SPECIES IDENTIFICATION OF FRESH AND COOKED MEAT BASED ON PCR-RFLP TECHNIQUE*

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Abstract

Adulteration of higher quality meat with cheaper or inferior quality meat is prevalent in the meat industry. Identification of meat from closely related meat species based on morphological features of meat is very difficult and it becomes even worse if meat is cooked or processed. The present study investigated the use of polymerase chain reaction- restriction fragment length polymorphism analysis (PCR-RFLP) of the mitochondrial cytochrome b (cyt-b) gene sequence for identification of fresh and pressure-cooked meats of cattle, buffalo, sheep, goat and pig. PCR amplification yielded a 609 bp fragments from both fresh as well as pressure cooked meat of all these species. The amplicons were digested with TaqI and AluI restriction enzymes, resulting a pattern that could identify and differentiate each of above species. Pressure cooking of meat did not affect the RFLP patterns of above species.

Keywords: *fresh meat, pressure cooked meat, mitochondrial DNA, cyt-b gene, PCR-RFLP*

Substitution of meat and meat products has always been a concern for various reasons such as wholesomeness, religious factors, public health and unhealthy competition in meat market (Arslan *et al.*, 2006). Proper labelling of meat and meat product is necessary to avoid fraudulent replacement of superior commercially valuable meat by cheaper or inferior meat of alternative species or replacement of animal protein with plant proteins (Ballin *et al*., 2009). Consumers are conscious about the source of food and expect it to be authentic. Thus, meat differentiation had become a significant element in food quality control procedures (Kumar *et al*., 2014).

Morphological character based identification is practiced to identify different species. However, when the time animal has been slaughtered for food, these markers are often removed from animal intentionally or destroyed. This creates problem for different

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government agencies who are involved in identification of species origin of an animal or products in order to enforce health related and or conservation regulations. The problem becomes more complicated if the meat of that animal has been processed (Bartlett and Davidson, 1992). Different techniques were followed for meat species detection which are based on morphological characters, protein based like electrophoresis techniques, spectrophotometry techniques and DNA based techniques like polymerase chain reaction. Techniques based on DNA is more advantageous over protein based techniques due to the fact that protein expression is tissue specific in nature and different processing and heating conditions of meat results in denaturation of proteins. DNA is more thermostable and less susceptible to disruption during different types of processing condition and furthermore, DNA is present in almost all cells. So, information getting from DNA by appropriate samples from same source will be identical regardless of the tissue of origin (Lockley *et al*., 2000). Mitochondrial DNA consists of sequences like D-loop region (Kumar *et al*., 2011 and Mane *et al*., 2011), mitochondrial RNA genes i.e. 18s, 16s and 12s (Mane *et al*., 2012; Girish *et al*., 2004 and Chen *et al*., 2010) and *cyt-b* genes (Hertanto *et al*., 2017 and Sari *et al*., 2017) which are commonly used as target sites for species detection in DNA based techniques. Mitochondrial *cyt-b* gene is one among most extensively sequenced genes and due to its sequence variability, this region of mitochondrial DNA is used to study the phylogenetic relationship among different organisms (Johns *et al*., 1998). Since DNA sequence analysis was costly PCR-RFLP technique is used as a routine analytical tool for species testing (Partis *et al.,* 2000). In the present study, PCR-RFLP assay based on *cyt-b* gene sequence is used to identify fresh as well as pressure cooked meat of cattle, buffalo, sheep, goat and pig.

