The Pharma Innovation Journal 2022; SP-11(6): 2742-2744

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(6): 2742-2744
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www.thepharmajournal.com
Received: 08-04-2022
Accepted: 10-05-2022

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Sequence variation of mitochondrial cytochrome b gene for species identification of chital meat and its differentiation from meat of domestic herbivores by PCR-RFLP

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Abstract
This paper describes the method to identify domestic herbivore species as well as Chital using sequence polymorphism of cytochrome b gene fragment of mitochondrial genome. Chital is prone to poaching for its meat, skin and antlers. In absence of authentic techniques for species identification, it becomes impossible to prosecute the wildlife crimes in the court of law. The present work was planned to study the species specific PCR-RFLP patterns for meat identification of chital and its differentiation from the meat of domestic herbivores of Central India. Meat samples of chital and cattle were collected from the carcasses during necropsy and meat of other domestic herbivores was collected from the local slaughter houses. Extracted DNA from each sample was used in the amplification of fragment of Cytochrome b gene using universal primers and then sequencing was done to select a specific restriction enzyme to distinguish between herbivore species. All the species produced characteristic PCR-RFLP profile with *Taq*I. The present study could efficiently differentiate chital and closely related domestic herbivore species and can be considered simple, more accurate, easy-to-use specific protocol for identification of species from biological samples.

Keywords: Cytochrome b gene, chital, meat

Introduction
India is one of the twelve mega-biodiversity countries in the world. Poaching is a major threat to birds, mammals, plants and reptiles and it has led Indian government to enact Wildlife (Protection) Act, 1972 and also to sign Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1976. Among wild herbivores, Chital is found abundant and is the major victim in cases of wildlife poaching. When the poachers are caught with the suspected meat, they claim it to be that of domestic herbivores. In such cases, authentic evidences are required to prosecute the poachers in the court of law.
There is lack of simple protocols to identify the origin of the seized meat samples. Among the various molecular biology based test used in the recent past, PCR-RFLP has been proposed to be one of the most efficient methods in terms of cost, detection power and applicability to large scale screening (Pereira *et al*., 2008) [6]. PCR-RFLP can reveal genetic variation between species (Partis et. al., 2000) [4]. However, very few literatures are available for PCR-RFLP assay targeting identification of wild and domestic herbivores. In this study, we report a PCR-RFLP assay for species identification of chital meat and its differentiation from the meat of four domestic herbivores (Goat, Sheep, Cattle and Buffalo) based on Cytochrome b gene fragment.

Materials and Methods
Sample collection and DNA extraction
Meat samples were collected from domestic herbivores including Buffalo (Bos bubalis), Goat (*Capra hircus*) and Sheep (*Ovisaries*) from local slaughter houses and from Cattle and Chital (*Axis axis*) during post mortem examination (Five meat samples each species). DNA was extracted using DNA easy Blood and Tissue Extraction kit (Qiagen, Germany) as per the manufacturer's instruction.

PCR Amplification and Sequencing

DNA samples were amplified by using cytochrome b gene universal primers Cytb1 CCAATGATATGAAAAACCATCGTT & Cytb2 GCCCCTCAGAATGATATTTGTCCTC (Janczewski *et al*., 1995) [2] in a Gradient thermal Cycler (Eppendorf, India) in a final volume of 50µl containing 40-50ng of extracted DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $Mgcl_2$,0.2 mM of each dNTP, 1 unit of Taq polymerase (Applied Biosystems), 0.5 µg/µl Bovine Serum Albumin (BSA) and 20 pmol of each primer. The amplification parameters were 95ºC for 20 minutes followed by 35 cycles of 95 º C for 1 min, 55º C for 1 min and 72 º C for 1 min with final extension at 72º C for 10 minutes prior to a 4 ºC hold. The PCR products were resolved on 1.5% agarose gel and visualized under UV light in the presence of Ethidium bromide dye.

Unused primers and dNTPs in the PCR products were cleaned by treating the PCR products with Exonuclease I *(ExoI*) and Srimp Alkaline Phosphatase (SAP) as per the manufacturer's instructions (ExoSAP-IT, USB, Cleveland, Ohio). The cleaned PCR products were sequenced (Applied Biosystems Genetic Analyzer) using 3.1 sequencing kit (Applied Biosystems) from both ends.

Restriction Fragment Length Polymorphism

The Cyt b sequences obtained from the domestic and wild herbivore samples were aligned using Clustal W (Thompson *et al*. 1994) [8] in MEGA 6 (Tamura *et al*. 2013) [7] to identify sequence variations and restriction profiling (Figure 1). Based on the restriction variations, restriction enzyme, *Taq*I (Fermentas Life Science, India) were selected for RFLP analysis of the PCR products. The PCR products were subjected to restriction digestion at 37 °C for 2 hours in a total volume 30 µl containing 17µl of PCR product, 20U of restriction enzyme, 3µl digestion buffer and 8µl of water. The digested products were visualized on 3% Agarose gel electrophoresis and stained with Ethidium bromide.

