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*Full Length Research Paper*

# Molecular traceability of the species origin of meats using multiplex PCR

**Ghorban Elyasi Zarringhabaie[1], Nasrollah Pirany[2]* and Arash Javanmard[3]**

[1]Department of Animal Science, East Azerbaijan Agriculture and Natural Resources Research Centre, Tabriz, Iran.
[2]Department of Animal Science, Faculty of Agriculture, University of Shahrekord, Shahrekord,Iran.
[3]Department of Genomics, North-West and West Agricultural Biotechnology Research Institute of Iran (ABRII), Tabriz, Iran.

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**The objective of this study was the designing of a fast and reliable multiplex polymerase chain reaction (PCR) identification system for testing the pure and mixed species origin of meat samples. For conducting this research, different primers were designed for each species according to the conserved region of mitochondrial cytochrome b (Cytb) gene. The results revealed different specific amplified fragments of pure meat sources for buffalo, goat, cattle and sheep species. After mixing different portions of the mentioned meat sources, this method was able to trace less than 10% of the other species of meat in the mixture. Then, it can be concluded that this procedure is simple, cheap, rapid, and efficient, and so it can be used in the meat industry.**

**Key words:** Food adulteration, meat origin species, Cytb, multiplex PCR.

## INTRODUCTION

In the last few decades, adulteration of food products has become a considerable problem in many countries as well as Iran. Adulteration may take the form of substitution of one cheaper meat with a costly expected species in food (Malmheden Yman and Emanuelsson, 1998). To protect consumer rights, the legislation of each country should therefore impose a labeling of food products declaring the species used in their manufacture. The increasing demand for transparency in the food industry derives either from socio-religious reasons (such as vegetarianism, preference for organic products, the absence of pork for Jews and Muslims), health concerns (such as absence of peanuts, lactose or gluten for individuals with particular allergies) or economic reasons has provoked a strong demand for appropriate detection methods that allow identification of different species in meat foods and/or of the different components in processed food.

Mainly, they are based on the analysis of certain biomolecules, such as proteins electrophoretic, isoelectric focusing, immunochemical, and HPLC methods (fatty acids and DNA), or they are based on the specific microscopic structure element determination. Species identification and authentication based on DNA analysis is more sensitive and reliable, since it is independent of the tissues being compared. In particular, due to the high mutation rate of mitochondrial DNA (mtDNA), 10 times greater than nuclear DNA, point mutations accumulate very quickly allowing the discrimination of closely related species (Jorde et al., 1998). Most of these methods make use of PCR amplification and direct sequencing of a conserved gene from the sample such as mt-12S rDNA (Rastogi et al., 2004), mt-16S rDNA (Borgo et al., 1996) and cytochrome b gene (Cytb). Cytb contains species-specific information, and it has been widely used in a considerable number of studies on phylogenesis and in studies dealing with forensic science and food inspection.

In mtDNA, Cytb is a functional gene located between genes responsible to produce $tRNA^{Glu}$ and $tRNA^{Thr}$ which encode partial cytochrome c oxidoreductase, a complex

*Corresponding author. E-mail: napirany@googlemail.com, n_pirani@tabrizu.ac.ir.

