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Full Length Research Paper

DNA species surveillance: Monitoring bushmeat poaching and trading in Kenya using partial cytochrome b gene

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DNA species identification has applications in such areas as forensic science, systematics, conservation genetics and agriculture. One key anthropogenic activity threatening large wildlife fauna is illegal exploitation. In Kenya, species identification of raw and processed meat products remains a constraint to effective enforcement of illegal trade in game meat (bushmeat) and products. We tested the reliability of a 321 bp mitochondrial cytochrome b (cyt b) region as a species identification tool for application in wildlife forensics. Query sequences were generated from known specimens of 14 Eastern African wildlife species, 13 representing commonly poached ungulates, and three domesticated species. These were compared, using Basic Local Alignment Search Tool (BLAST) algorithm, with NCBI GenBank reference sequences for species identity. These query sequences were subsequently deposited on Genbank. They represent a contribution to a diagnostic internal East African Wildlife reference cyt b database. The test species comprised: Cape buffalo, bushbuck, Guenther's dik-dik, common duiker, common eland, Grant's gazelle, hartebeest, impala, lesser kudu, plains zebra, Thomson's gazelle, common warthog, wildebeest, Maasai ostrich, cattle, goat and sheep. Additionally, cooked beef and pork samples were analyzed. The results show that, when conspecific sequences were available in the database, species discrimination was 100%. Phylogeny clustering of the species by maximum likelihood supported the species determination by BLAST. The second part of the study carried out a preliminary survey of the prevalence of illegal game meat sold in the dispersal area of Tsavo National Park, Kenya. Sixty two raw meat samples were randomly collected from small roadside retail outlets along the Nairobi-Mombasa highway (A109), a major transnational highway that transverses Tsavo National Park. The results indicate a 9.7% (n = 6) illegal game meat sale, comprising five Guenther's dik-diks and a Beisa oryx. A 2 km radius hotspot, with 83% (n = 5) of the bushmeat sales was identified just south of Tsavo East National Park.

Key words: East Africa, Kenya, bushmeat, poaching, wildlife conservation, species identification, mitochondrial cytochrome b gene.

INTRODUCTION

Bushmeat is the generic term for illegally hunted wildlife meat for sale or consumption as food. Bushmeat is a concern for wildlife conservation due to growing evidence that it is unsustainable (Wilkie et al., 1998; Milner-Gulland and Bennett, 2003; Bowen-Jones et al., 2003) and presents a more immediate threat to wildlife conservation

and species survival than habitat destruction (Redford, 1992).

Bushmeat consumption and trade is widespread, particularly in the tropical rain forests of Africa, South America (Wilkie and Carpenter, 1999; Morra et al., 2009; Redford, 1992; Brashares et al., 2004) and the savanna of Eastern Africa (Okello and Kiringe, 2004; Ndibalema and Songorwa, 2007). Reviews of the factors underlying declines in many bushmeat species in Africa (Bowen-Jones and Robinson, 2003) have identified key drivers of the bushmeat trade as increased human population, rising urban and declining rural incomes, loss of cultural values (Caspary, 2001), poverty, unemployment, food insecurity, settlement in wildlife areas, failure to provide stakes for communities in wildlife-based land uses, inadequate legislation and inadequate investment in enforcement measures (Lindsey et al., 2011). Further, a study covering Ghana, Cameroon, Tanzania and Madagascar (Brashares et al., 2011) suggested that wildlife consumption in Africa is influenced by economic and geographic drivers that present a continuum from subsistence-based rural consumption to mixed subsistence-commercial hunting for urban markets to the extreme case of hunting for the international trade in bushmeat. As the price of bushmeat rises with its movement across the rural to urban gradient, the characteristics of the consumer change as well. Wealthier households consume more bushmeat in settlements nearer urban areas, but the opposite pattern is observed in more isolated settlements (that is, poorer households consume more bushmeat in settlements further from urban areas).

The broad range of factors influencing the bushmeat trade and its importance in rural and urban livelihoods highlights the importance of a multi-disciplinary approach to solutions (Crookes and Milner-Gulland, 2006). Management of the bushmeat trade is therefore a complex issue requiring interventions that are context specific and culturally relevant, targeting both supply (for example, law enforcement and promotion of alternative income) and demand (for example, awareness initiatives to change preferences) (Crookes and Milner-Gulland, 2006). This study focuses on the former wildlife law enforcement intervention.

Law enforcement requires credible forensic evidence. It is imperative that illegal specimens confiscated by law enforcement agencies are unambiguously identified as to their species of origin. Such identification has traditionally relied on anatomical features. However, morphological identification is of limited value when, as is commonly the case, seized specimens are incomplete or processed and often lack diagnostic morphological characters.

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Development and application of DNA taxonomy (Baker and Bradley, 2006) presents a viable and reliable alternative species identification method for such specimens. An important prerequisite for this approach is the availability of a validated DNA sequence database. DNA is ubiquitous in all tissues and this allows recovery of DNA from a wide range of confiscated biological specimens. Due to its relative stability, DNA is recoverable in ancient sources (Pääbo, 1989) as well as heat or chemically treated, fermented or otherwise preserved samples (Kocher et al., 1989; Meyer et al., 1995).

