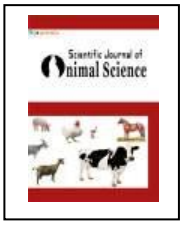
Sjournals
Scientific Journals
Scientific Journal of Animal Science (2014) 3(3) 87-94
ISSN 2322-1704
doi: 10.14196/sjas.v3i3.1137

Contents lists available at Sjournals
Scientific Journal of Animal Science
Journal homepage: www.Sjournals.com



Original article

Use of cytochrome oxidase 1 gene region: a molecular tool for the domestic and wildlife industry in Kenya

D. Mbugua*, C.N. Kimwele, J.O. Jung'a, E. Gatebe, F. Gakuya., M.J. Kinuthia, M.M. Mugambi, E.S. Kwamboka

*Corresponding author;

ARTICLE INFO

Article history:
Received 11 January 2014
Accepted 22 February 2014
Available online 15 March 2014

Keywords:
DNA species identification
CO1
Bold
PCR
NCBI
DNA bar coding
Wildlife
Bush meat
Meat substitution

ABSTRACT

Illegal substitution of meat products by traders either as closely related domestic species or as bush meat is a common occurrence in various parts of Kenya. This has implications on biosafety, food safety and consumer confidence and subsequently the meat and meat products industry both locally and export. In recent times, use of molecular techniques have seen increased application in wildlife conservation through conservation genetics in areas such as population genetics, evolutionary genetics, molecular ecology and wildlife forensics. We used DNA of the Cytochrome C Oxidase 1 gene region as a bar-coding technique for species identification. The accuracy of CO1 as a marker was tested using five known samples of wildlife species.Retail meat product substitution and bushmeat prevalence was estimated from 99 unknown meat samples that were randomly collected from meat traders in Nakuru County. The study validated the use of CO1 marker for species identification and illustrated use of the marker in identification of unknown tissue samples collected from the market survey.

1. Introduction

Species specification is important for quality control management of meat used as food, usually skeletal muscle, other organs like liver, kidneys, lungs and associated fat (Aberle et al., 2001). Fraudulent substitution is a common malpractice in the meat industry in which preferred domestic meat is substituted for another closely related domestic species or even wild animals e.g. chevon substituted by mutton, beef substituted with donkey or buffalo meat (Kang'ethe et al, 1986, Karisa et al, 2009).

Wildlife conservation plays a central role in tourism in Kenya. Tourism is Kenya's largest foreign exchange earning sector earning ksh73.68 billion in 2010 (Ministry of tourism, Kenya). Various conservation measures have been undertaken over the years ranging from wildlife protected areas to game farms and captive animal management bio-parks and animal orphanages.

In Kenya, an increasing large rural poor population around wildlife habitats may cause an increase in the utilization of bush meat. Poachers set indiscriminate snares that ensnare any species from non-threatened ostrich to the tiny dikdik antelope as well as threatened Kenyan endemic species such as the rare bongo or the roan antelope. The meat finds its way into urban centers like Nairobi and is sold for the pot. With a ready market for bush meat, poachers have no problem selling the "free meat" to village butcheries and truckers who ferry containers across the continent (Eating the unknown, Born Free foundation). Bush meat is very cheap in Kenya, unlike in West Africa where it is double the cost of domestic meat. A chunk of giraffe meat or a dikdik in Kenya goes for as little as Ksh 50 (US$ 0.5) (Eating the unknown, Born Free foundation). This low price does not reflect the actual economic value of wildlife as a natural resource, undervaluing it, a cost to the national economy. A whole chicken on the other hand, costs five to six times that price

In recent times, use of molecular techniques has seen increased application in wildlife conservation through conservation genetics in areas such as population genetics, evolutionary genetics, molecular ecology and wildlife forensics. DNA forensics primarily involves use of in species, population or individual identification. Such a process involves the comparison of a unknown specimens with that of a known specimens. DNA techniques are important for identification of confiscated wildlife specimen that lack diagnostic anatomic features. Use of molecular forensics can help identify the species from which the specimen is from especially by use of DNA barcode technique (Hajibabaei, M et al 2007) in combination with short tandem repeats (STRs), and this can significantly contribute to the enforcement of illegal trade in wildlife products and consequently reduce poaching. DNA-based methods are generally more advantageous and are currently widespread in the field of food authentication (Rasmussen and Morrissey 2008). In species identification, depository sequences from known species need to be available from depository data banks.

