GENETIC DIVERSITY AMONG WILD HERBIVORE SPECIES IN TANZANIA

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SUMMARY

Monitoring of genetic structure of populations is desirable in order to obtain information about the degree of genetic variation, which exits within and between the remaining populations. For wildlife species, this information is vital in planning future conservation and management strategies. However, currently, there are no published reports regarding the genetic structure/diversity within and between existing wildlife populations in Tanzania. The current study report a genetic data obtained by PCR-EFLP of conserved regions of mitochondrial DNA (mtDNA) using a single restriction enzyme. Two mtDNA markers, mt246 and mt700 targeting the control (D-loop) and cytochrome b gene regions of mitochondrial genome, and RsaI restriction enzyme were used to generate species-specific reference DNA fragment patterns using fresh meat from Wildebeest, Zebra, Thompson's gazelle, Impala, Reedbuck, Kongoni, Oryx, Warthog, Buffalo, Hippopotamus, Cattle, sheep, Goats and Pigs. The DNA fragment patterns were used to calculate genetic divergence, similarity and pair wise number of nucleotide substitutions between different wild and domestic species. The average percentage differences between species were found to be 59% using mt700 and 71%, using mt 246 markers, respectively, while the average percentage similarities using the same markers were 41% and 29%, respectively. The average number of pair wise nucleotide substitutions was 0.23 and 0.19 using mt700 and mt246, respectively. These data provide information on the genetic diversity among major wild herbivore species in Tanzania on these two genes and calls for further studies to explicitly elucidate the extent and trend of genetic diversity among wildlife population.

INTRODUCTION

Game meat provides an important source of protein in many African societies and for many years has been viewed as a dietary supplement. However, in recent years it has become a key source of food and legal tender in the drive for human survival in

eastern and Southern Africa (Barnett, 2000). This increase in illegal game harvesting coincide with a sharp rise in human population around protected areas and in some cases the resultant expansion in land-based activities have invaded these areas resulting into islands of wildlife populations (Hofer *et al.*, 1996). The illegal game meat trade occurs across virtually the whole of tropical Africa, Asia and the Neotropics, threatening a multitude of wildlife species (Barnett, 2000). In Tanzania the trade removes from Serengeti ecosystem approximately 4,458 resident and 111,691 migratory herbivores annually, equivalent to 11,950 tons of meat (Hofer *et al.*, 1996). In Uganda a drastic decline has been reported in the size of elephant population in the last 40 years, exacerbated by increase in the size of human population and poaching for ivory (Nyakaana and Arctander, 2001). One of the consequences for such a decline is likely to be associated with a reduction in the amount of genetic diversity in the surviving population due to increase effects of random genetic drift.

It is desirable to routinely monitor the genetic structure of any single wildlife species in order to obtain information about the amount of genetic variation within and between the remaining populations. This information is vital for the future conservation and management plans. Increasingly, genetically divergent populations are being recognized as appropriate units for conservation regardless of taxonomic status (Waits *et al.*, 1998). In Tanzania, additional efforts are required to preserve the unique Tanzanian wildlife diversity because the existing islands of wildlife population as a result of increasing human pressure are already under threat of extinction, *let alone* habitat fragmentation. There are currently no published reports regarding the extent of genetic diversity within and between the existing wildlife populations in Tanzania.

Molecular methods provide useful information for identifying genetic differentiation, diversity and reconstructing phylogenetic relationships among conspecific populations (Waits *et al.*, 1998). Mitochondrial DNA (mtDNA) analysis has been a powerful tool for detecting population genetic structure at the intra-specific level due primarily to its maternal mode of inheritance, lack or recombination, high mutation rate and the process of lineage extinction within populations (Cronin *et al.*, 1991). We describe here the extent of genetic diversity obtained in major Tanzanian wild-herbivore species, in Serengeti and Mikumi National Parks, using two mitochondrial DNA markers.