Mitochondrial cytochrome b (*cyt b*) gene is commonly used in species identification of vertebrate species including mammals, birds, amphibians, fishes and reptiles (Kocher et al., 1989; Irwin et al., 1991; Hsien et al., 2001; Verma and Singh, 2003; Tsai et al., 2007; Tobe and Lineacre, 2008). This is due to cyt b genes having a relatively fast mutation rate that results in significant interspecific variation and a relatively small variation within vertebrate species (Irwin et al., 1991; John and Avise, 1998; Hebert at al., 2004; Baker and Bradley, 2006). Further, there are homologous conserved flanking regions that have allowed the development of robust cyt b primers for the amplification of diverse representatives of the vertebrate phyla (Kocher at al., 1989; Irwin et al., 1991; Meyer et al., 1995; Hsieh et al., 2001; Verma and Singh, 2003).

Subsequently, considerable DNA identification of species of unknown samples has been carried out using the cyt b gene region (Meyer et al., 1995; Parson et al., 2000; Bradley and Baker, 2001; Brodmann et al., 2001; Verma and Singh, 2003; Tsai et al., 2007). Alternatively, the Barcode of Life Database (BOLD) (Ratnasingham and Herbert, 2007) uses cytochrome c oxidase I (CO1) region for molecular taxonomy. However, if one locus is to be used as a standard for mammalian species phylogeny and identification, a recent comparative study of CO1 and cyt b genes supports the use of cyt b over CO1 (Tobe et al., 2010).

MATERIALS AND METHODS

This study had two components. First, we tested the reliability of cyt b region for species discrimination and initiated an internal cyt b reference database for 14 large East African wildlife vertebrate species, 13 of which represented commonly poached ungulates. Secondly, we carried out a preliminary survey of the prevalence of bushmeat sales within the Tsavo National Park dispersal area.

Testing the reliability of cyt b species discrimination for 14 selected large East African wildlife vertebrate species

31 known samples of 14 East African wildlife species and three domesticated species were analysed: Cape buffalo: *Syncerus caffer,* bushbuck: *Tragelaphus scriptus,* Guenther's dik-dik: *Madoqua guentheri,* common duiker: *Sylvicapra grimmia,* common eland: *Tragelaphus oryx*, Grant's gazelle: *Gazella granti,*

Table 1. Primer sequences and the predicted size of amplification products.

Primer*	Sequence
Forward Mcb	(398) 5‘ TACCATGAGGACAAATATCATTCTG 3’
Reverse Mcb	(869) 5‘ CCTCCTAGTTTGTTAGGGATTG ATCG 3’

*Derived from *cyt b* gene sequence of blackbuck *Antelope cervicapra*. The numbers 398 and 869 refer to the position of 5' base of the primers in the complete *cyt b* gene sequence of blackbuck (NCBI accession no. AF022058) (Verma and Singh, 2003).

hartebeest: *Alcelaphus buselaphus,* impala: *Aepyceros melampus*, lesser kudu: *Tragelaphus imberbis,* Thomson’s gazelle: *Gazella thomsonii,* common wildebeest: *Connochaetes taurinus,* plains zebra: *Equus burchellii,* common warthog: *Phacochoerus africanus*, Maasai Ostrich: *Struthio camelus maasaicus*, cattle: *Bos taurus,* goat: *Capra hircus* and sheep: *Ovis aries.* Additionally, four cooked (two beef and two pork) samples were analysed.

All 31 test specimens were identified by Kenya Wildlife Service (KWS) and the University of Nairobi veterinarians based on diagnostic morphological characteristics that included an intact skeleton. Samples from cattle, sheep, goat, buffalo, eland, Grant’s gazelle, impala, Thomson’s gazelle and zebra were sourced from Marula and Soysambu game ranches, Kenya. These ranches culled specified game under KWS game cropping licenses. The samples of the bushbuck, dik-dik, duiker, hartebeest, warthog, wildebeest and ostrich were forensic samples from the KWS veterinary laboratory. A single lesser kudu sample was from a partially decomposed carcass found ensnared at Kasigau Ranch, Voi, Kenya. With the exception of the buffalo, bushbuck, dik-dik, lesser kudu and ostrich, two individuals were sampled per species. Approximately 0.5 g of tissue was collected as aseptically as possible and stored in 95% ethanol at -20°C before being transported, under KWS licence, to the Western Kentucky University (WKU) Biotechnology Centre for analysis. Additionally, two fried beef and two pork samples were collected in an *ad hoc* manner from the WKU cafeteria on four different occasions. The second beef and pork samples were stored at 4°C for three days before reheating at 180°C for 10 min. Similarly, the cooked samples were subsequently stored in 95% ethanol at -20°C before analysis.

DNA extraction

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s instructions. The DNA concentration was estimated using Nanodrop spectrophotometry (Thermo Fisher Scientific).