DNA bar-coding uses Cytochrome C Oxidase 1 (CO1) gene sequences to discriminate animal species (Hebert et al, 2003). The barcodes (sequences) generated are then compared with sequences held in the Barcode of Life Data Systems (BOLD) that manages the Barcode sequences. BOLD is an online system accessible to all for collecting, managing and analyzing DNA barcodes. DNA bar-codes have been proposed as a powerful new method for quickly identifying known species and discovering unknown species (Blaxter, 2003; Hebert et al., 2005; Marshall, 2005). DNA barcoding is designed purely to aid the recognition and identification of known species (Valentini et al. 2009; Casiraghi et al. 2010).

2. Materials and methods

The study aimed at validating the use of CO1 as a marker in wild animal species identification by use of five known wildlife species and identifying the prevalence of bush meat utilization within Nakuru town and its environs.

2.1. Study site and sampling

The study was conducted in Nakuru county in Rift valley province of Kenya purposively selected due to the many game parks, reserves and sanctuaries available in the area that host wildlife.

The areas to be sampled in the county were selected by stratified random sampling. The areas were sub-divided into urban areas, formal and informal settlements. The meat traders to be sampled in the different subdivided areas were then selected by simple random sampling technique.99 meat samples were collected. The meat samples were then aseptically sectioned and stored in cryo-vials containing 70% ethanol in the field and were later transported to the Kenya Wildlife Services (KWS) laboratory for refrigeration at 4oc for long term storage.

The second part of data collection involved the darting of selected wild animals; Impala (Aepyceros melampus), waterbuck (Kobus ellipsiprimn), buffalo (Syncerus caffer), Black rhino (Diceros bicornis) and elephant

(Loxodonta cyclotis). Five tissue biopsy samples were collected for each of the species under study and verified by a KWS veterinarian officer for authentication based on morphological diagnostic techniques of animals with intact skeletal structure. Where available, blood samples were obtained from restrained animals using a KWS veterinary officer in the field and from the KWS databank. Five blood samples of elephant and black rhino species were collected and stored in EDTA and kept in a cool box in the field. Five blood samples of elephant and black rhino species were collected and stored in EDTA and kept in a cool box in the field. The blood samples were later stored in a freezer at -20oc at KWS headquarters awaiting analysis. The collected tissue biopsy samples were stored in 70% ethanol in the field before being transferred for refrigeration at 4oc at KWS. Using a Dan-inject rifle, Biopsy darts were used to collect the tissue biopsy samples. The biopsy darts are designed to bounce back off the animal (Dan-inject, 2004) upon puncture. Only adult wild animals were sampled.

2.2. DNA Extraction

Total Genomic DNA from the meat samples was extracted using Zymo research DNA blood and tissue® extraction kit according to the manufactures instructions.

DNA concentration was estimated using a spectrophotometer (Eppendorf Biophotometer)

2.3. Polymerase chain reaction (PCR).

PCR was carried out to amplify a 700 bp DNA fragment from CO1 gene region. The universal conserved vertebrate BOLD primers used were: VF1D – t1 (5'TTCTCAACCAACCACAARGAYATYGG 3') and VR1D – t1 (5'TAGACTTCTGGGTGGCCRAARAAYCA 3'), (Ivanova et al. 2006).

