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Molecular characterization of the mitochondrial 16S rRNA gene of cattle, buffalo and yak

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ABSTRACT

A combination of polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP) and nucleotide sequencing is the most preferred and efficient method for characterization of different species, in terms of detection power and applicability to large scale screening. The present study was carried out with the aim of developing the molecular fingerprint of the mitochondrial 16S rRNA gene of Cattle, Buffalo and Yak. Blood samples were collected randomly from ten different animals of each species for mitochondrial DNA extraction. The extracted DNA was used for the amplification of the 16S rRNA gene using universal primers. The size of the amplified products was 600bp. RFLP studies were carried out by digesting the amplicons using restriction enzymes *viz*. *Alu*I, *Hinf*I and *Hae*III. The resulting RFLP pattern could easily identify and differentiate each of the species. Sequencing of the amplicons in all three species was carried out to confirm the variations at nucleotide level. Sequence analysis of the 16S rRNA gene using MEGA4 software and also PCR-RFLP revealed that the 16S rRNA gene can be used as a good candidate for a molecular marker.

Key words: PCR-RFLP, mitochondrial DNA, nucleotide sequence, 16S rRNA gene

Introduction

Molecular techniques developed during the last few decades have been able to provide authentic and reliable tools for identification of different genetic markers in various species of animals. These latest techniques are promising tools and can be used to overcome the drawbacks of many conventional methods for characterization of different species of animals. Development of a reliable method for differentiation of species is

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highly essential to ensure the authenticity of meat and meat products. Such a method needs to be cheap for routine application, must have repeatability and should be rapid.

Species identification can be achieved through different methods. They are mainly based on the analysis of certain biomolecules, such as protein-isoelectric focusing, immunochemistry, immunoassay and electrophoretic methods (ZERIFI et al., 1991), and determination of specific microscopic structural elements (KOOLMEES, 1999). However, each of these methods has major drawbacks, which are a consequence of their dependence on the ability to characterize proteins. Protein expression is tissue dependent and it may be denatured on processing and heating, leading to subsequent loss of analytical specificity (HUNT et al., 1997). Some techniques require blotting, staining, preparation of antibodies etc. Therefore, those techniques have limited applications and for these reasons nucleic acid based analysis is becoming more and more popular for the identification and differentiation of species (MEYER et al., 1995)

DNA has been exploited for species identification due to its stability at high temperatures and its structure being conserved within all tissues of an individual. This has resulted in the development of species-specific DNA probes (CHIKUNI et al., 1990; EBBEHOJ and THOMSEN, 1991), Polymerase chain reaction (PCR) assays (CHIKUNI et al., 1994; MEYER et al., 1994), Random amplified polymorphic DNA (RAPD) (WELSH and McCLELLAND 1990; WILLIAMS et al., 1990) and Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Both nuclear and mitochondrial genes have been targeted for species identification by PCR-RFLP (MATSUNAGA et al., 1999). Mitochondria are evolved from endosymbiotically incorporated organisms and have their own genome. 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. Sperm mitochondria disappear in early embryogenesis by selective destruction, inactivation or simple dilution by the vast surplus of oocyte mitochondria. Mitochondrial DNA is circular and double stranded (16,569 bp) with 37 genes. Among these, 22 genes encode for t-RNAs, 2 genes encode ribosomal RNAs (12S rRNA &16S rRNA) and 13 genes encode enzymes involved in the electron transport chain of oxidative phosphorylation and ATP production. In addition to the coding regions, there is only one non-coding region, the control region (CR), which is also known as the D-loop. The structure, genetic content and organization of mitochondrial DNA are strongly conserved among mammals. The target genes and DNA fragments used as markers for identifying animal species include the cytochrome *b* gene (MURUGAIAH et al., 2009), the 16S rRNA gene (MITANI et al., 2009), the 12S rRNA gene (GIRISH et al., 2005; FAJARDO et al., 2009; ROJAS et al., 2009; YIN et al., 2009; WANG et al., 2010) and the D-loop (BELLAGAMBA et al., 2001; DOOLEY et al., 2004). Mitochondrial genes are highly conserved in different species

of animals, which has enabled the designing of universal primers for amplification of the mitochondridal genes (ANTOINETTE et al., 1995). Polymerase chain reaction (PCR)-restriction fragment length polymorphism (BELLAGAMBA et al., 2001; MURUGAIAH et al., 2009), DNA sequencing (MITANI et al., 2009), species-specific primer amplification (ROJAS et al., 2009) and Real-time PCR assays (DOOLEY et al., 2004) have been widely used for species identification. Among these, the PCR-RFLP is regarded as one of the most efficient methods, in terms of cost, detection power and applicability to large scale screening. PCR-RFLP is a practical, simple and rapid technique for characterization of different species and breeds. There are hundreds to thousands of copies of mitochondrial DNA in each cell, thus providing as many DNA molecules as possible to minimize the risk of failure due to low DNA concentration with degraded templates.

