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DIFFERENTIATION OF MEAT SPECIES USING SPECIES-SPECIFIC REPEAT AND PCR-RFLP TECHNIQUE

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Abstract: In view of the ever increasing frauds like killing of cows for meat purpose, substitution of meat and presence of unknown animal species in food, it has become necessary to identify the species of animals to protect people from health, economic, religious and legal aspects. Considering the need of identification of domestic animals, PCR based molecular techniques were used. The DNA from Cattle, Buffalo, Sheep and Pig were isolated by alcohol-chloroform method and subjected to PCR assay using species specific primers to observe the band pattern. The present study revealed that PCR assay could differentiate Sheep, Pig, Cattle and Buffalo together. The PCR reaction for species specific primers with DNA extracted from blood as well as muscles samples of Cattle, Buffalo, Sheep and Pig revealed bands at 603; 603; 374 and ≤ 100 base pairs respectively. Therefore the samples were subjected for PCR-RFLP method, wherein it revealed bands at 191 and 169 base pairs for Buffalo samples while Cattle showed band at 359 base pairs. Thus RFLP was found necessary to differentiate Cattle and Buffalo species.

Keywords: Mitochondrial DNA, PCR-RFLP, Meat species.

INTRODUCTION

Meat adulteration in raw and processed products has been a wide spread problem in retail markets. Identification of species of meat used in processed meat products or ready to eat meat products have always been a concern for variety of reasons such as wholesomeness, adulteration, religious factors and also to control use of wild meat in hotel industries. The need for sensitive detection of Cattle, Buffalo, Pig and Sheep species in food is critical in response to the consumer demand. In forensic investigations, the PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of mitochondrial cyt-b gene is the most common promising molecular genetics approach to species identification (Pesole *et al.* 1999, Bellagamba *et al.* 2001, Pfeiffer *et al.* 2004, and Rastogi *et al.* 2007). The present study has been undertaken to identify the meat species from raw meat, based on species-specific repeat (SSR) and PCR- RFLP method.

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MATERIALS AND METHODS

The meat samples of cattle, buffalo, sheep and pig were collected from recognized slaughter house and local market. After collection, samples were kept at -20°C till further processing.

DNA Extraction

Genomic DNA including mitochondrial DNA was isolated from raw meat samples by alcohol phenol extraction method as described by Sambrook and Russell (2001) with slight modifications. Briefly, about 500 mg of muscle tissue was pulverized to powder and mixed with 1 ml Lysis buffer-50 mM Tris-HCL (PH-8.0), 10 mM EDTA (pH-8.0), 100 mM Nacl, 150 µg/ml proteinase-K and SDS to make final concentration to 2 per cent. The mixture was vortexed and allowed to digest at 56°C overnight DNA was successively extracted by equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), respectively. DNA was precipitated by adding double volumes of chilled ethanol (95%) in the presence of a high concentration of salt (10% 3 M sodium acetate). The DNA pellets were washed with 70% ice-cold ethanol, air-dried, subsequently dissolved in 50 µl nuclease free water for 2 hours, and stored at -20°C until used.

Quantitation of DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8% w/v agarose gel. The purity of DNA was checked using a UV spectrophotometer and the DNA samples (OD260:280 ratio) between 1.7 and 1.9 were considered good and were used for PCR amplification.

PCR amplification

Amplification of SSR and mitochondrial DNA cyt-b genes were performed following the described procedures using primer sequences (Neumax Scientific, Mumbai) shown in Table 1. In the present study, primer pairs based on SSR of cattle, buffalo, sheep and pig and mitochondrial DNA sequences of cattle and buffalo were used (Ahmed *et al.*, 2007).

The reaction mixture was prepared in a 500 µl PCR tube in a total volume of 50 µl containing 5 µl of 10X PCR buffer (with KCL), 2 µl of 25 mM $MgCl_2$ (2 mM), 1.5 µl of dNTP mix (10 mM), 0.5 µl of Taq DNA polymerase (2 units), 2µl each of forward and reverse primer (20 pmol), 1 µl of DNA template (50 ng) and nuclease free water (to make up the reaction volume to 50 µl). Initial denaturation done at 94°C for 5 min. followed by 35 cycles of denaturation at 94°C for 30 sec., annealing temperature as shown in table 1 for specific primer sequence and at 72°C for 30 sec then final extension at 72°C for 10 min.

PCR-RFLP

PCR amplified product of mitochondrial DNA cytochrome b gene of cattle and buffalo were subjected to restriction enzyme digestion with selected restriction enzyme taq 1. The digestion was performed by preparing reaction mixture after mixing 12 µl of PCR product with 2 µl of respective buffer (10X) and 10 U of restriction enzyme. Volume was made up to 20 µl nuclease free water. The mixture was incubated at appropriate temperature of 65^{o}C for one hour.