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#Chital     TAATAAAAAT TGTAAACAAC GCATTCATTG CCTCCGCCCC ATCAAATATT TCATCCTGAT GGAACTTCGG CTCTTTGCTA GGAGTCTGCT TAATTCTA [103]
#Goat       TAATAAAAAT TGTAAACAAC GCATTTATTG CTCCCACCCC ATCAAACATC TCATCATGAT GAAACTTTGG ATCCCTCCTA GGAATTTGCC TAATCTTA [103]
#Sheep      TAATAAAAAT TGTAAACAAC GCATTCATTA TCTCCGCTCC ATCAAATATT TCATCATGAT GAAACTTTGG CTCTCTCCTA GGCATTTGCT TAATTTTA [103]
#Cattle     TAATAAAAAT TGTAAACAAT GCATTCATCG CTTCCGCCCC ATCAAACATT TCATCATGAT GGAATTTCGG TTCCCTCCTG GGAATCTGCC TAATCCTA [103]
#Buffalo    TAATAAAAAT TCTAAACAAT GCATTCATTG CTCCCGCTCC ATCAAACATC TCATCATGAT GAAACTTTGG CTCTCTCCTA GGCATCTGCC TAATTCTG [103]

#Chital     CAAATTCTCA CGGGCTTATT CTTAGCAATA CACTATACAT CTGCAATAAC AGCATTCTCC TCTGTCACTC ATATCTGTCG AGACGTCAAC TACGGCTG [204]
#Goat       CAAATCCTGA CAGGCCTATT CCTAGCAATA CACTATACAT CCGCAATAAC AGCATTTTCC TCTGTAACTC ACATTTGTCG AGATGTAAAT TATGGCTG [204]
#Sheep      CAGATTCTAA CAGGCCTATT CCTAGCAATA CACTATACAC CTGCAACAAC AGCATTCTCC TCTGTAACCC ACATTTGCCG AGACGTAAAC TATGGCTG [204]
#Cattle     CAAATCCTCA CAGGCCTATT CCTAGCAATA CACTACACAT CCGCAACAAC AGCATTCTCC TCTGTTACCC ATATCTGCCG AGACGTGAAC TACGGCTG [204]
#Buffalo    CAAATCCTCA CCGGCCTATT CCTAGCAATA CACTACACAT CCGCAACAAC AGCATTCTCC TCCGTCGCCC ACATCTGCCG AGACGTGAAC TATGGATG [204]

#Chital     AATTATTCGA TATATGCACG CAAATGGAGC ATCAATTTTT TTATTTGCTT ATTTTTACAT GTAGGACGAG GTCTGTATTA CGGATCATAT ACCTTTTT [303]
#Goat       AATCATCCGA TACATACACG CAAACGGAGC ATCAATTTCT TTATCTGCCT ATTCATACAT ATCGGACGAG GTCTATATTA TGGATCATAT ACCTTTCT [303]
#Sheep      AATTATCCGA TATATACACG CAAACGGGGC ATCAATTTTT TTATCTGCCT ATTTATGCAT GTAGGACGAG GCCTATACTA TGGATCATAT ACCTTCCT [303]
#Cattle     AATCATCCGA TACATACACG CAAACGGAGC TTCAATTTTT TTATCTGCTT ATATATGCAC GTAGGACGAG GCTTATATTA TGGGTCTTAC ACTTTTCT [303]
#Buffalo    AATTATTCGA TACATACACG CAAACGGAGC TTCAATTTTT TCATCTGCTT ATATATACAC GTAGGACGAG GCATATACTA CGGATCATAT ACCTTTCT [303]


#Chital     AGAAACATGA AACATTGGAG TAATCCTCCT ATTTACAGTT ATAGCCACAG CATTTGTGGG ATACGTCCTA CCATGAGGAC AAATATCA [391]
#Goat       AGAAACATGA AACATTGGAG TAATCCTCCT GCTCGCGACA ATGGCCACAG CATTCATAGG CTATGTTTTA CCATGAGGAC AAATATCA [391]
#Sheep      AGAAACATGA AACATCGGAG TAATCCTCCT ATTTGCGACA ATAGCCACAG CATTCATAGG CTATGTTTTA CCATGAGGAC AAATATCA [391]
#Cattle     AGAAACATGA AATATCGGAG TAATCCTTCT GTTCACAGTA ATAGCCACAG CATTCATAGG ATAGGTACTA CCATGAGGAC AAATTACA [391]
#Buffalo    AGAAACATGA AACATCGGAG TAATTCTATT ATTCGCAGTA ATAGCCACAG CATTTATAGG ATACGTACTG CCATGAGGAC AAATATCA [391]
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Fig 1: DNA sequence alignment of fragment of the Cytochrome b gene of Chital and domestic herbivore species. The regions used for restriction analysis is shown highlighted with blue colour (*TaqI*-TCGA)