To date, no molecular signature or fingerprint has been developed for the indigenous livestock species of the North-East region of India. Molecular fingerprints of the mitochondrial DNA of Cattle, Buffalo and Yak of North Eastern India are a completely unexplored area. Keeping these facts in view, the present study was undertaken for mitochondrial DNA fingerprinting of amplified 16S rRNA gene by PCR-RFLP and its sequencing.

Materials and methods

Sample collection and extraction of mitochondrial DNA. Blood samples were collected randomly from three different domesticated species of animals, *viz*. Cattle (n = 10), Buffalo (n = 10) and Yak (n = 10), and transported to the laboratory in ice cold conditions. Samples were stored at -20 ºC until further processing. Mitochondrial DNA was extracted from the blood by following the standard phenol/proteinaseK method (SAMBROOK et al., 2001).The integrity of DNA was checked by 0.8% agarose gel electrophoresis immediately after extraction. Purity of DNA was judged on the basis of optical density ratio, at 260:280 nm.

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

Electrophoresis for confirmation of PCR products. To confirm the size of the amplified PCR product, 1% agarose gel electrophoresis was performed. The electrophoresis was carried out in horizontal submarine electrophoresis apparatus (Hoofer, USA) using 110 volt for 1 hour in 0.5X TBE buffer with a 100 bp DNA ladder. The amplified products were visualized under ultra-violet light in a transilluminator (Gel Logic 100, KODAK, USA). The PCR product was purified after confirmation of the size, and one part was sent for sequencing to the DNA Sequencing Facility, Department of Biochemistry, South Campus, University of Delhi, and the rest were used for restriction digestion.

Restriction Fragment Length Polymorphism (RFLP). The amplified fragments of the 16S rRNA gene of Cattle, Buffalo and Yak were digested with suitable restriction enzymes. Based on the analysis of the restriction sites of the published sequences, using NEB CUTTER software, three different restriction enzymes: *Alu*I, *Hinf*I and *Hae*III (MBI Fermentas), were selected for digestion of the amplicons. The reaction mixture was prepared by mixing 0.5 µL of restriction enzyme, 1.5 µL of the respective buffer, 10 µL of PCR product and 3 µL of nuclease free water to make the final volume up to 15 µL. Then the reaction mixture was incubated for 4 hrs at 37 ºC, and the digested products were visualized under a UV-light after electrophoresis in 2% agarose gel, along with the 50bp DNA ladder.

Analysis of the nucleotide sequences. The nucleotide sequence results were aligned in BLAST with other published sequences and the sequences without any error were taken for analysis. MEGA4 software (TAMURA et al., 2007) was used for construction of a phylogenetic tree.

Results

PCR amplification and RFLP. Isolated DNA was used for amplification of the 16S rRNA gene by universal primer. The size of the amplicons for 16S rRNA gene had the expected size of 600bp (Fig. 1).

Restriction fragment length polymorphism (RFLP) analysis of the mitochondrial 16S rRNA gene with *Alu*I enzyme in cattle led to the production of two fragments, approximately of 350bp and 250bp (Fig. 2). Similarly in Buffalo, enzyme *Hinf*I led to two fragments of 470bp and 130bp (Fig. 3) and in Yak *Hae* III produced 430bp, 90bp and 80bp fragments (Fig. 4).

