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Research Article

Development of Species-specific Polymerase Chain Reaction (PCR) Targeting on Mitochondrial D-loop for Identification of Buffalo and Goat Raw Meat

Anupam Singh*[1,2], Ashish Kumar Rai[2], Gyan Datta Tripathi[2], Veeveka Nand[2], Neha Gupta[2], Kuldeep Srivastava[2]

[1]*Sri Ramswaroop Memorial University, Barabanki, Uttar Pradesh, India*

[2]*Bansal Institute of Engineering and Technology, Lucknow, Uttar Pradesh, India*

***Corresponding author. E-mail: anupam4uk@gmail.com** (**A Singh**)

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Abstract: Today's, consumers are concerned about the meat they eat and also demand accurate labeling. Mitochondrial Analysis of DNA was the most frequently used DNA, because of its highly conserved sequences in various organism species. In this study, a rapid, reproducible and simple method for simultaneous identification of multiple meat species in a single step mitochondrial DNA based test has been developed based on the designing of species-specific primer. Meat samples of goat and buffalo were selected to verify the applicability of the technique. A species specific forward and reverse primer was designed with the help of the primer3 tool for amplification of mitochondrial D-loop region. The species-specific primers were verified *in silico* by SnapGene software. The two pairs of primers amplified the expected fragment of 338bp for buffalo and 450bp for goat. The change in the size of the PCR product was due to the existence of highly polymorphic regions within the buffalo and goat D-loop region. The tested species gives a unique band pattern for each species by using successful amplification of these polymorphic regions in the D-loop region. Overall, the simplicity of amplification of mitochondrial D-loop region could make this technique suitable for meat authentication in routine analysis.

INTRODUCTION

Advancement in meat production and an increase in buyer capacity of the consumers have led many buyers to be more selective in their food choices. The utilization of meat product is increasing continuously. Consumers inspect meat products thoroughly for many logics including allergic effect and religious value. Consumers are also discussed about some other issues such as improper labeling, cost of meat combination, and illegible products. As a national policy, the slaughter of cow is restricted for religious values of Hindus. In most of the country, mixing of buffalo and cow with beef meat is frequently used for economic reasons. This petty act of meat replacement wants to be blocked so as to secure religious values. Mixing of meat has also created health issues such as food allergy [1] and increased the risk of colon cancer [2,3]. DNA is a more stable and reliable as compared to a protein which is prone to denaturation under a higher temperature, so methods based on DNA amplification have been practiced in the present time [4,5]. PCR using species-specific primers would allow direct organism detection without the requirement of further analysis of the PCR products like sequencing [6]. PCR can be combined with a DNA sequencing or RFLP (restriction fragment length polymorphism) techniques. Multiplex PCR is most reliable and time preserving than the other methods [7, 8]. Therefore, genomic and mitochondrial genes, such as *cyt b* gene, D-loop, 16S rRNA and 12S rRNA gene, have been utilized regularly for species identification by multiplex PCR [9,10,11]. Thus, we propose to promote a decent biotechnological technique for identifying the orientation of meat. The objective of this project was to identify common mixing in different meat products such as buffalo and goat obtained from various markets in India by PCR.

MATERIALS AND METHODS

Sample collection and Preservation

Collected raw and fresh meat samples of goat and buffalo from the butchery of Lucknow were stored in clean and sterilized containers and stored at -20⁰C until used for further analysis.