The PCR volume of 50ul comprised approximately 2 µl (20nanograms) of template DNA; 10 picomoles of primer and Taq polymerase master mix (25 µl containing 0.4mM of each dNTP, 4.0 mM mgcl2, 1.5 units of Taq and Dream TaqTM buffer).

PCR cycling comprised 15 min at 95 ºC for initial denaturing followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 56.5 °C for 30 s and a final extension step at 72 °C for 1 min for 10 min at 72 ºC. PCR specificity was examined by 1.0% agarose gel using 2 µl of sample mixed with 3µl phenolphthalein blue dye from each reaction by utilizing ethidium bromide staining technique and visualized under ultra violet light.

Electrophoresis was done at 80 V for one hour and the resultant gel was visualized under ultra violet light (UV) transilluminator using an Uvitec gel documentation system.

2.4. DNA Purification

The P.C.R products obtained were purified using the GeneJETTM purification kit according to manufactures instructions. PCR products were purified to remove excess pre-PCR components like primers, dNTPs and buffers.

2.5. Sequencing

Purified amplicons were sequenced using Sanger's dideoxy terminator sequencing method by use of automated sequencing according to the manufactures instructions. Both forward and reverse direction sequences were derived. Resultant sequences were visualized using BioEdit® to detect and edit base-calling errors and forward and reverse sequences for each sample aligned to generate consensus sequences.

2.6. Use of bold and basic local alignment tool (BLAST) for sequence similarity search

The sequences were identified using BOLD database by use of species level Barcodes records search engine for sequences of more than 500 base pairs. The generated sequences were then identified through BLAST search for comparing with GenBank sequence database (NCBI) for identification of the species of origin of the specimen utilized. BLAST search was optimized to compare for highly similar sequences. E-value cut-off 0.0 was utilized for identification of highly significant matches.

3. Results

DNA was successfully extracted from all the five wildlife samples and 99 unknown meat samples from traders utilized. Extracted DNA was amplified and amplicons of approximately 680 base pairs of the CO1 gene were derived by use of the vertebrate universal primer. The derived amplicons were electrophoresed on 1.0 % agarose gel and the pictures of the visualized images taken as shown in the figure 1.1 below.

The PCR amplicons were purified by use of GeneJETTM purification kit according to manufactures instructions and successfully recovered amplicons were electrophored in 1.0% agarose gel and pictures of the visualized amplicons taken as shown in figure 1.2. After sequencing, approximately 680 base pairs long sequences were derived.

