Project Report

Nile crocodile genetic diversity and population structure, within the Kunene and Okavango rivers, Northern Namibia.

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Introduction

The Nile crocodile, *Crocodylus niloticus,* is found throughout sub-Saharan Africa, with its distribution limited to river basin formations (Hekkala *et al*. 2011). Within southern Africa the species has been studied based on behavioural and genetic diversity. However, available genetic information crucial in identifying management units for making informed conservation decisions is limited for this species.

In Namibia, the Ministry of Environment and Tourism have recently investigated the abundance and distribution of the Nile crocodile to establish management plans. The genetic assessment of the Kunene and Okavango Nile crocodile populations will help to establish effective management plans for the species.

The objectives of this study were:

- To estimate *C. niloticus* genetic diversity and population structure within the Kunene and Okavango river systems;
- To compare the Kunene and Okavango river systems' genetic diversity and population structure to the neighbouring river system in Malawi (Lower Shire river);and samples from unknown origin collected at a South African crocodile farm (Izintaba Crocodile Farm);
- To generate haplotype diversity and structure estimates of Nile crocodiles in the Kunene, Okavango and Lower Shire river systems;
- To determine whether the Kunene, Okavango and Lower Shire river populations cluster within the Western or Eastern clade;
- To develop a strategy for a management plan of the Kunene and Okavango river systems with regards to Nile crocodile conservation.

Materials and Methods

Sample Collection and DNA Extraction

A total of 139 Nile crocodile samples were collected from wild and wild-caught, ranch held live specimens. The samples originated from the different countries and regions: Botswana (Okavango Delta, n=29), Malawi (Lower Shire river North, n=27; Lower Shire river South n=25,), Namibia [Kunene, n=12; Okavango river (Bwabwatwa National Park n=20; Otjiwarongo Crocodile Ranch (known to descend from the Okavango population, n=13] and South Africa (Izintaba Crocodile Farm, origin unknown, n=13) (Fig 1). Total DNA was extracted using a standard CTAB protocol (Saghai-Maroof *et al*. 1984).

STR Selection and Analyses

Twelve loci were selected from previous publications for cross-species amplification in the Nile crocodile (Bishop *et al*. 2009; Miles *et al.* 2009) and amplified for each of the collected samples. These Short Tandem Repeats (STRs) were combined into three Polymerase Chain Reactions (PCRs) multiplexes based on annealing temperature, expected allele range and fluorescent labels. 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. Various population genetic analyses were carried out to determine population diversity and structure.

MtDNA Sequences and Analyses

Primers were designed using publically available sequences of the *C. niloticus* control region. All PCR 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. For the purpose of phylogeographic analyses, 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).

Results

Genetic diversity, Effective population size, Gene Flow and Structure

The two Lower Shire river populations (North and South) 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

Kunene (H_e=0.58, H_o=0.50, and R_s=4.10) and the three Okavango populations (He=0.59, H_o=0.58 and R_s=4.46, averaged across groups) (Table 1).

The effective population sizes, as assessed by the Linkage Disequilibrium (LD) estimates were generally high except for the Bwawatwa National Park (38.6; 17.7-432.7) and south Lower Shire () (43.4; 29.4-75.2) populations. Subsequently, due to the detection of gene flow within the Okavango and Lower Shire river systems, an overall LD estimate was determined for each river system, resulting in Okavango 404.6 (107.3-∞) and Lower Shire 143.5 (74.5-743.8). The Wilcoxon signed rank test indicated no presence of a recent bottleneck or population expansion (*P*-value > 0.05) in any of the populations, based on the Two-phased model.

