

Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations

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Abstract Conservation and management of widespread species can be improved if populations exhibiting genetic differentiation are recognized as local management units. Specimens of Nile crocodile (*Crocodylus niloticus*) corresponding to major river drainage systems from Eastern Africa and Madagascar, and a small set of samples from Western Africa, were analyzed using multilocus genotyping to evaluate the potential to discriminate among locations and to assign individuals to population of origin. Populations from all sampled regions exhibited marked levels of genetic and genotypic differentiation as assessed by significant F_{ST} values and Bayesian analysis of population structure. At the regional level, the majority (94%) of all specimens were successfully assigned to the population of origin using only four microsatellite loci. Three populations sampled within Madagascar required the use of 12 loci for successful assignment of greater than 84%. Our findings demonstrate a need for alternative management strategies that consider the biogeographic sub-structuring of Nile crocodiles associated with major river drainages in Africa and Madagascar.

Keywords Nile crocodile · *Crocodylus niloticus* · Genetic variation · Population divergence · Management units · African biogeography

Introduction

Estimates of genetic differentiation and information on isolated populations are important baseline data for effective conservation and management of wildlife. It has been suggested that populations with significant divergence in allele frequencies at nuclear or mitochondrial loci be considered separate demographic management units regardless of phylogenetic distinctiveness (Fraser and Bernatchez 2001; Moritz 1994). Identifying management units that correspond to population genetic structure could enhance monitoring and conservation of threatened, endangered, or commercially exploited species. Doing so, however, requires assessing geographic patterns of genetic variation among populations across a species' distribution.

The Nile crocodile (*Crocodylus niloticus*) is a widespread, commercially exploited species that is protected under the Convention on International Trade in Endangered Species CITES (Appendix I and II). It currently inhabits 47 countries throughout Africa including Madagascar (Fig. 1; Ross 1998). The skin from this species is considered to be a 'classic' skin by the leather industry due to the absence of osteoderms which negatively impact appearance during the dyeing process. Because of its use as a classic skin and its prominence as a trophy animal, *C. niloticus* declined dramatically beginning in the late 1800s until the mid twentieth century due to over-exploitation. With the implementation of CITES in 1972, legal protection led to recoveries in several regions and, subsequently, a burgeoning trade in *C. niloticus* skins (e.g., 80,000

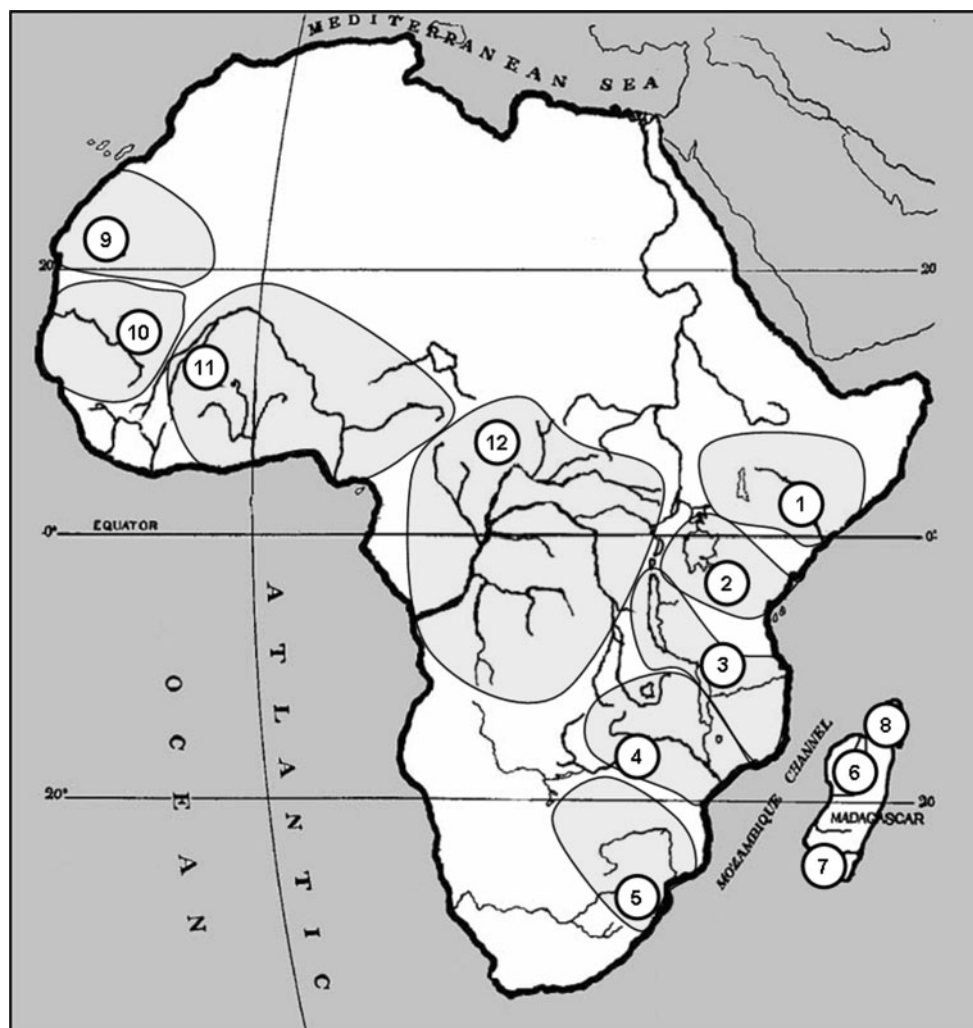
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Fig. 1 Map of Africa showing river drainages and localities of *Crocodylus niloticus* samples referred to in this study.

