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*Full Length Research Paper*

# The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification

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**Poaching is increasingly presenting challenge to conservational authorities in Africa. Accurate and reliable methods for the identification of poached wildlife meat when morphological features are missing, has been lacking in Africa. We describe a molecular based approach that has a potential of serving as a tool for game and domestic meat identification in Africa. A mitochondrial (mt246) marker and Rsa1 restriction enzyme were used in the PCR-RFLP species identification of game and domestic meat. Species-specific reference DNA fragment patterns were obtained using fresh meat from ten major wild herbivores, representing the highly targeted wild meat species in Tanzania and four domesticated animal species. With the exception of the zebra, all species produced unique monomorphic RFLP patterns that were species specific. These reference fragment patterns enabled identification of about 75% of unknown meat samples, demonstrating the ability of the technique in discriminating between and among wild and domestic species. The results provide preliminary promising fingerprints which need further validation for future use for the control of the up-surging bush meat trade in the continent.**

**Key words:** Mitochondrial DNA, PCR-RFLP, poaching, forensic, species identification.

## INTRODUCTION

The perceived high profile African wildlife issues have long overshadowed illegal bush meat trade, allowing it to grow unchecked for many years. Bush meat trade occurs across virtually the whole of tropical Africa, Asia and Neotropics, threatening a multitude of wildlife species (Barnett, 2000). Recent study indicates that, in Africa, bush meat trade is closely linked with other commerce such as charcoal tree logging, or illegal alcoholic brew, and in many cases follows the same trade routes (TRAFFIC project report, 2000). The dwindling ape population in Central Africa (Goodall, 2000) and the extensive defaunation in West Africa (Barnett, 2000) are among testimonies posing imminent threat to animal conservation in Africa. In Tanzania, the trade is estimated to be extensive, removing approximately 4,458 residents

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and 111,691 migratory herbivores annually, equivalent to 11,950 tons of meat from Serengeti ecosystem alone (Hofer et al., 1996).

There is therefore an urgent need to actively control the growing bush meat trade, and a clear research priority is to join effort in the stoppage of this long ignored illegal activity. A common problem in the wildlife enforcement in Africa is lack of reliable method for the identification of poached wildlife products such as skinned meat. Most bush meat in the region is transported skinned not only for the sake of hiding evidence, but also due to the cash value and separate markets for the skins of many species. Furthermore, in countries such as Botswana and Tanzania, most bush meat is transported to South Africa and Kenya as biltong (dried), making species identification even extremely difficult. We report here species identifications in a number of wild and domestic species using two mtDNA markers in polymerase chain reaction coupled with restriction fragment length polymorphism (PCR/RFLP) analyses using Rsa1 restriction enzyme. The mt246 marker targets the mitochondrial control (D-loop) of the mitochondrial genome. We further demonstrate the potential reliability of the DNA technique in game meat identification of species and their gender.

## MATERIALS AND METHODS

### Sample collection

Muscle tissues (5-10 g) 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 on ice, and finally frozen at -20°C in the laboratory for DNA analyses. The target animal species envisaged for this study included all large wild herbivore species, commonly hunted for meat. Hence, samples were obtained from Wildebeest (*Connochaetus taurinus taurinus*) (16), Zebra (*Equus burcheli*) (5), Thomson`s gazelle (*Gazella thomsonii*) (5), Impala (*Aepyceros melampus*) (4), Reedbuck (*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 also 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 RFLP patterns specific to each species. The obtained fingerprints were further tested on 20 smoke-dried game meat samples obtained from villages surrounding these parks.

### PCR and RFLP analyses

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 lysis buffer (TES, pH 8.0: 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) t 60°C for 2-3 h. This was followed by addition of 0.4 ml of potassium acetate per 1.0 ml solution, placed on ice for at least 30 min, and centrifugation at 12000 g for 10 min. The supernatant was washed 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).