MATERIALS AND METHODS

Sample collection

Muscle tissues (5-10 gm) were obtained from reference carcasses of the wild herbivore species (as road kills, predator kills/carion eater left-overs or disease-caused) from Serengeti (46) and Mikumi (12) National Parks (SNP and MNP, respectively) in Tanzania. Fresh tissues were transported in icebox, and finally frozen at -20°C in the laboratory for DNA extraction. The target animal species envisaged for this study included all wild herbivore species, commonly hunted for meat. However, during the conduct of this study, samples were obtained from Wildebeest, *Connochaetus taurinus taurinus* (16), *Zebra, Equus burcheli* (5), Thomson's gazelle, *Gazella thomsonii* (5), Impala *Aepyceros melampus* (4), Reed buck, *Redunca redunca* (2), Kongoni, *Alcelaphus buselaphus* (2), Oryx, *Oryx gazella* (2), Warthog, *Phacochoerus aethiopicus* (1), Buffalo, *Syncerus cafer* (1), and Hippopotamus, *Hippopotamus amphibius* (1). In addition, samples were obtained from domestic animals, Cattle, *Bovine* (5), Goats, *Caprine* (5), Sheep, *Ovine* (7) and Pig, *Porcine* (5). These reference samples were used to develop and validate DNA fragment patterns (fingerprints) specific to each species. These fingerprints were further tested on 20 (blind) smoke-dried game meat samples obtained from villages surrounding these parks, for species identification (data reported elsewhere). Following identification, the data were pooled together with those of the known species to calculate the genetic diversity indicators.

DNA Extraction.

DNA was extracted using the method described by Cronin *et al.* (1991) with slight modification. Briefly, total genomic DNA was extracted from 0.25 g of each sample by incubation in 0.5 ml of (TES, pH 8.0) lysis buffer (0.2 M tris-hydroxymethylaminomethane-Tris, 0.1 M ethylenediamine-tetraacetic acid-EDTA, and 1% sodium dodecyl sulphate-SDS) and 25 µl of protenase K (20 mg/ml) at 60°C for 2-3 hours. This was followed by addition of 0.4 ml of potassium acetate per 1.0 ml solution, placed on ice for at least 30 minutes, and centrifugation at 12000g for 10 minutes. The supernatant was purified three times with phenol-chloroform-isoamyl alcohol (24:23:1) and once with chloroform-isoamyl alcohol (23:1) prior to precipitation with equal volume of isopropanol (Sambrook *et al.*, 1989).

PCR and RFLP analyses.

Twenty microliter PCR reactions were performed using two separate mitochondrial primer sets (246 bp; HSF21 and LTPROBB13) described by Wasser *et al.* (1997) and (700 bp; H16498 and L15774) decribed by Kocher *et al.* (1989). The PCR reaction protocols used were as

previously described by Wasser *et al.* (1997). About 2-5 µl of the PCR products were electrophoresed on 15% polycrylamide (Scot-lab, Scotland) gels (PAG) in 1xTBE for 50 min at 200V and stained by silver nitrate solution. PCR positive samples were subsequently digested with RsaI enzyme (recognition sequence, 5'-GT▼AC-3') according to the manufacturer's procedure (Promega, Madison, WI, USA), electrophoresed on 15% PAG in 1xTBE for 50 min at 200V and stained by silver nitrate.

Data analysis

The data were analysed based on having or not having fragments and for the purpose of genetic diversity the following formulae were used.

Fragment sharing (F)

Fragment sharing was calculated as an indication of similarity of PCR-RFLP fragment patterns of animals between different species according to Gwakisa *et al.*, (1994) and Nei and Li (1979) using the following formula:

$F=2mxy/mx + my$.....(1)

Where mx and my are the numbers of restriction fragments resulting from digestion of sequences X and Y, respectively, and mxy is the number of fragments shared by the two sequences.

The expected proportion of shared fragments (F) can be expressed in terms of probability (G) that a restriction site has remained unaltered during time t by the approximate formula

$F=G4/(3 -2G)$ (2)

Where $G=e^{-r\lambda t}$, in which r is the number of nucleotide in the recognition site, λ is the rate of nucleotide substitution, and t is the time of divergence between the two sequences.

The number of base substitution per site (K)

The number of base substitutions per site between the sequences is:

$K = 2 \lambda t$ .....(3)

Hence, from equation (2), G can be estimated by

G = [F (3-2G)]¼ .....(4)

This equation can be solved by the process of iteration. As a first value, Nei, (1987) recommends G=F1/4. Usually very few iteration cycles are required. The estimate of G allows the estimation of K, using the following formula;

$K= -(2/r)\ln(G)$ .....(5)

Average percentage difference (APD)

This was calculated as an indication of variation in PCR-RFLP fragment patterns of animals between different species according to Gilbert *et al.* (1990) and Yukhi & O'Brien (1990) using the following two formulae:

Percentage difference (PD) = $(Nxy/Nx + Ny) \times 100$.....(6) $i = n$

Average percentage difference (APD) = $1/C \Sigma PDi$ .....(7) $i = 0$

Where Nxy is the number of fragments that differed between two sequences X and Y, Nx and Ny are the number of fragments

resulting from digestion of the two sequences X and Y respectively, and C is the number of interspecies pair-wise comparison.

