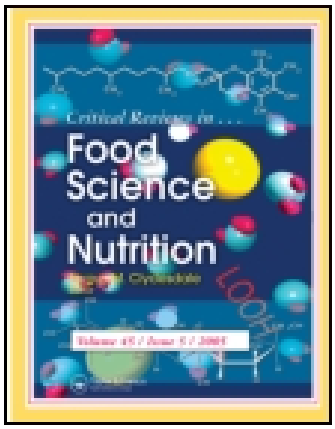


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# Determination of Sex Origin of Meat and Meat Products on the DNA Basis: A Review

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*Sex determination of domestic animal's meat is of potential value in meat authentication and quality control studies. Methods aiming at determining the sex origin of meat may be based either on the analysis of hormone or on the analysis of nucleic acids. At the present time, sex determination of meat and meat products based on hormone analysis employ gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS), and enzyme-linked immunosorbent assay (ELISA). Most of the hormone-based 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. On the other hand, the most common methodology to determine the sex of meat is unquestionably traditional polymerase chain reaction (PCR) that involves gel electrophoresis of DNA amplicons. This review is intended to provide an overview of the DNA-based methods for sex determination of meat and meat products.*

**Keywords** Sex determination, authentication, meat, meat products, PCR, DNA analysis

## *INTRODUCTION*

Sex has been important criterion which helps to establish the commercial value and classification of adult bovine carcasses particularly in western countries. All other quality factors being equal, sex is also an important factor for market value of beef. For example, meat from male castrated animals is usually more valuable than meat from cows of the same age (Tagliavini et al., 1993). Beef from male animals is commercially more valuable than beef from female animals because females are used for reproduction and are normally slaughtered only when aged, when the length and rigidity of muscle fibers is increased and the level of collagen is higher resulting in tougher meat of lower quality and thus lower commercial value. Many meat packing plants sell cow's meat as male meat, causing damage to various meat producing sectors and to consumers (Curi et al., 2002). Male beef is designated to be of higher quality than cow or heifer meat (Price, 1995) and therefore yields higher prices. Also, the European meat industry tends to raise entire male pigs and slaughter them before they are sexual matured to prevent boar taint in the meat (Lundstrom et al., 2009); this is also practiced in the United Kingdom and Ireland. Unfortunately, this procedure is not a guarantee to keep boar tainted meat from the market since other parameters like rearing systems, breed, and feeding can influence the development of boar taint (Thun et al., 2006). Hence, many meat processors wish to receive only meat from female pigs to guarantee the origin of specific meat products demanded by wholesalers, retailers, and consumers. To develop quality management systems, a highly sensitive, fast, and reliable PCR-based method is necessary to differentiate male and female pork tissue in meat and meat products, rather than to detect boar taint directly. Besides this, sex determination of cattle meat has been always of public interest in country like India where slaughter of cow (female cattle) is banned because of religious beliefs and laws thereby. To avoid unfair competition and to assure consumers of accurate labeling, it is necessary to have reliable methods for determining the gender of the meat.

To date, a range of different methodologies have been developed for determining the gender of meat, mainly based on detecting either hormone or DNA. Analysis of sex-specific hormones

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is a conventional method used to determine the sex of meat. The level of sex-specific hormones varies not only among individuals, but also within a given individual (Zeleny and Schimmel, 2002). It is therefore challenging to establish sex determination methods based on hormone analysis alone. However, gas chromatography-mass spectrometry (GC-MS) (Hartwig et al., 1997), high-performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) (Draisci et al., 2000), and enzyme-linked immunosorbent assay (ELISA) (Simontacchi et al., 1999) have been found effective to measure bovine sex hormones and thereby determine the sex. 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.

## *DNA-BASED METHODS*

Over the last few decades, DNA-based techniques, especially polymerase chain reaction (PCR) that involves gel electrophoresis of DNA amplicons for mammal 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. DNA-based molecular techniques developed over the last two decades have raised the hopes of developing authentic and reliable methods for sex determination, due to its presence as basic cellular component, the stability of DNA at high temperatures, and the fact that its structure is conserved within all tissues of an individual.