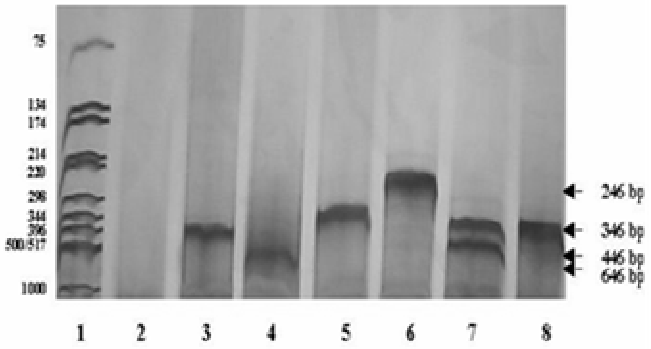


**Figure 1.** Polyacrlamide gel showing PCR amplification from representative reference species samples using mt246 marker. Lane 1 = Ladder, 2 = -ve DNA control, 3 = buffalo, 4 = caprine, 5 = porcine, 6 = +ve DNA control, 7 = reedbuck, and 8 = warthog.

20 µl PCR reactions were performed using two separate mitochondrial primer sets (246 bp; HSF21 and LTPROBB13) described by Wasser et al. (1997). 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% polyacrylamide (Scot-lab, Scotland) gels (PAG) in 1x TBE for 50 min at 200 V and stained by silver nitrate solution. In addition, the amplified mtDNA products were further digested with Rsa1 enzyme according to the manufacturer's procedure (Promega, Madison, WI, USA), electrophoreses on 15% PAG in 1x TBE for 50 min at 200 V and stained by silver nitrate.

## RESULTS

Four PCR fragments of 246, 346, 446 and 646 bp in size were amplified using mt246 primer, from reference wild and domestic species samples included in this study (Figure 1 and Table 1). With the exception of the Reedbuck and Hippo, all the other animal species amplified a single but variable-size fragment. Reedbuck amplified two fragments; the 346 and 446 bp, whereas hippo amplified three fragments; 246, 346 and 446 bp. Bovine, porcine, kongoni and warthog amplified the 346 bp fragment. The 446 bp fragment was amplified by wildebeest, thomson`s gazelle, impala, buffalo and oryx, the 646 bp fragment by ovine and caprine and 246 bp fragment by zebra. PCR amplification from 20 unknown samples revealed six fragments sized 176, 246, 276, 346, 376 and 446 bp (Table 2).

PCR-RFLP using mt246 maker produced 14 fragments of sizes ranging between 30-340 bp from reference samples, shared in different ways between species

**Table 1.** PCR amplification of a portion of mitochondrial DNA control (D-loop), using Mt246 primer.

| Species* | ID | PCR RESULTS | Fragment (bp) | | | |
|---|---|---|---|---|---|---|
| | | | 646 | 446 | 346 | 246 |
| Wildebeest (n = 16) | Wb | + | | | | |
| Zebra (n = 6) | Zb | + | | | | |
| Thomson`s g (n = 5) | Th | + | | | | |
| Impala (n = 4) | Im | + | | | | |
| Bovine (n = 5) | Bo | + | | | | |
| Caprine (n = 5) | Ca | + | | | | |
| Ovine (n = 7) | Ov | + | | | | |
| Porcine (n = 5) | Po | + | | | | |
| Buffalo (n = 1) | Bu | + | | | | |
| Reedbuck (n = 2) | Rb | + | | | | |
| Kongoni (n = 2) | Ko | + | | | | |
| Warthhog (n = 1) | Wa | + | | | | |
| Oryx (n = 2) | Or | + | | | | |
| Hippo (n = 1) | Hi | + | | | | |

n = Number of individual samples per species.
ID = Identification.
*Reference samples, whose identity were known and the fragment patterns they generated on restriction enzyme digestion were used to infer identities of unknown samples.