Analysis of PCR product

The products of PCR amplification were analyzed by agarose gel electrophoresis, PCR products 5 µl were mixed with 1 ul gel loading dye solution and loaded in a 1.5% agarose gel containing 0.5 µg/ml of gel ethidium bromide in tris borate EDTA (TBE) buffer. Electrophoretic separation of DNA fragments was done at 100 V for 60 min.

RESULTS AND DISCUSSION

PCR amplification of the SSR DNA sequence yielded 603 bp lengths in cattle and buffalo, <100 bp in pig, 374 bp in sheep (Fig. 1). The results showed that this technique can be used to identify various meat species i.e. cattle, buffalo, sheep and pig. These findings are in agreement with the observations of Lenstra *et al.,* 2001 and Ahmed *et al.,* 2007. During the present study, it was observed that species specific repeat (SSR) does not differentiate between cattle and buffalo species. For discrimination, it was necessary to use PCR-RFLP technique for the mitochondrial DNA region containing genes for cyt-b for which a universal primer amplifying in several mammalian species is available (Parson *et al.,* 2000). A single fragment with a size of 359 bp resulted from PCR amplification of the cyt-b gene in cattle and buffalo. The digestion of cytochrome b gene using taq 1 restricted enzyme yielded two fragments of 191 and 169 base pairs only with the buffalo (Fig. 2). Whereas in cattle, the cytochrome b gene amplified product was not digested and therefore, there was no change in its band pattern and thus cattle species could get identified from buffalo at its original band at 359 base pair (Abdel-Rahman *et al.,* 2007 and Ahmed *et al.,* 2007). These observations of the present study regarding two band patterns for two fragments at 191 bp and 169 bp for buffalo species is in agreement with the findings reported by earlier worker (Abdel Rahman *et al.,* 2007 and Jain *et al.* 2007).

The results showed that PCR amplification of species-specific genes from meat samples can effectively identify the species origin of the sample. However, the major limitation of the assay is its inability to discriminate between related animal species, namely cattle and

buffalo. Hence, a buffalo or cattle specific primer and/or probe must be designed to differentiate cattle and buffalo meat.

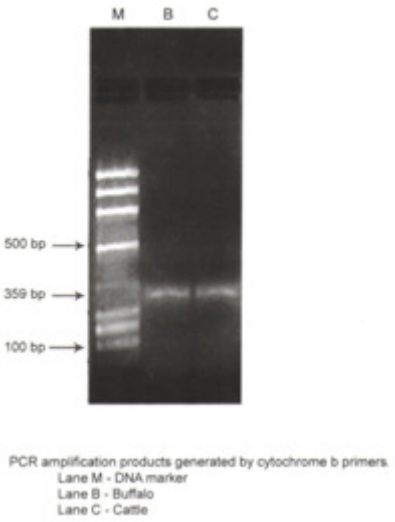

Fig. 1.

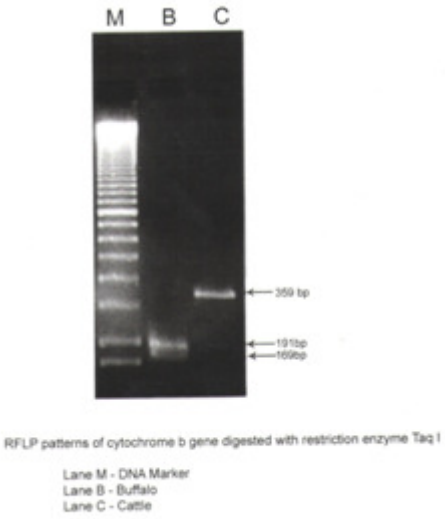

Fig. 2.

CONCLUSION

The SSR-PCR and PCR-RFLP could be applied as a useful molecular analytical method for identification and authentication of different mammals and control measures for any molecular-based evidence.

Table 1. Primer sequences of species-specific repeat and cytochrome-b gene' their annealing

Species	Primer Sequence	Temp.(^{O}C)	Product Size
Pig (Forward)	5' GGAGCGTGGCCCAATGCA 3'	57	<100
Pig (Reverse)	5' ATTGAATCCACTGCATTCAATC 3'	57	
Sheep (Forward)	5'GTTAGGTGTAATTAGCCTCGCGAGAA 3'	62	374
Sheep (Reversed)	5'AAGCATGACATTGCTGCTGCTAAGTTC 3'	62	
Buffalo & Cattle (Forward)	5' AAGCTTGTGACAGATAGAACGAT 3'	60	603
Buffalo & Cattle (Reverse)	5' CAAGCTGTCTAGAATTCAGGGA 3'	60	
Buffalo & Cattle (Cyto b)	5' CCATCCAACATCTCAGCATGATGAAA 3'	57	359
Buffalo & Cattle (Cyto b)	5' GCCCCTCAGAATGATATTTGTCCTCA 3'	57	

Temperatures and length of PCR product

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