



Molecular detection of goat and sheep meat origin using mitochondrial cytochrome b gene

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Abstract

The present study was conducted to discriminate between sheep and goat species meat origin utilizing mitochondrial cytochrome b (*Cyt b*) gene fragment. A total of 46 ear tissue and meat samples were collected from different slaughterhouses and farms of Mymensingh and Rangpur districts. Genomic DNA was extracted using GeNet Bio DNA isolation kit and DNA concentration and purity was quantified by NanoDrop spectrophotometer. Two pairs species specific primer were used to amplify *Cyt b* gene fragments. Selected primers were highly conserved across the breed within a species and worked well with the species of indigenous goat and sheep resulting similar size of the amplicons 330 and 585 bp respectively. The duplex PCR condition would enable to detect adulteration from goat and sheep mixed samples which revealed by two precise bands (330 and 585 bp) in a single reaction. This study suggests an accurate molecular technique for identification of sheep and goat meat species origin and differentiates species present in adulterate meat samples. In conclusion, this DNA based marker could be used for prevention of fraudulent practice in slaughterhouse and chain shops in Bangladesh.

Key words: goat, sheep, cytochrome b gene, PCR, agarose gel

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Introduction

Goat meat or chevon is the most popular red meat in Bangladesh. Chevon stands second in position among the produced red meat in Bangladesh which has been widely used in different festivals (). Consumers prefer goat meat for its better taste, texture and flavour. On the other hand, sheep meats (mutton) are generally not popular to the consumers of Bangladesh due to its characteristic odour. Therefore, price of chevon is about double than mutton. Goat and sheep population in Bangladesh is currently estimated to comprise 25.73 million goats and 3.21 million sheep (BER, 2015). Although a considerable number of sheep populations are available in Bangladesh, but there is no butcher shop or slaughterhouse who sell sheep meat in the market. In most cases, sheep are slaughtered by goat butcher and sold as chevon to the consumers. Moreover, adulteration may take place in the form of substitution of one cheaper meat with a costly expected species in value added products (Malmheden and Emanuelsson, 1998).

However, consumer faces unhealthy competition in meat market not protected from these malicious practices of meat adulterations. Meat adulteration and mislabelling are illegal and raise many healths, religious and economic issues. The detection of adulterations and identification of adulterants in meat products is important for the implementation of national standards as well as to protect the consumer preference (Haider *et al.*, 2012). Different DNA-based techniques have been used for animal species identification for the last two decades. Utilization of polymerase chain reaction of mitochondrial DNA (mtDNA) is modern method of animal species identification. The large variability of mtDNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species (Prado *et al.*, 2002). Genes to be targeted in mtDNA for amplification could be 12S, 16S and 18SrRNA, actin, cytochrome b, cytochrome oxidase-II, NADH dehydrogenase 5/6 and mtDNA D-loop (Kesmen *et al.*, 2009). In present study forward and reverse primers were selected from a published paper of Zarringhabaie *et al.* (2011) who used to amplify the fragment of *Cyt b* gene

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for goat and sheep species origin identification. The advantage of PCR based tests are higher accuracy, time saving, high sensitivity and flexibility compared to other methods (Amjadi *et al.*, 2012). Despite, the PCR based techniques have been used in developed worlds as well as in neighbouring countries to protect malpractice in slaughterhouses as well as in super markets but this kind of molecular approaches are totally absent in Bangladesh. Therefore, the objectives of this study were to adopt PCR based technique and to develop DNA marker from *Cyt b* gene for identification of goat and sheep species meat origin and detection of adulteration from mixed samples.

Materials and Methods

Sample collection, transportation and processing

Forty-six (46) ear tissue or meat samples of goat and sheep were collected from different regions of Mymensingh and Rangpur districts. Samples collected for this study purpose were submersed into 96% alcohol immediately after collection and transferred to the Animal Genetics Lab, Bangladesh Agricultural University, Mymensingh and stored at room temperature into a small plastic tube.

DNA extraction and quantification

Samples were trimmed to remove extra hair root and exogenous fat before DNA extraction. DNA was extracted according to the instructions followed by the GeNet Bio genomic DNA isolation kit. DNA concentration was assessed by 0.8% gel electrophoresis as well as NanoDrop spectrophotometer (Model ND1000). Purity of extracted DNA was assessed by calculating the OD260/OD280 nm ratios using NanoDrop (Model ND1000) spectrophotometer. Selected DNA

samples were homogenised with a concentration of ~ 50ng/μl by adding ddH₂O and stored at - 20°C for further studies.