RESULTS

Genetic similarities and divergences between animal species

Most of the unknown (smoked meat) samples were identified by PCR-RFLP (data reported elsewhere) and hence the results were pooled together with known samples in the calculation of genetic indicators described here. The results obtained from PCR-RFLP using Rsal restriction enzyme mapping allowed calculation of both genetic similarities and differences, based on the number of shared and unshared DNA fragments. A total of fourteen species (wildebeest-WB, zebra-ZB, thomson's gazelle-TH, impala-IM, bovine-BO, caprine-CA, ovine-OV, porcine-PO, buffalo-BU, reedbuck-RB, kongoni-KO, warthog-WA, oryx-OR and hippo-HI) were compared for genetic similarity. Based on results of restriction digestion of PCR amplified portion of mtDNA cytochrome b gene/control (D-loop) using mt700 marker (Table 1), most of the species shared DNA fragments 0%-44%, with an average of 41%. However, few species showed marked similarity, the highest being 86%, which was observed between bovine and porcine.

At the mtDNA control (D-loop) locus using mt246 marker (Table 2), most of the species showed fragment pattern sharing between 0%-40%, and an average of 29%, with few showing marked similarities of up to 75% (e.g ovine/caprine 75% and 67% for each of warthog/kongoni, bufallo/caprine, bovine/wildebeest and oryx/impala, respectively).

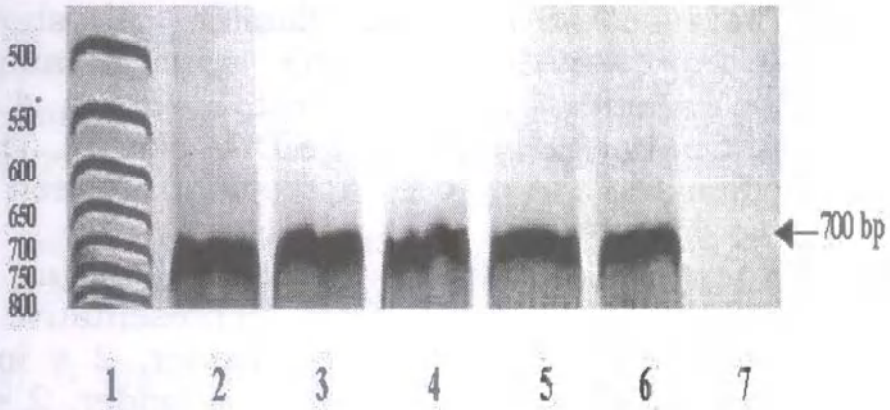
Table 1. *Index of genetic similarity between species revealed at *mtDNA* cytochrome b gene/control (D-loop) region following digestion with Rsal enzyme.

**	WB	ZB	TH	IM	BO	CA	OV	PO	RB	KO	WA	OR
ZB	0.5											
TH	0 5	05										
IM	0.3	0 0	0.0									
BO	0.4	0.2	0.2	0.2								
CA	0.4	0.3	0 6	0.5	0 4							
OV	0 4	0.1	0 3	0 5	0.6	0 5						
PO	0.4	0.2	0 2	0 4	0 8	0.5	0.7					
RB	0.3	0 0	0.3	0 6	0.5	0.8	0 6	0.6				
KO	0.8	0.4	0.4	0.2	0.5	0.3	0.3	0.5	0 2			
WA	0 4	0 2	0 4	0 2	0 3	0 4	0.2	0.2	0.2	0.5		
OR	0.5	0 1	0 3	0 2	0 5	0.1	0.6	0.4	0.2	0.6	0.2	
HI	0.4	0.4	0.6	0.2	0.5	0.6	0.3	0.5	0.4	0.5	0.5	0.2

