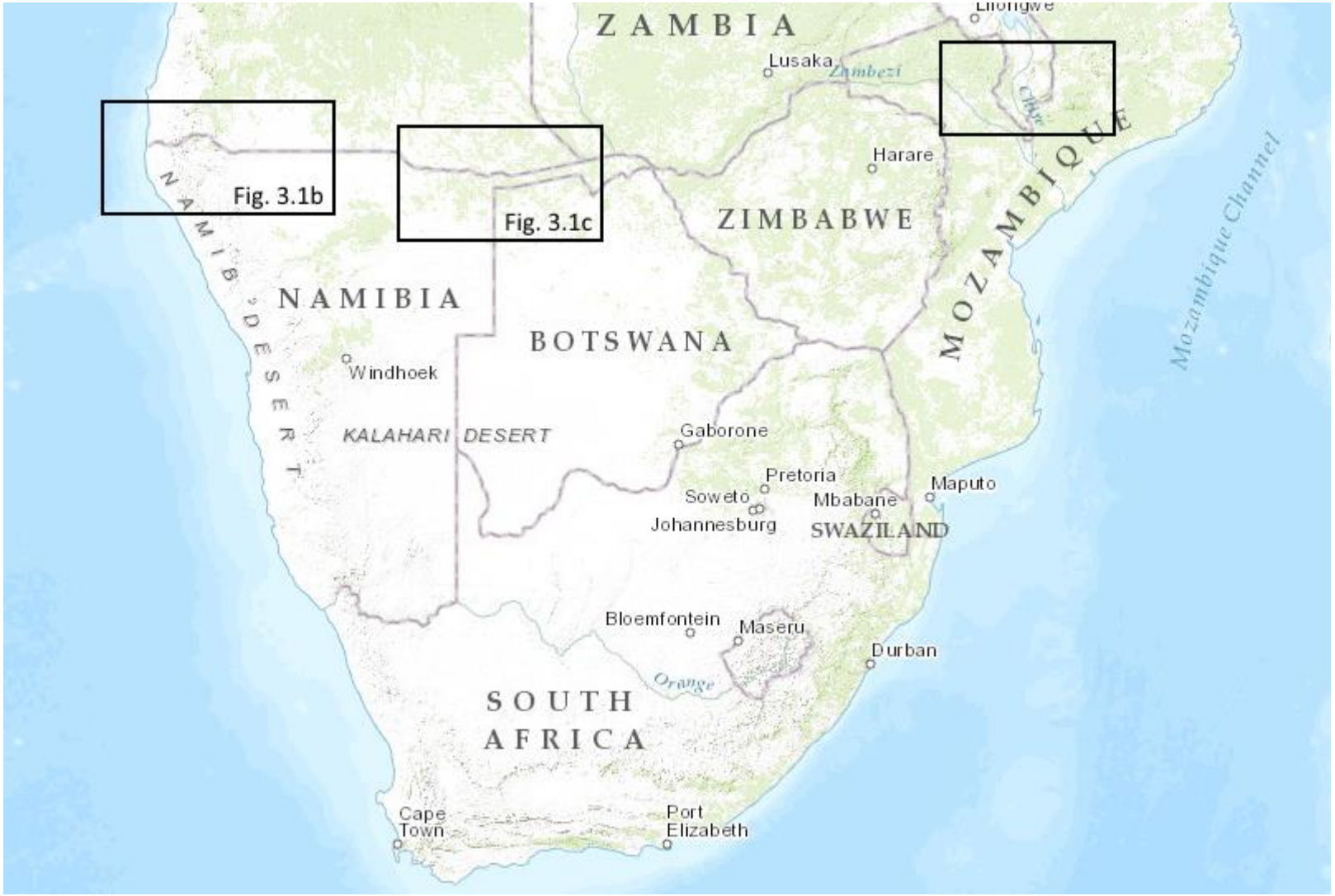

Figure S3.1 Map of Southern Africa river system indicating crocodile capturing sites. Within each of the three different river syetems, Fig. 3.1b Kunene river syetsm, Fig. 3.1c Okavango river systems and the third Shire river system with noexact GPS cooridiantes for crocodile capturing site locations.