Cyt b gene amplification by single and duplex PCR

The primer sequence information was obtained from a published paper by Zarringhabaie *et al.* (2011). The *Cytb* gene sequence of Black Bengal goat and indigenous sheep were retrieved from NCBI gene bank data base (accession no. AB110597.1 and AB006800.1) and were aligned by CLUSTAL OMEGA software (EMBL lab) to check the suitability of selected primers for indigenous sheep and goat species of Bangladesh. Two sets of primer pairs which were used for PCR amplification are listed in the Table 1. PCR amplification was performed by using gradient thermocycler (Biometra). Two types of PCR reaction was carried out which are given below:

i) Cyt b gene amplification by single PCR

PCR reaction was carried out in 20 μl volume comprising of 1X buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 1.0 μM of each primer, 2 μl (~ 50ng/μl) of genomic DNA and 0.3 U Taq DNA polymerase.

ii) Cyt b gene amplification by duplex PCR

Duplex PCR means both forward and reverse primers were added in mixed samples in one PCR reaction. PCR reaction was carried out in 20 μl volume comprising of 1X buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 1.5 μM of each primer, 3 (1.5+1.5) μl (~ 50ng/μl) of mixed genomic DNA and 0.5 U Taq DNA polymerase. The PCR amplification was performed using Biometra thermo-cycler and cyclic conditions comprised of initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing at 62°C for 60 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min.

Table 1. Two sets of primer pairs used for PCR amplification

Species	Type	Primer sequence	Reference
Goat	Forward	CGCCATGCTACTAATTCTTGTT	Zarringhabaie et al. (2011)
	Reverse	TGTCCTCCAATTCATGTGAGTGT	
Sheep	Forward	TACCAACCTCCTTTCAGCAATT	
	Reverse	TGTCCTCCAATTCATGTGAGTGT	

Agarose gel documentation of PCR product

The PCR products were electrophoresed at 120 V for 25 min in 2.0 % agarose gels. For this, 0.5x TBE buffer solution was used to prepare 2.0 % gel which stained with 5 µl (10 µg/µl) of ethidiumbromide (BioNEER, SouthKorea). The gel images were documented by UVsolo TS imaging system (Biometra, Germany).

Results and Discussion

DNA concentration and purity

The concentration and purity of extracted DNA is presented in Table 2. Purity of extracted DNA ranged from 1.82 to 1.93 in case of sheep and 1.83 to 1.95 in case of goat samples. The DNA concentration ranged from 74 to 700 ng/µl in case of sheep samples and 43 to 644 ng/µl in case of goat samples extracted from ear tissue but very low concentration was found for meat tissue samples which ranged from 11 to 24 ng/µl in case of sheep and 25 to 58 ng/µl in case of goat. Therefore, it is postulated that extraction methods need to optimise particularly for the amount of digestion enzyme (proteinase K) utilizing meat tissue samples by commercial genomic DNA extraction kit. In addition, the variation in concentration of extracted DNA might be due to differences in extraction methods, sources of sample and individual efficiency.

However, the amount of extracted DNA would be sufficient for PCR amplification and standardization of all samples were performed before heading to further amplification process. Tataurov et al. (2008) reported the concentration of DNA varied from 50 to 1410 µg/ml extracted from blood and meat products (meatballs) which are within the range of the present study. Satish

et al. (2009) measured DNA concentration from the cooked meat samples of ruminant and poultry species which ranged from 20 to 100 µg /500 mg, which was much lower than that of raw meats (100 µg to 500 µg/500 mg) of same species as well as from the concentration of the present study. The samples having optical density (OD) ratio at 260/280 ranged between 1.7 to 1.9 and not showing smearing on agarose gel electrophoresis were included for further reactions (Satish et al., 2009). In this study the minimum purity of extracted DNA was 1.82 which revealed that all the DNA samples were supposed to be free of proteins and RNA and suitable for PCR based study.

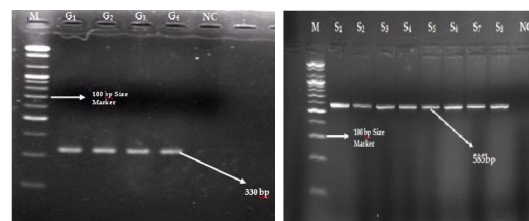


Figure 1. Agarose gel electrophoresis of single PCR amplification using *Cyt b* gene fragment for goat and sheep shown in 2.0% agarose gel. 330 bp and 585 bp for goat species specific band, M=100 bp DNA size marker, NC= negative control and G1-G4= goat samples, S1-S8=sheep samples.

***Cyt b* gene amplification by PCR**

1. *Cyt b* gene amplification by single PCR for species specific detection

Initially, independent primers were run using a pair of species-specific forward primer and reverse primers (common for two species). In 20 µl reaction volume, 1.0 µM of each primer was used in single PCR and 2.0 µl genomic DNA mixed with 18.0 µl PCR master mix.

Table 2. Summary of concentration and purity value of extracted DNA

Ear tissue and meat samples		Concentration of extracted DNA (ng/µl)			Purity of extracted DNA		
		Max	Min	Average	Max	Min	Average
Ear tissue	Goat(n = 20)	643.4	43.70	203.39	1.95	1.83	1.88
	Sheep(n = 16)	869.7	74.80	338.86	1.92	1.82	1.87
Meat tissue	Goat meat(n = 5)	58.3	24.90	37.66	1.93	1.89	1.91
	Sheep meat(n = 5)	24.4	11.10	18.63	1.93	1.87	1.90

The results showed expected different species specific amplified PCR product sizes of 330 and 585 bps for *Cyt b* gene fragment of goat and sheep species, respectively (Figure 1) for pure meat and ear tissue sample of those species. The exact fragment size was reported by Zaringabaie *et al.*, (2011) using sheep and goat meat samples of Iranian origin. The features revealed that selected primer sequence is highly conserved among the breeds of same species and could be utilized efficiently in Bangladeshi sheep and goat population.

