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Differentiation of Sheep and Goat Species by PCR-RFLP of Mitochondrial 16S rRNA Gene

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ABSTRACT

The present study was carried out with an aim to develop a method for differentiation of sheep and goat meat using PCR-RFLP. Tissue samples were collected randomly from ten animals of each species and used for mitochondrial DNA extraction. PCR amplification of 600 bp fragment of 16S rRNA gene was done using universal primer. RFLP studies were carried out by digesting the amplicons using restriction enzymes *viz*. *Alu*I and *Hha*I. Amplicons of Goat 16S rRNA gene was fragmented to 460bp and 140bp fragments by *Hha*I while the amplified gene of sheep was digested by *Alu*I into two fragments of 360bp and 240bp each. This resulting RFLP pattern of 16S rRNA could easily identify and differentiate meat of sheep and goat species.

Keywords: Mitochondrial DNA, PCR-RFLP, 16S rRNA gene.

Molecular methods developed during the past few years have been able to provide authentic and reliable tools for identification of different species of animals and birds. These latest techniques are able to overcome the drawbacks of many conventional methods used for characterization of different species of animals. Species differentiation is highly essential to ensure the authenticity of meat and meat products**.** But such methods should be cheap, repeatable and rapid. Species identification is usually based on protein-isoelectric focusing, immunochemistry, immunoassay and electrophoretic methods (Zerifi *et al.,* 1991) and determination of specific microscopic structural elements (Koolmees *et al*., 1999). But all these methods have their own drawbacks due to their dependency on protein characterization. Expression of protein is tissue dependent and they may denature on processing which leads to subsequent loss of analytical specificity (Hunt *et al*., 1997). Some other available techniques usually require blotting, staining, use of antibodies etc. These limit their usefulness and for these reasons nucleic acid based analysis is becoming more and more popular for the identification and characterization of different species (Meyer *et al*., 1995). DNA has been extensively used for species identification due to its structural stability at high temperature and is conserved within all tissues of an individual which helps in the development of species-specific DNA probes (Chikuni *et al*., 1990; Ebbehoj *et al.,* 1991), Polymerase chain reaction (PCR) assays (Chikuni *et al*., 1994; Meyer *et al*., 1994), Random amplified polymorphic DNA (RAPD) (Meyer *et al*., 1994; Welsh *et al*., 1990) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Mitochondria have their own genome and are evolved from endosymbiotically incorporated organisms. Mammalian mitochondrial DNA is strictly maternally inherited and is circular-double stranded (16,569 bp) with 37 genes. Among these 37 genes, 22 genes encode for t-RNAs, 2 genes encode ribosomal RNAs (12S rRNA &16S rRNA) and 13 genes encode enzymes involved in electron transport chain of oxidative phosphorylation and ATP

production. There is only one non-coding region which is also known as D-loop in addition to the coding region. Among mammals, the structure and the organization of mitochondrial DNA are highly conserved. DNA fragments or genes of mitochondria used as markers for identification of animal species include the cytochrome *b* gene (Williams *et al*., 1991), the 16S rRNA gene (Murugaiah *et al*., 2009), and the 12S rRNA gene (Mitani *et al*., 2009; Girish *et al*., 2005; Fajardo *et al*., 2009; Rojas *et al*., 2009; Yin *et al*., 2009) and the D-loop (Yin *et al.,* 2009; Wang *et al*., 2010). The highly conserved nature of these genes in different species of animals has enabled the designing of universal primers for their amplification (Bellagamba *et al*., 2001) and PCR-RFLP (Williams *et al*., 1991; Wang *et al*., 2010). DNA sequencing (Murugaiah *et al*., 2009), species-specific primer amplification (Fajardo *et al*., 2009) and Real-time PCR assays (Dooley *et al*., 2004) are the widely used method for species identification. Among all these techniques, PCR-RFLP is regarded as one of the most efficient method in terms of cost, detection limit and large scale applicability.

Considering these facts in view the present study was undertaken to develop a reliable and rapid method for differentiation of sheep meat from goat meat by targeting the mitochondrial 16S rRNA gene.

MATERIALS AND METHODS

Collection of Sample for extraction of mitochondrial DNA

Ten random tissue samples of both the species were collected from the slaughter house and transported to the laboratory in ice cold condition and were stored at -20ºC till further processing.

