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Full Length Research Paper

Species and gender differentiation between and among domestic and wild animals using mitochondrial and sex-linked DNA markers

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In many African countries accurate and reliable identification of poached wildlife products like carcasses or meat presents a big problem when morphological characters such as skin hair or bones are missing. We describe a molecular based approach that has a potential of serving as a forensic tool in game meat identification in Africa. A mitochondial DNA marker (mt700) and one restriction enzyme, Rsa1 were used in the PCR-RFLP species identification of game meat obtained from two National Parks in Tanzania. Species-specific reference DNA fragment patterns were obtained using fresh meat from ten wildlife and four domesticated species. All species except the zebra, produced unique monomorphic RFLP patterns. Collectively, these patterns demonstrate the potential ability of genetic techniques for discriminating between and among wildlife and domestic species. The reference PCR-RFLP fragments enabled species identification of about 79% of unknown meat samples. In addition, sex was also assigned to all of the samples following successful amplification of gender-specific, SRY and ZFY/X, chromosomal domains. Although the present study has been conducted on a limited range both in numbers and genetic diversity of wildlife species present in Africa, the results demonstrate the potential usefulness of the DNA approach in wildlife forensics in the continent.

Key words: Mitochondrial DNA, PCR, poaching, forensic, gender, species identification.

INTRODUCTION

Game meat is an important source of protein for those who live in rural Africa including Tanzania. However, in recent years, illegal game meat harvesting has become highly commercialized and unsustainable (Hofer et al., 1996). If the current unsustainable rate of illegal exploitation is to continue, the commercial game meat trade will undoubtedly decimate if not eliminate species distributed in small-localized populations, negatively impacting species survival and biodiversity. 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 west and central Africa for instance, bush meat causes extensive defaunation posing an immediate threat to animal conservation. This is evidenced by a dwindling in the ape population, as a result of unsustainable mean annual offtake that by far surpasses the annual rate of population increase (Goodall, 2000). In Tanzania this illegal trade is estimated to be extensive. Its economic benefits have recently drawn people to villages close to the protected areas, in large numbers, far above the national average

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of population growth (Hofer et al., 1996). In the Serengeti ecosystem alone, poachers remove approximately 4,458 resident and 111,691 migratory herbivores annually, equivalent to 11,950 tons of meat (Hofer et al., 1996).

There is a need for active enforcement of wildlife laws and financial support to maintain a “live protected area” system in Africa. However, a common problem in wildlife law enforcement is identifying the species of origin of poached carcasses, meat or blood when morphological features such as skin, hair, tail or bone are not available. Different approaches have been used in developed countries, ranging from immunological, protein electrophoretic procedures to the current most widely used DNA techniques (Mc Clymont et al., 1982; Cronin et al., 1991).Immunological and protein (allozyme or general protein) electrophoretic procedures have been used with considerable success (Bunch et al., 1976; McClymont et al., 1982; Wolfe, 1983; Mardine, 1984; Pex and Wolfe, 1985; Dratch, 1986). However, immunological tests often are not sensitive enough to distinguish closely related species. Furthermore, electrophoretically detectable protein polymorphisms sometimes are lacking in certain populations or species and may not be species-specific.

DNA techniques using either nuclear (nDNA) or mitochondrial (mtDNA) markers show great potential for use in conservation biology and wildlife management (Cronin et al., 1991; Foran et al., 1997; Paxinos et al., 1997; Waits et al., 1998). Of the two, mtDNA analysis usually serves as a powerful tool for detecting population genetic structure at both inter- and intraspecific level due primarily to its maternal mode of inheritance, lack of recombination, high mutation rate and the process of lineage extinction within populations (Brown et al., 1982; Avise et al., 1984).

We report here species identification of a number of wild and domestic species using a mtDNA marker in polymerase chain reaction coupled with restriction fragment length polymorphism (PCR/RFLP) analyses using RsaI enzyme. This marker (mt700) targets the mitochondrial cytochrome b gene/control (D-loop). 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), 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 14 smoke-dried, unidentified game meat samples obtained from villages surrounding these parks.