2. *Cyt b* gene amplification by duplex PCR for detection of adulteration in mixed meat samples

Having confirmed species specificity of each primer independently, a duplex PCR was standardized by mixing all primers in a single reaction but targeting DNA of single species. Electrophoresis band pattern of PCR amplicon on 2.0% agarose gel resulted clear cut differentiation between goat (330 bp) and sheep (585 bp) when run with single species and multiple band pattern (330 and 585 bp) when run with mixed DNA samples of goat and sheep (Figure 2). In that case, duplex PCR was optimised by changing the concentration of each primer (1.5 μ M), genomic DNA (1.5 μ l for each species) and polymerase enzyme (0.5U/reaction). This result revealed that selected primers worked well in duplex manner and discriminate sufficiently the species meat origin from the mixed samples.

This method is sensitive and is possible to trace each species meat from adulterate samples. The amplicon sizes resulted by duplex PCR using mtDNA *Cyt b* gene for goat and sheep species in 2% agarose gel are shown in Figure 2. Several studies were conducted on molecular traceability of species origin from the samples of meat and meat products using mitochondrial *Cyt b* gene where they employed different molecular methods like PCR, PCR-PFLP, PCR-SSR, Real time PCR and FINS. All of these methods traced species origin and adulteration in meat and meat products efficiently. For example, Zarringabaie *et al.* (2011) reported different specific amplified fragments of pure meat sources for buffalo (124 bp), goat (330 bp), cattle (472 bp) and sheep (585 bp) species and this method was able to

trace less than 10% of the other species of meat in the mixture. This finding conforms to this present investigation where similar amplicon sizes were found.

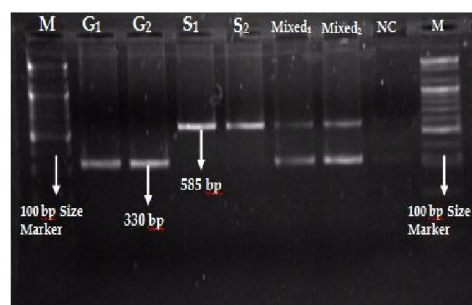


Figure 2. Agarose gel electrophoresis of amplicon by duplex PCR using mtDNA *Cyt b* gene for goat and sheep species. Here M=100 bp DNA size marker, G1-G2 for goat and S1-S2 for sheep samples. Mixed samples contain sheep and goat DNA with equal proportion. Electrophoresis was done in 2.0% agarose gel and specific amplified product sizes 330 and 585 bp for *Cyt b* gene fragment of goat and sheep respectively, NC=negative control.

In another investigation, Satish *et al.* (2009) worked on mitochondrial *Cyt b* gene based PCR to identify and differentiate species from the cooked meat of sheep, goat, cattle, pig and chicken. These animal species produced DNA fragments of 331, 157, 274, 398 and 227 base pairs respectively and clearly differentiated the species origin of meat. The amplified fragments were different from the present study as they used different primer sets of *Cyt b* gene. The use of PCR techniques to amplify the mitochondrial *Cyt b* gene in identifying pig DNA in foods has been reported by several authors (Chandrika *et al.*, 2009; Tanabi *et al.*, 2007). Nuraini *et al.* (2011) used *Cyt b* gene and detected the presence of rat meat in a mixture of beef meatballs at concentration of 15% which was labelled as 100% beef originated. Buffalo, cattle, goat and sheep species also detected on mitochondrial *Cyt b* based on PCR-RFLP methods (Ahmed *et al.*, 2005; Zahran *et al.*, 2015). Amjadi *et al.* (2012) used PCR-RFLP technique in mitochondrial *Cyt b* gene and detected 80% of the collected samples were contaminated by prohibited ruminant residuals. Species identification using PCR-RFLP of a mitochondrial *Cyt b* segment has been well documented (Partis *et al.*, 2000).

All above discussions revealed that *Cyt b* gene is a widely used marker for meat species identification and detection of adulteration from mixed meat samples and also support our study. Single and duplex PCR in mitochondrial *Cyt b* gene used in this study is an easier method than PCR-RFLP, PCR-SSR, PCR-FINS and Real time PCR. The advantage of the present molecular methods is that there is no need to use restriction enzyme and RFLP methods to distinguish specific electrophoresis fragment on agarose gel and the post PCR analysis is much simpler than other methods. Also, this PCR based method is reliable, highly sensitive and specific, as well as cost effective and less time consuming.

Conclusion

Our DNA based study showed an accurate analytical technique for identification of goat and

sheep species meat origin by conventional PCR and meat adulteration between these two species through duplex PCR analysis without further using any enzymatic digestion. Therefore, it can be suggested as a useful laboratory tool especially for meat traceability and has also commercial impact for prevention of malpractice in slaughterhouse, meat products factory and chain shops of Bangladesh.

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