Polymerase chain reaction (PCR) amplification

Primer pairs, designated as *Mcb* (Table 1) were used for the PCR (Saiki et al., 1988) and sequencing reactions. For each individual sample, 20 ng genomic DNA was used. In a total volume of 50 µl, the following reagents and their respective concentrations were used; 0.5 µM each primer, 1.5 mM $MgCl_2$, 0.05 U/µl *Taq* polymerase and 200 µM dNTPs. The cycling conditions were: initial denaturation at 95°C for 10 min; subsequent 35 cycles of denaturation at 95°C for 45 s; annealing at 51°C for 1 min; extension at 72°C for 2 min and a final extension of 72°C for 10 min (Verma and Singh, 2003). The PCR products were analysed using a 1.5% agarose gel electrophoresis.

DNA sequencing

Prior to the sequencing, the amplicons were purified with an Ultraclean DNA cleanup kit (MOBIO) according to the manufacturer’s instructions. Big-Dye terminator cycle sequencing (Applied Biosystems) was carried out in an ABI 3730 automated sequencer. Sequencing was carried out in both forward and reverse directions. The resultant sequences were then assembled using ContigExpress (Vector NTI 9.0, Invitrogen, Carlsbad, CA) and visually examined to detect base-calling errors. The forward and reverse sequences were then aligned to generate consensus sequences.

Sequence similarity search using the Basic Local Alignment Search Tool (BLAST) program

The generated query consensus sequences were compared through a BLAST search of GenBank sequence databases (NCBI/EMBL/DDBJ/PDB) to identify their species origin (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST was optimised for highly similar sequences (megablast). A strict e-value cut-off of 10^{-6} was used.

Preliminary survey of the prevalence of bushmeat sales in the Tsavo dispersal area

A preliminary survey of the prevalence of bushmeat sales in small retail outlets within the dispersal area of Tsavo National Park was carried out. Meat samples were purchased as domesticated species meat, either beef or chevon. Using a stratified random sampling method, 62 raw meat samples (Table 5) were obtained from towns located along the Nairobi-Mombasa highway (A109) that traverses the Tsavo National Park dispersal area. Approximately, 0.5 g of tissue was collected as aseptically as possible and stored in 95% ethanol before being transported to the International Livestock Research Institute (ILRI) Nairobi for storage at -20°C and analysis. DNA extraction, PCR, sequencing and BLAST protocol was as described previously. Four known positive controls (Grant’s gazelle, eland, impala and buffalo) were used (Table 2).

RESULTS

Testing the reliability of cyt b species discrimination for 14 selected large East African wildlife vertebrate species

The *Mcb* primers were aligned to homologous sections of *cyt b* gene of the test species and determined to have an

Table 2. Positive control species used in the retail market survey analysis.

Control species	Test species NCBI Accession Number	Identified species	E-value	Species Identity NCBI Accession number	Identity (%)
Gazella granti	AY534343	*G. granti*	0.0	AF034723.1	84
Tragelaphus oryx	FJ785344	*T. oryx*	0.0	AF036278.1	97
Aepyceros melampus	FJ785385	*A. melampus*	0.0	AF034966.1	98
Syncerus caffer	FJ785373	*S. caffer*	0.0	AF036275.1	97

Table 3. Identity of *Mcb* forward and *Mcb* reverse primer set, designed from *Antelope cervicapra* (Accession no. AF022058) to homologous sequences of test species.

Species	NCBI Accession number	*Mcb* Forward (398)	*Mcb* Reverse (869)
Bos taurus	V00654	100	96.2
Capra hircus	DQ089480	96.0	92.3
Ovis aries	DQ097430	100	96.2
Syncerus caffer	AF036275	96.0	84.6
Tragelaphus scriptus	AF036277	100	92.3
Madoqua guntheri	AF022071	92	96.2
Sylvicapra grimmia	AF153905	91.7	88.5
Tragelaphus oryx	AF036278	91.7	96.2
Gazella granti	AF034723	92.0	88.5
Alcelaphus buselaphus	AF016640	96.0	92.3
Aepyceros melampus	AF036289	96.0	92.3
Tragelaphus imberbis	AF036279	92.0	92.3
Phacochoerus africanus	Z50090	92.0	84.6
Connochaetus taurinus	AF016638	92.0	88.5
Equus burchelli	-	Unavailable	Unavailable
Gazella thomsonii	-	Unavailable	Unavailable
Struthio camelus masaicus	U76055	92.0	80.8

identity of between 84.6 to 100% (Table 3). The primers successfully amplified a consensus sequence region of 321 bp across all the test species. This region was used as the standard test fragment size. A total of 20 mtDNA haplotypes were detected across the test species. Two intraspecific site variations were evident in goat (substitution T, C at position 89 and A, G at position 191; NCBI accession nos. FJ785333 and FJ785334), four in sheep (substitution C, T at position 7; A, C at position 9; T, A at position 10 and G, A at position 43; NCBI accession nos. FJ785335 and FJ785336) and one in the common eland (single substitution T, C at position 10; NCBI accession nos. FJ785343 and FJ785344). Despite a small sample size, there were high levels of inter-specific variations; 137 site variations with a haplotype diversity of 0.974 (calculated by the formula given by Nei, 1987).