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 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) at 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 minutes, 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).

PCR and RFLP analyses

Twenty microliter PCR reactions were performed using mitochondrial primer set (700 bp; H16498 and L15774) described 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% polyacrylamide (Scot-lab, Scotland) gels (PAG) in 1X TBE for 50 min at 200 V and stained by silver nitrate solution. Sex determination of wild and domestic species was accomplished by PCR amplification of single copy chromosomal sex determining genes, SRY and ZFY/X using the following primer sets; ZFY/X-P1-5EZ and ZFY/X-P2-3EZ (442 bp) and SRY-Y53-3C and SRY-Y53-3D (224 bp) described by Wasser et al. (1997). About 2-5 µl of the gender PCR products were electrophoresed on 10% PAG in 1X TBE for 50 min at 200 V and stained by silver nitrate.

In addition, the amplified mtDNA products were further digested with RsaI 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.

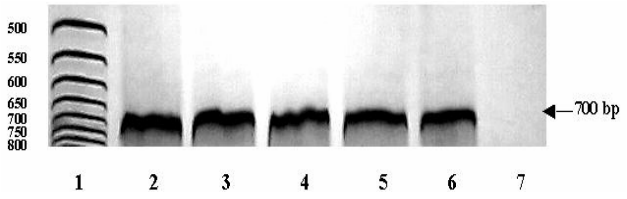
RESULTS

Based on PCR-RFLP fragment pattern findings, there was no marked genetic difference between Serengeti and Mikumi animals, hence reference samples belonging to the same species were pooled and are presented together in this paper.

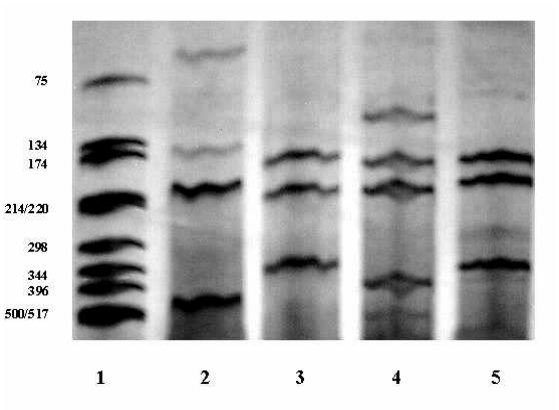
PCR/RFLP using mt700 marker

A 700 bp region of the mitochondrial genome, was successfully amplified from all samples obtained from

(a)



(b)



(c)

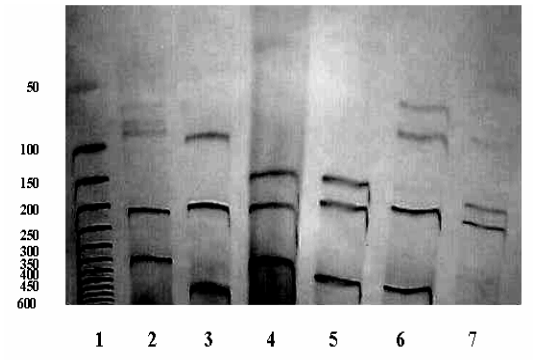


Figure 1. a) A representative polyacrylamide gel showing PCR amplification from representative reference species samples using mt700 marker. Lanes 1 = ladder, 2 = wildebeest, 3 = zebra, 4 = +ve DNA control, 5 = impala, 6 = bovine and 7 = - ve DNA control. The samples were run in duplicates and the percent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

reference wild (wildebeest, zebra, thomson`s gazelle, impala, reedbuck, kongoni, warthog, oryx and hippopotamus), domestic (bovine, caprine, ovine and porcine) species and all unknown samples. All the species produced a similar size PCR fragment (Figure 1a).

