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Detection of species-specific genetic markers in goat, sheep and pig by molecular characterization of *Cytochrome b* gene

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ABSTRACT

Present work was designed to know the inter-species genetic variation by detecting polymorphisms and analysing the nucleotide sequences of *cytochrome b* gene. Blood samples were collected from apparently healthy adult animals of indigenous goat, sheep and pig of Asom, and mitochondrial DNA was extracted for amplification by PCR. One half of the PCR product was used for restriction digestion while the other half after purification was sent for sequencing. Species specific restriction patterns were observed for *cytochrome b* gene amplicon upon digestion with *Alu*I, *Hinf*I, and *Hae*III. The nucleotide sequence results were further analyzed using ClustalW and Mega5 softwares for unambiguous species typing. The percent similarity study showed that goat *cytochrome b* gene had 89.64 and 82.12% similarity with sheep and pig respectively. Phylogenetic tree also revealed close relationship of goat and sheep *cytochrome b* gene. Lowest pair wise distance was observed between goat and sheep (0.123) followed by goat and pig (0.227). Pig and sheep *cytochrome b* gene showed the highest degree of pair wise distance (0.255) between the nucleotides. From the present study, it can be concluded that RFLP and sequence analysis of *cytochrome b* gene provided the information about the extent of inter-species genetic diversity and can be used as a powerful tool for genetic traceability of species.

Key words: *Cytochrome b*, Mitochondrial DNA, PCR-RFLP, Restriction enzymes

Identification of different species of animals is important from the point of health, food security, ethical and economic reasons. So, it is necessary to know the uniqueness and degree of genetic diversity of one population from the other populations within a species and also between the species (Halliburton 2004). Utilization of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) which is accurate, rapid and easy to perform, is the mostly used PCR based technique for species diversity study. In PCR-RFLP technique, mitochondrial genes are more preferred than nuclear since they are highly conserved in different species of animals owing to their maternal inheritance (Antoinette *et al.* 1995). Moreover, several thousand copies of the mitochondrial DNA genome are present in each cell. This increases the probability of achieving a positive result even in the case of samples suffering severe DNA fragmentation due to intense processing conditions (Bellagamba *et al.* 2001). Among the mitochondrial genes, *cytochrome b* (Meyer *et al*. 1995), *12S rRNA*, *16S rRNA* (Borgo *et al.* 1996) and *D-loop* (Murray *et al.* 1995) have been used for study of genetic diversity, as these are species specific.

Cytochrome b, a component of respiratory chain complex III, is found in the mitochondrion of eukaryotes and in aerobic prokaryotes. These complexes are involved in electron transport and generation of ATP, thus playing a vital role in the cell (Esposti *et al.* 1993). The amino acid sequence of *cytochrome b* gene is highly conservative, but because of degeneracy of the genetic code, the genes for *cytochrome b* differ at least in a few nucleotides even in much related species. Another advantage of the *cytochrome b* gene is the possibility of amplifying certain segment of the gene from any vertebrate species using only one pair of primers (Kocher *et al.* 1989). It is also used in studies of molecular evolution (Kocher *et al.* 1989, Montgelard *et al*. 1997, Prusak *et al*. 2004) and forensic medicine (Barlett and Davidson 1992).

Present work was designed to detect species-specific genetic markers for goat (*Capra hircus*), sheep (*Ovis aries*) and pig (*Sus scrofa domesticus*), by identifying polymorphisms of the amplified mitochondrial *cytochrome b* gene and its characterization by sequencing.

MATERIALS AND METHODS

Blood samples were randomly collected from apparently healthy indigenous animals of goat, sheep and pig,

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maintained at institute farms and different villages of Asom.

DNA extraction from blood samples: Approximately 5 ml of blood was aseptically drawn from 10 adult animals of each species, into vacutainer tubes containing EDTA (2.7%) as anticoagulant. The samples were stored at –20°C until processing. Total DNA was isolated from blood using a standard phenol/chloroform/isoamyl alcohol extraction protocol (Sambrook and Russel 2001). Extracted DNA was run in 1% agarose gel electrophoresis to know its integrity, and the concentration was estimated by checking the optical density (OD) at 260 and 280 nm in a UV spectrophotometer. The samples having OD ratio (260 nm/ 280 nm) 1.7 to 1.9 were used for amplification by PCR.