## *HYBRIDIZATION ASSAY*

Hybridization techniques which lack a PCR step to amplify template DNA, but use PCR-generated probes for specifically detecting the isolated template DNA, were originally developed for species identification in meat (Buntjer et al., 1995; Janssen et al., 1998), but can easily be adapted to meat sexing using the respective primers. Briefly, DNA is either isolated or purified by proteinase K treatment followed by binding and elution from a DNA binding resin or simply by extracting DNA in 0.5 M NaOH, and spotting the aliquot of DNA on a positively charged nylon membrane. Hybridization is performed by means of PCR-generated probes (DIG-labeled), specific for either a bovine satellite DNA and/or bovine male-specific BRY-1 repeat (Schwerin et al., 1992; Appa Rao et al., 1995). Detection of the probe is accomplished by means of an anti-DIG-IgG conjugate and Fast-Violet B as the coloring agent. Down to about 10 ng of template, DNA can be detected using this approach. However, DNA hybridization is less sensitive compared to PCR, and prone for sample cross contamination. DNA hybridization requires relatively inexpensive equipment but it is laborious and time consuming. DNA testing by hybridization is relatively cumbersome and so other DNA-based approaches for sex determination have been evolved using PCR.

## *POLYMERASE CHAIN REACTION (PCR)*

The PCR is an in vitro enzymatic method which allows several million fold amplification of a specific DNA sequence within few hours. This technique was invented by Mullis et al. (1986) and is not only useful for molecular biologist and geneticist but also useful for forensic experts and food technologists. Now days, PCR assay is gaining immense popularity in determination of sex origin of meat. This is a sensitive, cost effective, precise, authentic, and potentially applicable technique for authentication of meat and meat products due to its lesser complexity and fast reliable nature. The PCR assays are also proving useful in solving the issues of traceability of live animals and derived products (Cunningham and Meghen, 2001). The ingredients required for successful PCR amplification are template DNA, pair of forward and reverse oligonucleotide primers, all four deoxynucleotide triphosphates, a thermostable DNA polymerase enzyme, and reaction buffer. The PCR techniques involve denaturation, primer annealing, and elongation steps for a set number of times depending on the degree of amplification required. This generates billions of copies of desired DNA segment from picograms quantities of starting DNA in matter of few hours. However, the other parameters are also important for successful amplification of desired PCR products, such as DNA quality, primer concentration, $Mg^{++}$ concentration, annealing temperature, and final extension periods (Macpherson et al., 1993; Meunier and Grimont, 1993).

To date, different PCR-based methods, most of which have been developed for embryo sexing applications, are routinely applied. These includes methods amplifying Microsatellite, Y-chromosome-specific sequences, the zinc finger protein gene (*ZFX/ZFY*), Y-encoded, testis-specific protein (*TSPY*), sex determining region of the Y chromosome (*SRY*), the amelogenin gene (*AMELX/AMELY*), or DEAD box polypeptide 3 gene (*DDX3X/DDX3Y*).

### *Microsatellite and Y-chromosome-specific Sequences*

Microsatellite is a polymorphic DNA sequence that can be used as a molecular marker (O'Brien et al., 1993). It is a repeating nucleotide marker, also known as short-tandem repeats. The repeated sequence of microsatellite is usually less than 100 bp. The polymorphic state of microsatellite is determined by two repeated nucleotide of CA or GT which represent 0.5% of the genome (Jeffreys et al., 1985). Peura et al. (1991) developed and reported an extremely accurate PCR-based assay for sex determination in cattle embryos based on amplification of the bovine satellite and Y-chromosome-specific sequences. The bovine satellite-specific primer recognizes sequences that are amplified both in males and females, while the bovine Y-chromosome-specific primer pairs amplified and the Y-chromosome-specific sequence in males only. Similar findings were reported by Appa Rao et al. (1993) in buffalo (*Bubalus bubalis*) embryos and Appa Rao et al. (1994) in Indian zebu and crossbred cattle embryos.