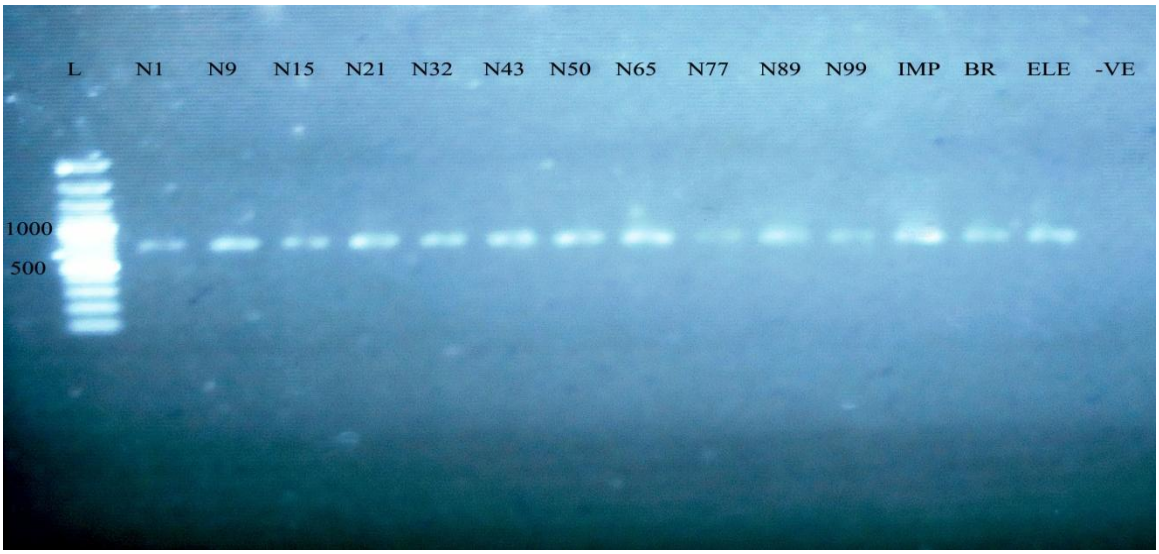


Fig.1.1. An agarose gel image of CO1 PCR products at approximately 680 base pairs for both wildlife and domestic animals biopsy samples.

Key: L=ladder, N1-N99=Domestic animals meat samples, IMP=common impala, BR= black rhino, ELE= elephant, -VE= Negative control.

3.1. Validating use CO1 gene as a marker for species identification

BLAST results showed wildlife samples had maximum identity of above 98% with 80% of all the sequences having a maximum identity of 99%. All sequences had an E-value of 0.00 indicating highly significant matches. All the samples that had been morphologically identified during samples collection in the field were accurately identified to their true species as illustrated in the table 1.1. The sequences were similarly identified using BOLD database by use of species level Barcodes records search engine for sequences of more than 500 base pairs with similar results.

Table 1.1
Identity of sampled wildlife animals using BLAST and BOLD database.

Samples species	Identified species (BLAST)	Accession #	E-value	Max identity %	Identified species by BOLD	Top hit Similarity score
Buffalo	Syncerus caffer	JQ235544.1	0.0	99	Syncerus caffer	99.35%
Black rhino	Diceros bicornis	FJ905814.1	0.0	98	Diceros bicornis	99.51%
Elephant	Loxodonta cyclotis	JN673264.1	0.0	99	Loxodonta cyclotis	99.21%
Water buck	Kobus ellipsiprimn	JN632651.1	0.0	99	Kobus ellipsiprimn	98.73%
Impala	Aepyceros melampus	JN632592.1	0.0	99	Aepyceros melampus	98.44%

Phylogenetic and molecular evolutionary analyses of the derived sequences from the sampled wildlife were conducted using MEGA version 5 (Tamura et al, 2011). Fourteen reference samples for each species by use of their accession numbers identified above derived from NCBI were utilized in deriving the Phylogenetic tree to illustrate the clustering of each sample to its true specie as shown in the figure 1.2. Bootstrap method for testing phylogeny was utilized by use of 1000 bootstrap replications (Felsentein, 1985). Resultant Phylogenetic clustering of sample species by use of maximum likelihood (Tamura and Nei, 1993) confirmed the identification of species by BLAST.