Exact species identity was observed in all but two test species (Table 4). The percentage sequence similarity ranged from 95 to 100% (Table 4) indicating that the results obtained using this method was accurate and reliable. The DNA recovered from the cooked pork samples was degraded. Subsequently, the first sample sequenced only the reverse strand while the second sample sequenced a short 191 bp consensus sequence. Repeat PCR and sequencing did not improve the results. However, these samples, as well as the cooked beef samples were accurately identified as *Sus scrofa* and *Bos Taurus*, respectively (Table 4). Using evolutionary analysis conducted in MEGA5 (Tamura et al., 2011), a phylogenetic clustering of species by Maximum Likelihood (Tamura and Nei, 1993) was congruent to the species determination by BLAST (Figure 1). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Preliminary survey of the prevalence of bushmeat sales in the Tsavo dispersal area

From the 62 samples purchased randomly from retail

Table 4. *Mcb* cytochrome b region species identification test results using known voucher samples.

Test species	NCBI accession number*	BLAST E-value	Specimen identity as determined by BLAST	Sequence similarity (%)	NCBI accession number
Bos. taurus 1	FJ785331	1e-166	*Bos taurus*	100	GU947021.1
Bos taurus 2	FJ785332	1e-166	*Bos taurus*	100	GU947021.1
Capra hircus 1	FJ785333	5e-176	*Capra hircus*	99	HM209303.1
Capra hircus 2	FJ785334	2e-179	*Capra hircus*	100	HQ996593.1
Ovis aries 1	FJ785335	2e-164	*Ovis aries*	100	HM209296.1
Ovis aries 2	FJ785336	1e-166	*Ovis aries*	100	HM236175.1
Syncerus caffer 1	FJ785337	3e-163	*Syncerus caffer*	99	D82888.1
Syncerus caffer 2	FJ785338	3e-163	*Syncerus caffer*	99	D82888.1
Tragelaphus scriptus	FJ785339	1e-166	*Tragelaphus scriptus*	100	EF137968.1
*Sylvicapra grimmia*1	FJ785341	1e-161	*Sylvicapra grimmia*	99	FJ807613.1
*Sylvicapra grimmia*2	FJ785342	1e-161	*Sylvicapra grimmia*	99	FJ807613.1
Madoqua guentheri	FJ785340	1e-141	*Madoqua guentheri*	95	AF022071.1
Tragelaphus oryx 1	FJ785343	6e-165	*Tragelaphus oryx*	99	HQ122589.1
Tragelaphus oryx 2	FJ785344	3e-163	*Tragelaphus oryx*	99	HQ122589.1
Gazella granti 1	FJ785345	3e-158	*Gazella granti*	98	AF034723.1
Gazella granti 2	FJ785346	3e-158	*Gazella granti*	98	AF034723.1
Alcelaphus buselaphus 1	FJ785347	6e-160	*Alcelaphus buselaphus*	98	AF028822.1
Alcelaphus. buselaphus 2	FJ785348	6e-160	*Alcelaphus buselaphus*	98	AF028822.1
Aepyceros melampus 1	FJ785349	1e-166	*Aepyceros melampus*	100	HQ122593.1
Aepyceos. melampus 2	FJ785350	1e-166	*Aepyceros melampus*	100	HQ122593.1
Tragelaphus. imberbis	FJ785351	1e-166	*Tragelaphus imberbis*	100	AF036279.1
Struthio camelus massaicus 1	FJ785364	6e-165	*Struthio camelus*	99	HQ122573.1
Struthio camelus massaicus 2	FJ785365	6e-165	*Struthio camelus*	99	HQ122573.1
Gazella. thomsonii 1	FJ785352	4e-122	*Antilope cervicapra*	98	FJ556559.1
Gazella thomsonii 2	FJ785353	4e-122	*Antilope cervicapra*	100	FJ556559.1
Phacochoerus africanus 1	FJ785354	1e-166	*Phacochoerus africanus*	100	DQ409327.1
Phacochoerus africanus 2	FJ785355	1e-166	*Phacochoerus africanus*	100	DQ409327.1
Connochaetes taurinus 1	FJ785356	1e-166	*Connochaetes taurinus*	100	AF034969.1
Connochaetes taurinus 2	FJ785357	1e-166	*Connochaetes taurinus*	100	AF034969.1
Equus burchellii 1	FJ785358	3e-138	*Equus sp. (E. grevyi)*	97	HQ122608.1
Equus burchellii 2	FJ785359	3e-138	*Equus sp. (E. grevyi)*	97	HQ122608.1
Bos taurus 1 (Cooked beef)	FJ785360	1e-166	*Bos taurus*	100	GU947021.1
Bos taurus 2 (Cooked beef)	FJ785361	1e-166	*Bos taurus*	100	EU177857.1
Sus scrofa 1 (Cooked pork)	FJ785362	2e-164	*Sus scrofa*	100	GU135837.1
Sus scrofa 2 (Cooked pork)	FJ785363	1e-94	*Sus scrofa*	100	DQ534707.2