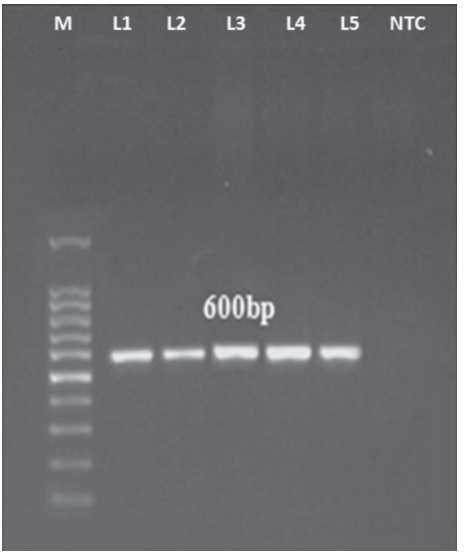

Fig. 1. 1% agarose gel electrophoresis of PCR products of 16S rRNA gene. M: 100bp Marker; L1- L5: PCR Products of Cattle, Buffalo and Yak

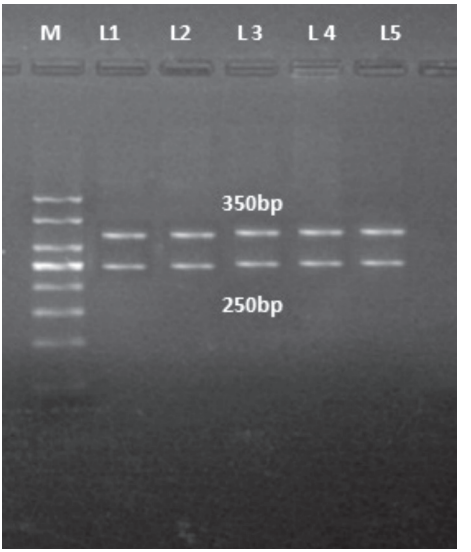

Fig. 2. 2% agarose gel electrophoresis of 16S rRNA gene RFLP of Cattle by *Alu*I M: 50bp Marker; L1-L5: RFLP products

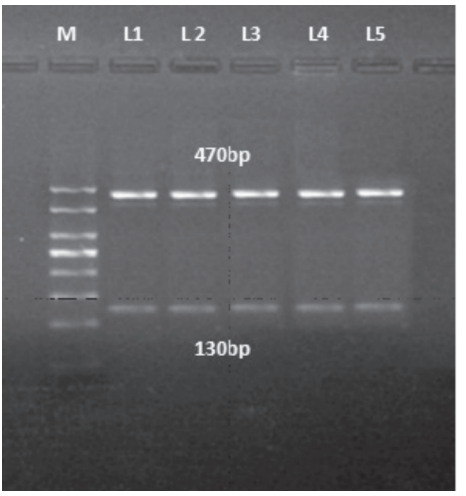

Fig. 3. 2% agarose gel electrophoresis of 16S rRNA gene RFLP of Buffalo by *Hinf*I, M: 50bp Marker; L1-L5: RFLP products

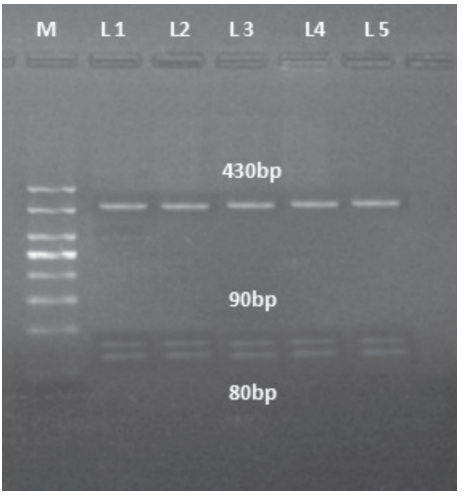

Fig. 4. 2% agarose gel electrophoresis of 16S rRNA gene RFLP of Yak by *Hae*III, M: 50bp Marker; L1-L5: RFLP products.

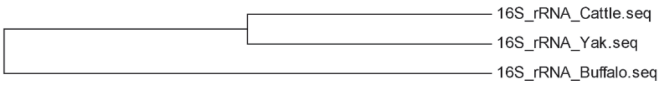

Fig. 5. Phylogenetic tree of the 16S rRNA gene of cattle, buffalo and yak

Analysis of the sequences. The sequences were separately aligned in BLAST to discover the biological relation and closeness of our sequence to other published sequence. Subsequently, MEGA4 software was used for analysis of the sequences. The nucleotide sequences were submitted to the NCBI Gene bank and were assigned the accession numbers KC208029, JX666612 and KR677383 respectively for Cattle, Buffalo and Yak. Phylogenetic analysis of our partial 16S rRNA gene sequence showed that the Cattle 16S rRNA gene is comparatively more closely related to Yak than to Buffalo (Fig. 5).