DNA Extraction

Genomic DNA was isolated from the goat and buffalo meat samples by Phenol-chloroform methods as described by Sambrook and Russel (2001). The tissue samples (75 mg) were dissected into very small pieces or pulverized in liquid nitrogen and 10 volumes (w/v) of DNA lysis buffer (fresh meat) (pH 8.0) containing Ribonuclease-A @ 100 µg/ml (20 µg/ml) was added and incubated at 37⁰C for 1 hour. Added proteinase-K solution (20mg/ml) and again incubated at 50⁰C for not less than 3 hours or overnight. An equal volume of Tris-saturated phenol (equilibrated with 0.1M Tris, pH8.0) was added and the components of the tubes were subjected to gentle mixing end to end for 10 min and centrifuged at 6,500 RPM for 15 min. The upper phase was accumulated and then washed twice with chloroform: phenol: isoamyl alcohol (24:25:1) mixture. The upper phase again collected into a microcentrifuge tube containing 1/5 volume of 10M ammonium acetate and double volume of 100% absolute ethanol and was mixed well for precipitation of DNA. The mixture containing visible DNA threads was centrifuged at 10,000 RPM for 10 min. The DNA pellet was washed twice with 70 % alcohol, dried over a dry bath at 60° C and then dissolved in 1X TE (Tris-EDTA) buffer (50- 100 µl) or nuclease-free water. The quality of DNA was checked on 1%agarose gel (Fig. 1).

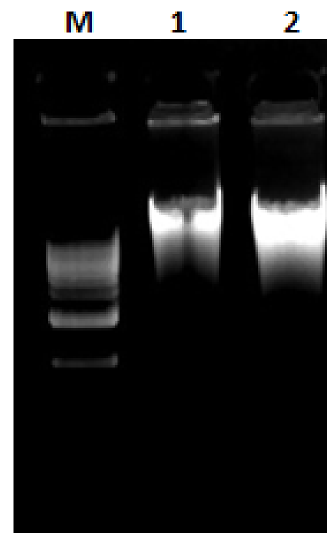

Figure. 1: Agarose gel Electrophoresis of genomic DNA isolated from meat samples, where Lane M, 1 and 2 represent Marker (1kb), buffalo and goat respectively

Primer Design

Mitochondrial sequences of buffalo and goat were collected from the NCBI database. The NCBI accession numbers of buffalo (*Bubalus bubalis*) and goat (*Capra hircus*) were respectively AF475278.1 and KP776458.1. Specific primer sets (sequences are provided in Table1) were designed with the same annealing temperature with Primer3 on the basis of mitochondrial D-loop region sequence. (Hsieh et al., 2001) The species-specific primers were verified *in silico* by SnapGene software.

Table.1 Sequences of primers used for PCR of D-loop region

S.No	Name	Primer	Sequences(5'- 3' direction)	No. of Bases	Tm	GC %
1	BUF	F	ATAGCACATTTAAGTCAAATCCATT	25	54	29.16
2	BUF	R	GGCCATAGCTGAGTCCAAG	19	57	57.89
3	GOA	F	CCGCGTGAAACCAGCA	16	57	62.5
4	GOA	R	AGCGTGTTTAAAACGGTGG	19	57	47.36

Primer validation by *in silico* method

SnapGene tool (http://www.snapgene.com) were utilized for validating species-specific primers, by using species-specific sequences retrieved from NCBI for D-loop region. The sequences of D-loop region of buffalo (Accession no. - AF475278.1) and goat (Accession no. - KP776458.1) were retrieved from NCBI, used for validation of designed species-specific primers. *In silico* primer, validation results are given in fig. 2 and fig. 3 for Buffalo and goat respectively.