**Abbreviations: Cytb,** Cytochrome B; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism.

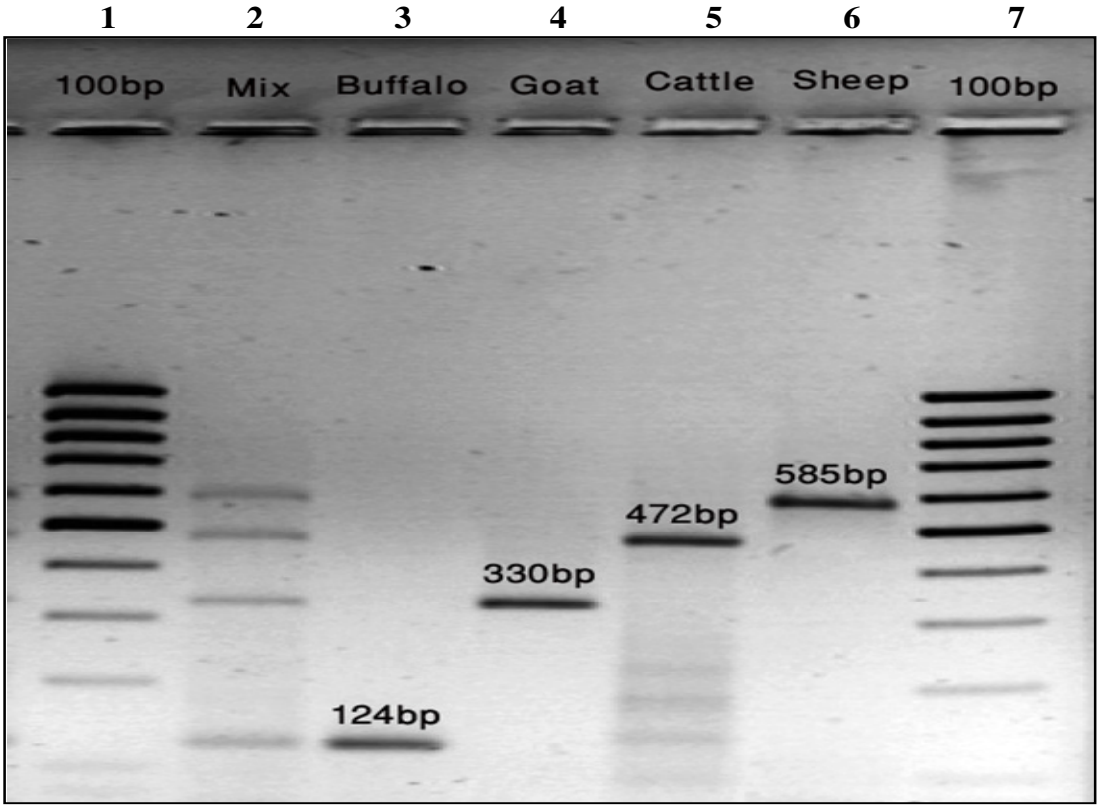


**Figure 1.** PCR product sizes of Cytb in pure and mixed meat of different investigated species.

enzyme in oxidative phosphorylation (Leonard and Schapira, 2000; Southern et al., 1988). Many investigations of vertebrate cytb gene have focused on inheritance and evolution (Antoinette and Greg, 2001; Moritz et al., 1987). The application of PCR seems to give the most satisfactory results (Teletchea et al., 2005). Prevention of fraudulent labeling of meat constitutes an important part of food regulatory control and quality assurance systems. Therefore, the objective of this study was to evaluate the different meat species origin using multiplex PCR technique.

## MATERIALS AND METHODS

### Samples

In total, 100 raw meat samples were collected and transferred to the laboratory in a chilled condition using an ice container. The tissue was cleaned of extraneous fat, connective tissue and blood vessels and then stored in liquid nitrogen for 1 h. Our initial hypothesis was based on this idea that all samples species origin and their relevant sexes were clear due to butchers claim.

### Preparation of the meat mixtures

The samples of meat for each species were minced and prepared separately by adding 10, 20, 30 and 40% (w/w) of sheep, cattle, buffalo and goat meat to each sample. The mixtures of meat were prepared in a total weight of 250 g following mixing. A 2 g portion of each sample was taken separately from five different areas of each mixture.

### DNA extraction and PCR amplification

DNA was extracted from samples using Promega commercial kit. Purity of the extracted DNA was assessed by calculating the $OD_{260}/OD_{280}$ nm ratios using nanodrop (Model ND1000). For primer designing, the mitochondrial Cytb gene sequences (accession numbers) for each species were taken from GenBank; *Bubalus bubalis* (AB529514.1), *Capra hircus* (AB110597.1) *Bos taurus* (EF693798.1) and *Ovis aries* (AB006800.1). Then the sequences were aligned (Figure 3) using BLAST, MAFFT and CLUSTW online multiple alignment tools (www.ebi.ac.uk.) and primers were designed using Primer Premier 5.0 (Perimer Biosoft International, Palo Alto, CA) software. Primer sequence and their corresponding amplified size are shown in Table 1 and Figure 1.

PCR was carried out in 25 µl volume comprising of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.01 mM of each primer (Fermantaz), 50 ng of genomic DNA, and 0.2 U Taq DNA polymerase (Cinnagen, Iran). The PCR protocol was done using Biometera thermo-cycler (T-Personal Model; Germany) comprising of initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation for 45 s at 94°C, annealing at 62°C for 1 min, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were electrophoresed at 85 V for 45 min in 2.5% agarose gels, and viewed under UV light after staining with Ethidium bromide. The sizes of PCR products were determined in relation to a 100 bp DNA

**Table 1**. Primer sequence, PCR product size and specific forward primer for identification of species using multiplex PCR.

| Specific species primer | Primer sequence (5´- 3´) | PCR product size (bp) |
|---|---|---|
| Common-R | TGTCCTCCAATTCATGTGAGTGT | - |
| Buffalo-F | TCCTCATTCTCATGCCCCTG | 124 |
| Goat-F | CGCCATGCTACTAATTCTTGTT | 330 |
| Cattle-F | TCCTTCCATTTATCATCATAGCAA | 472 |
| Sheep-F | TACCAACCTCCTTTCAGCAATT | 585 |

