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Article

Molecular detection of cattle and buffalo species meat origin using mitochondrial cytochrome b (*Cyt b*) gene

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Abstract: The study was conducted to adopt PCR based technique for identification of species origin from meat samples of cattle and buffalo using mitochondrial cytochrome b (*Cyt b*) gene fragment. A total of 42 ear tissue and meat samples were collected from different slaughterhouses and farms of Mymensingh, Bogra and Rangpur districts and stored in 96% ethanol at room temperature. Genomic DNA was extracted from all samples using GeNet Bio genomic DNA isolation kit. The average DNA yield of considered samples was found 204.57 ng/µl where the purity ranged from 1.82–1.99. Two (2) pair species-specific primers were used to amplify *Cyt b* gene fragments of 472 bp and 124 bp for cattle and buffalo, respectively. The PCR results revealed different species specific amplified fragments which could discriminate between cattle (472 bp) and buffalo (124 bp) species precisely from pure and mixed samples of those species. This study suggests an accurate molecular technique for identification of cattle and buffalo species meat origin and differentiates species present in adulterated meat samples. In conclusion, this DNA based technique could be utilized for prevention of malpractice in slaughterhouse and chain shops and thereby to protect consumer's right.

Keywords: beef; buffalo meat; genomic DNA; mitochondrial *Cyt b* gene; PCR

1. Introduction

Adulteration of food products has become a considerable problem in many countries as well as in Bangladesh by the fraudulent incorporation. Adulteration of meat may take the form of substitution of one cheaper meat with a costly expected species (Yman and Emanuelsson, 1998), for example buffalo meat (buffen) mixed with beef, which is a very old and common practice in Bangladesh. Beef is the most popular red meat in the world preferred for its taste, flavor and texture. Therefore, it is most expensive meat and consumers are ready to pay more prices for its delicacy. Whereas buffalo meat is relatively coarse and less popular compare to beef. Currently, there are about 1.46 million buffalo in Bangladesh (BER, 2015). Although a considerable number of buffalo population is available in Bangladesh and transportation of these species to the metropolitan cities is a common scenario of our country, but it is hard to find butcher shop or slaughterhouse in the market who sells buffalo meat and we found beef only. Under this circumstance, identifying the species origin of meat became a burning issue these days in Bangladesh.

Different DNA-based techniques used for animal species identification which include DNA hybridization of meat samples (Lenstra *et al.*, 2001), PCR and its variants (Matsunaga *et al.*, 1999a and b), PCR-RFLP (Girish *et al.*, 2005), polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) (Weder *et al.*, 2001) and PCR-sequencing (Bartlett and Davidson, 1992). The high copy number and sequence diversity favor the utilization of mitochondrial genes over nuclear genes for species identification purposes (Kocher *et al.*, 1989). DNA extracted from several mitochondrial genes including cytochrome b (*Cyt b*) (Verma and Singh,

2003), the 12S and 16S rRNA (Fajardo *et al*., 2006), and the displacement loop (D-loop) (Lopez *et al*., 1996) have been targeted for species identification. Since last two decades, mitochondrial *Cyt b* gene sequence analysis has been using for identifying animal species origin of closely related taxa (Satish *et al*., 2009; Zarringhabaie *et al*., 2011; Farag *et al*., 2015) and has been widely used for authentication of meat species in all kind of fresh and precooked products and the method is simple, cheap, efficient and highly specific (Zarringhabaie *et al*., 2011).

In Bangladesh, adulteration in food and food products has reached maximum height during the recent years where meat industry not untouched. PCR based techniques which have been widely used in developed worlds even in the neighboring countries to trace adulteration in slaughterhouse and supermarkets but molecular technique based detection is totally absent in Bangladesh. Moreover, so far we know, there is no published scientific report on authentication of meat animal species origin in Bangladesh using PCR based approach. Therefore, the present study was carried out to adopt PCR based technique in detection of cattle and buffalo species and to develop DNA based molecular marker from *Cyt b* gene fragment for identification of meat origin and detection of adulteration in mixed samples.