**Table 2.** PCR amplification of a portion of mitochondrial DNA control (D-loop) of unknown samples, using Mt 246 primer.

| Sam* | PCR RESULTS | Fragment (bp) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 446 | 376 | 346 | 276 | 246 | 176 |
| 1 | + | | | | | | |
| 2 | + | | | | | | |
| 3 | + | | | | | | |
| 4 | + | | | | | | |
| 5 | + | | | | | | |
| 6 | + | | | | | | |
| 7 | + | | | | | | |
| 8 | + | | | | | | |
| 9 | + | | | | | | |
| 10 | + | | | | | | |
| 11 | + | | | | | | |
| 12 | + | | | | | | |
| 13 | + | | | | | | |
| 14 | + | | | | | | |
| 15 | + | | | | | | |
| 16 | + | | | | | | |
| 17 | + | | | | | | |
| 18 | + | | | | | | |
| 19 | + | | | | | | |
| 20 | + | | | | | | |

Un = Unknown.
ID = Identification.
*Samples whose identities were not known.

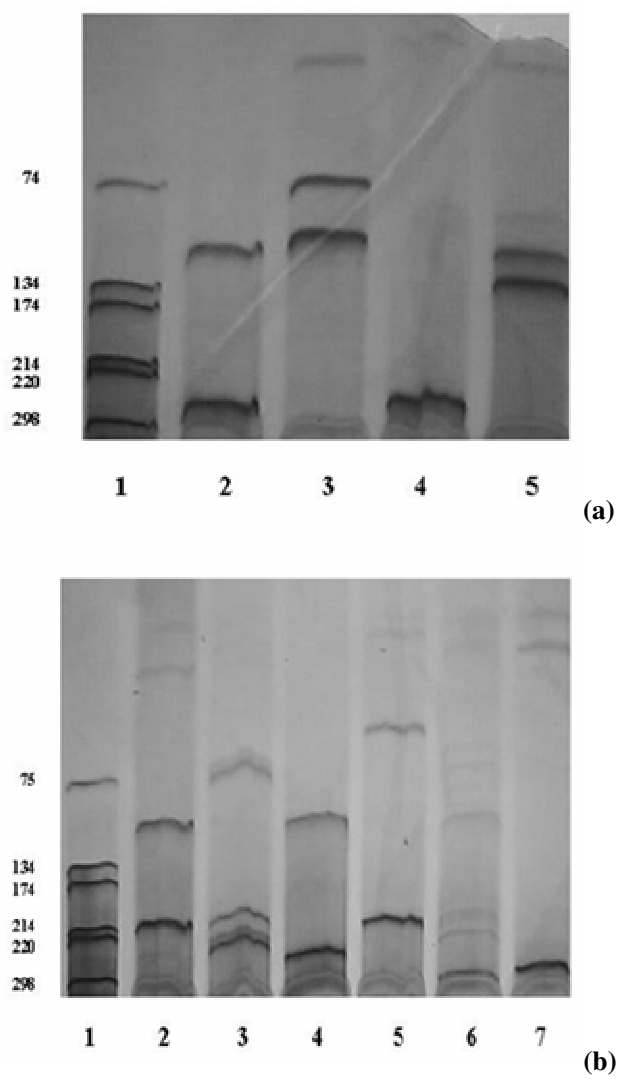


**Figure 2.** Polyacrylamide gel showing RFLP patterns obtained following restriction digestion of mt246 PCR products from representative reference samples using Rsa1 enzyme; (a) Lane 1 = ladder, 2 = wildebeest, 3 = zebra, 4 = bovine and 5 = porcine; (b) Lane 1 = ladder, 2 = buffalo, 3 = thomson`s gazelle, 4 = wildebeest, 5 = impala, 6 = caprine, and 7 = ovine.

(Figures 2a-b, Table 3). The zebra species produced two different fragment patterns (100, 75, 50 bp and 120, 100 bp) representing intraspecies variations. The rest of the species produced monomorphic fragment patterns that were species-specific. From unknown samples, 13 fragments of sizes ranging from 30-300 bp was generated (Table 4).