Materials and Methods

Fresh muscles samples approximately, 50-100 g, six each from slaughtered cattle, buffalo, pig and goat were collected in duplicates from Meat Technology Unit, Mannuthy and from sheep from the Corporation slaughter house, Thrissur. One set of each sample was subjected to pressure cooking at 105 °C for 15-20 minutes. Genomic DNA was isolated from the meat samples by phenol-chloroform method as described by Sambrook and Russel (2001) with slight modifications. The tissue samples (200 mg) were cut into very small (<1 mm) pieces, triturated and 10 volumes (w/v) of DNA lyses buffer (fresh meat- 10 mM Tris–HCl,100 mM EDTA and 0.5% SDS) (pH 8.0) to which Proteinase-K (SRL, India; 20 mg/mL) was added @ 200 µg/mL and incubated at 55 °C overnight. Resultant slurry was treated with Ribonuclease-A @ 100 µg/mL and incubated at 37 °C for 1 h which was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and again with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated by 100 % ethanol and 1 M ammonium acetate solution and was dissolved in TE buffer (10 mM Tris–HCl, pH 7.4 and 0.1 mM EDTA). DNA concentration was determined by measuring the absorbance at 260 nm. DNA from pressure cooked meat was also extracted similarly except that DNA lysis buffer (cooked meat) was used instead of DNA lysis buffer (fresh meat).

Polymerase Chain Reaction

Universal primers VPH-F (5'ATCCGACACAACAACAGCATT CTCCT 3') and VPH-R (5'GCTGGGGTGTAGTT GTCTGGGTCTC3') targeting for mt *cyt-b* gene were used for PCR amplification of DNA from both fresh as well as pressure cooked meat, as described by Kumar *et al.* (2014). Amplification was carried out in 0.2 ml PCR tubes containing 1µL of DNA template, 1µL (20 pmol) each of forward and reverse primers (Sigma-Aldrich, USA), 10 µL of 2X Taq PCR Smart mix (Origin) and nuclease free Millipore water was used to make the reaction volume to 20 µL. Amplification was carried out in a thermal cycler (Bio-Rad, USA) with following conditions: 5 minutes initial denaturation at 95º C followed by 35 cycles of denaturation at 95º C for 45 sec, annealing at 55.6º C for 45 sec, extension at 72º C for 1 min and final extension was done at 72º C for 5 min. PCR products were analysed by horizontal submarine gel electrophoresis in 2% agarose gel with ethidium bromide as stain.

Sequencing and restriction site mapping

Amplified product of *cyt-b* gene from cooked meat samples of cattle, buffalo, goat, sheep and pig were sequenced by automated Sanger sequencing technique at SciGenom, Pvt. Ltd, Cochin. Sequences were analysed and edited with EMBOSS merger software and restriction sites present in the sequences were mapped by using Nebcutter software.

Restriction fragment length polymorphism analysis

AuI and *TaqI* were the restriction enzymes selected for differentiation of different meat sources based on mapped restriction sites (Table 1). According to supplier's guide, reaction mixtures for restriction digestion of mt *cyt-b* gene were prepared. For restriction digestion of *AluI*, reaction mixture was prepared by mixing 4 µL of PCR product with 2 µL of 10X Tango buffer (Thermo Scientific) and 1µL of *AluI* (Thermo Scientific). Finally, reaction volume was made up to 15 µL by adding nuclease free Millipore water and incubated at 37º C for 4 hours. Reaction mixture was prepared for restriction digestion of *TaqI* by mixing 4 µL of PCR product along with 1.5 µL of 10X Tango buffer and 2 µL of *TaqI* (Thermo Scientific). Finally, volume was made up to 15 µL by adding nuclease free Millipore water and incubated at 65 ºC for 4 hours. Digested products were visualized under electrophoresis in 2.25 % agarose gel along with 100 bp ladder.

Results and Discussion

The PCR based RFLP technique was used in the present study to identify the meat sources efficiently. Concentration of DNA isolated from fresh and cooked meat was 1.32 to 1.69 mg/µL and 1.50 to 1.80 mg/µL, respectively. DNA samples exhibiting OD_{260}: OD_{280} ratio in the range of 1.8 - 2.0 were considered good and subjected to PCR. On quality analysis it was observed that genomic DNA extracted from pressure cooked meat showed shearing while compared to that of fresh meat (Fig.1). However, the sheared DNA did not affect the PCR amplification. PCR products visualized under gel electrophoresis showed that DNA from both fresh as well as pressure cooked meats had the same intensity of bands with size of 609 bp (Fig.2). Matsunaga *et al*. (1999) reported that different processing conditions like high temperature and pressure cooking resulted in DNA fragmentation which failed to amplify the DNA fragment size more than 450 bp. But in present study partial *cyt-b* gene of size 609 bp got amplified from the DNA which was isolated from pressure cooked meat.

