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Differentiation of sheep and goat species by PCR-RFLP of mitochondrial 12S 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 both the species and used for mitochondrial DNA extraction. PCR amplification of a partial 456bp fragment of 12S rRNA gene was done using universal primer for both the species. The PCR-RFLP studies on 12S rRNA gene in goat and sheep using *BspT*I and *Apo*I restriction enzymes respectively, lead to production of 323bp and 133bp fragment in goat and 320bp and 136bp fragments in sheep. The resulting RFLP pattern of 12S rRNA could easily identify and differentiate meat of sheep and goat species.

Keywords: Mitochondrial DNA, PCR-RFLP, 12S rRNA gene

1. Introduction

Species identification, differentiation and authentication of various animal tissues has been possible with the use of several authentic and reliable molecular tools, using mitochondrial and nuclear markers have been developed in the last few years [1]. A cheap, repeatable and rapid molecular method is highly essential to ensure the authenticity of meat and meat products**.** These methods are promising and are able to overcome the drawbacks of many conventional methods used for characterization of different species of animals. PCR is a simple and rapid technique for species identification [2] that has been used as most powerful tool since last many years, due to its specificity and sensitivity to detect single copy of DNA in sample [3]. DNA has been extensively used for species identification and authentication due to its structural stability at high temperature and is conserved within all tissues of an individual [1] which helps in the development of species-specific DNA probes [4, 5], PCR with species-specific primers [6, 7, 8, 9], restriction fragment length polymorphism (PCR-RFLP) [10], randomly amplified polymorphic DNA (RAPD) [11], amplified fragment length polymorphism (AFLP) [12], terminal restriction fragment length polymorphism (Terminal-RFLP) [13], quantitative PCR (qPCR) assays [14], DNA hybridization [7, 15] and nucleotide sequencing [16, 17]. All these techniques have their own advantages and limitations. Mitochondria are double membrane organelles present in the cytoplasm evolved from endosymbiotically incorporated organisms and have their own genome. There are hundreds to thousands copies of mitochondrial DNA in each cell [18]. Unlike nuclear DNA, mitochondrial DNA is continuously replicated, even in terminally differentiated cells, such as nerve cells and cardiomyocytes. Mammalian mitochondrial DNA is thought to be strictly maternally inherited. The target genes and DNA fragments used as markers for identifying animal species cytochrome *b* gene [19], the 16S rRNA gene [20] the 12S rRNA gene [17, 21, 22, 23, 13] and the mitochondrial DNA control region, *i.e.* the D-loop [24, 25]. PCR amplification and sequencing of a conserved gene from the sample e.g. mt-12S rDNA [26] mt-16S rDNA [27] use of oligonucleotide probes [28] microsatellites analysis [29], SSCP of PCR amplified fragments [30]. This study was carried out for differentiation and identification of sheep meat from goat meat by PCR-RFLP of mitochondrial 12S rRNA gene.

2. Materials and Methods

2.1 Collection of sample for extraction of mitochondrial DNA

Tissue samples of apparently healthy sheep and goat were collected from the slaughter house and transported to the laboratory in ice cold condition in PBS and were stored at -20ºC till further processing. Tissue samples were then used for extraction of mitochondrial DNA by following standard phenol/proteinase K method [31].

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.

2.2 PCR amplification of 16S rRNA gene and confirmation

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 a partial fragment of mitochondrial 12S rRNA gene [32]. 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 (denaturation at 94ºC for 45s, annealing at 60ºC for 45s and extension at 72ºC for 1 min) and final extension for 10 min at 72ºC. The PCR products were analyzed by electrophoresis in 1% agarose gel with ethidium bromide staining.

2.3 Restriction fragment length polymorphism (RFLP)

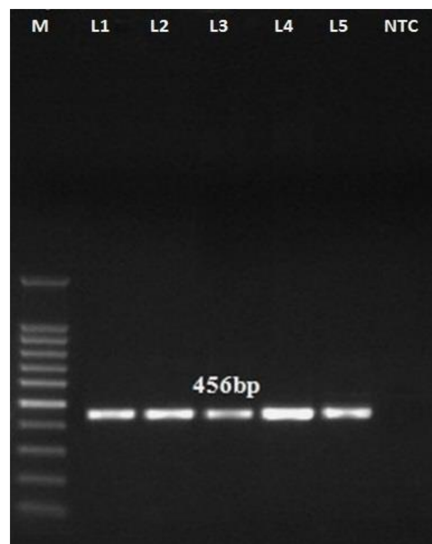
Based on the analysis of the restriction sites of the published sequences using NEB CUTTER software two different restriction enzymes *viz. BspT*I and *Apo*I (Fermentas) were selected for digestion of purified amplicons of 12S rRNA gene of goat and sheep respectively. The reaction mixture was prepared 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-transilluminator.