Gene amplification by polymerase chain reaction: A pair of universal primers; forward (F: 5'- CCATCCAACATCTC AGCATGATGAAA 3') and reverse (R: 3'-ACTCCTGTTT ATAGTAAGACTCCCC–5'), was used as per Bravi *et al.* (2004), for amplifying the mitochondrial *cytochrome b* gene. The PCR reactions were carried out in a total volume of 50 µl reaction mixture containing 25 µl Taq green PCR master mix (2X), 2 µl template DNA, 1 µl each of forward and reverse primer (10 pmole/µl) and 21 µl of nuclease free water. PCR tubes containing mixture were briefly spanned @ 2,000–3,000 rpm for 30 sec. After 5 min of initial denaturation (94°C), 32 cycles were run in a thermal cycler each comprising 50 sec denaturation (93°C), 40 sec annealing (51°C) and 60 sec extension (72°C), and final extension for 10 min (72°C). The amplifications were confirmed by 1.5% agarose gel electrophoresis.

Polymorphism detection by PCR-RFLP: One half of the PCR product was used for restriction digestion by different enzymes viz. *Alu*I, *Hinf*I and *Hae*III. Selection of *Alu*I, *Hinf*I, and *Hae*III was based on their putative ability to discriminate between the species of interest, after analyzing the theoretical restriction patterns of these enzymes for the targeted fragment with Webcutter 2.0. PCR products of 7.5 µl were separately digested by 1.5 µl of 10 units of *Alu*I, *Hinf*I and *Hae*III in 3 tubes. Agarose gel electrophoresis (2%) was run to resolve the different restriction fragments. The gel was visualized and documented by gel documentation system and the size of the restriction fragments was analyzed by comparing with the marker.

Sequencing and analysis: The other half of the amplicon after purification was sent for sequencing to DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, India. The nucleotide sequences were analyzed and compared with other sequences using ClustalW and Mega5 softwares.

RESULTS AND DISCUSSION

A single fragment of 358 bp resulted from PCR amplification of DNA samples in all the three species, which is in accordance to the reports of Parson *et al*. (2000), Bravi *et al*. (2004) and Farag *et al*. (2015). We selected the 358 bp fragment of *cytochrome b* for species identification because this fragment has by far the widest taxonomic representation in nucleotide databases as reported by Guo *et al*. (2005). Also, previous research (Hsieh *et al*. 2003) showed that about 300 bp fragment size of *cytochrome b* gene is sufficient for discriminating between close species.

When PCR product of goat *cytochrome b* gene was treated with *Alu*I*, Hinf*I and *HaeIII,* the results revealed from the present experiment have been summarized in Table 1 and Fig 1. Results obtained by using the three enzymes are totally in agreement and support of those obtained by Bravi *et al.* (2004) and Farag *et al*. (2015), who reported that the PCR- RFLP method allowed them to identify many domestic species like cattle, horse, donkey, pig, sheep, dog, cat, rabbit, chicken, buffalo and camel through the different restriction patterns of *Alu*I, *Hinf*I and *Hae*III, on *cytochrome b* gene fragment (358 bp). They concluded that this method may be useful as a first assessment process in forensic