Comparison of the fragment pattern homology between reference and unknown samples enabled identification of 15 out of 20 (75%) samples. The remaining 5 samples could not be identified with certainty because their fragment patterns were unique as compared to the reference fragment patterns.

## DISCUSSION

This study has generated results of mitochondrial DNA fragment analyses for fourteen animal species, ten of which were wild (wildebeest, zebra, thomson`s gazelle, impala, buffalo, reedbuck, kongoni, oryx, warthog and hippopotamus) and four were domestic (bovine, caprine, ovine and porcine). Although numerous loci could be used for the genetic identification of species in this study, the control (D-loop) region of the mitochondrial genome was used for two reasons. The D-loop and adjacent control region do not encode proteins and typically have a high mutation rate, increasing their likelihood of discriminating between species (Li and Graur, 1991). Also the D-loop region has also been well characterized both phylogenetically and at the molecular level (Foran et al., 1988; Kocher et al., 1989; Lopez et al., 1996; Foran et al., 1997).

The ten wildlife species involved in this study represent some of the major herbivore species, typically hunted for meat in Tanzania. Their samples were collected from Serengeti and Mikumi national parks, located in the northern and eastern geographical zones, respectively, in Tanzania. As the sampling regimen used relied entirely on samples from natural mortality and leftovers from predator kills, it was difficult to obtain enough samples of some species for this study. This constrains the reliability of results obtained in some species. To discriminate meat products of the species examined at a level of certainty that will hold up in a court of law, one has to be certain that the probability of mis-assignment is extremely low. The only way to do this is to have a data set of reference samples from each species that is sufficiently large to assure that all possible variants of each species have been accounted for. The size of the reference sample set should be determined by the variability found within each species across the region. Future studies are aimed at fulfilling this goal. However, present results show that the primer set used in this study already hold great promise for reliably differentiating between and among meat obtained from wildlife versus domestic animals species.

The mt246 primer revealed polymorphic PCR fragments of sizes ranging from 176 bp to 646 bp, which were highly variable in size between species. The 246 bp fragment was observed in the zebra alone. Caprine and ovine generated the largest fragment of 646 bp. Multiple PCR fragments were observed in hippopotamus (446, 346, and 246 bp) and reedbuck (446 and 346 bp). Similar findings have been described in felids using primers flanking the same region of mitochondrial genome but with different sequences (Foran et al., 1997). This has been explained to be a result of occurrence of >1 size class of mitochondrial DNA (heteroplasmy) providing multiple PCR fragments. These inconsistent (variable) mt246 PCR results at present can be explained by unknown natural variation in the subpopulations or random mutational process in the mtDNA genome, given

**Table 3.** Fragment patterns obtained following digestion of PCR amplified portion of MtDNA control (D-loop) with Rsa1 enzyme.

| Species* | PCR-RFLP products (bp) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 340 | 298 | 250 | 240 | 214 | 134 | 120 | 100 | 75 | 60 | 50 | 45 | 40 | 30 |
| Wb (n = 16) | | | ■ | | | | | ■ | | | | | | ■ |
| Zebra (n = 4) | | | | | | | | ■ | ■ | | ■ | | | |
| Zebra (n = 2) | | | | | | | ■ | ■ | | | | | | |
| Th (n = 5) | | | | ■ | ■ | | | | ■ | | | | | |
| Impala(n = 4) | | | | | ■ | | | | | ■ | | | ■ | ■ |
| Bo(n = 5) | | | ■ | | | | | | | | | | ■ | ■ |
| Ca (n = 5) | | ■ | | ■ | | | | ■ | | | ■ | | | ■ |
| Ovine (n = 7) | | ■ | | | | | | | | | ■ | | | ■ |
| Po (n = 5) | | | | | | ■ | | ■ | | | ■ | | | |
| Bu (n = 1) | | | | | ■ | | | ■ | | | ■ | | | ■ |
| Rb (n = 2) | | | | | ■ | | ■ | | ■ | | ■ | | ■ | ■ |
| Ko (n = 2) | ■ | | | | | | | | | | | | | ■ |
| Wa (n = 1) | ■ | | | | | | | | | | | | | |
| Oryx (n = 2) | | | | ■ | | | | | ■ | ■ | | | ■ | ■ |
| Hippo (n = 1) | | ■ | | | | | | ■ | ■ | | | ■ | | |

*Reference samples.