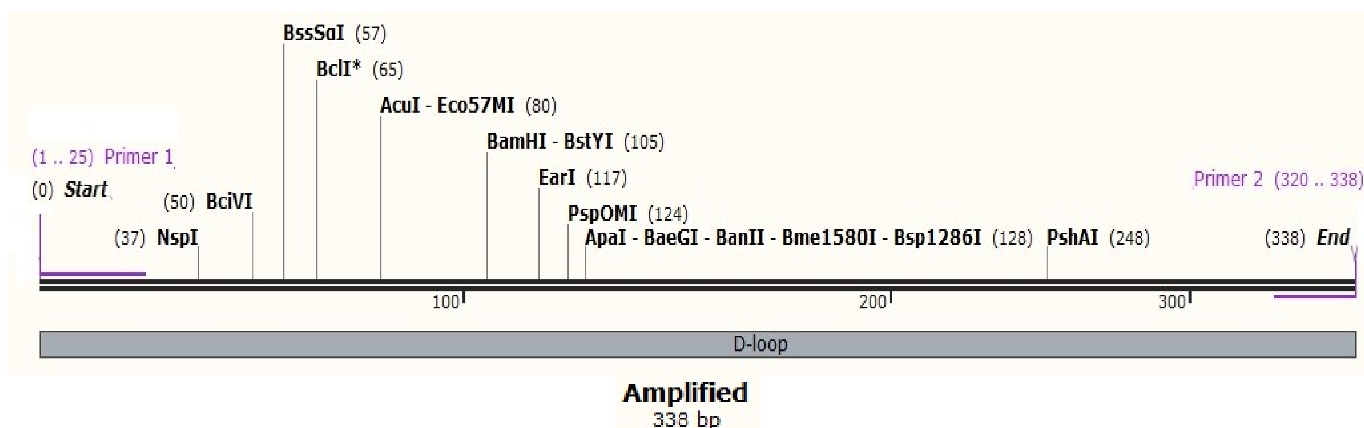

Figure. 2: *In silico* Primer validation of buffalo's species-specific D-loop region.

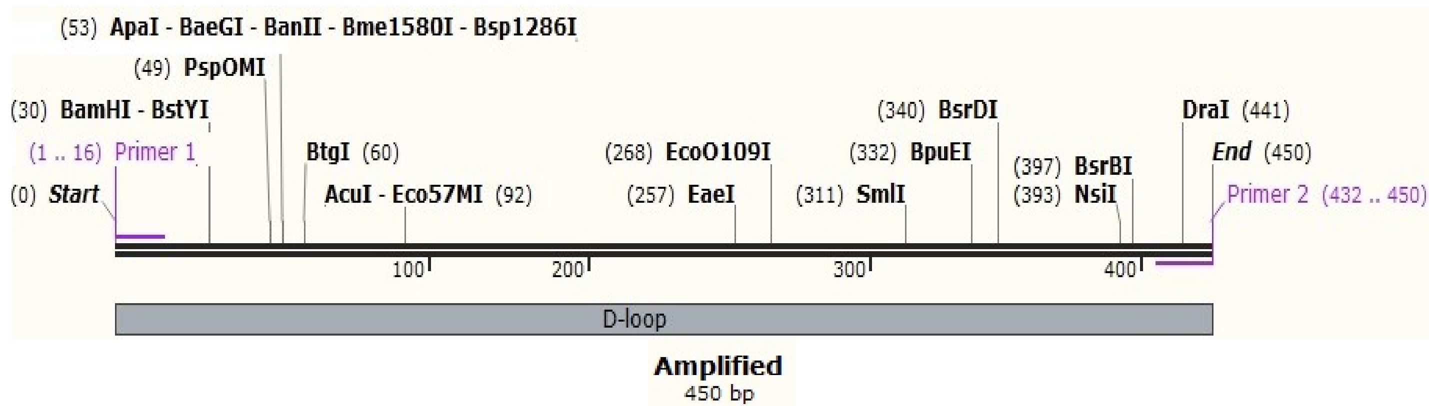

Figure. 3: ***In silico*** **Primer validation of goat's species-specific D-loop region**

Primer Synthesis

Species-specific designed primers were purchased from Micelles Life Sciences (P) Ltd. Initially, primers were in the desalted state, followed by diluting the primers (in concentration 100pm/µl) with distilled water as mentioned by the manufacturer and stored at -20^{0}C for further analysis.

PCR amplification of D-loop region

The reaction mix contained 1X polymerase buffer with 1.5mM $MgCl_2$, 0.5µM of BUF primers and 0.5µM GOA primers (listed in Table 1), 200µM of each dNTPs, 1U of Taq DNA polymerase and template DNA in a final volume of 25µl. The PCR optimization used for D-loop region comprises of pre-denaturation at 94 ^{0}C for the duration of 5 min., denaturation (35 cycles) at 94 ^{0}C for the duration 30sec., annealing (35 cycles) at 52^{0}C for 30sec., extension (35 cycles) at 72^{0}C for 30 sec. and the final extension at 72^{0}C for 5 min.