Discussion

Mitochondria possess extra-chromosomal genetic material and have many desirable features, such as a 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 and their sequencing has been a reliable and authentic method for forensic analysis (KOCHER et al., 1989). Among the mitochondrial genes, the 16S rRNA gene has been proven to be a good representative for species identification (ROJAS et al., 2009). Recent advancement in the field of molecular biology has made it possible to use mitochondrial DNA as a tool for taxonomic species determination and forensic analysis. PCR amplification, RFLP and sequencing of 16S rRNA gene are a good tool for identification of different species and breeds of animal.

The present study was conducted using PCR-RFLP and sequencing to identify the polymorphism of the 16S rRNA gene in Cattle, Buffalo and Yak. To know the RFLP pattern of the 16S rRNA gene, the PCR products of Cattle were digested with *Alu*I, Buffalo with *Hinf*I and Yak with *Hae*III. Each of the enzymes could easily differentiate one species from the other two. The amplified 600bp fragment of 16S rRNA gene of Cattle, upon digestion with enzyme *Alu*I, produced two fragments of 350bp and 250bp. Similarly, digestion of the amplified 16S rRNA gene of Buffalo with *Hinf*I produced fragments of 470bp and 130bp. Restriction digestion of the amplicons of Yak with *Hae*III produced fragments of 430bp, 90bp and 80bp. Phylogenetic analysis of the 16S rRNA gene revealed the closeness of Cattle and Yak, as both are in close proximity with each other in the tree.

On the basis of nucleotide variation, CHEN et al. (2010) developed an efficient PCR-RFLP method for detection of different species, targeting the mitochondrial 12S rRNA gene. CHIKUNI et al., (1994) differentiated sheep and goat meat by PCR-RFLP analysis of

Satellite I DNA using an *Apa*I restriction enzyme. The RFLP pattern of the melanocortin gene was used as a marker for differentiation of Hanwoo meat from Holstein and Angus meat (CHUNG et al., 2000). The RFLP pattern generated from restriction analysis enables identification of the species origin of meat.

Conclusion

From the present study it may be concluded that universal primers may be used for amplification of the mitochondrial 16S rRNA gene of our indigenous domestic animals. Mitochondrial DNA fingerprinting (PCR-RFLP) of the 16S rRNA gene may show the uniqueness and differences of one species from another. PCR-RFLP of the 16S rRNA gene may be used to settle disputes or deception that arise between consumers and sellers regarding illegal substitution of superior quality meat with cheaper meat and it may also be applied for forensic analysis. Sequencing of the 16s rRNA gene of different species provides information on genetic variations at the nucleotide level which may be regarded as a good candidate for molecular markers.

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SAŽETAK

Kombinacija lančane reakcije polimerazom (PCR) s polimorfizmom duljine restrikcijskih fragmenata (RFLP) i utvrđivanjem slijeda nukleotida (sekvenciranje) najpoželjniji je i učinkovit postupak za otkrivanje širokog raspona varijacija pri karakterizaciji različitih vrsta. Cilj ovog istraživanja bio je razviti molekularni otisak za gen koji određuje mitohondrijske 16S rRNA goveda, bivola i jaka. Za izdvajanje mitohondrijske DNA nasumično je prikupljeno po 10 uzoraka krvi različitih životinja unutar svake vrste. Izdvojena DNA iskorištena je za umnažanje 16S rRNA gena uz uporabu univerzalnih početnica. Veličina umnoženih proizvoda iznosila je 600 bp. RFLP analize provedene su digestijom amplikona pomoću restrikcijskih enzima *viz. Alu*I*, Hinf*I *i Hae*III. Dobiveni RFLP uzorak mogao je lako prepoznati i razlikovati svaku od vrsta. Sekvenciranje amplikona u sve tri vrste provedeno je s ciljem da se potvrde varijacija na razini nukleotida. Analiza sekvencija pomoću računalnog programa MEGA4 i metoda PCR-RFLP pokazala je da se gen 16S rRNA može upotrijebiti kao dobar kandidat za molekularni biljeg.

Ključne riječi: PCR-RFLP, mitohondrijske DNA, sekvenciranje, 16S rRNA gen