Numbers correspond to those in Table 1; 1. Kenya; 2. Tanzania; 3. Malawi; 4. Zimbabwe; 5. SAfrica; 6. MadNW; 7. MadSE; 8. MadANK; 9. Mauritania; 10. Gambia; 11. Burkina Faso; 12. Democratic Republic of Congo



annually in 1993) predominantly from ranching and captive breeding (Ross 1998).

The Nile crocodile harvest program under CITES authority has been proposed as a model for sustainable management of wildlife that emphasizes ranching as a viable alternative to wild harvest (Hutton 2000). Countries are given quotas under this harvest program and are permitted to export cropped skins from wild populations under the caveat that the future development of crocodile management programs will emphasize a shift towards ranching. Ranching has the potential to reduce impacts on wild populations because eggs, which exhibit naturally high rates of mortality, are collected and juveniles are raised in captivity for eventual commercial use. Since 1990, five countries (South Africa, Ethiopia, Kenya, Somalia, and Tanzania) have transferred from quota based harvesting to the CITES Appendix II ranching systems. Uganda and Madagascar retain CITES Appendix II status under the quota system. The wild harvest of crocodiles is discouraged under CITES ranching criteria, but still exists in Malawi,

Tanzania and Mozambique. Ongoing wild harvest of Nile crocodiles is currently legal in several West African nations including Cameroon, Congo, Democratic Republic of Congo, Sierra Leone, Togo, Sudan, and Chad, but international trade remains illegal (Ross 1998).

While Nile crocodiles are not currently considered threatened, insufficient data are available to adequately assess the status of populations for 25 out of 39 (64%) countries reporting exports for trade (Thorbjarnarson et al. 1992). Among those countries for which data do exist, Nile crocodiles are considered to be severely depleted in six (30%), somewhat depleted in 12 (60%), and not depleted in two countries (10%). Local extirpation is likely widespread (Ross 1998), so it is imperative to evaluate current levels of genetic variation and to develop methods for identifying the source of material in commercial trade.

Historically, a number of morphological differences among Nile crocodile populations have been observed, and several subspecies have been proposed. Although not currently recognized, these subspecies include *C. n. africanus*

(East African Nile crocodile), *C. n. chamses* (West African Nile crocodile), *C. n. corviei* (South African Nile crocodile), *C. n. madagascariensis* (Malagasy Nile crocodile), *C. n. niloticus* (Ethiopian Nile crocodile), *C. n. pauciscutatus* (Kenyan Nile crocodile), *C. n. suchus* (Central African Nile crocodile; Fuchs et al. 1974; King and Burke 1989). Despite previously recognized variation within the species, reliably delineating genetic boundaries has proved difficult. Such efforts have been hampered by a combination of extreme morphological plasticity and low levels of intra-specific genetic variation observed using mitochondrial DNA markers (Hekkala 2004).