Table 1. Restriction enzymes with their detailed description of the fragments that could discriminate commonly used meat animal species**.**

Species	Restriction enzyme digestion of 609 bp PCR product					
Species	*TaqI*			*AluI*		
	RE cleavage positions	Fragments (bp)		RE cleavage positions	Fragments (bp)	
		Expected	Diagnostic		Expected	Diagnostic
Beef	315	294 315	294 315	92 545	64 92 453	64 92 453
Buffalo beef	70	70 539	70 539	92	92 517	92 517
Chevon	43 315 446	43 131 163 272	131 163 272	Not present	609	609
Mutton	315	294 315	294 315	350	259 350	259 350
Pork	44 343	44 266 299	266 299	261 288	27 261 321	261 321

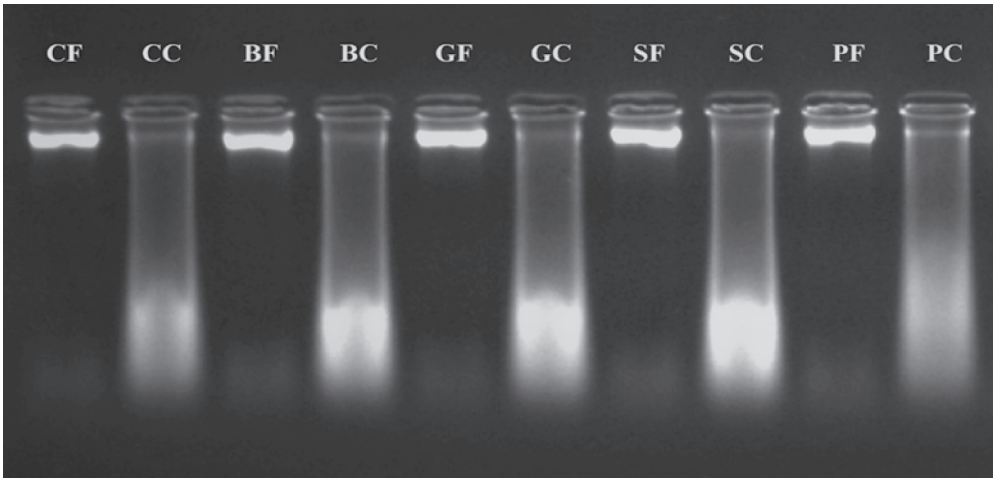

Fig.1. DNA isolated from fresh and cooked meet resolved on 0.8 per cent agarose gel

