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A duplex PCR assay for sex determination of meat from cattle, buffalo, sheep and goat by amplification of the male specific *SRY* gene

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ABSTRACT

Sex determination of domestic animal's meat is of potential value in meat authentication and quality control studies. The objective of this study was to develop a simple and accurate PCR-based sex determination protocol, which can be applicable to cattle, buffalo, sheep and goat meat. Duplex PCR assay, including the SRY gene and the GAPDH house keeping gene, was optimized by using genomic DNA extracted from meat samples of known sex. It was possible to identify the sex of animals by amplifying both gender-specific (SRY) and autosomal (GAPDH) genes simultaneously in the duplex reaction, with the male yielding two bands and the female only one band. The protocol was subjected to a blind test showed 100% concordance, proving its accuracy and reliability.

Key words: Duplex PCR, GAPDH gene, Sex determination, SRY gene

Sex determination of domestic animal meat is of potential value in meat authentication and quality control studies. Male beef is designated to be of better quality than cow meat because cow meat is usually derived from animals at the end of their useful reproductive and productive life, and therefore male carcass yield higher prices particularly in European countries (Zeleny and Schimmel 2002). Also, cow (female cattle) slaughter is banned in many parts of India because of religious beliefs. To implement the regulations pertaining to such issues and to assure consumers of accurate labeling meat analysts need to have reliable, authentic and simple method for determining the sex of meat. To date, a range of different methodologies have been developed for determining the sex of meat, mainly based on detecting either hormone or DNA (Zeleny and Schimmel 2002). In general terms, hormone-based methods include immunochemical determination using ELISA (Simontacchi *et al*. 1999), and chromatographic techniques with mass-spectrometric detection, such as high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC–MS/MS) (Draisci *et al.* 2000) and gas chromatography-mass spectrometry (GC-MS) (Hartwig *et al*. 1997). Most of the methods proved to be highly specific and sensitive, but were not performed on a regular basis for meat sexing due to the technical limitations or the expensive equipments required.

Over the last few decades, DNA-based techniques, especially polymerase chain reaction (PCR)-based methods for meat sexing have received particular attention, and have proved to be reliable, sensitive, and fast (Tagliavini *et al*. 1993, Appa Rao *et al*. 1995). DNA regions that differ between male and female individuals are essential features in PCR sex determination. These methodologies were developed mainly based on amelogenin gene (*AMELX* and *AMELY*) (Ennis and Gallagher 1994, Chen *et al.* 1999, Fontanesi *et al.* 2008) and zinc finger gene (*ZFX* and *ZFY*) (Aasen and Medrano 1990, Kirkpatrick and Monson 1993, Zinovieva *et al*. 1995, Lockley *et al.* 1997). Alternatively, the bovine-specific repetitive sequence BRY-1 (Matthews and Reed 1992) and the single copy sequence BOV97 M (Miller and Koopman 1990) are also used in the analysis for the sexing of cattle. More recently, based on real time PCR, both SYBR Green (Ballin and Madsen 2007) and TaqMan (Parati *et al*. 2006) technology for bovine sex determination have been reported. Moreover, studies have demonstrated that *SRY* gene can also be used as a target DNA for embryos sexing by PCR amplification (Lu *et al.* 2007, Fu *et al*. 2007, Shi *et al.* 2008). The purpose of the present work was to develop a fast, reliable, and straightforward protocol for the sexing of cattle which is equally applicable to buffalo, sheep and goat meat also based on the male specific *SRY* gene using PCR technique.

MATERIALS AND METHODS

Sample selection and DNA extraction

Meat samples from both male and female cattle, buffalo,

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sheep and goat were collected. The collected samples were transported to the laboratory under refrigeration, and were stored frozen at –20 °C prior to analysis. We extracted the genomic DNA from tissue samples using commercial kit according to the manufacturer's instructions. Subsequently, the quality of genomic DNA was assessed by agarose gel electrophoresis using 0.8% agarose stained with ethidium bromide. The purity and concentration of DNA was estimated spectrophotometrically using Spectrophotometer at 260 and 280 nm wavelengths.

Design of PCR primers

Based on the information obtained from the alignment of the entire encoding region of cattle, buffalo, sheep and goat *SRY* gene sequences (GenBank Accession Nos. Z30327.1, EU016229.1, Z30265.1 and Z30646.1, respectively), we designed a pair of primers *SRY-FW* and *SRY-RV* targeting the high-motility-group (HMG) box of *SRY* gene (Fig. 1). This set of primers was potentially suitable for the amplification of a male-specific DNA fragment of 119-bp in length, providing the unambiguous determination of male and female meat from cattle, buffalo, sheep and goat. Also, another pair of primers *GAPDH-FW* and *GAPDH-RV* targeting the *GAPDH* gene was used in the present work. The amplicon was expected to yield a 332-bp fragment in both male and female meat, as a positive amplification control. Information on the four primers *(SRY-FW, SRY-RV, GAPDH-FW, and GAPDH-RV)* used in this study is shown in Table 1. The synthesized PCR primers were obtained commercial.