Here we use 12 non-coding nuclear microsatellite loci developed for congeners (FitzSimmons et al. 2001) to estimate the extent of genetic differentiation within *C. niloticus* and to evaluate whether microsatellite markers can be used to assign individuals to population of origin. Information on intraspecific genetic variation will guide more effective management of *C. niloticus* by potentially delimiting demographic groups, and demonstrating whether microsatellites enable reliable assignment testing will increase opportunities for monitoring trade by potentially adding genetic markers to the small number of survey and reporting tools currently available.

Materials and methods

Specimens and extractions

We obtained tissue or blood samples from 143 *C. niloticus* in mainland Africa and Madagascar from 2000 to 2003 (Fig. 1; Table 1). Tissue samples of crocodiles from East

Africa were collected from four different harvested populations in Kenya, Malawi, Tanzania and Zimbabwe. Blood samples were obtained from live animals in South Africa, Madagascar, and the Western Sahara during ongoing ecological studies. Additional tissue samples were collected from carcasses during surveys for bushmeat in the Democratic Republic of Congo. All samples were collected under the appropriate CITES Scientific Authority for each country of origin. Samples were stored in Lysis buffer consisting of 0.1 M Tris (Trizma® base), 0.1 M EDTA, 0.01 M NaCl, 0.5% weight/volume SDS (sodium dodecyl sulfate) at room temperature in the field and subsequently transferred to long term storage at –80°C.

Laboratory protocols

DNA isolation followed the Qiagen DNeasy Tissue kit manufacturer’s protocol for blood or tissue, respectively and DNA template was stored at –20°C during use. We successfully extracted DNA from 98 individuals and 30 samples were initially screened for variation at 18 crocodile specific microsatellite loci described in FitzSimmons et al. (2001). Of the 18 loci tested, only two loci, Cj128 and CUI 108, did not successfully amplify in *C. niloticus*. Locus Cj122, Cp10, and CUD78 proved to be monomorphic for all individuals tested and Cj105 exhibited multiple PCR products. The remaining 12 loci—C119, Cj104, Cj107, Cj127, Cj128, Cj35, Cj101, Cj131, Cj16, Cj18, Cuc20, and Cud68—amplified consistently and were informative for use with *C. niloticus*.

All PCR amplifications of the 12 microsatellite loci included approximately 5.0 ng template DNA, 1.0 mM mixed forward and reverse primer, 1.5 mM Qiagen brand

Table 1 Geographic locations of *Crocodylus niloticus* specimens sampled from African and Madagascar river drainages

Region	Geographic location (drainage basin)	Locality	Tissue	N (collected)	N (included in analysis)
EAfrica	1. Kenya (Lake Turkana Basin)	Tana River	Dried skin	17	17
	2. Tanzania (Ruaha River Basin)	Lake Rukwa	Dried skin	14	12
	3. Malawi (Excluded)	Salima Bay	Dried skin (salted)	8	0
	4. Zimbabwe (Zambezi River Basin)	Lake Kariba	Dried skin	11	11
	5. SAfrica (Limpopo River Basin)	Lake St. Lucia	Blood	13	13
Madagascar	6. MadNW (Betsiboka River Basin)	Besalampy River	Blood	30	11
	7. MadSE (Mandrare River Basin)	Fort Dauphin	Blood	27	13
	8. MadAnk (Mahavavy River Basin)	Ankarana Caves	Blood	15	15
WAfrica	9. Mauritania (Senegal Basin)	Chitemeya-Nuochotte	Fresh muscle	4	1
	10. Gambia (Gambia River Basin)	Kedougou	Fresh muscle	1	1
	11. Burkina Faso (Niger River Basin)	Unknown	Fresh muscle	1	1
	12. Democratic Republic Congo (Congo-Zaire Basin)	Edzala, Lukuela	Dried skin	2	2
Total				143	98