Extraction of Mitochondrial DNA

Tissue samples were then used for extraction of mitochondrial DNA by following standard phenol/ proteinase K method (Sambrook *et al*., 2001). Briefly, the tissue samples were homogenised in PBS (Phosphate Buffer saline) using pestle and mortar and centrifuged at 1000×*g* for 20 min. Supernatant was discarded. Cell Pellet was treated with cell lyses buffer and kept in ice for 10 min. Then it was centrifuged at 1000×*g* for 15 min and supernatant was discarded. This step was repeated 3-4 times until the pellet was free of reddish tinge. DNA extraction buffer (3 ml) was added per 10 ml of blood, vortexed and incubated at 37°C for 30 min. Subsequently, 200µl of 10% SDS per 10ml of blood and proteinase-K (0.1 mg/ml) were added, mixed and incubated at 5°C for 3-4 hr. Contents of tubes were extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v). Finally, the aqueous phase was mixed with 0.2 volume of 1M ammonium acetate solution and 2 volume of absolute alcohol. Precipitated DNA was transferred to a sterile 1.5 ml microcentrifuge tube and washed twice with 70% ethanol. DNA pellet was air dried for 1 hr to remove traces of ethanol and subsequently dissolved in suitable volume of 1X TE buffer and stored at -20°C.The integrity of the extracted DNA was checked on 0.8% agarose gel and the purity was determined on the basis of optical density ratio at 260:280 nm.

PCR amplification of 16S rRNA gene

Universal Primers (Forward: 5′-CGC CTG TTT ATC AAA AAC AT-3′ and Reverse: 5′-CTC CGG TTT GAA CTC AGA TC-3′) were used for amplification of mitochondrial 16S rRNA gene (Guha *et al*., 2007). The amplification was carried out in 0.2 ml PCR tubes containing 5µl of 10x PCR buffer (100 mM Tris–HCl, pH 9.0, 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin), 1µl of 10mM dNTP mix, 1µl (10pmol) each of forward and reverse primers, 1U of Taq DNA polymerase, 50ng of purified DNA and nuclease free water to make the volume up to 50µl. The cycling conditions were as follows: 5 min at 94ºC for initial denaturation, followed by 30 cycles of amplification (45s at 94ºC, 45s at 60ºC and 1 min at 72ºC) and final extension for 10 min at 72ºC.

Gel Electrophoresis for confirmation of PCR products

Agarose gel (1%) electrophoresis was performed to confirm the size of the amplicons. Electrophoresis was carried out in a horizontal submarine electrophoresis apparatus (Hoofer, USA) using 100 volt for 45 minutes in 0.5X TBE buffer with 100 bp DNA ladder and the gel was visualized under ultra-violet light in a transilluminator (Gel Logic 100, KODAK, USA). After confirmation of the size, PCR product was purified using the Qiazen Gel Extraction Kit (QIAquick Gel Extraction Kit). For this,

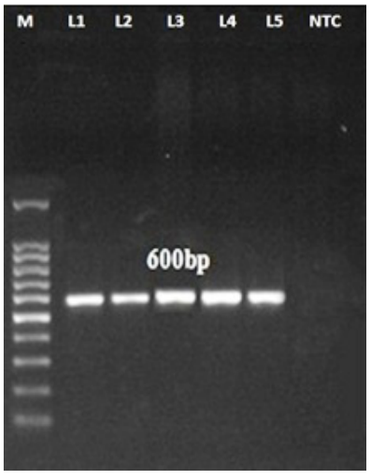

Figure 1: 1% Agarose gel electrophorsis of PCR Products of sheep and goat 16S rRNA gene. M: 100bp Marker. L1-L5: PCR products, NTC: Non Template Control

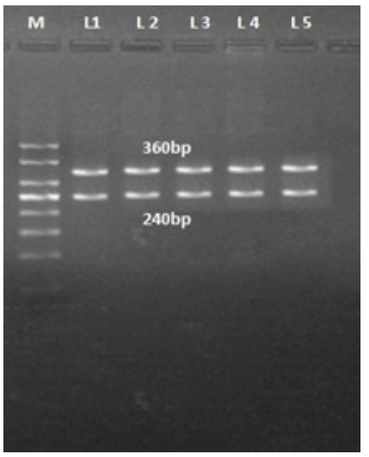

Figure 2: Agarose gel electrophoresis (1.5%) of sheep 16S rRNA gene after digestion by *Alu* I , M: 50 bp Marker; L1-L5: RFLP products (correct)

the specific 600 bp band was excised from the gel and processed according to the protocol provided along with the kit. Purified PCR product was then used for subsequent restriction enzyme digestion as well as for sequencing.