Amongst the main four sampling locations (Kunene, Okavango, Lower Shire and South Africa), all the pair-wise F_{ST} values were statistically significant (P <0.05) suggesting significant subdivision. However, within the two wild Okavango populations and the two Lower Shire populations F_{ST} values were non-significant (*P*-value >0.05) thus suggesting the occurrence of gene flow. The genetic distance (estimated as pairwise F_{st} value) between the Kunene and the Okavango (average F_{st}= 0.130, P<0.05) was the lowest between all pairs of populations. The Kunene-Lower Shire pair (average F_{st}= 0.211, P<0.05) and the Kunene-South Africa pair (F_{st}= 0.162, P<0.05) populations showed moderate genetic differentiation. The Okavango-Lower Shire pair (average F_{st}= 0.166, P<0.05) showed higher genetic differentiation compared to the Okavango-South Africa pair (average F_{st}= 0.142, P<0.05).

When considering the Kunene and Okavango populations (east southern Africa) against those of the Lower Shire and South Africa (west southern Africa), AMOVA analysis revealed minor partitioning of the genetic variation between east and west southern Africa (F_{ct}=0.126, P<0.05, 13%), and a high degree of variation within each region (F_{st}=0.178, P<0.05, 82%). Bayesian clustering analysis revealed the presence of two genetic clusters in the total dataset (ΔK being the highest for K=2) (Fig. 2a).

Further AMOVA analysis between the Kunene and the Okavango revealed minor partitioning of the genetic variation between the Kunene and the Okavango (F_{ct}=0.069, P<0.05, 14%), and a high degree of partitioning of the variation within the regions (F_{st}=0.115, P<0.05, 83%).

The Bayesian clustering analysis including just the Kunene and Okavango revealed the presence of three populations of which two were found to overlap in the Okavango river (ΔK being the highest for K=3) (Fig. 2b).

Mitochondrial Analysis

Two lineages previously described across Africa (Schmitz *et al.* 2003; Hekkala *et al*. 2011) represent the two main Nile crocodile genetic clades, designated 'Eastern' and 'Western' as. The populations from the Kunene, Okavango and Lower Shire river systems clustered within the 'Eastern' clade. The Kunene and Okavango showed strikingly low haplotype diversity with a single haplotype observed in a total of 65 individuals collected from four locations (Fig 3a) , and no haplotype diversity observed among the two rivers (h=0; π=0). The Lower Shire river (Malawi) showed the highest haplotype diversity among the three river systems (h=0.332±0.083; π=0.015±0.008) with four haplotypes observed in a total of 47 individuals (Fig 3b). The Kunene and Okavango sequences clustered separately from the Lower Shire river sequences by two mutational steps.

Discussion

The development of molecular genetic makers (STRs) for the Nile crocodile through cross-species amplification proved useful in assessing the levels of diversity and depicting the population genetic structure for this species in Southern Africa (Kunene, Okavango and Lower Shire river systems, and a commercial crocodile farm in South Africa). The mean observed heterozygosity in all sampling locations were comparable to neighbouring river basins studied in previous works (Bishop *et al*. 2009; Hekkala *et al*. 2010). Interestingly, the Okavango population showed no evidence of a genetic bottleneck even though it has experienced a large reduction in size in the past due to over exploitation (Ross 1998). This result does not support the reported loss of diversity in the Okavango Delta found in a previous study (Bishop *et al*. 2009) possibly owing to the sampling size and considering more effective diversity estimates for the Nile crocodile population by comparison of census size and effective population size in the previously mentioned study.

It was previously shown that the Nile crocodile populations are structured relative to river basin formations (Hekkala *et al*. 2011), thus suggesting that no gene flow was to be expected between the Kunene and Okavango river systems. Indeed, our findings revealed significant population differentiation (pairwise F_{st} = 0.130, P<0.05 averaged across the Okavango river groups) between these river systems suggestive of a land barrier to gene flow (Fig 2b). Furthermore, the Bayesian clustering analysis detected three populations, one in the Kunene and two in the Okavango river system. Genetic structure analysis among the Kunene,

Okavango, and Lower Shire river systems and South Africa commercial populations revealed two populations with restricted gene flow (Fig 2a).