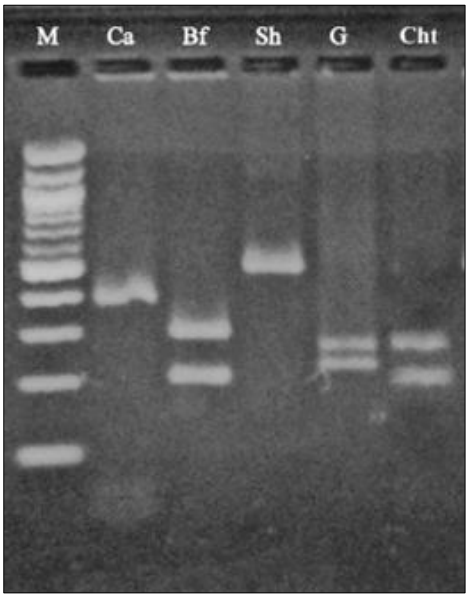
Results

All samples were successfully amplified to expected size of 474 bp. For *Taq*I, herbivore species were divided into three sub groups as- Non-cut, Single cut and double cut depending on the number of recognition sites of the enzyme.

For *Taq1*, Chital had two restriction sites (260bp, 187bp and 27bp), while the four domestic herbivores could be differentiated by using *Taq*1 into three groups; Sheep as non-cut, Goat and buffalo with single cut (1 restriction site producing two fragments of ~250bp and 224bp&~280bp and 194bp respectively) and Cattle with double cut (2 restriction sites producing three fragments of ~370bp, 60bp and 44bp). The number of restriction sites and fragment length patterns are given in Table 1and Plate 1.

Table 1: PCR-RFLP pattern for Chital and domestic herbivore species

Species	*Taq*I
Cattle	2 (370/60/44)
Buffalo	1 (280/194)
Sheep	NC
Goat	1 (250/224)
Chital	2 (260/187/27)



M - 100 bp DNA ladder, Ca-Cattle, Bf-Buffalo, Sh-Sheep, G-Goat & Cht-Chital

Plate 1: RFLP patterns of PCR amplified products of wild and domestic herbivore species after digestion with *TaqI* restriction enzyme

Discussion

Protein-based methods for species identification are less sensitive and often show cross reactivity in closely related species. Pascoal *et al*. (2004) [5] proved that PCR-RFLP method is rapid and easy-to-perform two step analytical approach to achieve qualitative meat species identification in raw and even in cooked food products containing one or more different species. Development of simple and authentic method for detecting the species origin of a wide variety of wild meat continues to be a major challenge before the wildlife forensic experts.

In this study, PCR amplification of the Cytochrome b (Cytb) gene followed by restriction fragment length polymorphism analysis (PCR-RFLP) was used to differentiate Chital and four domestic herbivore species.For most domestic animals, many PCR-based methods have been proposed for species identification (Mahajan *et al*., 2011) [11]. Recently, poultry and livestock animals have been distinguished by PCR-RFLP method based on 12S rRNA gene polymorphism (Chen *et al*., 2012) [1]. However, there are very few PCR-RFLP assays for differentiation of wild herbivores and domestic herbivores. The PCR-RFLP method can be carried out without requirement of very costly equipments compared to DNA sequencing.

Mitochondrial DNA owns several advantages over nuclear DNA. Mitochondrial DNA is presented in thousands of copies per cell and possesses many points of mutations allowing the discrimination of even closely related species. Mitochondrial DNA is maternal inheritance and therefore is free of heterozygosity (Unseld *et al*., 1995; Lockley and Bardsley, 2000) [10, 3]. Cytochrome b gene is located on the mitochondrial genome which is used very often in studies concerning meat species identification and therefore sequence data of many vertebrate and invertebrate species are available. In comparison with other mitochondrial markers, the Cytochrome b gene demonstrates greater level of nucleotide variation in shorter sequences (Tobe *et al*., 2010) [9]. The present PCR-RFLP assay takes into account of the intra-species variations to make species identification more reliable and robust.

Conclusion

The extent of wildlife crime is unknown but it is on the increase and has observable effects with the dramatic decline in many species of flora and fauna. Wild herbivore species, especially Chital, are being poached for their meat on the large scale. Development of PCR-RFLP assay can efficiently differentiate chital and closely related domestic herbivore species with the restriction enzymes i.e. *Taq*I.

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