outlets in the dispersal area of Tsavo National Park ecosystem, six were identified as game meat (bushmeat) species. The species were identified as five Guenther's dik-diks: *M. guentheri*, and one Beisa oryx: *Oryx beisa*. This represented 9.7% (n = 6) of the samples purchased (Table 5). All the samples identified as bushmeat originated at close proximity to the protected National Park. A 2 km radius hotspot representing 83% (n = 5) of the bushmeat sales was delineated just south of Tsavo East National Park (Figure 2), all the species identified here were Guenther's dik-diks (Table 2). The outlier sample identified as game meat species, a *B. oryx*, was purchased in the dispersal area of Chyulu National Park (Figure 2).

DISCUSSION

Mitochondrial genome is one of the most extensively studied, as its abundance and small size makes it easy to isolate and purify (Wolf et al., 1999). With the exception of rare presence of paternal mtDNA in offspring (Cree et

Table 5. *Mcb* cytochrome b region species identification of raw meat samples purchased from small retail outlets along Nairobi-Mombasa highway. The town listing order is incremental distance with reference to Nairobi.

Sample No.	Area	Putative species	Identity species	NCBI accession number
1	Machakos	*Bos taurus*	*Bos taurus*	JN817351.1
2	Machakos	*Bos taurus*	*Bos taurus*	JN817351.1
3	Makutano	*Capra hircus*	*Capra hircus*	GU229281.1
4	Sultan Hamud	*Capra hircus*	*Ovis aries*	GU233796.1
5	Emali	*Capra hircus*	*Ovis aries*	DQ903224.1
6	Emali	*Bos taurus*	*Bos taurus*	JN817351.1
7	Machinery	*Bos taurus*	*Bos taurus*	GU249569.1
8	Machinery	*Bos taurus*	*Bos taurus*	GU249569.1
9	Machinery	*Bos taurus*	*Bos taurus*	AB110596.1
10	John Mulwa	*Bos taurus*	*Bos taurus*	GU229278.1
11	Suncity	*Bos taurus*	*Bos taurus*	GU249569.1
12	Kimongo	*Bos taurus*	*Bos taurus*	GU249569.1
13	Kimongo	*Bos taurus*	*Bos taurus*	JN817351.1
14	Makindu	*Bos taurus*	*Bos taurus*	JN817351.1
15	Makindu	*Bos taurus*	*Bos taurus*	GU249569.1
16	Kitui road	*Capra hircus*	*Capra hircus*	GU229281.1
17	Masimba	*Capra hircus*	*Capra hircus*	GU229278.1
18	Chyulu	*Capra hircus*	*Capra hircus*	GU229278.1
19	Chyulu	*Capra hircus*	*Capra hircus*	GU229278.1
20	Chyulu	*Capra hircus*	*Oryx beisa*	DQ138210.1
21	Mbuizau	*Capra hircus*	*Ovis aries*	DQ903224.1
22	Kibwezi	*Capra hircus*	*Capra hircus*	EU259132.1
23	Kibwezi	*Capra hircus*	*Capra hircus*	GU229278.1
24	Kibwezi	*Bos taurus*	*Bos taurus*	GU249569.1
25	Kibwezi	*Capra hircus*	*Capra hircus*	GU229281.1
26	Kibwezi	*Capra hircus*	*Capra hircus*	GU229278.1
27	Kibwezi	*Capra hircus*	*Capra hircus*	EU259132.1
28	Kambu	*Capra hircus*	*Capra hircus*	GU229278.1
29	Kambu	*Capra hircus*	*Capra hircus*	AB110596.1
30	Mangelete	*Capra hircus*	*Capra hircus*	GU229278.1
31	Mtito Andei	*Capra hircus*	*Capra hircus*	AB110596.1
32	Mtito Andei	*Capra hircus*	*Capra hircus*	GU229278.1
33	Mtito Andei	*Bos taurus*	*Bos taurus*	GU249569.1
34	Mtito Andei	*Bos taurus*	*Bos taurus*	JN817351.1
35	Mtito Andei	*Capra hircus*	*Ovis aries*	FJ218141.1
36	Mtito Andei	*Capra hircus*	*Ovis aries*	DQ903224.1
37	Manyani	*Capra hircus*	*Capra hircus*	GU229281.1
38	Manyani	*Capra hircus*	*Capra hircus*	GU229278.1
39	Sofia Voi	*Bos taurus*	*Bos taurus*	GU249569.1
40	Sofia voi	*Bos taurus*	*Bos taurus*	JN817351.1
41	Ghazi Voi	*Capra hircus*	*Capra hircus*	GU229278.1
42	Tausa	*Capra hircus*	*Capra hircus*	AB110596.1
43	Voi	*Capra hircus*	*Capra hircus*	AB110596.1
44	Kasigau	*Bos taurus*	*Capra hircus*	GU229278.1
45	Kasigau	*Bos taurus*	*Capra hircus*	GU229278.1
46	Kasigau	*Bos taurus*	*Capra hircus*	GU229278.1
47	Kasigau	*Capra hircus*	*Capra hircus*	FJ455852.1
48	Rukanga	*Capra hircus*	*Capra hircus*	GU229278.1

Table 5. Contd.