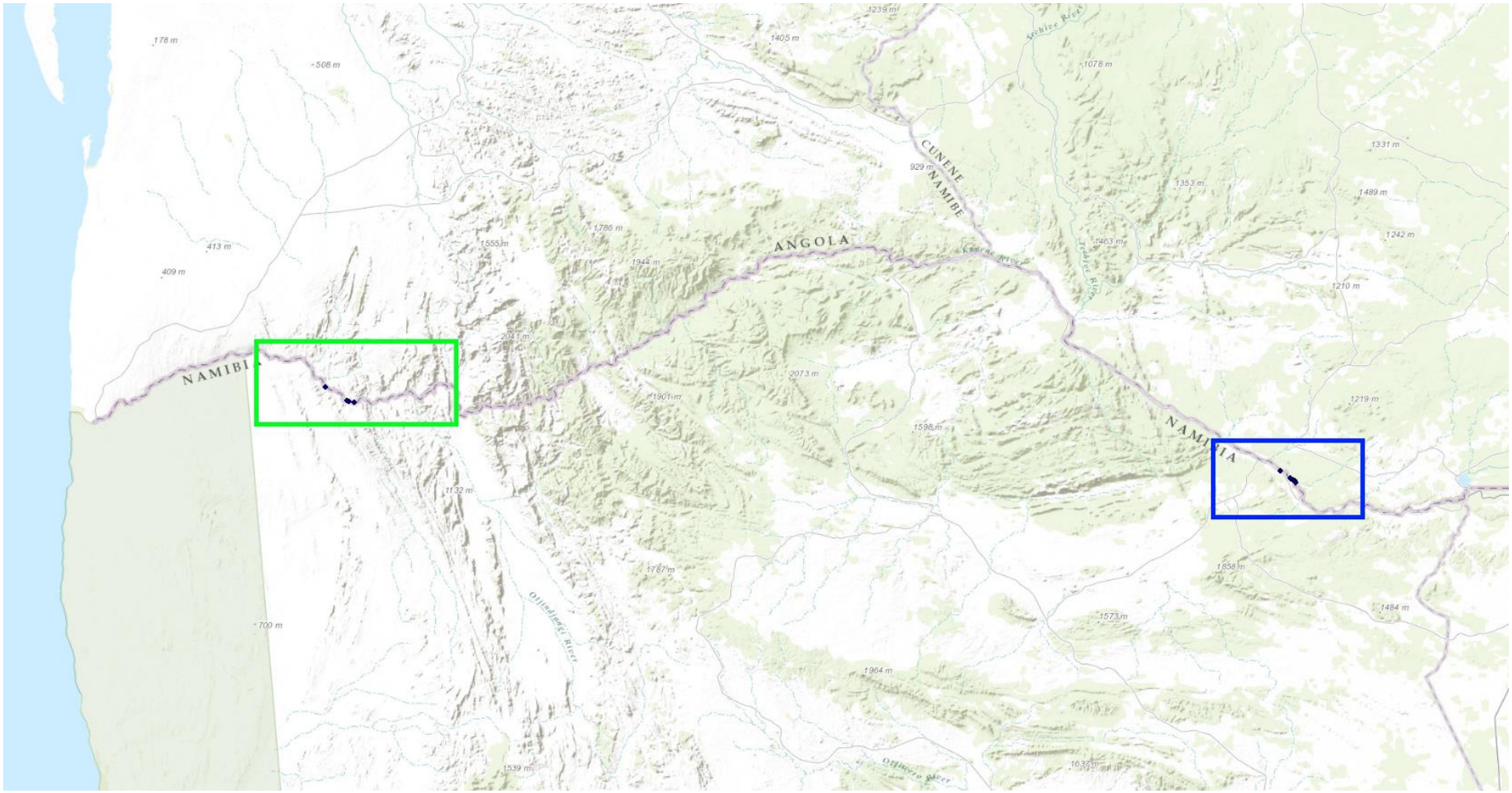

Figure S3.1b Map of the Lower Kunene river system and crocodile capturing sites. On the left (green square) capturing site Serra Cafema and on the right (blure sqaure) capturing site East of Swart Boois drift. Blue dots indicate sampling location of Nile crocodile individuals used within this study.