Numbers correspond to map locations in Fig. 1

dNTPs, 1 unit Qiagen TaqGold polymerase, and 1× Qiagen PCR buffer containing 1.5 mM MgCL in a final volume of 11 µl. Samples were amplified on an ABI 9700 thermocycler using an initial denaturing of 4 min at 94°C, followed by 31 cycles of 1 min at 94°C, 1 min at TA as in FitzSimmons et al. (2001) and 1:30 min extension at 72°C. All programs included a final extension of 4 min at 72°C. PCR products were separated on an ABI Prism 3700 Genetic Analyzer with a Rox 500 internal size standard. Allele sizes were called using ABI GENEMAPPER 3.7 and confirmed by visual inspection.

Analytical approaches

Intraspecific genetic diversity measures (Expected heterozygosity (H_e), observed heterozygosity (H_o), number of alleles (A) and allelic richness (a) were estimated using *GENEPOP v3.1* (Raymond and Rousset 1995). Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were estimated with probability tests computed using the Markov chain method (1,000 iterations, Guo and Thompson 1992) available in *GENEPOP v3.1* (Raymond and Rousset 1995). Population structure was assessed with an analysis of molecular variance (AMOVA) using *ARLEQUIN v3.1* (Excoffier et al. 1992; Schneider et al. 2000). In order to identify groups of populations based on genetic differences, we iteratively grouped sampling localities into regions to maximize the among-group variance (Brown et al. 2007).

Correspondence of geographically separated populations as discrete genetic units was examined using the Bayesian genotype clustering method as implemented in *STRUCTURE v2.0* (Pritchard et al. 2000). The number of genotype clusters (k) is determined by HWE across all loci and gametic phase equilibrium between pairs of loci within groups. The value of K was iteratively set between one and ten, with ten being representative of the number of sample locations, where locations with ≤ 4 samples were grouped together with geographically proximate locations (e.g., all Western Africa locations). Values of $P(X | K)$ and $L'(K)$ were obtained from three iterations of a Markov chain Monte Carlo simulation (MCMC) involving 1.0×10^6 repetitions following an initial burning of 5.0×10^4 repetitions. We examined $P(X | K)$ values and note the break in the slope of the distribution of $P(X | K)$ values because this metric can be a good predictor of the uppermost hierarchical level of genetic structure among sampled populations (Evanno et al. 2005). Due to limitations on sampling distributions and sample sizes in the current study, these analyses only provide qualitative estimates of genetic distinctiveness among individuals from the sampled locations, rather than estimates of genotypic differentiation for population assignment.

Using the Paetkau et al. 1995 frequency based assignment test in *GENECLASS v2.0* (Piry et al. 2004), individual Nile crocodile specimens were assigned to sample locations. Confidence estimates for assignments were generated from 1,000 bootstraps. Individuals are said to be “mis-assigned” when their genotypes are more likely in populations other than the one they were sampled from. To eliminate bias, the contribution of the specimen to be classified was removed from the estimation of the allelic distributions (Waser and Strobeck 1998).

Results

Amplification success and estimates of genetic diversity

Many samples were degraded or contaminated during extended periods between collection and extraction. However, of 143 samples collected, we successfully extracted and amplified DNA from 98 individuals (Table 1). Estimates of allelic richness ranged from 2.18 to 2.78, and H_e ranged from 0.4344 to 0.5727 with estimates of genetic diversity across all measures appearing to be higher across Eastern Africa than in Madagascar (Table 2). Of the 12 microsatellites used in the study, five loci exhibited private alleles among individuals sampled from East Africa. Sample sizes from West Africa (WAfrica) populations were too small to evaluate population level frequencies, however, we found evidence of private alleles at six loci among individuals from this region. Individuals from the isolated Ankarana Caves (MadAnk) population in Madagascar exhibited the lowest level of heterozygosity and multiple fixed alleles.