Restriction Fragment Length Polymorphism (RFLP)

Based on the analysis of the restriction sites of the published sequences using NEB CUTTER software, two different restriction enzymes *viz. Alu*I and *Hha*I (Fermentas) were selected for digestion of the amplified fragments of the 16S rRNA gene of both sheep and goat. The reaction mixture was prepared by mixing 0.5µl of restriction enzyme, 1.5 µl of respective buffer, 10 µl of PCR product and 3µl of nuclease free water to make the final volume 15 µl. Reaction mixture was then incubated for 4 hrs at 37ºC. The digested products were separated in 1.5% agarose gel electrophoresis and visualized under UV-light**.**

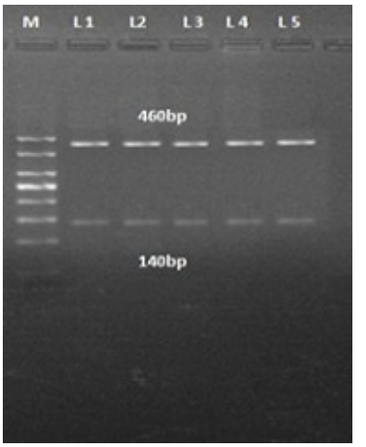

Figure 3. Agarose gel electrophoresis (1.5%) of Goat 16S rRNA gene after digestion by *Hha*I, M: 50bp Marker; L1-L5: RFLP products.

Sequencing of the PCR products

PCR product was purified and sent to DNA sequencing facility, South Campus, University of Delhi for sequencing to verify the restriction enzyme recognition sites at nucleotide level.

RESULTS

Extraction of DNA, amplification and RFLP

Universal primer was used for amplification of 16S rRNA gene. At the initial stage of the study annealing temperature was standardized and optimum amplification was recorded at 60ºC. For confirmation of the size, the amplified products were separated in 1% agarose gel at 100V for 45 minutes. The size of the PCR products for 16S rRNA gene had the expected size of 600 bp (Fig. 1).

To determine the RFLP pattern of the 16S rRNA gene in both the species, PCR products of sheep and goat were digested with *Alu*I and *Hha*I. In sheep, the amplified 600

bp fragment of 16S rRNA gene was digested by enzyme *Alu*I producing two fragments of 360 bp and 240 bp (Fig. 2); while in case of goat, the amplicon was digested by *Hha*I into two fragments of 460 bp and 140 bp size (Fig. 3). Many researchers have also reported the successful use of PCR-RFLP technique to study the polymorphism of 12S rRNA (Girish *et al*., 2005) and 16S rRNA gene (Chakraborty *et al.,* 2005) in different animal species. Our finding is in agreement with other reports in respect of 16S rRNA gene in different species (Shekhar *et al*., 2011).

DISCUSSION

Mitochondria possess extra-chromosomal genetic material and have many desirable features such as high copy number as compared to single copy number nuclear genes. Hence, mitochondrial DNA sequences are preferred for forensic, molecular and zoological studies. PCR amplification of mitochondrial genes, RFLP and their sequencing has been a reliable and authentic method for forensic analysis. Among the mitochondrial genes, the 12S rRNA gene and 16S rRNA gene have been proven to be a good representative for species identification. PCR amplification, RFLP and sequencing of 16S rRNA gene are the useful tools for identification of different species and breeds of animal. In our present study, we developed a PCR-RFLP of 16S rRNA gene for differentiation of sheep and goat species.

RFLP pattern of the 16S rRNA gene was determined by digesting the 600bp PCR products of sheep and goat with enzyme *Alu*I and *Hha*I. Sheep 16S rRNA gene upon digestion with enzyme *Alu*I produced two fragments of 360bp and 240bp and digestion of goat 16S rRNA gene with *Hha*I produced 460bp and 140bp fragments. Earlier researchers also used successfully different restriction enzymes to characterize the polymorphism in 16S rRNA genes in different domestic animals and our finding is in good agreement with them (Guha *et al.*, 2007; Mitani *et al*., 2009). Verification of the RFLP pattern obtained from the restriction digestion is very important to omit the possibility of any false positive or false negative results due to mutation or individual variation. Therefore, sequencing of the amplicon is highly essential for unambiguous species typing and characterization and in our study we were able to verify the restriction enzyme recognition sites in the sequenced fragments. From the present study it can be concluded that PCR-RFLP analysis of 16S rRNA gene can be used to differentiate these two species and can be applied directly to detect fraudulent substitution of goat meat with sheep meat or vice versa. Besides this, it may have direct application in forensic as well as vetero-legal cases.

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