The results of the PCR-RFLP are summarized in Figure 1 and Table 1. The fragment pattern for a particular species represents the number (n) of individuals studied as indicated for each species. Fifteen DNA fragments of sizes between 30 to 450 bp were generated. The zebra population produced three unique fragment patterns representing intraspecies variations (Table 1). The remaining reference species produced monomorphic fragment patterns that were species-specific. Bovine and porcine presented the closest fragment patterns, which differed by the presence of 30 bp restriction fragment for porcine that was absent in the bovine. Similarly, 13 fragments of sizes between 30-400 bp were obtained from PCR-RFLP of unknown samples (Table 2). Four fragments (55, 60, 90 and 450 bp), which were obtained in reference samples were not observed in unknown samples, whereas two fragments (250 and 300 bp) were obtained in unknown samples but not in reference samples. By comparing the fragment patterns between unknown (Table 2) and reference (Table 1) samples, 11 out of 14 (79%) unknown samples were identified based on fragment pattern homology with reference samples. The remaining 3 samples could not be identified due to the uniqueness of their fragment patterns, which did not match any of the reference samples.

Sex determination

The SRY and ZFX/Y protein coding genes were successfully amplified from DNA isolated from all reference and unknown tissue samples (Figure 2, Table 3). DNA from male muscle tissues amplified both SRY (224 bp) and ZFY (442 bp), or SRY alone in some cases, while female amplified ZFX gene only. Amplification of these genes enabled correct sex assignment to 100% (n = 82) of samples used in this study.

DISCUSSION

This study has generated results of mitochondrial DNA fragment analyses for thirteen animal species, nine of which were wild (wildebeest, zebra, thomson`s gazelle, impala, 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 cytochrome b gene/control (D-loop) region of the mitochondrial genome was used for several reasons. The cytochrome b gene has only moderate intraspecific variation and occurs in high copy numbers and hence is well-suited for use in species identification (Kohn and Wayne, 1997). In addition, loci from the D-loop and

Table 1. Fragment patterns obtained following digestion of mt700 PCR products from known reference species samples with RsaI enzyme.

Species	PCR-RFLP PRODUCTS (bp)														
	450	400	344	220	200	150	100	90	80	75	60	55	50	45	30
Wb (n = 15)			■		■		■			■	■			■	
Zebra (n = 1)			■	■						■		■	■		
Zebra (n = 3)			■	■						■			■		
Zebra (n = 2)			■	■			■								
Th (n = 5)		■			■	■	■	■	■	■			■		
Impala (n= 4)	■					■					■			■	■
Bo (n = 5)			■		■	■									
Ca (n = 5)		■			■	■	■						■	■	■
Ov (n = 7)			■		■	■		■	■					■	■
Po (n = 5)			■		■	■									■
Rb (n = 2)		■			■	■								■	■
Ko (n = 2)			■		■		■				■				
Wa (n = 1)	■				■		■								
Oryx (n = 2)			■		■			■	■	■	■				
Hippo (n = 1)					■	■	■						■		

n = Number of samples per species, Wb = wildebeest, Bo = bovine Ca = caprine, Ov = ovine, Po = porcine, Rb = reedbuck, Ko = kongoni, Wa = warthog, Hippo = hippopotamus. Shaded cell implies presence of a fragment.

Table 2. Fragment patterns obtained following digestion of mt700 PCR products from unknown samples with Rsa1 enzyme and species identified.

Unidentified game meat*	Spp[ID]	PCR-RFLP products (bp)												
		400	344	300	250	220	200	150	100	80	75	50	45	30
1	Wb		■				■		■		■		■	■
2	-	■					■		■					
3	Zb		■			■			■					
4	-		■	■					■					
5	Zb		■			■					■	■		
6	Zb		■			■					■	■		
7	Zb		■			■					■	■		
8	Zb		■			■			■					
9	Po		■				■	■						■
10	-				■	■	■						■	
11	Zb		■			■			■					
12	Zb		■			■			■					
13	Zb		■			■			■					
14	Wb		■		■		■		■	■	■		■	■

[ID]Species identified.
*Samples whose identities were not known.
Shaded cell implies presence of a fragment.