Table 2 Intraspecific genetic diversity measures (expected heterozygosity (H_e), observed heterozygosity (H_o), number of alleles (A) and allelic richness (a) for *Crocodylus niloticus* populations from sampled locations)

Population	n	Loci typed	H_e	H_o	A	a
MadAnk	15	8	0.43	0.47	3.25	2.19
MadSE	13	10	0.47	0.41	2.70	2.18
MadNW	11	12	0.49	0.44	2.58	2.49
SAfrica	13	11	0.48	0.48	3.45	2.32
Tanzania	12	6	0.48	0.61	3.00	2.78
Zimbabwe	11	12	0.57	0.54	3.33	2.45
Kenya	17	12	0.52	0.42	3.25	2.40
WAfrica	6	10	0.40	0.25	1.72	NA

Abbreviations correspond to those in Table 3

Population genetic structure and assignment testing

Despite small sample sizes, variation in allele frequency distributions, including fixed and private alleles among individuals from some locations, indicated high levels of genetic differentiation among sampled populations. Pairwise F_{ST} values and values obtained from AMOVA tests (Table 3a, b) yielded population differentiation measures that indicate highly significant geographic structuring among Nile crocodile populations from all drainages at both the regional and sub-regional scale. F_{ST} values at the regional level (across Africa) ranged from 0.31 to 0.59 (Table 3a; $P < 0.0001$), and sub-regional values (within Madagascar) ranged from 0.17 to 0.23. West African samples (WAfrica) showed the highest levels of genetic divergence relative to all other populations ($F_{ST} = 0.46$ – 0.60). While this finding is likely an artifact of the small number of samples that were available to characterize this region, it nonetheless supports previously identified levels of genetic divergence between Eastern and Western populations

(Hekkala 2004; Schmidt et al. 2003). Hierarchical analysis of molecular variance (AMOVA) resulted in higher values for groupings by region (All Mainland, WAfrica, EAfrica, Mad) than by sample location (Table 3b).