**Table 4.** Fragment patterns obtained following digestion of PCR amplified portion of MtDNA control (D-loop) of unknown samples, with Rsa1 enzyme and species identified.

| Sam* | Spp≅ | PC-RFLP products (bp) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 300 | 250 | 214 | 134 | 120 | 100 | 90 | 75 | 60 | 50 | 45 | 40 | 30 |
| 1 | Wb | | ■ | | | | ■ | | | | | | | ■ |
| 2 | - | | | ■ | | ■ | ■ | ■ | ■ | | | | | |
| 3 | Zb | | | | | ■ | ■ | | | | | | | |
| 4 | - | | | | ■ | | | ■ | | | | | | |
| 5 | - | ■ | | | | | | | ■ | | | | | |
| 6 | Zb | | | | | | ■ | | ■ | | ■ | | | |
| 7 | Zb | | | | | | ■ | | ■ | | ■ | | | |
| 8 | Zb | | | | | ■ | ■ | | | | | | | |
| 9 | Po | | | | ■ | | ■ | | | | ■ | | | |
| 10 | - | | ■ | | | | | | | | | ■ | | ■ |
| 11 | Zb | | | | | ■ | ■ | | | | | | | |
| 12 | Zb | | | | | ■ | ■ | | | | | | | |
| 13 | Zb | | | | | ■ | ■ | | | | | | | |
| 14 | Wb | | ■ | | | | ■ | | | | ■ | | | ■ |
| 15 | - | | | ■ | | | ■ | | | | | | | ■ |
| 16 | Im | | | ■ | | | | | | ■ | | | ■ | ■ |
| 17 | Wb | | ■ | | | | ■ | | | | | | | ■ |
| 18 | Zb | | | | | | ■ | | ■ | | ■ | | | |
| 19 | Zb | | | | | | ■ | | ■ | | ■ | | | |
| 20 | Wb | | ■ | | | | ■ | | | | ■ | | | ■ |

≅ Species identified.
*Samples with unknown identities.

the behavior of the D-loop across species. The likelihood of the PCR artifacts can however, not be ignored. Therefore sequencing of these fragments is envisaged in future studies, not only to rule-out/confirm this possibility

but also to provide an insight on evolutionary forces in these populations resulting in high level of heterogeneity. Sequencing will also confirm/rule-out the possibilities of insertion, duplication, heteroplasmy non-target nuclear loci and mtDNA pseudogene.

PCR analysis of unknown samples collected around National parks and game controlled areas in the country, using the mt246 primer, revealed 176, 276 and 376 bp PCR products that were not observed in reference samples. This finding potentially indicates the high degree of polymorphism exhibited in the region and the need to extend the study to include more numbers of respective species and other commonly poached wild mammal species. This will not only broaden the knowledge on the degree of polymorphic alleles exhibited by the region, but also provide reference species-specific patterns for all meat-target species potentially widening the chance of identification and hence the accuracy of this technique.

The RFLP fragments of mt246 PCR products using Rsa1 enzyme, revealed diagnostic fragment patterns that distinguished all the reference species genotyped in the present study. Intra-species fragment pattern polymorphism was obtained in the zebra alone. The total percent of positively identified unknown samples using the PCR-RFLP pattern of mt246 marker was 75%, demonstrating the potential usefulness of the methodology in the discrimination between species and point to the need for further studies to validate the technique by taking an account of all species that are target for meat in the region and genotype sufficient samples for each species.

In conclusion, this study has demonstrated the potential of using the mtDNA markers in the game meat species identifications and discrimination between game and domestic meat. If this preliminary study is carried further to a validation, it is likely to be an important conservational too in the fight against the intensifying bush meat trade in the African continent.

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