PCR amplification

Polymerase chain reaction (PCR) was performed in 25 µl of reaction mixture containing 50–100 ng of genomic DNA, 200 µm of each dNTP, 1.5 mM $MgCl_2$, 5 pmoles of each primer, 1 unit Flexi DNA polymerase, 1x PCR- colored buffer and nuclease free water to make a final volume. Amplification was performed on a PTC-200 DNA thermal cycler, using 0.2 ml reaction tubes. The PCR programme consisted of 5 min denaturation at 94°C, followed by 34 cycles of denaturation (94°C, 45 sec), annealing (55°C, 45 sec) and primer extension (72°C, 60 sec). The final cycle was followed by extension at 72°C for 10 min and indefinite hold time at 4°C.

Gel electrophoresis

The PCR product obtained was analyzed by agarose gel electrophoresis in 2% agarose gels stained with ethidium bromide. A 100 bp DNA ladder was electrophoresed simultaneously in order to assess the size of amplification product. The gels were visualized in automatic gel documentation system and the size of the amplicon was determined using software available with the gel documentation system.

RESULTS AND DISCUSSION

Duplex PCR was carried out in a single reaction set with the four primers SRY-FW, SRY-RV, GAPDH-FW and GAPDH-RV. The DNA extracted from the male meat samples generated two amplification fragments of 332 and 119-bp in

Bos 142 GAGAGCAGCCAGGACCACGTCAAGCGACCCATG**AACGCCTTCATTGTGTGGTCTC**GTG 199
Bub 142 GAGAGCAGCCAGGACCACATCAAGCGACCCATG**AACGCCTTCATTTTGGGGTCTC**GTG 199
Cap 175 GAGAGCAGCCAGAACCACGTCAAGCGACCCATG**AACGCCTTCATTGTGTGGTCTC**GTG 232
Ovi 175 GAGAGCAGCCAGGACCACGTCAAGCGACCCATG**AACGCCTTCATTGTGTGGTCTC**GTG 232

Bos 200 AACGAAGACGAAAGGTGGCTCTAGAGAATCCCAAAATGAAAAACTCAGACATCAGCAA 257
Bub 200 AACAAAAACAAAAGTTGGCTCTAGAGAATCCCAAAATGAAAAACTCAAAGATCAGCAA 257
Cap 233 AACGAAGACGAAAGGTGGCTCTAGAGAATCCCAAACTGCAAAACTCAGAGATCAGCAA 290
Ovi 233 AACGAAGACGAAAGGTGGCTCTAGAGAATCCCAAATTGCAAAACTCAGAGATCAGCAA 290

Bos 258 GCAGCTGGGATAT**GAGTGGAAAAGGCTTACAGATGC**TGAAAAGCGCCCATTCTTTGAG 315
Bub 258 GCAGCTGGGCTAT**GAGGGAAAAAGGCTTACAGATGC**TGAAAAGCGCCCATTCTTTGAG 315
Cap 291 GCAGCTGGGATAC**GAGTGGAAAAGGCTTACAGATGC**TGAAAAGCGCCCATTCTTTGAG 348
Ovi 291 GCAGCTGGGATAC**GAGTGGAAAAGGCTTACAGATGC**TGAAAAGCGCCCATTCTTTGAG 348

Fig. 1. Partial DNA sequences alignment of the encoding region of cattle (*Bos taurus* Z30327.1), buffalo (*Bubalus bubalis* EU016229.1), sheep (*Ovis aries* Z30265.1) and goat (*Capra hircus* Z30646.1). The primers SRY-FW and SRY-RV are indicated by bold letters.

Table 1. The information on the four primers used for sexing of meat

Primer name	Gene	Primer length (bp)	Sequence (5'- 3')	Primer combination	Amplicon size
SRY-FW	*SRY*	22	AACGCCTTCATTGTGTGGTCTC	SRY-FW, SRY-RV	119 bp (only in male)
SRY-RV	*SRY*	23	GCATCTGTAAGCCTTTTCCACTC		
GAPDH-FW	*GAPDH*	20	CGGGAAGCTCGTCATCAATG	GAPDH-FW,	332 bp (both male and female)
GAPDH-RV	*GAPDH*	22	CCGGGCTCTCACATTCCTAAGT	GAPDH-RV	

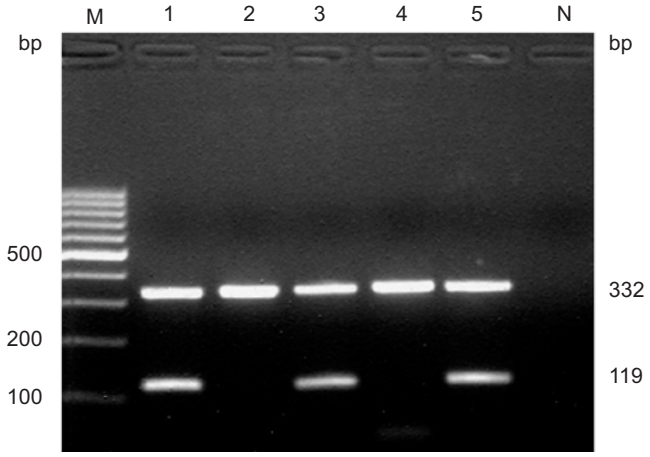

Fig. 2. Electrophoretic pattern of amplified fragmnts fromthe cattle meat DNA using SRY-FW, SRY-RV, GAPDH-FW and GAPDH-RV primers. Samples are: male (Lanes 1,3 and 5) and female (Lanes 2 and 4). Lane M, Marker of molecular weight, 100-bp ladder, N, Non-template control.