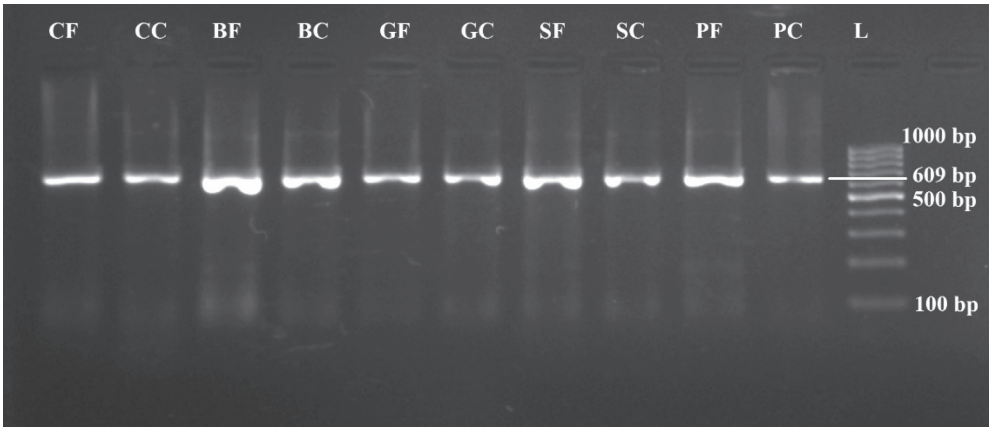

Fig. 2. PCR amplification pattern of *cyt-b* gene of cattle, buffalo, goat, sheep and pig with the primer pair VPH-F and VPH-R. CF-Cattle fresh meat sample, CC-Cattle cooked meat sample, BF- buffalo fresh meat sample, BC-buffalo cooked meat sample, GF- goat fresh meat sample, SF-sheep fresh meat sample, SC- sheep cooked meat sample, PF- pig fresh meat sample, PC- pig cooked meat sample and L- ladder 100 bp

This might be due to the presence of several copies of mt DNA per cell and at least few copies of mt DNA would be available for successful amplification even though when severe degradation of isolated DNA from processed meat might have occurred. Karabasanavar *et al*. (2014) succeeded to amplify mt D-loop sequence of 712 bp size from pork which was autoclaved (121ºC, 15 Psi, 30 minutes) and had given same opinion. PCR products were sequenced by using automated Sanger sequencing technique. Even though DNA sequencing and analysing is an accurate and authentic technique, it is not an economical one to use for routine identification of meat species (Partis *et al*., 2000). So, PCR-RFLP is simple, rapid and more economical technique used for routine meat species identification. Merged sequences were analysed for restriction site mapping and same is enlisted in Table 1. Based on the restriction mapping, *AluI* and *TaqI* restriction endonuclease enzymes were used. When partial *cyt-b* gene of 609 bp was subjected to *TaqI,* it yielded two fragments of similar size (294 and 315 bp) in case of cattle and sheep, two fragments of different sizes in case of buffalo (70 and 539 bp) and pig (266 and 299 bp) and three fragments of different sizes (131, 163 and 272 bp) in case of goat (Fig.3). So *AluI* was further used to differentiate cattle and sheep samples, which was found to produce three fragments (64, 92 and 453 bp)