49	Buguta	*Capra hircus*	*Capra hircus*	GU229278.1
50	Marungu	*Capra hircus*	*Capra hircus*	GU229278.1
51	Kibauni village	*Capra hircus*	*Madoqua guentheri*	AF030598.1
52	Maungu	*Capra hircus*	*Capra hircus*	GU229278.1
53	Maungu	*Capra hircus*	*Capra hircus*	GU229278.1
54	Taita village	*Capra hircus*	*Madoqua guentheri*	AF030598.1
55	Miasenyi	*Capra hircus*	*Madoqua guentheri*	AF030598.1
56	Makina	*Bos taurus*	*Bos taurus*	JN817351.1
57	Makina	*Capra hircus*	*Madoqua guentheri*	AF030598.1
58	Meli Kubwa	*Capra hircus*	*Madoqua guentheri*	AF030598.1
59	Meli Kubwa	*Capra hircus*	*Capra hircus*	GU229281.1
60	Umoja	*Capra hircus*	*Capra hircus*	EU259132.1
61	Taita village	*Bos taurus*	*Bos taurus*	JN817351.1
62	Samburu	*Capra hircus*	*Capra hircus*	GU229278.1

al., 2009), the inheritance pattern is almost exclusively maternal with absence of recombination (Hayashi and Walle, 1985). Analysis is therefore simplified due to general lack of heterozygosity. Presence of cyt b nucleotide substitutions that may represent synapomorphic character states defining phylogenetic lineages, permits inference on species variation (Tobe et al., 2010) using sequence data. Subsequently, *cyt b* gene has been extensively used to discriminate among fish (Akimoto et al., 2006), mammalian, avian and reptilian vertebrate species (Parson et al., 2000; Verma and Singh, 2003; Murugaiah et al., 2009).

In Kenya, suspected poached game meat is usually encountered at various stages of processing or as falsified meat either in commercial outlets or on transit to such outlets (KWS, personal communication). Application of *cyt b* gene species identification may be a valuable tool to assist KWS in enforcement and surveillance measures countering illegal harvesting and marketing of bushmeat. For *cyt b* region to be a reliable and accurate tool for species identification, a validated East African reference database generated from baseline voucher samples that are rigorously acquired and tested is needed.

Using *Mcb* primers (Verma and Singh, 2003), the first part of this study tested the reliability of species level discrimination by a 321 bp *cyt b* gene region as well as generating verified sequences for input into a validated East African species reference database. The species used for this part of the study were large vertebrate wildlife at a high risk of illegal game cropping. The results show that NCBI's GenBank, with an estimated sequence database of close to 100 million, presents a potentially reliable bioinformatic tool for cyt b species identification. A strict e-value cut-off (10^{-6}) BLAST algorithm was used to search the NCBI GenBank reference sequence database for the best alignment hit (top hit) to all or part of the query sequence. This method is reported to perform better than other weighted methods of BLAST (Ross et al., 2008). Where conspecific sequences were available in the database, the use of the conserved single locus *Mcb cyt b* gene region reliably and accurately identified the species of the selected group of large and diverse East African wildlife fauna. Where no conspecific sequences were available in the database, false positives occurred as evident in the misidentification of plains zebra: *E. burchellii* as Grevyi's zebra: *Equus grevyi* and Thomson's gazelle: *G. thomsonii* as blackbuck: *Antilope cervicapra*. This was consistent with Ross et al. (2008) suggestion that BLAST is a reliable method if conspecific sequences which are available in the database, otherwise false positives would occur.

It is therefore imperative that a validated and local reference cyt b region sequence database be generated as a stand-alone as well as a parallel database to GenBank. This is important because GenBank as the source database is not validated. For instance, in the event that a GenBank input sequence of a species is incorrect, a correct query sequence would not "hit", not as a result of an erroneous query voucher specimen but because of the incorrect reference sequence on GenBank. A reference database for East African wildlife species therefore needs to be established. These limitations indicate that GenBank database should not be the basis for the "gold" standard but can be used to corroborate the internal East African database. In this study, we used a more rigorous protocol for establishing known baseline samples whose sequences were then deposited in GenBank. These sequences are representative of an initial internal reference East African vertebrate wildlife cyt b database. More work is required to generate reference sequences from voucher specimens to build a comprehensive database covering