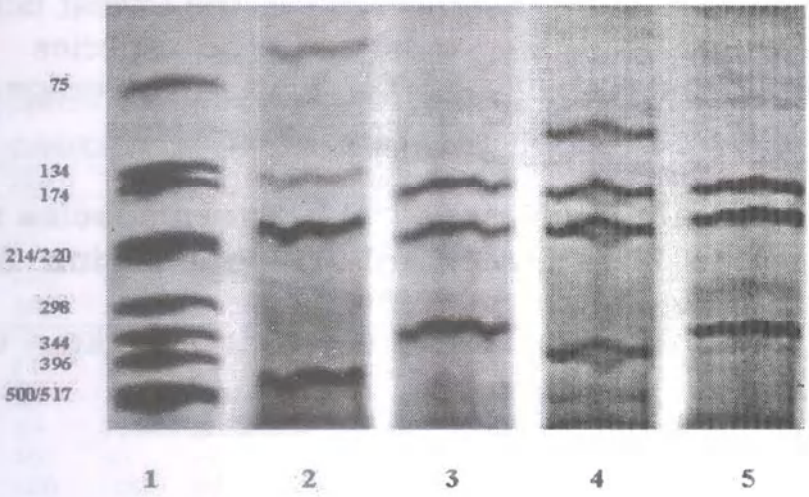
*Index of genetic similarity was calculated as an indication of similarity of PCR - RFLP fragment patterns of animals between species, based on fragment sharing.

Table 2. *Index of genetic similarity between species revealed at the mtDNA control (D-loop) region following digestion with Rsal enzyme.

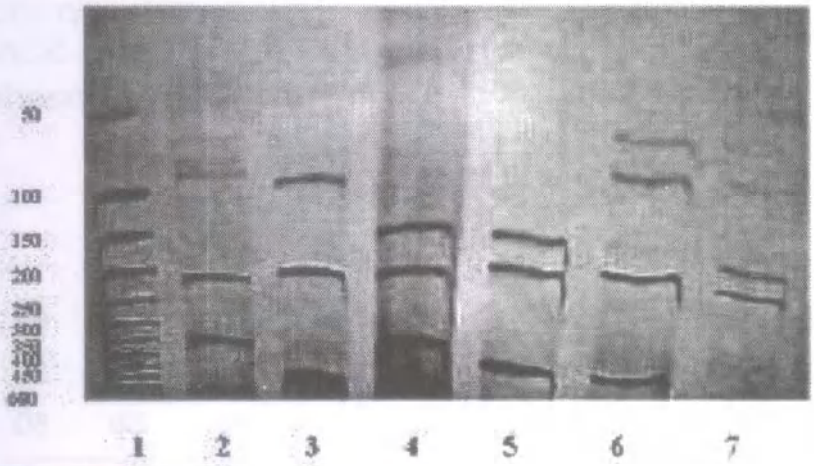
**	WB	ZB	TH	IM	BO	CA	OV	PO	BU	RB	KO	WA	OR
ZB	0.2												
TH	0.0	0.2											
IM	0.2	0.0	0.2										
BO	0.6	0.0	0.0	0.5									
CA	0.5	0.4	0.5	0.2	0.2								
OV	0.3	0.2	0.3	0.2	0.3	0.7							
PO	0.3	0.5	0.3	0	0	0.5	0.3						
BU	0.5	0.5	0.2	0.5	0.2	0.6	0.5	0.5					
RB	0.2	0.6	0.2	0.6	0.4	0.3	0.4	0.2	0.6				
KO	0.4	0.0	0.4	0.3	0.4	0.2	0.4	0.0	0.3	0.2			
WA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6		
OR	0.2	0.2	0.2	0.6	0.5	0.4	0.2	0	0.2	0.5	0.2	0	
HI	0.2	0.5	0.2	0	0	0.4	0.2	0.2	0.2	0.2	0	0	0.2


500
550
600
650
700
750
800
700 bp
1 2 3 4 5 6 7

(a)


75
134
174
214/220
298
344
396
500/517
1 2 3 4 5

(b)


90
100
150
200
1 2 3 4 5 6 7

(c)