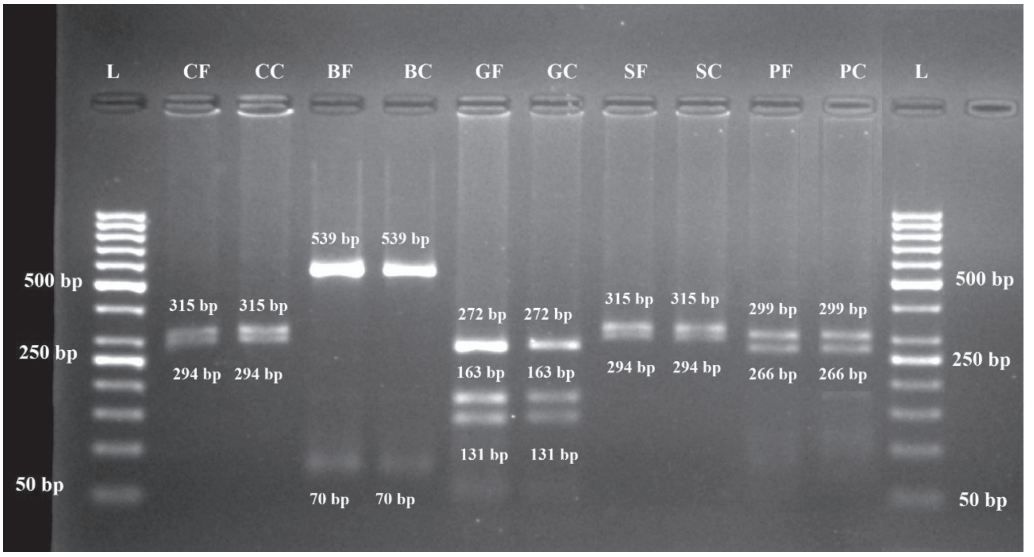

Fig. .3. *TaqI* digestion pattern of 609 bp fragment of *cyt-b gene* of cattle, buffalo, goat, sheep and pig. CF-Cattle fresh meat sample, CC-Cattle cooked meat sample, BF- buffalo fresh meat sample, BC- buffalo cooked meat sample, GF- goat fresh meat sample, SF-sheep fresh meat sample, SC- sheep cooked meat sample, PF- pig fresh meat sample, PC- pig cooked meat, L- ladder 50 bp

in case of cattle (Fig. 4) and two fragments (259 and 350 bp) in case of sheep. These findings were similar to the findings of Kumar *et al*. (2014). Findings suggest that pressure cooking of meat did not affect the restriction profile of *cyt-b* gene in case of cattle, buffalo, goat, sheep and pig. Girish *et al.* (2005) also succeeded to identify the autoclaved meat by using PCR-RFLP technique.

It can be concluded that, meat of four closely related species used for commercial meat production i.e., cattle, buffalo, sheep and goat along with pig can be qualitatively identified and differentiated by PCR-RFLP of mitochondrial *cyt-b* gene. This method can be applied with good repeatability and equal efficiency to identify the species of both fresh and pressure-cooked meats.

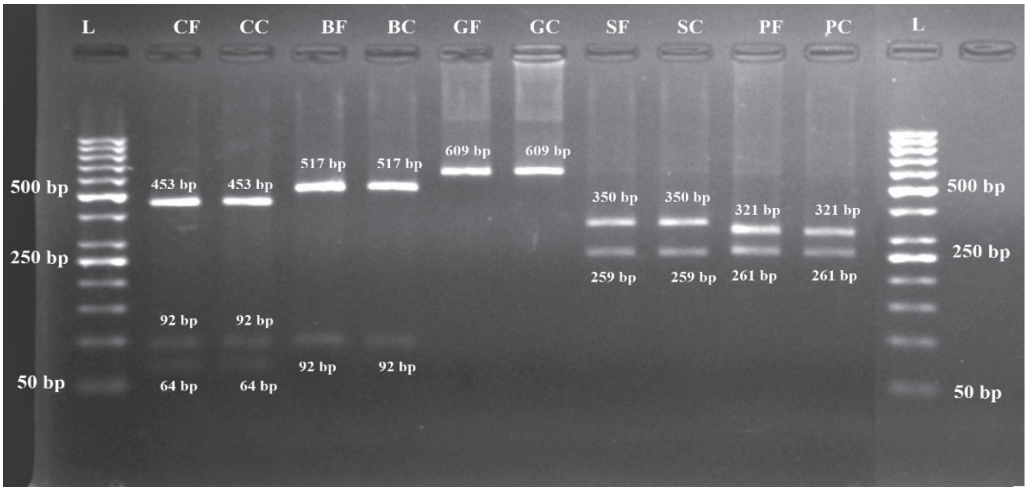

Fig. 4. *AluI* digestion pattern of 609 bp fragment of *cyt-b gene* of cattle, buffalo, goat, sheep and pig. CF-Cattle fresh meat sample, CC-Cattle cooked meat sample, BF- buffalo fresh meat sample, BC- buffalo cooked meat sample, GF- goat fresh meat sample, SF-sheep fresh meat sample, SC- sheep cooked meat sample, PF- pig fresh meat sample, PC- pig cooked meat sample and L- ladder 100 bp

CF-Cattle fresh meat sample, CC-Cattle cooked meat sample, BF- buffalo fresh meat sample, BC- buffalo cooked meat sample, GF- goat fresh meat sample, SF-sheep fresh meat sample, SC- sheep cooked meat sample, PF- pig fresh meat sample and PC- pig cooked meat sample

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