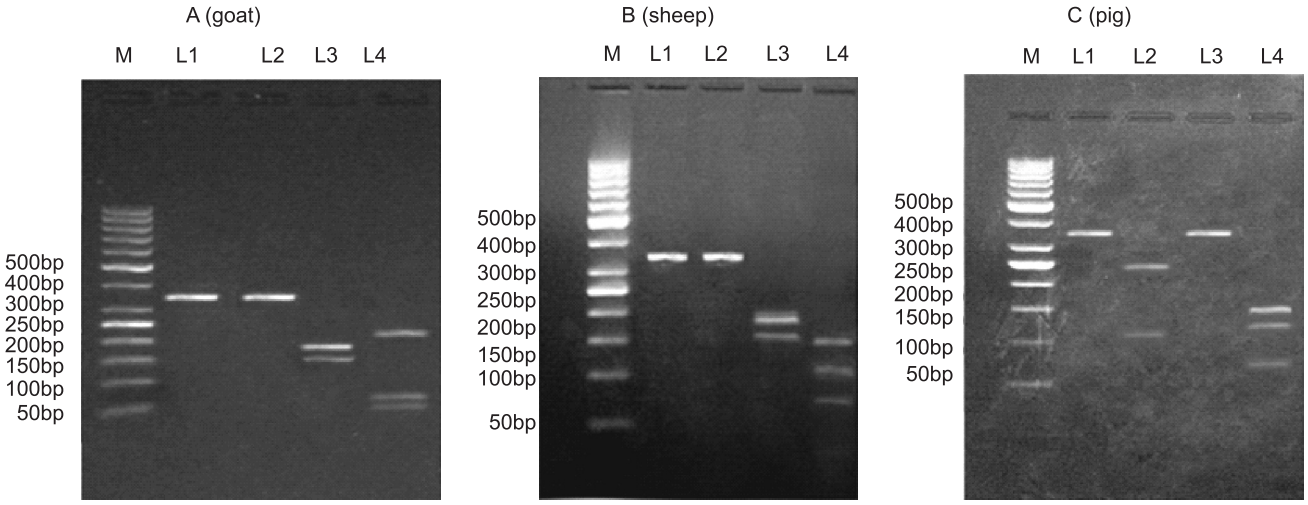

Fig. 1. PCR-RFLP patterns of mitochondrial *cytochrome b* gene of A (sheep), B (Goat), C (Pig). M, 50 bp+ DNA ladder; L1, PCR product (358bp) for A, B, C; L2, digestion with *Alu*I (no restriction site for A and B; for C- 115 bp, 243 bp); L3, digestion with *Hinf* I (161bp, 197 bp for A and B; for C- no site); L4, digestion with *Hae*III (for A- 54 bp, 74 bp, 230 bp; for B- 74 bp, 124 bp, 160 bp; for C- 74 bp, 132 bp, 152 bp).

Table 1. *Alu*I, *Hinf*I and *Hae*III restriction patterns for *cytochrome b* gene in goat, sheep and pig

Species	*Alu*I	*Hinf*I	*Hae*III
Goat	no site	161 bp, 197 bp	54 bp, 74 bp, 230bp
Sheep	no site	161 bp, 197 bp	74 bp, 124 bp,160 bp
Pig	115 bp, 243 bp	no site	74bp, 132 bp, 152 bp

Table 2. Per cent identity matrix of *cytochrome b* gene at nucleotide level created by Clustal 2.1

1. Pig	100.00	81.51	82.12
2. Sheep	81.51	100.00	89.64
3. Goat	82.12	89.64	100.00

evidence and testing for species level identification. Earlier researchers have also successfully used PCR-RFLP technique using *Alu*I, *Hinf*I, *Hae*III to study polymorphisms in *cytochrome b* gene of different domestic animals (Pfeiffer *et al.* 2004, Tsai *et al.* 2007, Minarovic *et al.* 2010). *Alu*I was used to yield specific restriction profiles which helped in direct identification of buffalo and cattle raw meat on the basis of genetic diversity. Similarly, *Hae*III was used to yield specific restriction sites that allowed a direct identification of goat and sheep raw meat (Ahmed *et al.* 2005). Ali *et al.* (2012) used *Alu*I in swine-specific PCR-RFLP assay targeting a 109 bp mitochondrial *cytochrome b* gene for detection of pork in commercial meat products. The conserved sequences of the mitochondrial DNA were used to study interspecific and intergeneric comparisons (Lockwood *et al.* 1993). Restriction enzymes (*Bam*HI, *Eco*RI, *Sac*I and *Taq*I) have been shown to be useful in obtaining RFLP patterns for haplotype individual identification on the basis of genetic diversity (Spike *et al.* 1996). Our findings are in good agreement with the findings of the above workers.

Nucleotide sequence results were aligned and compared with available *cytochrome b* gene sequence entries in nucleotide database using BLAST network. The multiple alignments of *cytochrome b* gene sequence results of goat, sheep and pig, using ClustalW software, gave substantial evidence of the polymorphisms in the restriction sites and

Fig. 2. Multiple alignments of the *cytochrome b* gene sequence result of goat, sheep and pig, using ClustalW software, showing the polymorphic restriction sites. *Alu*I has restriction site at AGCT, *Hinf*I has restriction site at GAATC, and *Hae*III has restriction site at GGCC.

their potential use as marker. The percent similarity study showed that goat *cytochrome b* gene had 89.64 and 82.12% similarity with sheep and pig respectively and that between sheep and pig was 81.51% (Table 2).

PCR-RFLP and sequencing indicated certain uniqueness and some inter-species diversity between the three species under study. The PCR-RFLP used in the present experiment was found to be simple, reliable and quick in identifying variations in the *cytochrome b* gene by generating certain species-specific restriction sites. Analysis of the nucleotide sequences further revealed the extent of inter-species polymorphisms at the DNA level.

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