Based on the limited sample set of the present study a single mitochondrial haplotype was observed among the Kunene and Okavango samples, suggesting that not enough time has passed to fix population-specific mutations that render the two river systems divergent. Contrastingly, four haplotypes were found in the Lower Shire river sample, suggesting a more diverse population (Fig 3a and Fig 3b).

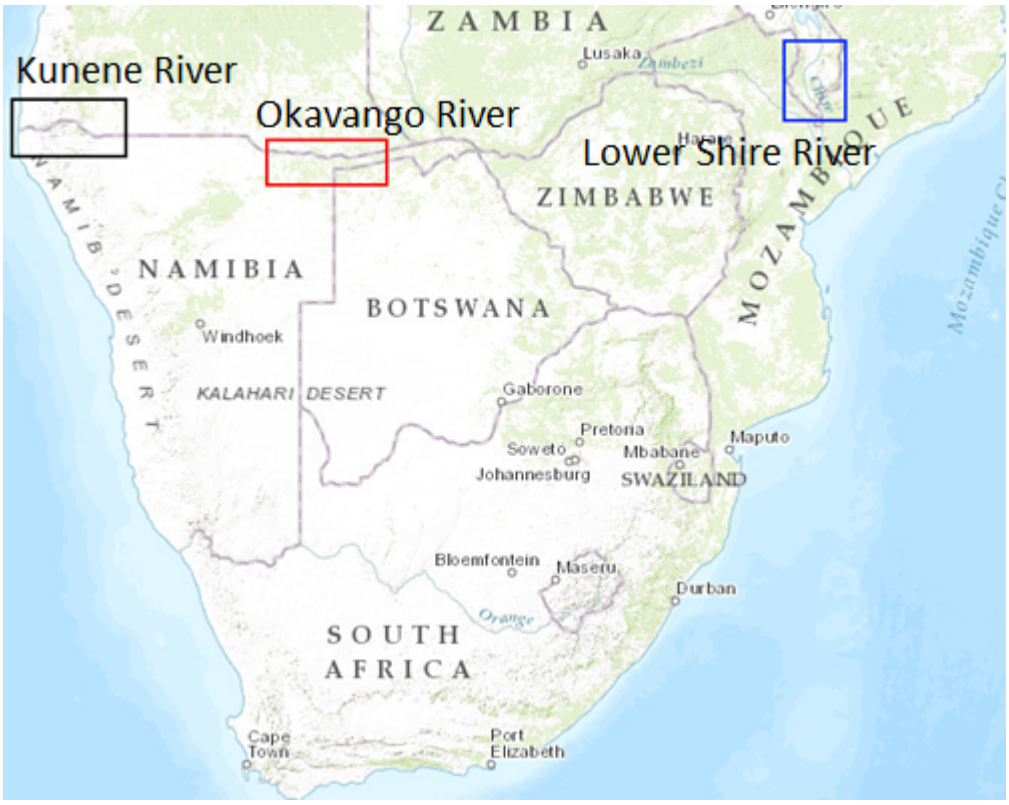


Figure 1 Map showing the three sampling locations of wild Nile crocodiles in Southern Africa. Kunene (black), Okavango (red) and Lower Shire river (blue).