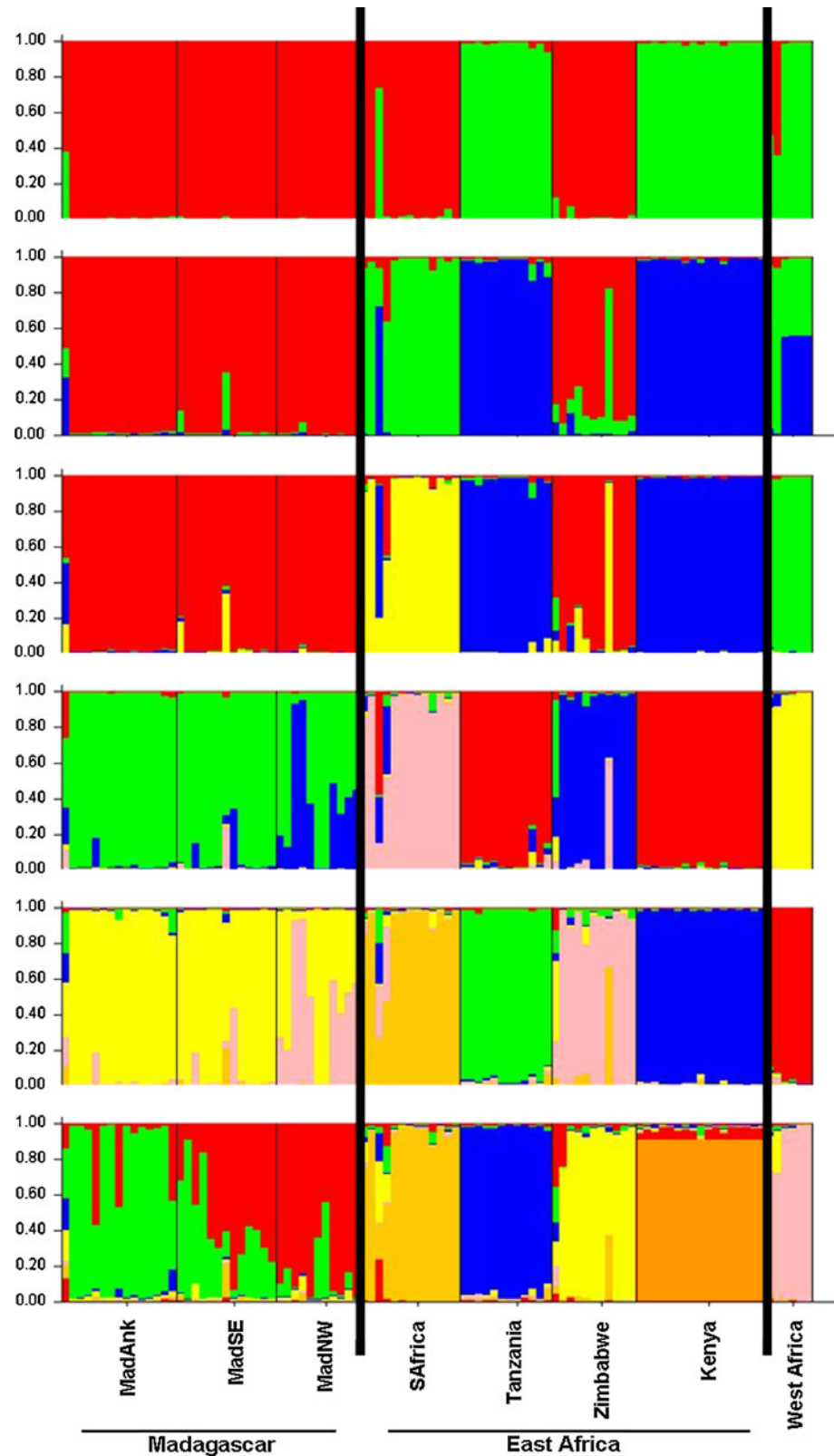
Values of $L'(K)$ estimated by *STRUCTURE v2.0* indicate that seven distinctive groups ($K = 7$) occur within the data set. While the break in the slope of the distribution of $P(X | K)$ values at 2 likely reflects differences between Madagascar and mainland Africa, the maximum $L'(K)$ suggests that seven groups occur within the data set with partitions corresponding to four major drainages in Eastern Africa; Kenya (Lake Turkana), Tanzania (Ruaha River), Zimbabwe (Zambezi River), and South Africa (Limpopo River), two sub-groupings in Madagascar and one Saharan/West African cluster (Fig. 2; $K = 2$ – 7). Madagascar populations exhibited additional signatures of differentiation among the sampled localities in river basins in the southeast (MadSE, Mandrare River), northwest (MadNW, Betsiboka River) and northern (MadAnk, Mahavavy River) regions (Fig. 2; $K = 7$).

Table 3 (a) Estimates of pairwise F_{ST} values among sampled locations; all values are significant at $\alpha = 0.05$. (b) AMOVA results for seven regional sites in mainland Africa and Madagascar

Population	MadAnk	MadSE	MadNW	SAfrica	Tanzania	Zimbabwe	Kenya
(a)							
MadSE	0.2100						
MadNW	0.2329	0.1730					
SAfrica	0.3100	0.3402	0.3651				
Tanzania	0.4540	0.4314	0.4729	0.4174			
Zimbabwe	0.3074	0.2664	0.1692	0.3017	0.4140		
Kenya	0.3427	0.3691	0.3650	0.3762	0.3307	0.3199	
WAfrica	0.5944	0.5237	0.5427	0.5226	0.4816	0.4883	0.4562
Component	Partition variance		% Total	F_{ST}	P		
(b)							
AllMainland vs. MAD							
Among groups	15.12180		10.32	0.22833	0.00000		
Between populations	18.33872		12.51				
Within populations	113.08552		77.17				
EAfrica vs. MAD							
Among groups	16.14948		10.66	0.23632	0.00000		
Between populations	19.63669		12.97				
Within populations	115.64357		76.37				
EAfrica and MAD vs. WAfrica							
Among groups	-17.20780		-13.78	0.09424	0.00000		
Between populations	28.97353		23.21				
Within populations	113.08552		90.58				