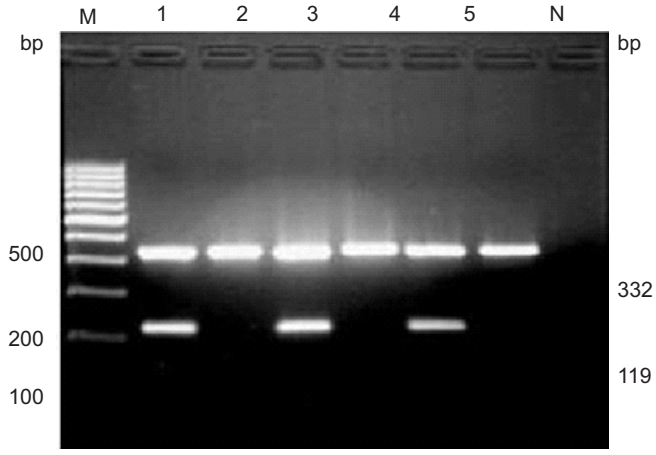

Fig.3. Electrophoretic pattern of amplified fragmnts fromthe cattle meat DNA using SRY-FW, SRY-RV, GAPDH-FW and GAPDH-RV primers. Samples are: Buffalo male (Lanes 1) Buffalo female (Lane-2), sheep male (Lanes-3), Sheep female (Lane-4), goat male (Lane-5) and goat female (Lane-6). Lane M, Marker of molecular weight, 100-bp ladder, N, Non-template control.

length, whereas the DNA extracted from the female meat samples generated only one amplification fragment of 332-bp (Fig. 2,3), suggesting that the amplicon of 332-bp is common to both male and female meat samples, and the amplicon of 119-bp is only in male. Therefore, the male-specific amplification fragment of 119-bp can be used to differentiate the meat from male and female, and the amplification fragment of 332-bp common to male and female may be used as a positive control for the sexing of meat in practice.

In mammals, the SRY gene located on the non-recombining region of the Y chromosome is the dominant gene in sex determination (Koopman *et al.* 1991) and consists of a single exon containing the HMG box. Studies have demonstrated that the HMG-box of the SRY gene is a highly conserved sequence among species, making it an ideal target for the development of DNA-based sex determination protocol (Lu *et al*. 2007). Furthermore, several methods were reported for the embryo sexing based on the HMG-box of SRY gene in different domestic animals, such as bovine (Lu *et al*. 2007), goat (Shi *et al.* 2008), pig (Sathasivam *et al*. 1995). Therefore, the HMG box of SRY gene was chosen as target sequence of PCR amplification to differentiate the sexing of cattle, buffalo, sheep and goat meat in the present work. More recently, based on HMG box of *SRY* gene, another method was reported for the sex identification in six bovid species, including yak (*Bos grunniens*), cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), sheep (*Ovis aries*) and mithun (*Bos frontalis*) using a duplex PCR technique (Prashant *et al*. 2008), where a fragment of 218-bp of GAPDH gene was co-amplified as positive control. The assay was accurate, reproducible and could be of potential use for sex determining of the bovid species, but the test was performed only on the genomic DNA isolated from blood samples.

At the present time, males were usually identified by amplifying a specific DNA sequence of Y chromosome in the gender determination of mammal meat. However, an absence of amplification of a fragment does not necessary mean that the sample analyzed is from female, because PCR itself is a quite sensitive technique, and under suboptimal condition it might give a negative amplification even in the presence of target template DNA (Kudo *et al*. 1993). In order to circumvent possible false negative amplification in the practical analysis of meat samples, one DNA fragment, which is present in both male and female, should be amplified in the same reaction to act as an endogenous control (Dervishi *et al.* 2008). As a rule, an autosomal sequence is always used to meet this purpose (Mara *et al.* 2004). In the present work, however, a 332-bp fragment of *GAPDH* gene, which is common to both male and female, was co-amplified with primer pair *GAPDH-FW* and *GAPDH-RV* designed in our study as an internal control (Yin *et al.* 2009).

A duplex PCR method with subsequent gel electrophoresis able to identify the sex of cattle, buffalo, sheep and goat meat was proposed by amplifying the target sequence of male-specific SRY gene. Two amplification fragments of 332-bp (common to both male and female) and 119-bp (only in male) can be detected simultaneously in a single reaction set without further analysis of requiring RFLP or sequencing. The method proved to be reliable, cheap and is potentially suitable for routine analyses.

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