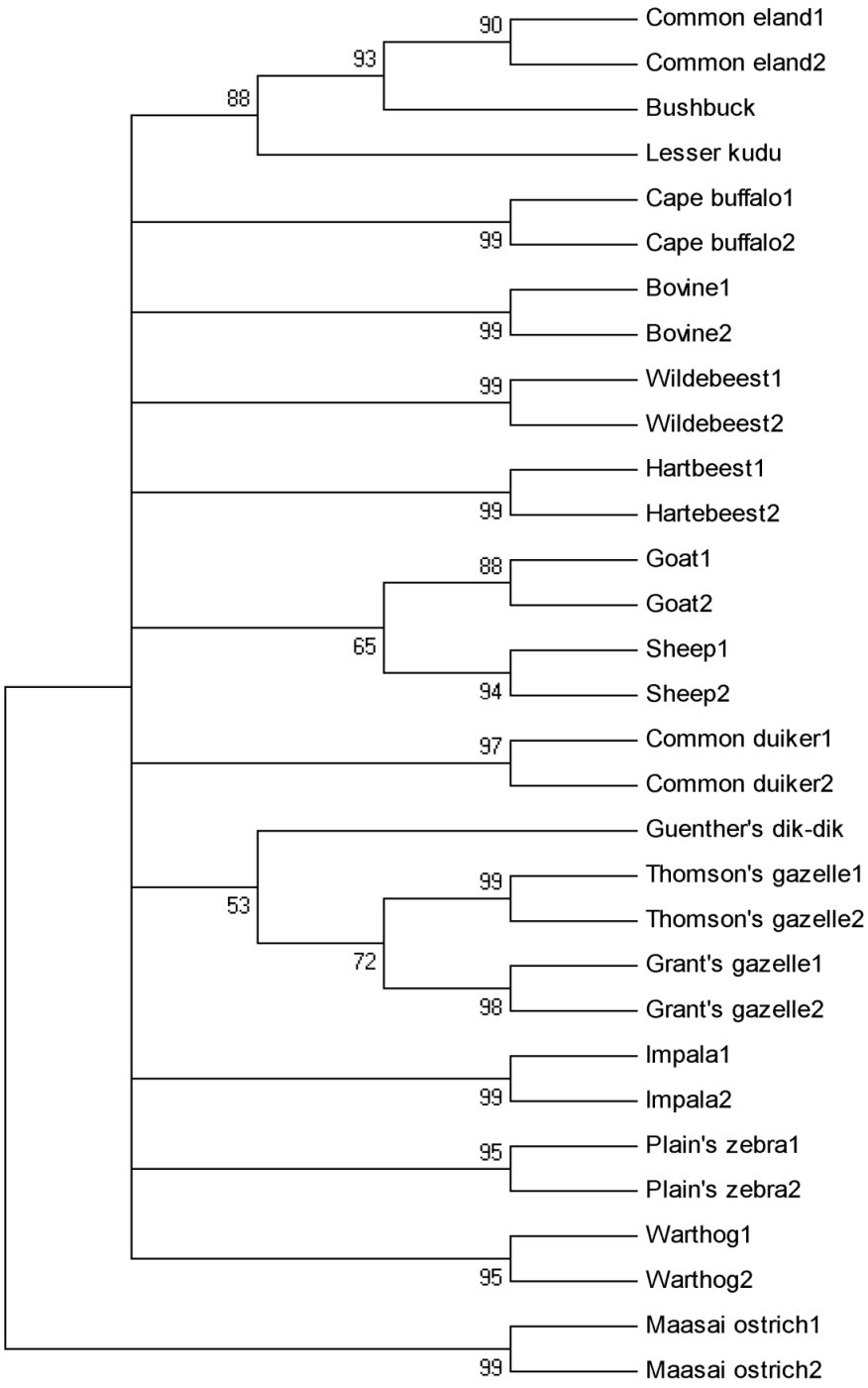

Figure 1. 321 bp mtDNA (cyt b) region phylogenetic anaylsis of the test species by maximum likelihood method based on the Tamura Nei model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

more East African vertebrate species.

This method is reliable as it showed versatility in its ability to use a single conserved primer pair to accurately identify, where conspecific sequences were available in the database, diverse East African species from varied sources of biological material - preserved, partially