2. Materials and Methods

2.1. Sample collection and processing

A total of 42 ear tissue and meat samples of cattle and buffaloes were collected from different slaughterhouses of Bogra, Rangpur and Mymensingh District of Bangladesh. Collected samples were stored in 96% ethanol at room temperature. The samples of ear tissues for each species were separately prepared by cleaning of all hair roots and crushed with surgical blade and forceps. The tissue was cleaned off extraneous fat, connective tissue and blood vessels.

2.2. DNA extraction and quantification

Genomic DNA was extracted from all samples using GeNet Bio genomic DNA isolation kit (GeNet Bio, South Korea) according to the manufacturer's instructions. Extracted DNA was stored at -20^{0}C with appropriate labeling for further use. Concentration and purity of the extracted DNA was assessed by agarose gel electrophoresis and using NanoDrop spectrophotometer (Model ND2000).

2.3. Polymerase chain reaction (PCR)

The primer sequence information was selected from a published paper by Zarringhabaie *et al.* (2011) which is shown in Table 1. The *Cyt b* gene sequence information of indigenous cattle (472 bp) and buffalo (124 bp) were retrieved from NCBI gene bank data base having of accession number (*Bubalus bubalis*, AB529514.1 and *Bos taurus*, EF693798.1) and were aligned by CLUSTALW software (EMBL lab) to check the suitability of primers in indigenous cattle and buffalo species of Bangladesh.

PCR amplification was carried out in 20 µl reaction volume comprising 2 µl and 3 µl of genomic DNA in conventional and duplex PCR, respectively. The concentration of PCR ingredients in master mix contained 1x buffer (GeNet Bio Inc., South Korea), 1.5 mM $MgCl_2$, 0.2 mM dNTP (GeNet Bio Inc., South Korea), 1.0 µM of each primer (Integrated DNA Technologies, South Korea), and 0.3 U Taq DNA polymerase (GeNet Bio Inc., South Korea) for each reaction. For duplex PCR, master mix contained 1x buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 1.5 µM of each primer and 0.5 U Taq DNA polymerase used for each reaction. The gene amplification by duplex PCR was tested on DNA samples from each species as well as equally mixed DNA samples of two species. The thermal profile was comprised of initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 60 s and extension at 72°C for 45 s, a final extension at 72°C for 10 min and holding temperature at 12°C for an indefinite time. The PCR products were visualized by 2.0% agarose gel electrophoresis in 0.5x TBE buffer at 120 V for 30 minutes, which stained with 5 µl (10 µg/µl) of ethidium bromide (Bio NEER, South Korea) and images of gel were captured by UVsolo TS imaging system (Biometra, Germany).

3. Results and Discussion

3.1. DNA yield and purity

The average concentration of extracted DNA was found 204.57 ng/µl (Table 2) for all collected samples and purity of those samples were ranged from 1.82–1.99 (A260/A280). In cattle sample the average concentration of DNA obtained 189.31 ng/µl, where ear tissue samples averaged 208.73 ng/µl and meat samples averaged 97.08 ng/µl and the purity of cattle DNA samples was 1.82–1.94. In case of buffalo sample, the average yield of DNA was 223.04 ng/µl, where ear tissue samples possessed 272.86 ng/µl and meat samples contained 36.2 ng/µl and

the purity of buffalo DNA samples were 1.84–1.99. Ali *et al.* (2012) reported the average purity of DNA samples, using A260/A280 optical density, were 1.82–1.99 which is exactly similar to the current study. A ratio of ~1.8 is generally accepted as "pure" for DNA as "rules of thumb" where extracted protein and DNA absorb maximum light at 260 and 280 wavelengths, respectively. Mahajan *et al.* (2011) reported the DNA concentration ranged between 200–2600 µg/ml with the purity of 1.6 to 1.9 of cattle and buffalo meat using the method described by Cronin *et al.* (1991).It was reported that DNA extraction methodology must be resulted in DNA purities and concentrations between 1.6-2.0 and 25-1000 ng/µl, respectively (Di Pinto *et al.*, 2005). Farag *et al.* (2015) found the average yield of DNA of 90.77 ng/µl and the purity was 1.8 in average, from buffalo blood samples. These results also support the present findings. In general, the concentration of extracted DNA was relatively low from meat samples which indicated that the processing of meat tissues were not perfectly done and 20 µl Proteinase K is not sufficient for complete tissue lysis. Shahriar *et al*. (2011) also stated the same problem while using proteinase K for DNA extraction. The yield of extracted DNA also varies greatly on sample source, personal skills and DNA extraction methods. However, the amount of extracted DNA would be sufficient for PCR amplification and standardization of all samples were performed before going further amplification process.