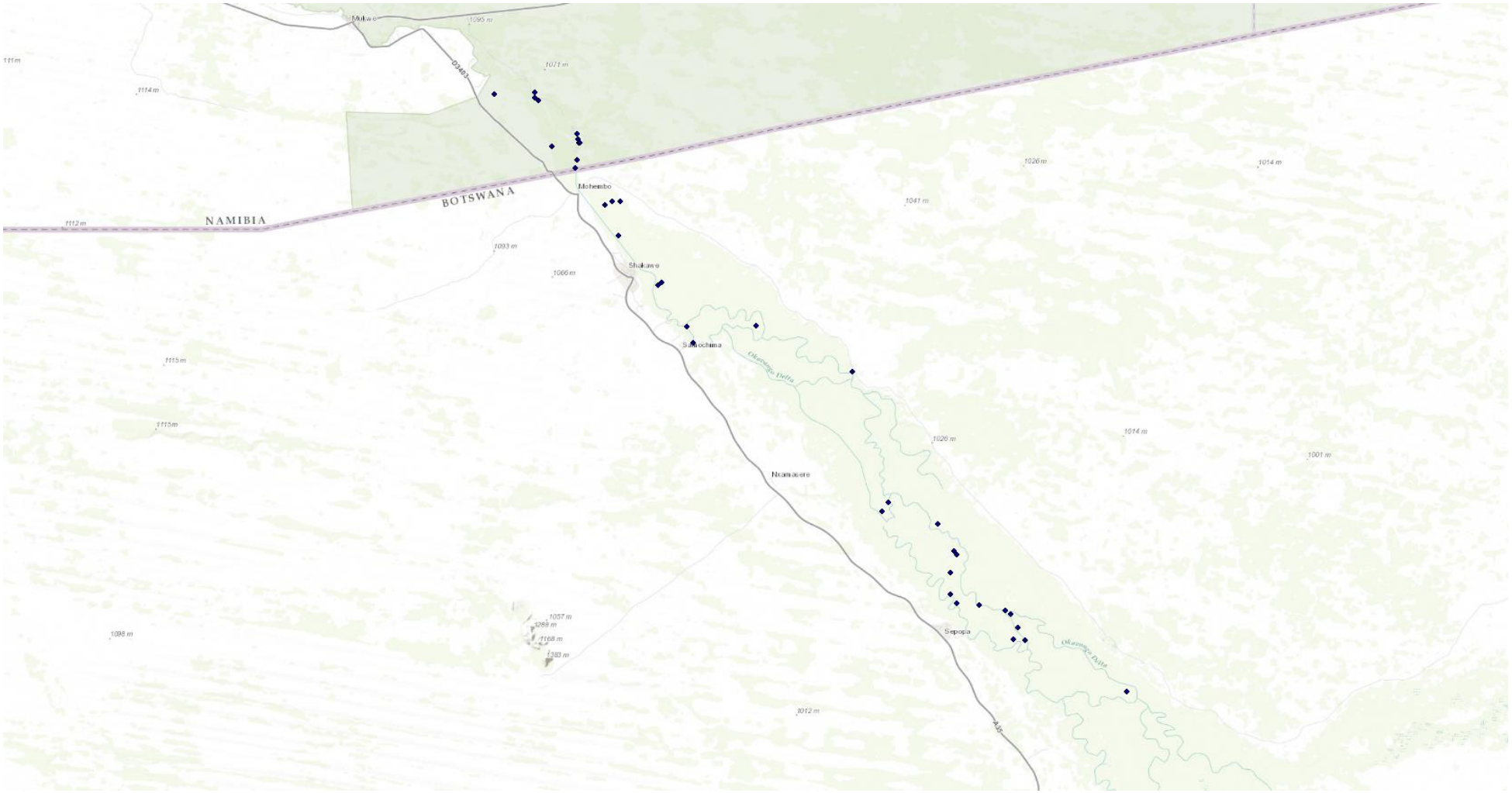

Figure S3.1c Map of the Okavango river system from Namibia and the Okavango Delta in Botswana. Blue dots indicate sampling location of Nile crocodile individuals used within this study.