Figure 1. a) A representative polyacrylamide gel showing PCR amplification from representative reference species samples using mt700 marker. Lane1 = ladder, 2 = wildebeest, 3 = zebra, 4 = +ve DNA control, 5 = impala, 6 = bovine and 7 = - ve DNA control. The samples were run in duplicates and the percent agreement between duplicates was 100%;
Fig 1(b) and (c) show RFLP patterns obtained following restriction digestion of mt700 PCR products from representative reference samples using Rsa1 enzyme. (b) Lane 1 = ladder, 2 = impala, 3 = bovine, 4 = caprine, and 5 = ovine; (c) Lane 1 = ladder, 2 = oryx, 3 = warthog, 4 = porcine, 5 = reedbuck, 6 = kongoni, and 7 = hippopotamus

A total of thirteen species were compared for the divergence at mtDNA cytochrome b gene/control region locus (Table 3). Results shows that, while few species revealed <50% genetic divergence, the lowest being 14%, most of the species revealed >50%, with an average of 59% divergence

Table 3. *Index of genetic divergence (%) between species revealed at mtDNA cytochrome b gene/control (D-loop) region following digestion with Rsal enzyme.

**	WB	ZB	TH	IM	BO	CA	OV	PO	RB	KO	WA	OR
ZB	50											
TH	50	50										
IM	64	100	100									
BO	56	78	78	75								
CA	54	69	38	50	60							
OV	54	85	69	50	40	43						
PO	60	80	80	56	14	45	27					
RB	64	100	64	40	50	17	33	33				
KO	20	60	60	78	43	63	63	50	78			
WA	56	78	56	75	67	60	80	71	75	43		
OR	45	60	64	80	50	83	33	56	80	33	75	
HI	60	60	40	78	43	33	63	50	56	50	43	78

*Index of genetic divergence was calculated as an indication of variation of PCR - RFLP fragment patterns of animals between species, based on unshared fragments.

At the mtDNA control (D-loop) region locus (Table 4), a total of fourteen species were compared for their genetic divergence. The result obtained revealed divergence ranging from 25%-

100% with an average of 71% between these species, where most of species showed >60% genetic divergence.

Number of nucleotide substitution between animal species

Tables 5 and 6 show the pair wise number of nucleotide substitution between different wild and domestic species at two different loci of the mitochondrial DNA, the cytochrome b gene/control (D-loop) region and control (D-loop) region. Results show that the differences in the number of nucleotide substitution between species studied was high at both loci. At the cytochrome b gene/control (D-loop) the number of nucleotide substitution varied between 0 - 0.53 with an average of 0.23 while at control (D-loop) ranged between 0 - 0.40 with an average of 0.19

Table 4. *Index of genetic divergence (%) between species revealed at mtDNA control (D-loop) following digestion with RsaI enzyme.

**	WB	ZB	TH	IM	BO	CA	OV	PO	BU	RB	KO	WA	OR
ZB	71												
TH	100	71											
IM	71	100	71										
BO	33	100	100	43									
CA	50	56	75	78	75								
OV	67	71	100	71	67	25							
PO	67	43	100	100	100	50	67						
BU	43	50	71	50	72	33	43	43					
RB	78	40	56	40	56	64	56	78	40				
KO	60	100	100	67	60	71	60	100	67	75			
WA	100	100	100	100	100	100	100	100	100	100	33		
OR	75	78	50	33	50	60	75	100	78	45	71	100	
HI	71	50	71	100	100	56	71	71	75	80	100	100	78

*Index of genetic divergence was calculated as an indication of variation of PCR-RFLP fragment patterns of animals between species, based on unshared fragments.

Table 5. Pair wise number of substitutions between wild and domestic species at mtDNA cytochrome b gene to control (D-loop) region.

BO	0.2	0.4	0.4	0.3								
CA	0.2	0.3	0.1	0.1	0.4							
OV	0.2	0.5	0.3	0.1	0.1	0.1						
PO	0.2	0.4	0.4	0.2	0.0	0.1	0.0					
RB	0.2	0	0.2	0.1	0	0.0	0.1	0.1				
KO	0.0	0.2	0.2	0.4	0.1	0.2	0.2	0.1	0.4			
WA	0.2	0.4	0.2	0.3	0.2	0.2	0.4	0.3	0.3	0.1		
OR	0.1	0.4	0.2	0.4	0.1	0.4	0.1	0.2	0.4	0.1	0.3	
HI	0.2	0.2	0.1	0.4	0.1	0.1	0.2	0.1	0.2	0.1	0.1	0.4

DISCUSSION

The average DNA fragment sharing between species revealed at cytochrome b gene/control (D-loop) was 41%, while at control (D-loop) was 29%. The lower the degree of fragment sharing between species, the higher the ability of the technique to distinguish between species and the vice versa. Bovine and porcine as well as caprine and ovine exhibited the highest level of fragment sharing of 86% and 75% at cytochrome b gene/control (D-loop) and control (D-loop).