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). The cytochrome b gene and D-loop regions

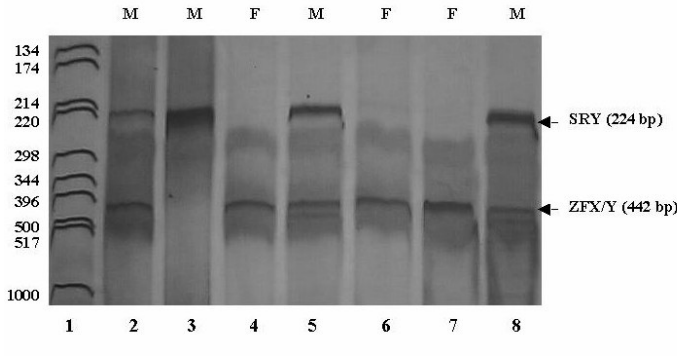


Figure 2. A representative polyacrylamide gel showing gender amplification of sex determining genes from all samples. Lane 1 = Ladder, 2, 3 and 5 are males, 4 and 6 are females. Lanes 7 and 8 are female and male positive controls, respectively.

Table 3. Species, number of individuals per species and sex assignment as revealed by the amplification of SRY and ZFX/Y protein encoding genes.

Species	Male	Female	Total
Wildebeest	13	3	16
Zebra	2	4	6
Thomson`s	3	2	5
Impala	3	1	4
Bovine	4	1	5
Caprine	4	1	5
Ovine	0	7	7
Porcine	3	2	5
Buffalo	0	1	1
Reedbuck	2	0	2
Kongoni	1	1	2
Warthog	0	1	1
Oryx	1	1	2
Hippo	1	0	1
Unknown	14	6	20
Total	**51**	**31**	**82**

have 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 nine 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 and calls for additional studies to validate these results. 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 mtDNA primer set used in this study already hold great promise for reliably differentiating between meats obtained from wildlife versus domestic animals.

The mt700 primer specifically amplified DNA from all of the samples used in this study by giving a consistent 700 bp fragment. Digestion of the mt700 PCR products with RsaI enzyme generated fragments of different sizes ranging from 450-30 bp, which were sufficiently diagnostic. These findings support the argument by Kohn and Wayne (1997) that the moderate intraspecies variation of mitochondrial DNA cytochrome b gene is well suited for use in species identification. Intraspecies polymorphism was only detected in the Zebra, where three distinct fragment patterns were observed using this marker. This intraspecific polymorphism was probably due to mutational changes in fragment patterns in the zebra. However, since the number of zebra samples obtained for this study was small, additional zebra samples are required from within and between different geographical areas to validate and establish species uniqueness and intraspecific variation. Although the mt700 PCR-RFLP patterns were able to differentiate all reference species included in this study, the zebra data is still subject to further analysis, which will involve sequencing of respective fragments.

To identify the sex of animals from which samples were obtained, sex-determining genes, SRY and ZFX/Y, were amplified from DNA obtained from all samples. Using the sex specific primers (SRY-Y53-3 and ZFY/X-P-EZ, Wasser et al., 1997), ZFX (442 bp) alone was expected to be amplified in females, and both SRY (224 bp) and ZFY (442 bp) or SRY (224 bp) in males. In this study, the primers amplified the ZFX (442 bp) alone in females while in most of the male samples (70%) both the SRY (224 bp) and ZFY (442 bp) were amplified. SRY was amplified alone in only a few males (30%) (Figure 2). Amplification of SRY gene alone in male DNA samples is a common observation and has been explained to be a result of low quality of the template DNA (Wasser et al., 1997). Amplification of these genes enabled correct

assignment of sex to 100% (n = 82) of samples collected from different wild and domestic species. Similar findings using the same primers were previously reported by Wasser et al. (1997) in American black bears.