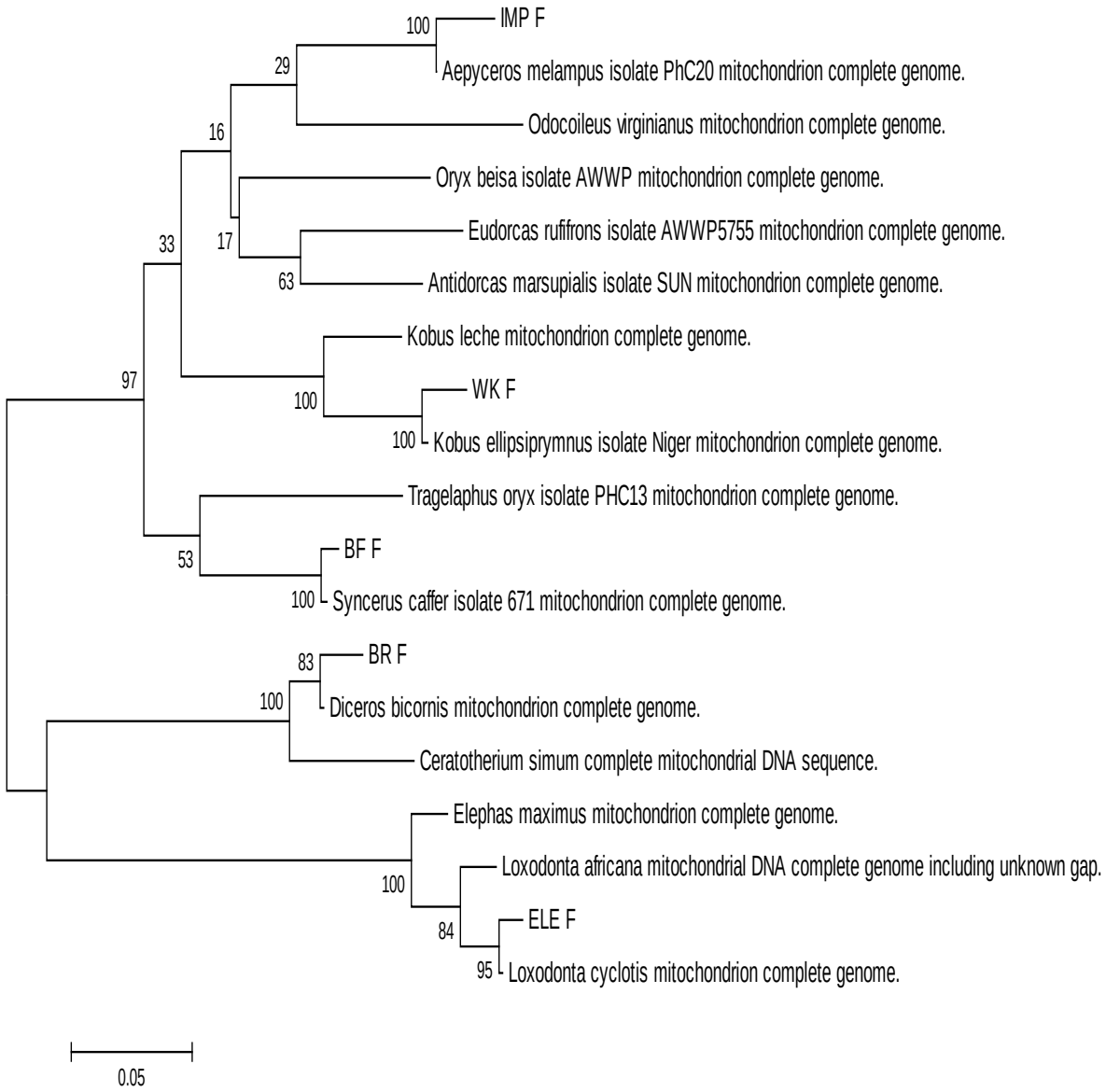


Fig. 1.2. Phylogenetic tree analysis of the test species in relation to their reference species in relation to other closely related species derived from NCBI by use of maximum likelihood Tamura Nei model. The percentage of the replicate trees that associated species clustered together in the bootstrap test using 1000 replicates is indicated next to the branches.

Key: WK F=waterbuck, IMP F=impala, BF F-buffalo, BR F= black rhino and ELE F= elephant.

3.2. Identifying the prevalence of bush meat sale and meat substitution by traders in nakuru and its environs

From the 99 unknown species samples collected, no bush meat was detected indicating the prevalence of bush meat utilization in Nakuru and its environs at the time of sampling to be 0%. Species substitution between goat and sheep by meat traders was identified to be prevalent in the region especially in the urban areas but also present in formal and informal settlements. No substitution was observed for sale of beef. However, substitution of mutton for chevon was prevalent in all zones surveyed of formal settlements (1), informal settlements (1) and urban areas (18). From the collected samples, 66.67% of samples collected were beef, 13.13% were mutton and 20.20% of the samples collected were chevon but after identification through BLAST, 66.67% were identified as beef and 33.33% as mutton while no samples were identified as chevon.