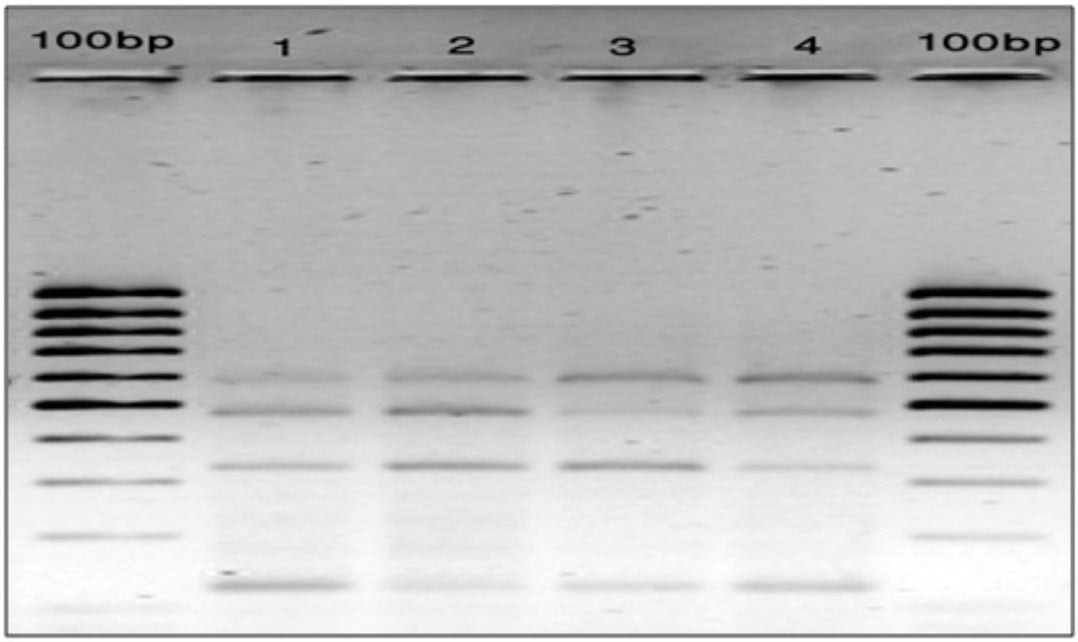


**Figure 2**. PCR product size of Cytb in the different mixed meat of the investigated species. The percentage of sheep, goat, cattle and buffalo meat respectively for sample 1 are: 10, 20, 30 and 40%; sample 2: 20, 30, 40 and 10%; sample 3: 30, 40, 10 and 20%; sample 4: 40, 10, 20 and 30%.

size standard (Fermentas) using a computer software BIO 1D++.

## RESULTS AND DISCUSSION

The results of the PCR products are shown in Figures 1 and 2. The results show expected different specific amplified PCR products sizes of 124, 330, 472 and 585 bps for Cytb gene of buffalo, goat, cattle and sheep species, respectively (Figure 1) for pure and equally mixed meat of those species. The result of this study also show that designed primers worked well in multiplex manner and this method was sensitive and it was possible to trace each species meat when its portion in the mixture was less than 10% (Figure 2). Species identification of meat and meat products is important because of health, ethical, and economic reasons (Winter et al., 1990). The risk and threat of food adulteration and mislabeling have become a large concern and challenge for the food control sectors and consumers. Therefore, to enhance food security, fast and reliable detection methods are essential for the food industry. In order to enable food control authorities to supervise compliance with labeling requirements, suitable detection methods, which could allow unambiguous identification of animal or fish in a foodstuff, are prudent. Many meat products nowadays may contain several species in different proportions mixed together and undetectable by the naked eye or by eating. The advantage of the present molecular methods is that there is no need to use restriction enzyme and restriction fragment length polymorphism (RFLP) methods to distinguish specific electrophoresis fragment on agarose gel.

In recent years, many investigators applied various typing methods including PCR amplification (Tanabe et al., 2007) to identify species in meat. For example, Meyer et al. (1994) detected 0.5% pork in beef using the duplex PCR technique. Their results revealed that PCR was the