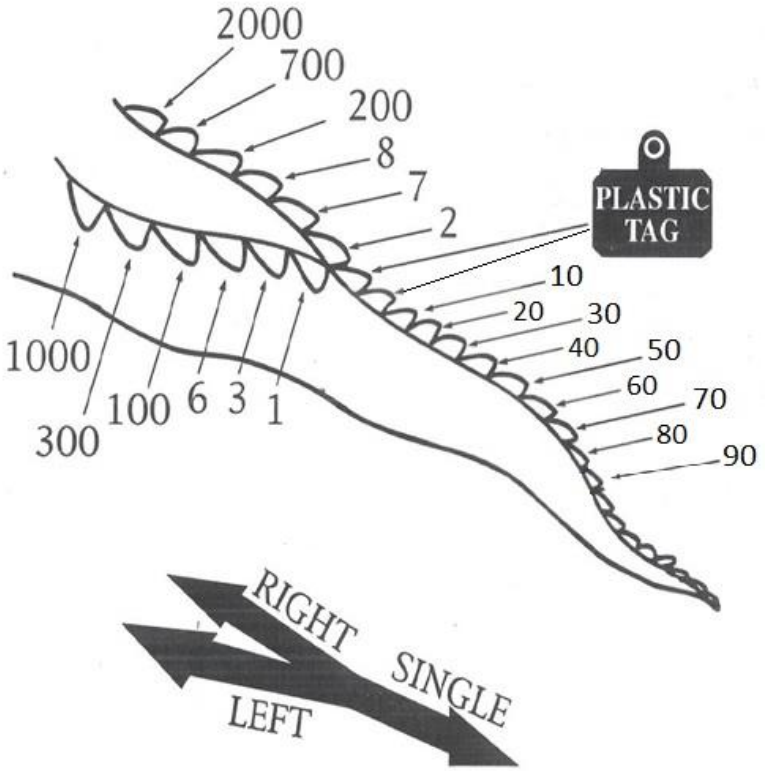

Figure S3.2 Scute cut removal system for Nile crocodile individual identification in the wild. Drafted from Leslie et al., 1997

Mitochondrial DNA sequences were aligned using ClustalW function implemented in Geneious v7.1 (Kearse *et al.* 2012) and constructed a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree within the Geneious Tree Builder using the Tamura-Nei genetic distance model.