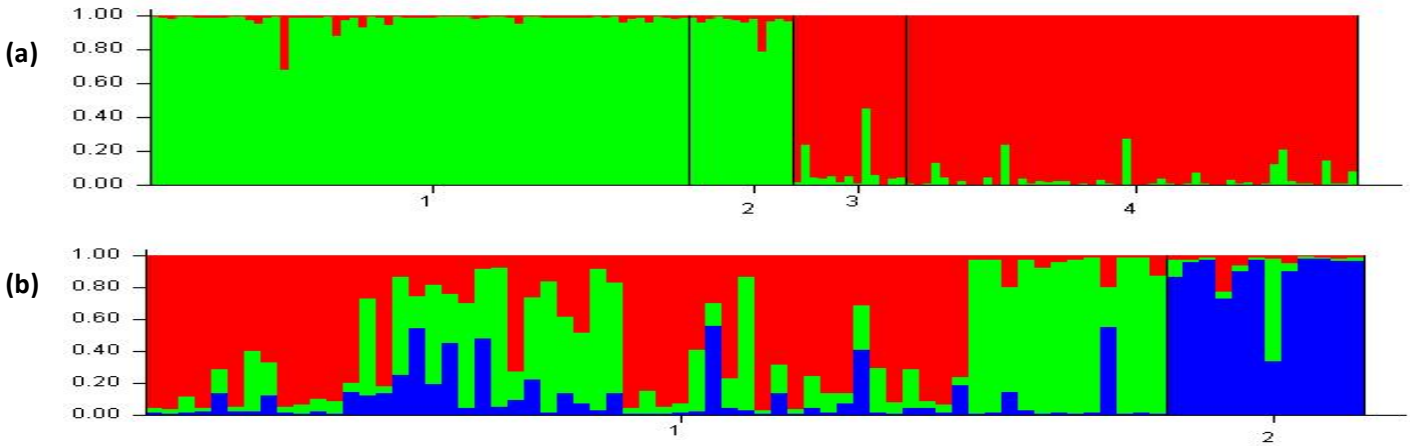


Figure 2 Genetic structure of Crocodylus niloticus populations based on Bayesian clustering analyses [Structure software v2.3.4 (Pritchard et al. 2000)] a) Genetic clusters in Southern Africa (complete dataset) , K = 2 and (b) Genetic clusters in the Kunene and the Okavango samples. Populations: (1)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