3.2. *Cyt b* gene fragment amplification by conventional and duplex PCR for detection of species origin

In recent years, many investigators applied various molecular methods including PCR amplification (Tanabe *et al.*, 2007) to identify species in meat and adulteration in meat and meat products. In this study, the specificity of the primer sets selected was performed initially in a conventional (single) PCR reaction using two species-specific primer sets. Having confirmed species specificity of each primer independently, a duplex PCR was standardized by mixing two primer sets in a single reaction targeting DNA of two different species for detection of species meat origin using mitochondrial *Cyt b* gene fragments. The results of conventional PCR revealed different expected specific amplified PCR products sizes of 124 and 472 bp for *Cyt b* gene of buffalo and cattle species respectively (Figure 1). The duplex PCR was tested on DNA samples from each species as well as DNA mixture of the mixed two species and the results showed expected different specific amplified PCR products sizes of 124 and 472 bp for *Cyt b* gene of buffalo and cattle species, respectively (Figure 2) for pure and mixed samples of those species. The result of this study also shows that selected primers are highly conserved across the breed/population within a species and equally worked well in duplex manner using samples of indigenous cattle and buffalo. In duplex PCR two different fragments were observed in case of mixed sample which is exactly similar with conventional PCR amplicon. This method was sensitive and it was possible to trace each species meat when its portion in the mixture was less than 10% (Zarringhabaie *et al.*, 2011). The responsive size for each animal species from either tissue or meat was detected in this study to correspond to each animal species without cross-contamination. The identification of animal species from inter-species DNA mixed from tissue and meat in duplex PCR approach was adopted and performed efficiently. The result indicated that DNAs from all species were specifically detected (Figure 2).

Mitochondrial *Cyt b* gene (~1,143 bp) has been using as the most useful marker in recovering phylogenetic relationships and for identifying animal species origin among closely related taxa (Randi *et al.*, 2001; Satish *et al.*, 2009; Gupta *et al.*, 2012). 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-RFLP, PCR-SSR, real time PCR and FINS. All of those methods traced species origin and adulteration in meat and meat products efficiently. For example, Zarringhabaie *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. This finding conforms to the present investigation where similar amplicon sizes were observed. 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. Gupta *et al.* (2012) found 113 bp and 152 bp *Cyt b* gene fragment for cattle and buffalo species respectively. Jain *et al.* (2007) found species-specific DNA fragments of 157, 227, 274, 274, 331, 398 and 439 bp from goat, chicken, cattle, buffalo, sheep, pig and horse meats respectively, using multiplex PCR targeting *Cyt b* gene fragment. The amplified fragment sizes were different from the present study as they used different primer sets of *Cyt b* gene fragments. The approach of duplex (Gupta *et al.*, 2012) or multiplex-PCR (Zarringhabaie *et al.*, 2011) using mitochondrial *Cyt b* gene can be applied to detect authentication with equal efficacy to fresh, cooked or putrefied mixed samples of animal origin.

Table 1. List of primers used for conventional and duplex PCR.