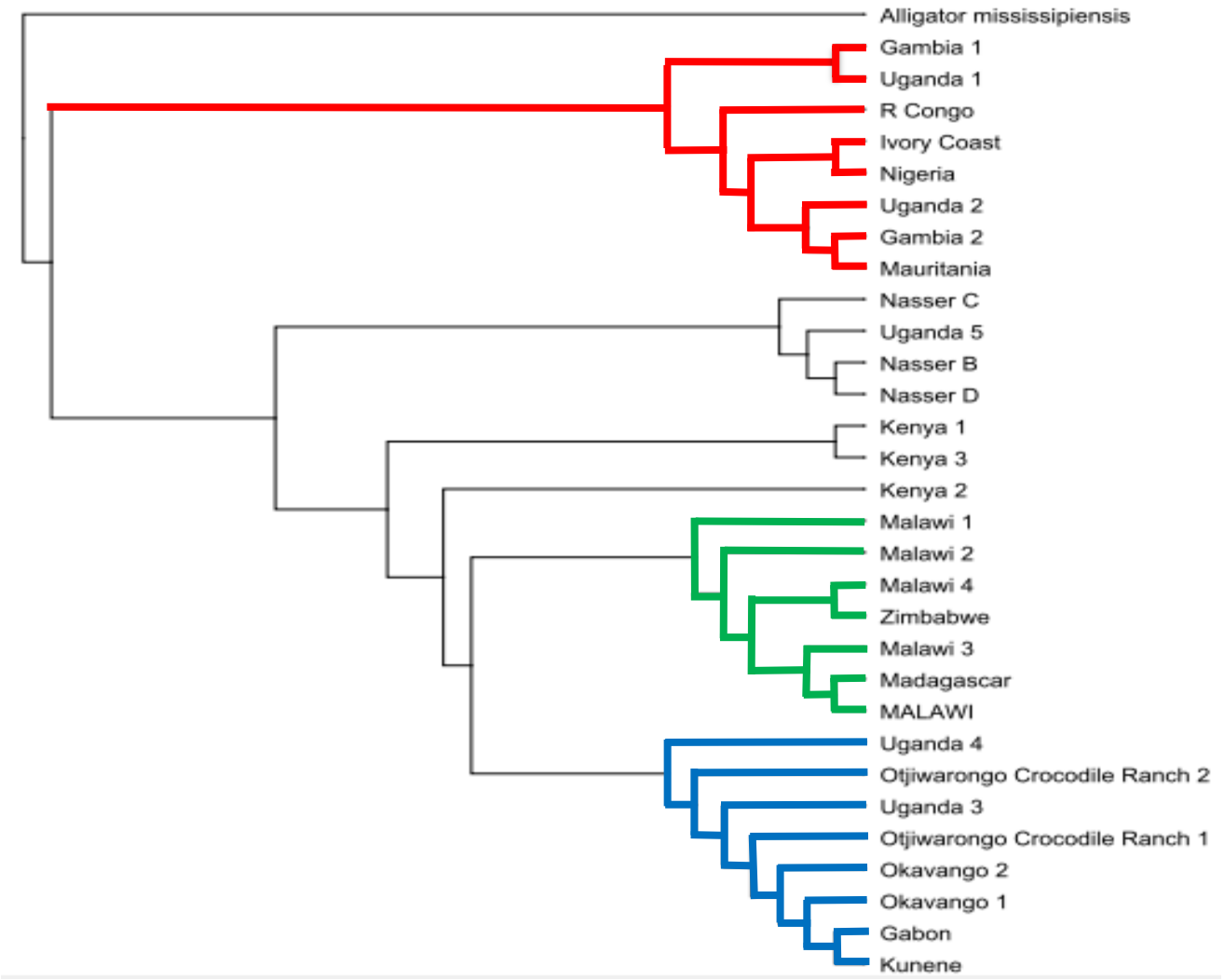

Figure S3.3 An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree of mtDNA control region sequences used within the study for the Lower Kunene, Okavango, Lower Shire and publically available sequences (Hekkala *et* al. 2011) for the Nile crocodile in Africa, considering *Alligator mississipiensis* as the outgroup. Redlines indicate the separation of the western Nile crocodile clade as described by Schmitz *et al*. (2003) and the black lines the eastern clade. Of which the eastern clade consist of a further two lineages within Southern and eastern Africa. Lineage 1: Green and Lineage 2: Blue for Southern Africa.