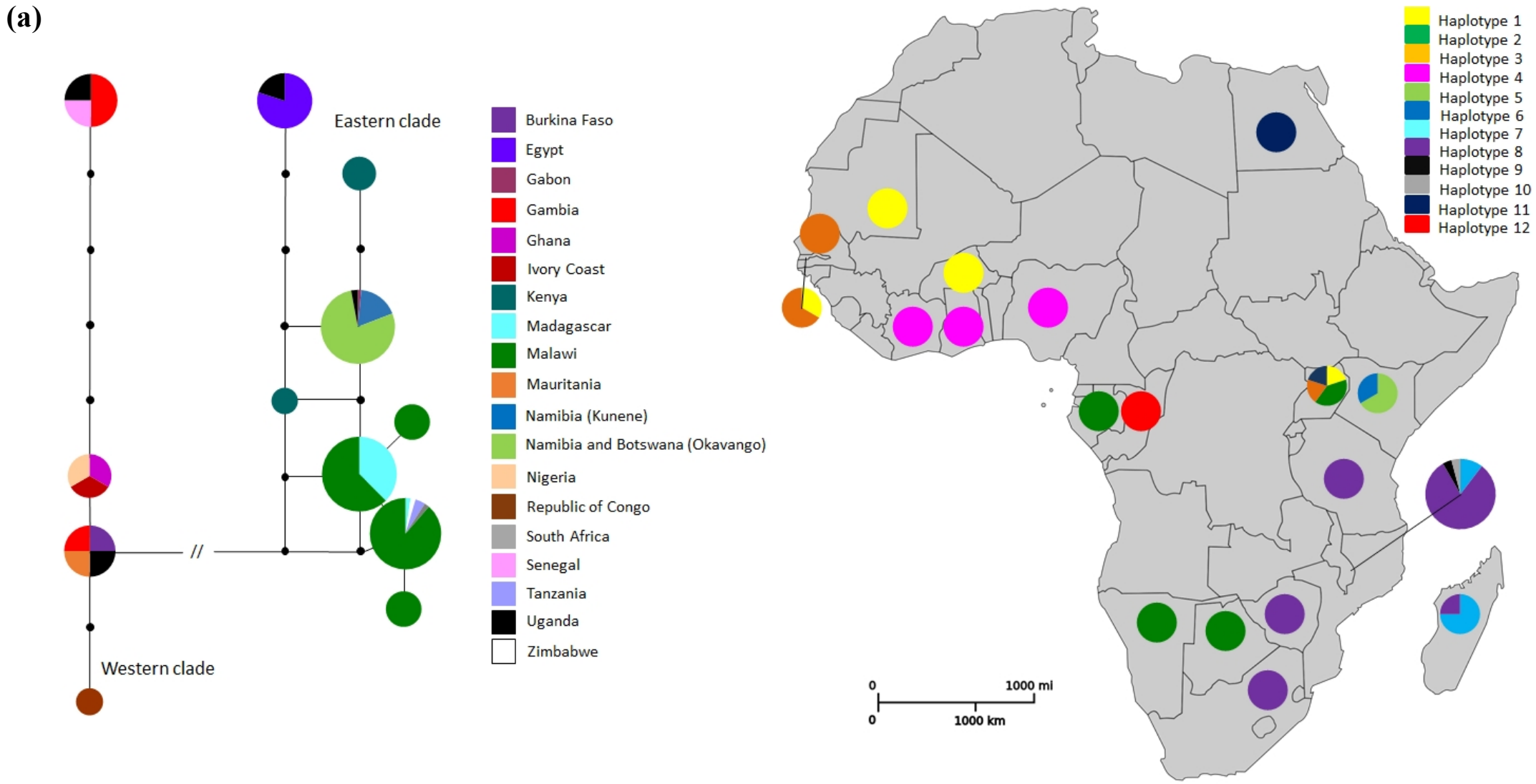


Figure 3 (a) The Medium-Joining haplotype Network depicting two groups of haplotypes, namely the Western and Eastern clades. Haplotype colours correspond to the countries where the samples were collected. Circles represent mtDNA haplotypes, lines connecting haplotypes represent a single substitution 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 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 Kunene and Okavango from the lower Shire river population, which shares haplotypes with the surrounding countries.

Table 1. Genetic diversity measures and Hardy-Weinberg Equilibrium test in Souther crocodile populations. N - number of individuals, An - number of alleles, He heterozygosity, Ho - observed heterozygosity, HWE - Hardy Weinberg Equilibrium test - mean allelic richness, Fis - mean frequency of inbreeding coefficient. Values were est on genotypes determined using 11 nuclear STR markers.

Population	N	An	He	Ho	HWE (P)	Rs	Fis
Panmixia	139	11.1	0.712	0.607	0.003	10.848	0.144
Kunene river	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	6.0	0.624	0.613	0.442	4.749	0.011
Otjiwarongo Crocodile Farm	13	4.3	0.562	0.535	0.537	4.137	0.043
Okavango river (overall)	62	5.182	0.595	0.584	0.531	4.456	0.015
Lower Shire river (North)	27	6.9	0.664	0.617	0.337	5.519	0.098
Lower Shire river (South)	25	6.9	0.684	0.625	0.367	5.540	0.071
Lower Shire river (overall)	52	6.909	0.674	0.621	0.352	5.529	0.085
South Africa commercial crocodile farm	12	3.8	0.519	0.586	0.273	3.734	-0.104

CONCLUSIONS

- The genetic diversity of Nile crocodile populations within the Kunene and Ok systems was comparable to previously published neighbouring river systems (2009; Hekkala *et al*. 2010);
- Nile crocodile populations within the Kunene and Okavango river systems d divergence, as indicated by no mtDNA mutational differences, however S showed that the Okavango sample was sub structured in two populations
- Comparison of the Kunene and Okavango samples to South Africa and I indicated two separate populations, in accordance to a previous study that occurrence of two genetic clusters in Southern Africa (Hekkala *et al*. 2011).
- The Nile crocodile populations in all three river systems conformed to the 'E crocodile clade.
- Based on our findings, the Kunene and Okavango river systems are isolated and will require population-monitoring specific to each river system.

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