Gel visualization of amplified PCR Product

Amplified DNA sample was separated by 1% agarose gel electrophoresis and each sample produced a characteristic band pattern at constant voltage 90V for 45 minutes. After running the gel, DNA bands with separate size were visualized under UV light and documented by gel doc.

RESULTS AND DISCUSSION

Meat contamination may be identified by using PCR based approaches that have tested their calibre in determining meats of domesticated animals, meat products and various fish varieties [12,13]. This study used the mitochondrial D-loop region for amplification and the PCR products were analyzed. The approach based PCR technique was able to determine the source of meat of some domestic animals [14]. D-loop region variability has also become helpful in the detection of meat species utilizing multiplex PCR as a tool [15]. The two pairs of primers amplified the expected fragment of 338bp for buffalo and 450bp for goat (Fig. 4). The change in the size of the PCR product was due to the existence of highly polymorphic regions within the buffalo and goat D-loop region. The tested species gives a unique band pattern for each species by using successful amplification of these polymorphic regions in the D-loop region.

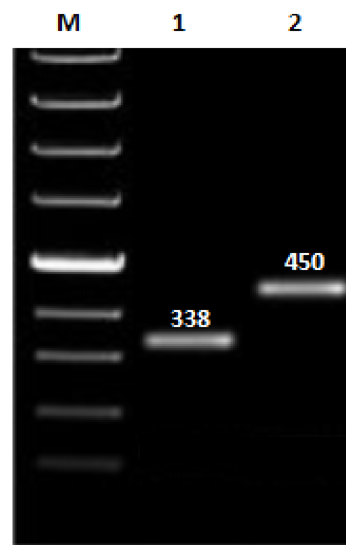

Figure. 4: Agarose gel electrophoresis of PCR products amplified with species-specific primers. Lane 1 represents product from buffalo (338bp) and Lane 2 represent product from goat (450bp). M - 100bp DNA ladder.

Throughout the past couple of decades, a lot of processes dependent on DNA research are used to track adulterations of food products of animal source. The absolute most extended methods demand PCR amplification of the conserved gene fragment by a set of species by either using universal primers or amplification of DNA with specific primers for identification using a specified target organism. Several methods have been reported for differentiation of milk species like electrophoresis, polyacrylamide gel electrophoresis, isoelectric focusing, and capillary electrophoresis. Also, HPLC and ELISA are useful for species in identification in meat and meat products.Selection of highly polymorphic regions within the buffalo and goat mitochondrial D-loops, showing low homology between the 2 species, allowed us to design specific primer pairs for detection of cow and goat DNA.

In this study, two different set of primers were used to amplify *D-loop* gene (buffalo and goat) from different species one by one. The PCR using primer produced a single band of expected size in each species independently, confirming the species specificity of primers.

CONCLUSION

The present study was undertaken to develop a rapid and reliable molecular method to check the purity of species-specific meat at meat collection centres to cater the need of meat industry as demand for the production of specialized meat health products. Thus, the manufactures and consumers will be protected against the meat adulteration and will able to ensure meat products availability of choice. The mitochondrial D-loop region was targeted to develop PCR amplification for detection of species – specific meat of buffalo and goat by using two species-specific primers. Specific band of amplified DNA product generated by the selected primers resulted from which the species origin can be visually inferred, making this technique especially suitable for routine analysis.

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CONFLICT OF INTEREST: **None**

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About Author

Mr. Anupam Singh received B. Tech. (Biotechnology) and M. Tech. (Biotechnology) from Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P. He has worked as Asst. Professor at S.D. College of Engineering and Technology, Muzaffarnagar. Currently, he is working as an Associate Professor and Head in the Department of Biotechnology, Bansal Institute of Engineering and Technology, Lucknow U.P, India. He has published 15 research papers in various national and international journals of repute.