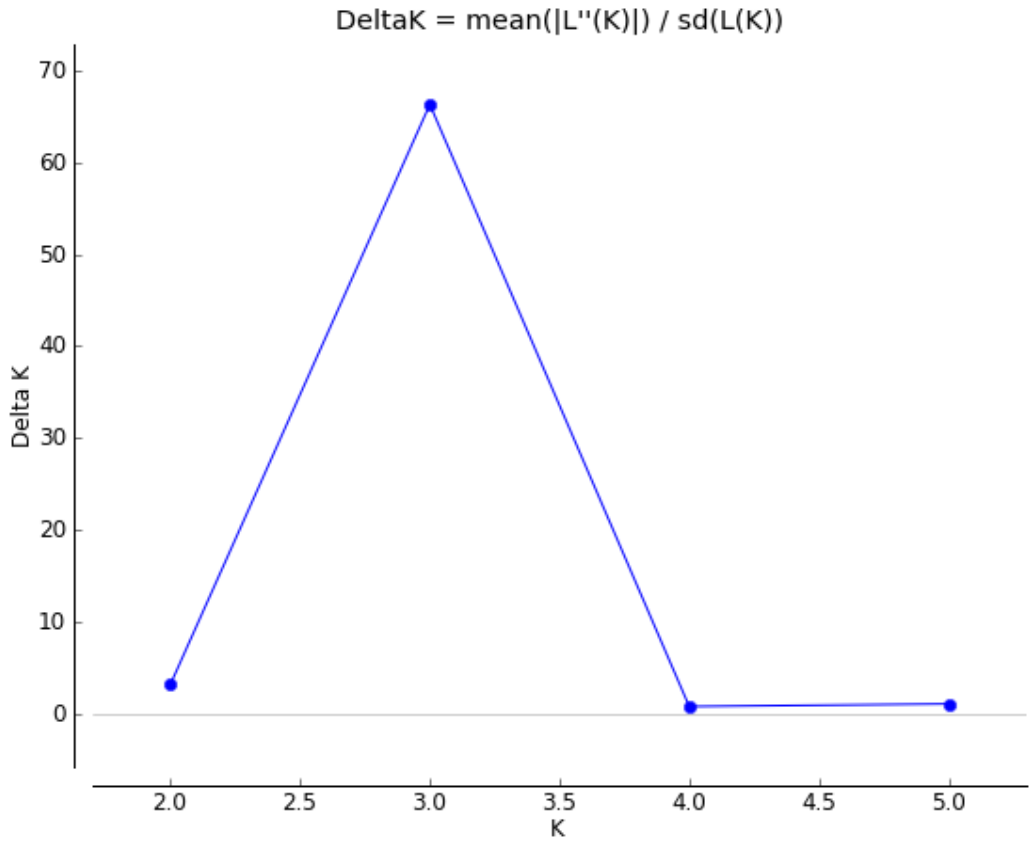

Figure S3.4 Delta K vs K for number of population detection without prior assumption of populations in the Kunene and Okavango river populations. Results indicate two distinct populations present within the Kunene and Okavango river systems in Northern Namibia.

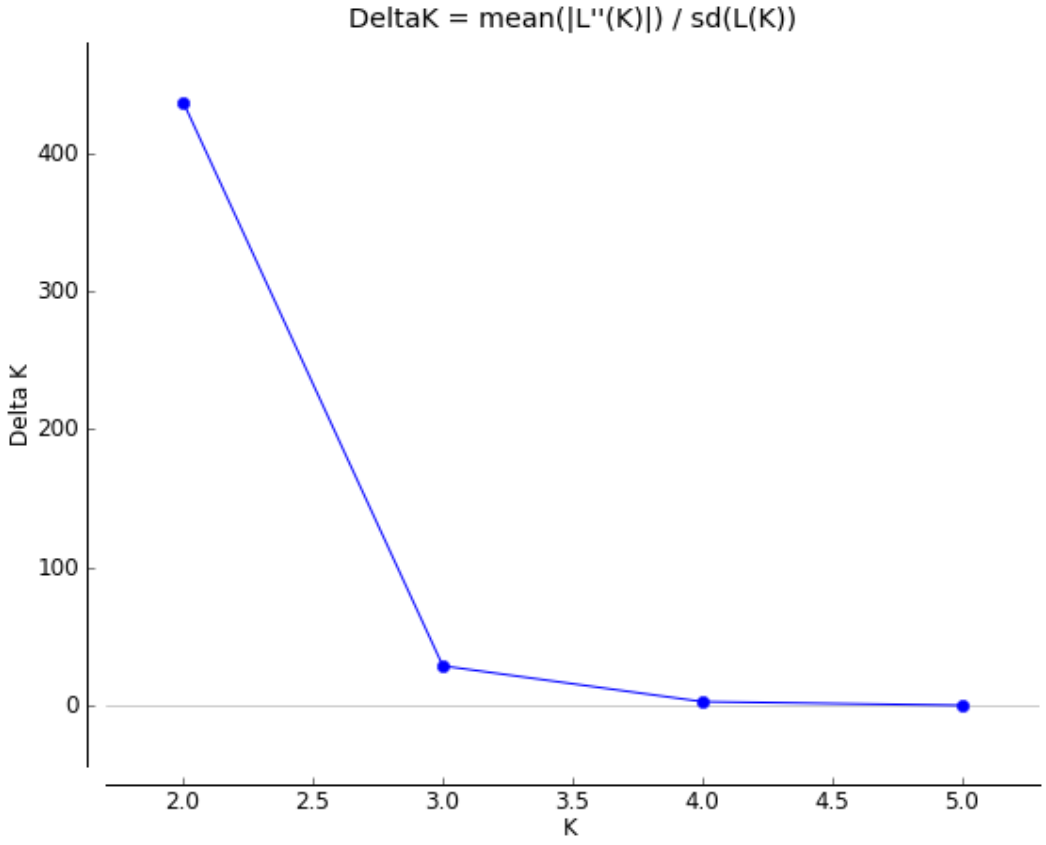

Figure S3.5 Delta K vs K for number of population detection without prior assumption of populations in Southern Africa rivers, Kunene, Okavango, Shire and South Africa Rivers. Results indicate two distinct populations present within Southern Africa