In conclusion, this study showed the potential of using the mtDNA markers in the game meat species identifications and discrimination between game and domestic meat, although more studies are envisaged in future to validate the findings presented here. In addition, the gender PCR allowed additional information to be obtained on the sex of the species under investigation.

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REFERENCES

Avise JC, Neigel JE, Arnold J (1984). Demographic influences on mitochondrial lineage-survirorship in animal populations. J. Mol. Evol. 20: 99-105.

Barnett R (2000). The Utilisation of Wild Meat in Eastern and Southern Africa, In Trade Review, Food for Thought, Traffic- East/Southern Afr. WWF-IUCN. pp.167-188.

Brown WM, Prager EM, Wang A, Wilson AC (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. J. Mol. Evol. 18: 225-239.

Bunch TD, Meadows RW, Fotle WC, Egbert LN, Spillete JJ (1976). Identification of Ungulate hemoglobins for law enforcement. J. Wildlife Managt. 40: 517-522.

Cronin MA, Palmisciano DA, Vyse ER, Cameron DG (1991). Mitochondrial DNA in Wildlife Forensic Science;Species Identification of tissues. Wildlife Soc. Bull. 19: 94-105.

Dratch PA (1986). A marker for Red deer –Wapiti hybrids. Proceedings of the New Zealand Soc. Anim. Prod. 46: 179-182.

Foran DR, Crooks KR, Minta SC (1997). Species identification from scat: an unambiguous genetic method. Wildlife Soc. Bull. 25(4): 835-839.

Foran DR, Hixon J, Brown W (1988). Comparison of ape and human sequences that regulate mtDNA transcription and D-loop DNA synthesis. Nucleic Acids Res.16: 5841-5861.

Goodall J (2000). "Foreword" in Bushmeat: Africa's conservation Crisis, WSPA, March 2000.

Hofer H, Campbell KL, East M, Huish SA (1996). The impact of game meat hunting on target and non-target species in the Serengeti. In: The exploitationof mammal populations: (Edited by Taylor VJ and Dunstone N) Chapman and Hall, London. Weinheim. Newyork. Tokyo. Melbourne. Madras. pp. 118-146.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. Proceedings of the Natural Academy of Sciences USA 86: 6196-6200.

Kohn MH, Wayne RK (1997). Facts from feces revisited. Tree 12(6): 223-227.

Li W–H, Graur D (1991). Fundamentals of molecular evolution. Sinauer Associates, Inc. Sunderland, Massachusetts. pp. 284.

Lopez JV, Cervarlo S, O`Brien S (1996). Complete nucleotide sequence of domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (NUMT) in the nuclear genome. Genomics 33: 229-246.

Mardini A (1984). Species identification of tissues of selected mammals by agarose gel electrophoresis. *Wildlife Society Bulletin* 12: 249-251.

Mc Clymont RA, Fenton M, Thompson JR (1982). Identification of Cervid tissue and hybridization by Serum albumin. J. Wildlife Mangt. 46: 540-544.

Paxinos E, Mcintosh C, Ralls K, Fleischer R (1997). A non-invasive method for distinguishing among canid species: amplification and enzyme restriction of DNA from dung. *Molecular ecology* 6: 483-486.

Pex Jo's, Wolfe JR (1985). Phenotyping phosphoglucose Isomerase in West coast Cervids for species identification and individualization. J. Forensic Sci. 30: 114-118.

Sambrook J, Fritch EF, Maniatis T (1989). Molecular cloning: a laboratory mannual 2nd Edition. Cold spring Habor Laboratory, New York. pp. 8,3-15,113.

Waits LP, Talbot SL, Ward RH, Shields GF (1998). Mitochondrial DNA Phylogeography of the North American Brown Bear and Implications for Conservation. Conservation Biol. 12 (2): 408-417.

Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997). Techniques for amplification of fecal DNA methods to field studies of Ursids. Mol. Ecol. 6: 1091-1097.

Wolfe JR (1983). Elecrophoretic differentiation between Alaskan brown and black bears. J. Wildlife Managt. 47: 268-271.