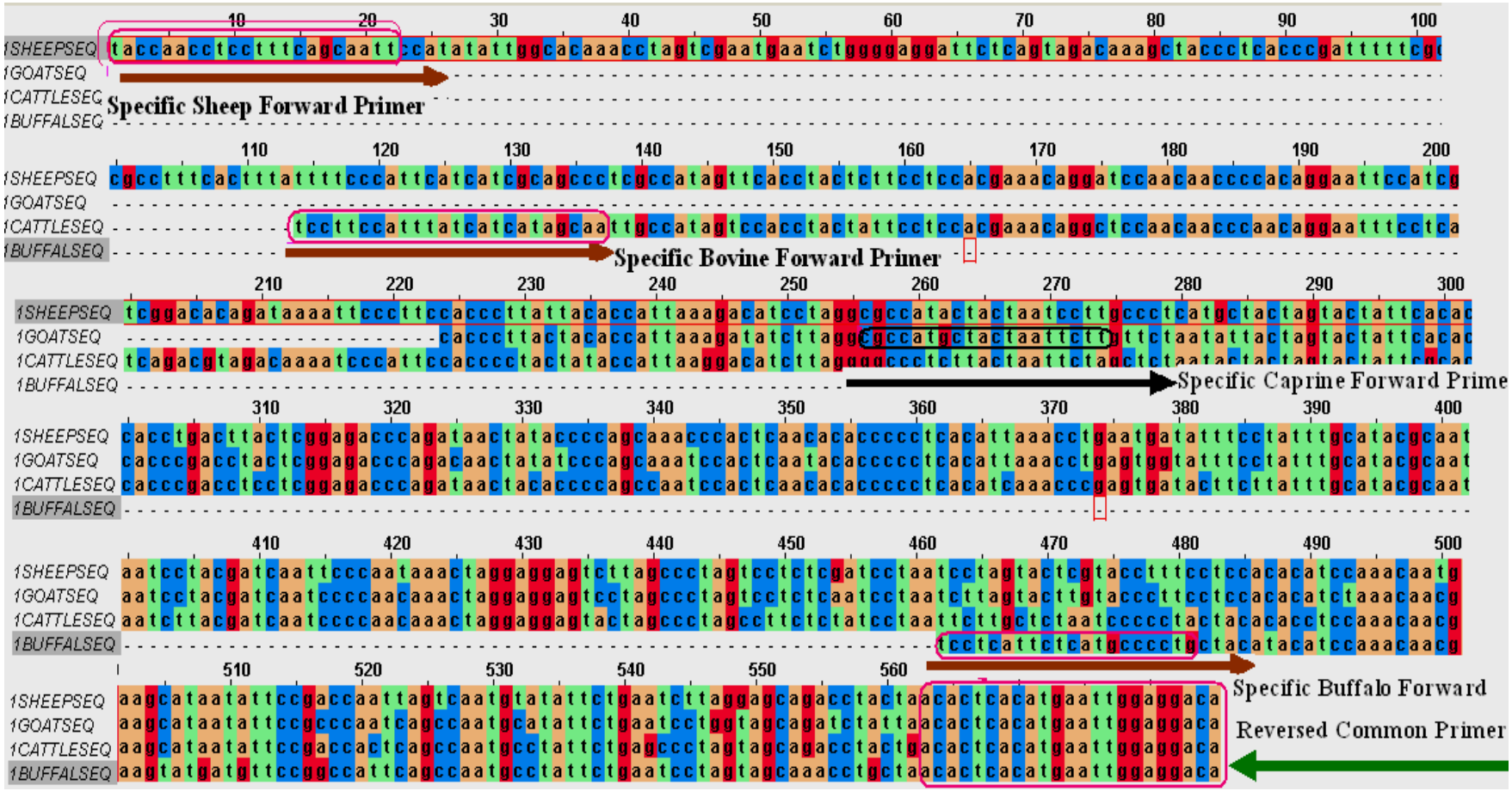


**Figure 3.** Alignment of Cytb sequence between the four investigated species and position of the designed primers and PCR product size, respectively. Numbers are showing length of amplified fragments in multiplex PCR and boxes are indicators for length and annealing position of the different primers. Different colors also are showing similarity and dissimilarity of sequences in different investigated species.

method of choice for identifying meat species in muscle foods. They also detected 0.01% sow's protein in processed meat products using the nested-PCR technique. Partis et al. (2000) also detected 1% pork in beef labelled meat. Hopwood et al. (1999) detected 1% chicken in lamb using PCR-RFLP. Results of this study support the findings of Meyer et al. (1994, 1995), Hopwood et al. (1999), and Partis et al. (2000) who reported that PCR could be used for identification of meat mixes at 1 and 0.5% levels. In meat processing, it may be inevitable that one species of meat may be contaminated with another during meat operations, such as cutting and grinding via knives, grinders, choppers, and cutting boards.

PCR analysis of such samples may show positive results for a violation due to its high sensitivity (Antoinette and Greg, 2001; Teletchea et al., 2005) even though contamination was unintentional and at a very low level. Therefore, a precaution should be exercised when interpreting the results of species identification by PCR and analysis of multiple samples should be taken from each lot for an objective evaluation.

## Conclusion

In conclusion, this study suggests an accurate analytical technique for sheep, goat, beef and buffalo meat identification, based on PCR analysis of the Cytb gene of mitochondrial DNA for enforcement of labeling regulations. This technique is useful and feasible to trace meat adulteration and differentiate species present in mixed meat. Therefore, it can be suggested as a useful laboratory tool for species identification, especially for meat traceability and Halal authentication.

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