Table 6. Pair wise number of substitutions between species at mtDNA control (D-loop) region.

**	WB	ZB	TH	IM	BO	CA	OV	PO	BU	RB	KO	WA	OR
ZB	0.3												
TH	0	0.3											
IM	0.3	0	0.3										
BO	0.1	0	0	0.1									
CA	0.2	0.2	0.3	0.4	0.3								
OV	0.2	0.3	0	0.3	0.2	0.0							
PO	0.2	0.1	0	0	0	0.1	0.2						
BU	0.1	0.1	0.3	0.1	0.3	0.1	0.1	0.1					
RB	0.4	0.1	0.2	0.1	0.2	0.2	0.2	0.4	0.1				
KO	0.2	0	0	0.2	0.2	0.3	0.2	0	0.2	0.3			
WA	0	0	0	0	0	0	0	0	0	0	0.1		
OR	0.3	0.4	0.1	0.1	0.1	0.2	0.3	0	0.4	0.1	0.3	0	
HI	0.3	0.1	0.3	0	0	0.2	0.3	0.3	0.3	0.4	0.4	0	0.4

regions respectively. The average percentage difference between the species was found to be 59% and 71% at cytochrome b gene/control (D-loop) and control (D-loop), respectively. By comparing the two loci, mtDNA cytochrome b gene/control region and mtDNA control region, it was observed that some of the species

whose fragment patterns were closely similar at one locus, showed lowered similarity at the other. An example can be inferred from comparison between bovine and porcine. At mtDNA cytochrome b gene/control region locus, their similarity was 86% while at the mtDNA control region locus, was 0%. Generally, some of the species, whose fragment pattern similarity was 0% at one locus, had varying degree of similarity at the other. A good example is similarity between warthog and the rest of the species. Based at mtDNA control (D-loop) region, its similarity with the rest of the species was 0% except the kongoni, whereas, at mtDNA cytochrome b gene/control (D-loop) its similarity with the rest of the species ranged between 20-57%. This is probably due to varying degrees of nucleotide substitutions, between the two regions, control (D-loop) being a non coding region typically has much higher degree of substitution than cytochrome b gene.

In practice, the higher the percentage difference between species, the greater the ability of the technique to distinguish between species and the vice versa. The approach used in this study was found to provide authentic genetic differentiation between livestock breeds of cattle (Gwakisa *et al.*, 1994, 1997). This approach may find use in phylogenetic studies of animals whether of the same species but separated geographically or of closely related species. The value of such genetic comparison may indicate the extent of genetic diversity between and within species. The data presented in this study clearly showed that, the average similarities between species involved in the study were 41% and 29% at cytochrome b gene and control (D-loop), while the average percentage difference between them were 59% and 71% at cytochrome b gene and control (D-loop), respectively. The average numbers of nucleotide substitution between species were 0.23 and 0.19 at cytochrome b gene and control (D-loop), respectively. A study conducted in nine-banded armadillos (*Dasypus novemcinctus*) from Paraguay revealed a nucleotide diversity ranging between 0.003 to 0.005 (Frutos and Van Den Bussche, 2002). The average numbers of substitution per site between species reported here is high indicating that the two mitochondrial loci studied mutates relatively quickly increasing the possibility of existence of genetic differences between species. Similar approach was used to study the genetic profile of book trout in U.S and find that, the book trout populations in tributaries to Lake Huron are not depauperate of genetic diversity (Burnham-Curtis, 1996). Furthermore, Danzmann *et al.*, 1991 employed mt DNA analyses for the genetic discrimination of wild and

hatchery populations of brook charr, and concluded that, the potential for introgression of hatchery fish into population was low.

Acknowledging small sample sizes for some species, the data presented here provide some insights on the extent of genetic diversity between and within wildlife species in Tanzania. To authenticate and improve the accuracy of these results, additional sampling covering a wide range of geographical areas will need to be accomplished to augment the sample sizes and accurately describe the population genetics of these species in terms of heterozygosity, nucleotide diversity and population pair wise distance.

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