Table 1.2
Putative and confirmed species from samples collected.

Putative species	Number collected	Identified species by BLAST	Number identified
Bos Taurus	66	Bos Taurus	66
Ovis aries	13	Ovis aries	33
Capra hircus	20	Capra hircus	0
Total	99		99

Table 1.3
Classification of meat substitution by traders according to the areas sampled.

Zone	Cases of meat substitution (chevon for mutton)
Urban areas	18
Formal settlements	1
Informal settlements	1
Total	20

4. Discussion

The introduction of DNA barcoding has led to the identification of CO1 as a suitable marker for use in animal species identification (Herbert et al, 2003) and consequently an important part of forensic investigation especially in conservation of wildlife (Kumar et al, 2012) and also in non forensic applications in the identification of fish (Ward et al, 2005), birds (Herbert et al, 2004), insect (Hajibabea et al, 2006) and primates (Lorenzo et al, 2005). According to Herbert et al 2003, the CO1 mitochondria gene is widely accepted for species identification due to distinct ability of possessing a wide range of Phylogenetic signals than other mitochondria genes. The core function of species identification is in the comparative matching of samples sequences generated from identified species to their reference sequence carried out either through DNA sequences similarity searches (Atschul et al, 2006) or by Phylogenetic reconstruction (Baker and Palumbi, 1994).

This study successfully validated the use of CO1 gene as a marker for the identification of Kenyan wild animal species by use of both DNA sequences similarity searches and Phylogenetic reconstruction. Wild animals sampled were identified by veterinary staff from the Kenya wildlife services (KWS) for authentication through morphological characteristics diagnostic methods of animals with intact skeletons. Biopsy tissue samples from the identified wild animals were utilized to successfully extract DNA, amplify using CO1 (vertebrae) primer and generate sequence for comparative analysis with reference sequences available in NCBI database. Use of BOLD database and BLAST by use of highly similar sequences optimization search engine identified the sample sequences to their true species as illustrated in table 1.1. A maximum identity of 99% for four of the sequences and 98% for one of the sequences proved the high similarity from the comparative studies enabling successfully identification of their specific species. Phylogenetic reconstruction using maximum likelihood (Tamura and Nei, 1993) by use of reference samples from NCBI database aligned sample sequences to their species identity confirming results generated through BLAST. According to Ratnasingham and Herbert 2007, BLAST may fail to identify the nearest match reference sequence as it depends on both sequence similarity and length of sequences with longer sequencer better identified. In contrast, BOLD-ID search utilizes Markov models that increases the speed and accuracy of matching sequences while returning a probability based match profile to indicate the likely sources of

sample specimens (Ratnasingham and Herbert, 2007). To incorporate such considerations, sample sequences were further identified using BOLD-ID search that confirmed all species identified using BLAST with high similarity scores of over 98% for all sequences (Table 1.1).

5. Concluction

This study successfully utilized CO1 marker as a forensic tool for identification of species from tissue samples collected from a market meat traders' survey. Through comparative analysis with available sequences in both the NCBI and BOLD databases, specific species of were identified and compared with the putative species identified during sample collection. There was no bush meat detected among the samples collected from Nakuru County. However, substitution of species by meat trader was prevalent in the region specifically the substitution of mutton for chevon. Of the three most commonly traded types of meat in by Kenyan meat traders, mutton is the least accepted meat. People perceptions that mutton has higher fat content as compared to beef and chevon and allergic reaction by some people after eating mutton are some of the likely reasons for low preference of the type of meat as compared to the others (Kang'ethe et al, 1986). Such reasons are the driving force behind the identified common practice of substituting mutton for chevon by meat traders. From the results of the study, substitution was identified to be highly prevalent in urban regions than in formal and informal settlements. Such practices could be associated with the preference for chevon by most people living in urban areas and with higher cost associated with chevon as compared to mutton; meat traders are likely tempted to substitute chevon for mutton in an effort to meet the demand for chevon while gaining more profits.