2.4 Sequencing of the PCR products

The presence of the respective restriction sites were also verified by sequencing the purified PCR amplicons at DNA sequencing facility, South Campus, University of Delhi

3. Results

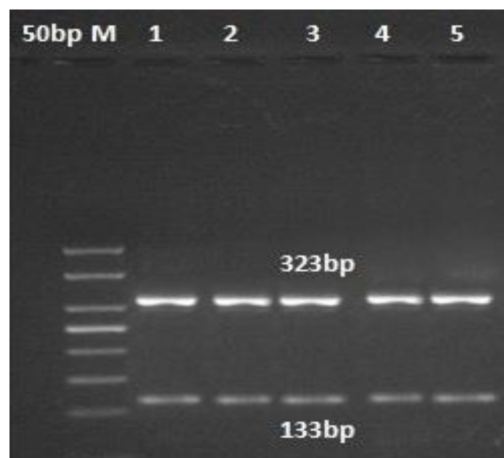
The yield of extracted DNA and OD (260:280 nm) ratio for goat were found to be115ng/µl and 1.86; for sheep were 83ng/µl and 1.99 respectively. Isolated DNA was used for amplification of 12S r RNA gene by using universal primer. At the initial stage of the study annealing temperature was standardized and optimum amplification was recorded at 60ºC. For confirmation of the size, the amplified products were separated in 1% agarose gel at 90V for 45 minutes. The size of the PCR amplicons of 12S rRNA gene had the expected size of 456bp (Figure 1) for both the species.



M: 100bp Marker, L1-L5: PCR products, NTC: Non Template Control

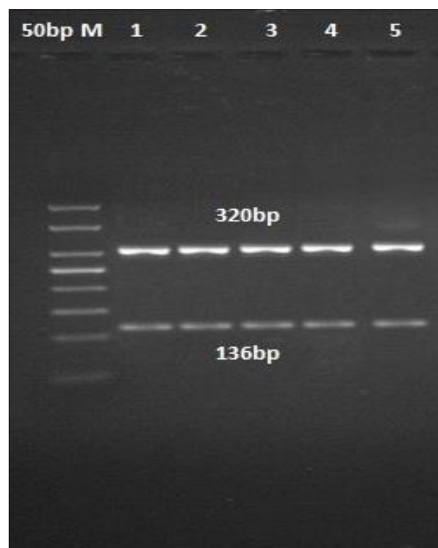
Fig 1: 1% Agarose gel electrophorsis of PCR amplicons of Goat and Sheep12S rRNA gene.

The PCR-RFLP studies on 12S rRNA gene of goat and sheep using *BspT*I and *Apo*I restriction enzymes respectively, lead to production of 323bp and 133bp fragment in goat (Fig. 2) and 320bp and 136bp fragments in sheep (Fig 3).



M:50bp Marker, L1- L5: RFLP products.

Fig 2: Agarose gel electrophoresis (1.5%) of *BspT*I digested 12S rRNA gene of Goat



M: 50bp Marker, L1-L5: RFLP products.

Fig 3: Agarose gel electrophoresis (1.5%) of *Apo*I digested 12S rRNA gene of Sheep

4. Discussion

Amplification of mtDNA using universal primers followed by sequencing represents a very useful tool in species identification [33]. PCR-RFLP of the mitochondrial 12S rRNA gene is highly repeatable, cheaper and quicker than other methods of species identification [34]. PCR-RFLP has been proven to be a practical, simple and rapid technique [34, 35]. Mitochondrial DNA (12S rRNA and 16S rRNA gene) analysis has been used to observe interspecies and intraspecies variations and taxonomic classification of animals [36]. PCR amplified products were sequenced to determine the effectiveness of each mtDNA locus for species identification [33]. PCR amplification and sequence analysis of a partial fragment of mt 12S rRNA gene of different species including sheep and goat showed 456 bp amplicon and found as an ideal, authentic and unambiguous qualitative method for meat species identification [37]. PCR- RFLP of 456 bp PCR amplified product of sheep and goat with *Alu* enzyme produced 246 and 210 bp fragments, restriction enzyme digestion with *ApoI* and *BspTI* produced 329 and 127 bp; 323 and 133 bp fragments respectively [38]. From the present study it can be concluded that PCR-RFLP analysis of mitochondrial 12S rRNA gene can be used as a marker to distinguish sheep meat from goat meat. Besides this, it may have direct application in forensic as well as vetero-legal cases.

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