AllMainland = EAfrica (Kenya, Tanzania, Zimbabwe), SAfrica, and WAfrica (Senegal, Democratic Republic of Congo, Mauritania, and Chad), EAfrica = Kenya, Tanzania, Zimbabwe, South Africa), MAD = Madagascar (MadAnk, MadNW, MadSE)

Fig. 2 Genetic subdivision among sampled *Crocodylus niloticus* populations based on Bayesian cluster analysis using 12 microsatellite loci. Individual genotypes are proportionally assigned to clusters (K) from $K = 2$ to $K = 7$ (top to bottom). Localities correspond to those in Table 1



GENECLASS analysis with 12 loci resulted in all individuals assigned to the geographic location of origin 94% of the time at both $\alpha = 0.01$ and $\alpha = 0.05$. In addition, four

loci (Cj16a, Cj18a, Cj35a, Cj128a) exhibited sufficient variation to successfully assign 84% of individuals to location of origin at $\alpha = 0.05$. Within Madagascar, all

individuals were successfully assigned to the three sub-populations at $\alpha = 0.05$ using all 12 loci.

Discussion

Despite previous assumptions of uniformity across its distribution, and a high capacity for dispersal, the Nile crocodile appears to exhibit extensive population genetic structure. Our analyses revealed levels of genetic variation in Nile crocodile populations suggesting demographic and biogeographic structuring throughout the species' range. Traditional measures of population differentiation, such as pairwise F_{ST} values, were high relative to those found in comparable studies of African mammals (Brown et al. 2007; Comstock et al. 2002) and other large ectotherms (Ciofi et al. 1999). Within crocodylians, measures of intraspecific differentiation for *Alligator mississippiensis* (Glenn et al. 2002) and *Crocodylus moreletii* (Ray et al. 2004) were lower compared to estimates from *C. niloticus* populations in Africa and Madagascar. It has been hypothesized that the lower values found in *A. mississippiensis* are attributable to a recent bottleneck (Glenn et al. 2002) and localized sampling may explain values observed in *C. moreletii* (Ray et al. 2004). In contrast, Nile crocodile populations exhibit levels of intraspecific genetic differentiation that are often attributed to interspecific differentiation in other groups.

The observed patterns of genetic differentiation in Nile crocodiles appear to correspond to major river basins in Eastern Africa and support previously hypothesized differences between mainland Africa and Madagascar (Grandidier 1872). The results of hierarchical analyses of molecular variance indicate regional subdivisions corresponding to West Africa, East Africa and Madagascar, while Bayesian analysis of population structure identified clusters of East African *C. niloticus* populations according to drainages in Kenya, Tanzania, Zimbabwe and South Africa (the Turkana, Ruaha, Zambezi and Limpopo river basins, respectively).

Crocodile populations in Madagascar exhibit genetic clustering consistent with isolation from mainland Africa by the Mozambique Channel. The weaker divergence observed between populations sampled in northwestern Madagascar (MadNW) and Zimbabwe (Zambezi river drainage) across the Mozambique Channel ($F_{ST} = 0.0162$), than that observed in comparison with other Malagasy populations (MadSE vs. Zimbabwe and MadAnk vs. Zimbabwe; $F_{ST} = 0.2664$ – 0.3074 , respectively) may reflect relatively recent colonization. Prior studies have identified flooding from East African rivers as a possible mechanism for colonization of Madagascar for several endemic groups found on the island (Poux et al.

2005; Stankiewicz et al. 2006; Tattersall 2006; Yoder et al. 1996).

The presence of geographic structure in *C. niloticus*, as evidenced by microsatellite allele frequency variation and the presence of private alleles among individuals from different regions, is consistent with previous reports of distinct evolutionary lineages within this species based on mtDNA (Hekkala 2004; Schmidt et al. 2003) and nuclear intron sequence data (Hekkala 2004). Evidence of congruent biogeographic patterns of genetic variation in other African vertebrates (Agnès 2003; Brown et al. 2007; de-Menocal 2004; Moodley and Bruford 2007; Vrba 1995) also suggests that historical events have likely shaped contemporary patterns of geographic variation (Avice 1994). Population genetic structure in *C. niloticus* may also reflect behavioral and ecological constraints on dispersal. Although *C. niloticus* is generally thought to be a highly vagile species, male crocodylians are highly territorial and defend sun-exposed, sandy beach sites attractive to females for egg laying (Hutton 1989). Limited access to such sites may select for natal philopatry, which could give rise or contribute to geographic patterns of genetic structure. Several behavioral studies of crocodylians support this hypothesis (Hutton 1987; Ogden 1978; Rodriguez 2007). More extensive comparative analysis of fine scale genetic variation within and among populations will be necessary to disentangle the influence of ecological and historical factors on genetic variation in *C. niloticus*.