Species	Type	Primer sequence (5'-3')	Reference
Cattle	Forward	TCCTTCCATTTATCATCATAGCAA	Zarringhabaie *et al.* (2011)
	Reverse	TGTCCTCCAATTCATGTGAGTGT	
Buffalo	Forward	TCCTCATTCTCATGCCCCTG	
	Reverse	TGTCCTCCAATTCATGTGAGTGT	

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

Species	Type of sample	DNA concentration (ng/µl)			Purity of extracted DNA		
		Min	Max	Average	Min	Max	Average
Total sample		18.2	913.6	204.57	1.82	1.99	1.87
Cattle	Ear tissue	69.0	596.1	208.73	1.82	1.89	1.86
	Meat sample	61.6	118.0	97.08	1.89	1.94	1.92
Buffalo	Ear tissue	116	913.6	272.86	1.84	1.92	1.87
	Meat sample	18.2	69.4	36.2	1.85	1.99	1.92

Legends: ng= nanogram; µl= microliter.

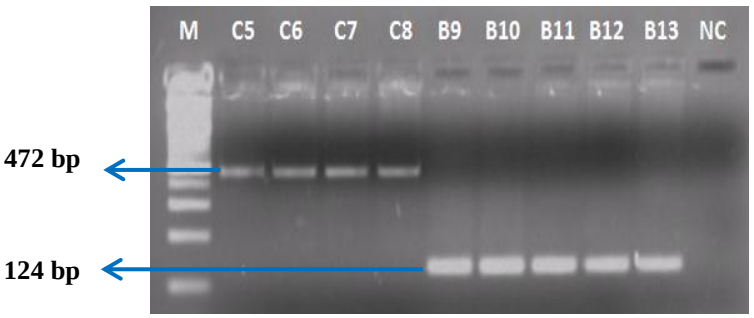

Figure 1. PCR products shown in 2.0% agarose gel. This figure represents, M= 100 bp DNA size marker, C5~C8 represent cattle samples and B10~B13=represent buffalo samples, NC=Negative control.

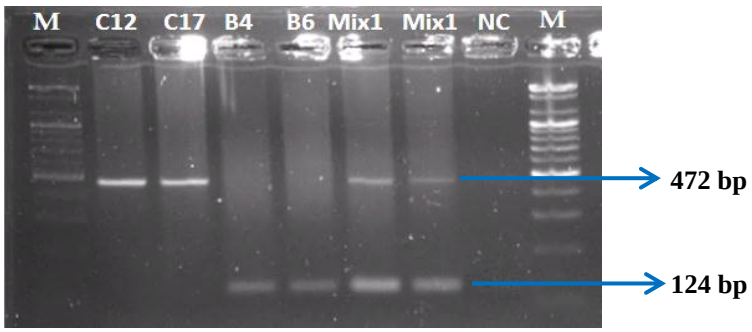

Figure 2. Duplex PCR products shown in 2.0% agarose gel. This figure represents, M= 100 bp DNA size marker, C12~C17=represent cattle samples and B4~B6=represent buffalo samples, Mix=Mixture of sample of cattle and buffalo with equal proportion, NC=negative control.

Buffalo, cattle, goat and sheep species also detected on mitochondrial *Cyt b* based on PCR-RFLP methods using restriction enzymes *Alu*I and *Hae*III for cleavage of amplified products resulting 2 fragments for buffalo and cattle species and 3 fragments for the species of sheep and goat (Ahmed *et al.*, 2005). Notably, the advantage of the present molecular methods is that there is no need to use restriction enzymes and RFLP methods to distinguish specific electrophoresis fragment on agarose gel. The optimized duplex PCR protocol enabled to discriminate meat species between cattle and buffalo. This protocol would be enough to detect adulteration in pure and mixed meat of those species. Moreover, the sensitivity, high specificity and reliability of the duplex PCR assay suggested this method could be a feasible and ideal for rapid analysis on industrial meat and meat products or in foodstuff.

4. Conclusions

Mitochondrial *Cyt b* gene could be used as excellent molecular marker for detecting species origin of meat as the conventional and duplex PCR revealed expected different specific amplified PCR products, sizes of 124 and 472 bp for *Cyt b* gene of buffalo and cattle species respectively, for pure and mixed sample of those species. This adopted highly specific and reliable technique has commercial impact that could be utilized as a laboratory tool for detecting species origin of meat and control fraudulent practices in slaughterhouse and chain shop.

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Conflict of interest

None to declare.

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