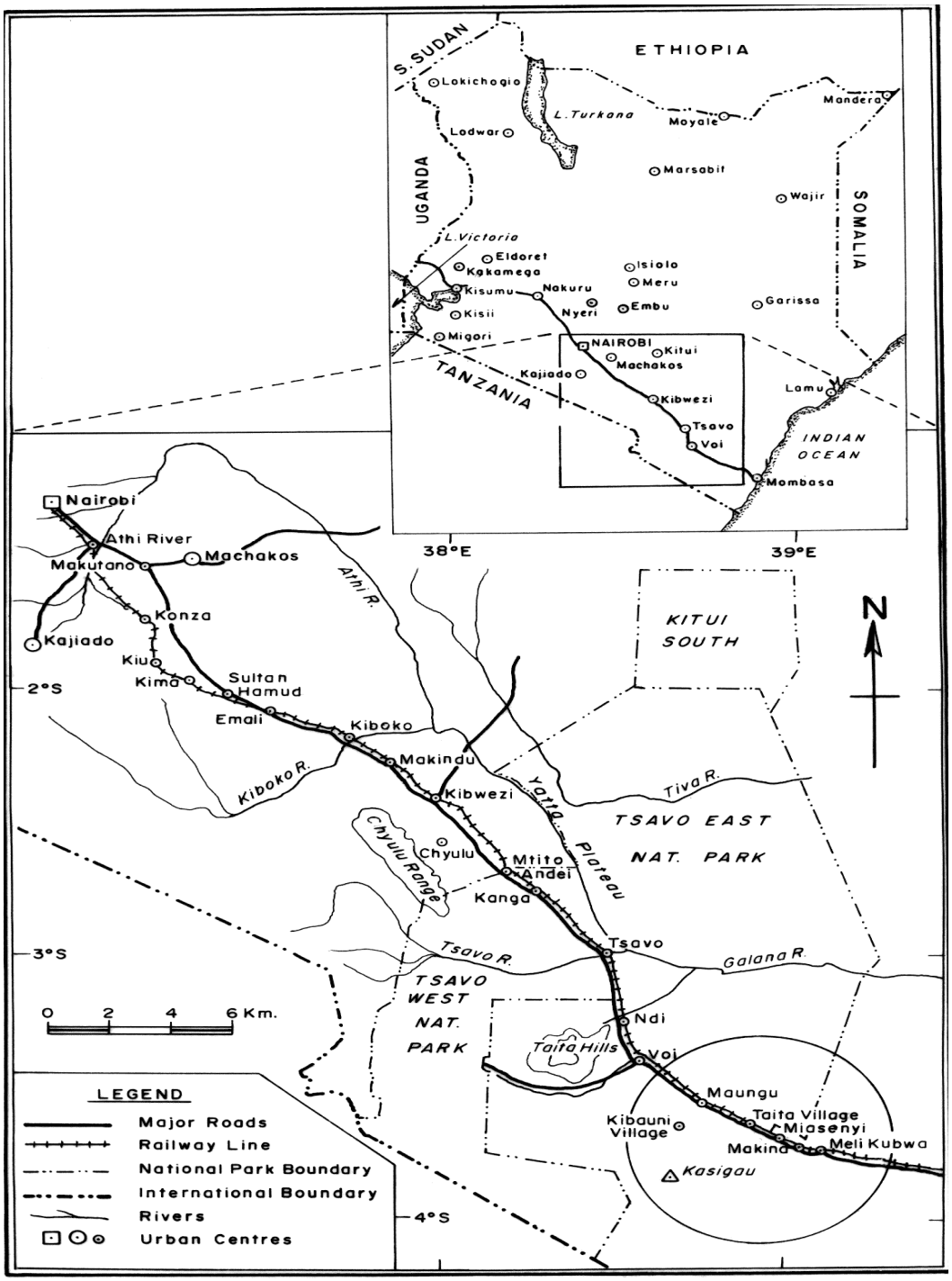

Figure 2. Sample collection centers along the Nairobi-Mombasa highway. The encircled 2 km radius area at the bottom right indicates a hotspot where 83% of the samples identified as bushmeat were purchased.

decomposed and cooked.

In the second part of this study, we carried out a preliminary survey of the prevalence of local bushmeat trade in Kenya. We determined the species origin of 62 raw meat samples randomly purchased from small retail outlets located along A109, the Nairobi - Mombasa

highway traversing the Tsavo National Park and its dispersal area (Figure 2). Tsavo National Park, comprising Tsavo East and West, covers a total area of 22,812 km^2 (KWS, 2012). Six samples were identified as game meat species; of these six samples, five were Guenther's dik-diks, *M. guentheri*, and one a *B. oryx*, *O. beisa.* This represented a 9.7% (n=6) of the total (N=62) meat samples purchased. 83% (n=5) of the samples identified as bushmeat (n=6) were purchased within a hotspot area comprising small centres within a 2 km radius adjacent to Tsavo East National Park on the South-Eastern border (Figure 2). The other positive sample was purchased at Chyulu centre, located at the dispersal area of Chyulu National Park (Figure 2). This finding suggests that the proximity of human settlements to the protected wildlife conservation area influences the availability of wildlife, presenting an opportunity for illegal harvesting. Human settlement adjacent to wildlife areas is therefore a potential driver for bushmeat consumption and trade.

Cyt b gene sequence data and comparative analysis against a validated database provides an accurate and reliable identification tool for game meat species. Such a method can be widely applied to diverse biological specimens such as raw, degraded, decomposed (Tsai et al., 2007) processed (cooked) or otherwise preserved tissue. The creation of a validated database for East African wildlife species would be useful in providing credible forensic evidence necessary for KWS, as the Kenyan wildlife law enforcement agency to, not only help curb illegal game meat trade, but also enhance the capacity to regulate, manage and monitor any legal game meat outlets to ensure licence requirement compliance.

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