Acknowledgement

We are highly grateful to the sponsors, the National Council for Science and Technology (NCST) for the funding that made this study possible. We acknowledge the Institute of Biotechnology Research (IBR) at Jomo Kenyatta University of Science and Technology (JKUAT) where the lab analyses were done. We also wish to extend our gratitude to the Kenya wildlife service (KWS) for the permission to collect wildlife tissue samples and technical assistance in acquiring the biopsy samples. We are also indebted to John Kariuki and Moses Yongo of KWS, Richard Rotich of IBR and Jackson Mugweru of the University of Nairobi for their technical advice and support.

References

Altschul, S.T., Madden, A., Schäffer, J., Zhang, Z., Zhang, W., Miller, S., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res., 25 (17). pp. 3389–3402.

Arbele, E.D., Forrest, J.C., Gerrad, D.E., Mills, E.W., 2001. Principle of meat science. Kendal/Hunt publishing.

Baker, C.S., and S.R. Palumbi, Which whales are hunted—a molecular genetics approach to monitoring whaling. Sci., 265 (5178) (1994), pp. 1538–1539.

Blaxter, M., 2003. Molecular sustematics: counting angels with DNA. Nature (London)., 421, 122-124.

Casiraghi, M., Labra, M., Ferri, E., Galimberti, A., De Mattia, F., 2010. DNA barcoding: a six-question tour to improve users' awareness about the method. Brief. Bioinformatics., 11, 440–453.

Eating the unknown., Born Free Foundation., 2004. A survey of meat sold in butcheries in informal settlements in Nairobi.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolut., 39, 783-791.

Hebert, P.D.N., 2005. DNA bar-coding Australia's fish species. Phil.Trans. R. Soc. Lond. B. Biol Sci., 360, 1-11. Kress,

Ivanova, N.V, DeWaard, J.R., Hebert, P.D.N., 2006. An inexpensive,automation-friendly protocol for recovering high-quality DNA. Molecular Ecol. Notes., 6, 998–1002.

Kang'ethe, E.K, Gathuma, J.M., Lindquist, K.J., 1986. Identification of species of origin of fresh cooked and canned meat products using antisera to thermo stable muscle antigens by ouchterlory's double diffusion test. J. sci. food agr., 37, 157 – 164.

Karisa, B.K., Kimwele, C.N., Skilton, R.A., Jung'a, J.O., Hanoette, O., 2008. Estimating the prevalence of game meat utilization in commercial outlets in Nairobi using cytochrome B sequences. Int. soc. Anim. Genet.

Kumar, U.S., Ratheesh, R.V., Thomas, G., George, S., 2012. Use of DNA barcoding in wildlife forensics: a study of sambar deer (Rusa unicolor). Forest Sci. Technol., 8(4), 224-226.

Lorenzo, J.G., Jackson, W.E., Beck, J.C., Hanner, R., 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials, Phil. Trans. R. Soc., B 360 (1462), pp. 1869–1877.

Marshall, E., 2005. Taxonomy. Will DNA barcodes breathe life into classification? Sci., (Washington D.C), 307, 1037.

Ministry of tourism., 2011. Kenya. Tourism performance overview. www.tourism.go.ke, accessed on 21/11/.

Rasmussen, R.S., Morrissey, M.T., 2008. DNA-based methods for the identification of commercial fish and seafood species. Compr. Rev. Food Sci. Food Saf., 7, 280–95.

Ratnasingham, S.R., Herbert, D.N., 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). Mol. Eco. Notes., 7, 355-364.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol., 10, 512-526.

Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends Ecol. Evolut., 24, 110–117.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Valentini, A., Pompanon, F., Taberlet, P., 2009. DNA barcoding for ecologists. Trends Ecol. Evolut., 24, 110–117.