Our findings have important implications for the conservation and monitoring of harvested Nile crocodile populations. Despite the apparent recovery and ubiquity of populations in East Africa, the geographic range of the Nile crocodile remains fragmented due, in part, to increasing aridity and anthropogenic land use changes. Recent estimates suggesting that between 250,000 and 500,000 Nile crocodiles remain in the wild, along with range maps routinely depicting a distribution roughly circumscribed by the equatorial belt, belie the fact that the species can be locally rare (Ross 1998). Populations are known to occur sparsely throughout Madagascar as well as the northwestern and central Sahara (Ross 1998; Shine et al. 2001), and little is known regarding distributions and population status in the densely forested Congo. Early reports by Chapin (Schmidt 1919), for example, note with some surprise that this “larger cousin” of the African dwarf crocodile was found at low densities in the upper reaches of the Congo. The assumption that all populations constitute a single polytypic species has resulted in the Nile crocodile being listed at LRLc (Low Risk least concern) by the IUCN Red List (Ross 1998). Evidence of strong geographic differentiation within the species, however, suggests the presence of demographically independent groups, some of which may correspond to evolutionary lineages (Hekkala 2004)

that have been previously described as distinct entities (Fuchs et al. 1974; King and Burke 1989).

Estimates of intraspecific genetic variation and identification of demographically independent populations are important baseline data for effective conservation and management of wildlife, particularly for tracing the origins of materials taken from heavily exploited species (Comstock et al. 2003). It is clear that complete genetic characterization of a population requires large numbers of samples particularly when the data are to be used as an enforcement tool. Despite concerns that small sample sizes may result in mis-assignment of, or failure to assign, samples when using standard Bayesian, frequency or distance based algorithms, the data presented here are robust to methodological differences and represent preliminary steps toward developing tools for monitoring trade. We recognize the limitations of our small sample sizes and that additional work will be necessary to ascribe observed patterns of genetic differentiation to the appropriate management units, but our findings strongly suggest that the current IUCN listing may underestimate threats to local populations.

Our population assignment results provide a baseline for developing more robust monitoring programs to gauge potential impacts of farming and ranching practices that involve translocations of individuals among sites. A recent review of crocodile ranching programs conducted for CITES by the IUCN Crocodile Specialist Group suggests that monitoring solely by import and export quotas is inadequate to ensure a sustainable trade in crocodile skins. Based on the data from 15 countries operating ranching programs under the provisions of Resolution Conf. 11.16, it is apparent that no Party complies fully with the reporting requirements (Ross 1998).

For effective management of any wild species, data on the status of wild populations constitutes a basic requirement and is also the most onerous, expensive, and time-consuming activity in managing wildlife populations. For species exploited by harvest programs, like the Nile crocodile, a description of the pattern of intraspecific variation provides an objective way to delimit demographic units for management.

The development of reliable genetic assignment protocols would strengthen trade-monitoring efforts by adding genetic profiling to the tool-kit now available (Allendorf et al. 2008; Comstock et al. 2003; Mills 2007). Costs associated with these methods are decreasing rapidly and may therefore reduce costs and increase compliance for monitoring international trade in listed species. In the long term, it would be beneficial for the CITES Secretariat to fund and maintain long term genotyping libraries, such as these, to which individuals can be added over time until the library encompasses localities fully representative of

geographic distributions. In the interim, the seven genotype clusters observed in our dataset suggest that a review of management units (Fraser and Bernatchez 2001; Moritz 1994) for the purpose of population monitoring in the Nile crocodile is warranted.

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