Miller and Koopman (1990), Schroder et al. (1991), and Agrawala et al. (1992) developed duplex PCR using primers for BOV97M, a bovine Y-specific DNA fragment, and for actin, as the internal control. Subsequent agarose gel electrophoresis exhibits bands of 97 bp only in male and 220 bp in both the sexes, respectively. Machaty et al. (1993) also developed duplex PCR using primers for OY11.1, a bovine Y-specific repeated sequence, and the bovine-specific satellite DNA sequence 1.715 serving as internal control. Subsequent agarose gel electrophoresis exhibits bands of 173 bp only in male and 216 bp in both the sexes, respectively. Tagliavini et al. (1993) developed a simple procedure that allows unambiguous establishment of the sex origination of samples of either fresh or frozen bovine meat, based on the amplification by PCR of bovine Y-chromosome-specific sequences. Two sets of previously described oligo nucleotide primers [BRY.1 (Matthews, 1990) was derived from single copy sequence and BOV97M (Miller, 1990) was derived from repetitive sequence] were amplified separately. All male meat samples showed one band (either BRY.1—307 bp or BOV97M—97 bp as per primers used), whereas no band was observed in female samples.

Appa Rao et al. (1995) developed a simple and reliable method for accurate identification of male and female raw meats in cattle, buffalo, sheep, and goat using the PCR technique. A set of bovine Y-specific primers (Peura et al., 1991) were used in his study. Upon PCR amplification, male samples yielded one 300 bp band, whereas no band was observed in female samples. Bredbacka et al. (1995) designed primers to amplify the highly repetitive (approximately 60,000 copies) btDYZ-1 region of the bovine Y-chromosome for sexing of bovine embryos and also reported that the accuracy of sex determination using PCR was 100%. Park et al. (2001) developed a method for rapid sexing of preimplantation bovine embryo using consecutive and multiplex PCR. The BOV97M and bovine 1.715 satellite DNA sequences were selected for amplification of male- and bovine-specific DNA, respectively. In consecutive and multiplex PCR, the first 10 PCR cycles were done with male-specific primer followed by an additional 23 cycles with bovine-specific primer. Upon PCR amplification, a male-specific band of the BOV97M (141 bp) in males and a common band of bovine 1.715 satellite DNA (216 bp) in both males and females was observed.

Weikard et al. (2001) developed a PCR-based method for sex determination of bovine DNA samples and embryo biopsies, using only one primer pair that amplify both the male-specific sequence FBNY (127 bp) and the sex independent control, microsatellite marker FBN17 (136–140 bp). Synteny mapping assigned the male-specific sequence to bovine chromosome Y (BTAY), whereas FBN17 was mapped to bovine chromosome 2. Localization of FBNY on BTAY was confirmed by fluorescence in hybridization of two BAC clones containing the male-specific sequence. There was no amplification of the male-specific target sequence FBNY in sheep, pig, goat, mice, man, and several wild species of the tribe Bovine. The bovine male-specific fragment was detected in dilutions containing as little as 10 pg genomic DNA and in blastomeres from embryo biopsies. This assay does require neither restriction endonuclease digestion of the PCR product nor additional-nested PCR steps. Owing to the advantage of parallel amplification of the autosomal locus FBN17, no additional control fragment is necessary to detect PCR failure.

Curi et al. (2002) developed a PCR-based sex determination in bovine carcass by amplification of male-specific BRY.1 region and sexually neutral GH region. Upon PCR amplification, a male-specific band of the BRY.1 region (307 bp) in males and a common band of sexually neutral GH region (223 bp) in both males and females was observed. Mahanem et al. (2006) developed a PCR-based sex determination in Sahiwal-Friesian crossbred cattle based on amplification of DYS6, (Y-specific microsatellite loci located at Y-chromosome) using sperm and blood samples. Upon PCR amplification, male samples yielded one 130 bp band, whereas no band was observed in female samples.

### *Zinc Finger X-linked/Y-linked (ZFX/ZFY) Gene*

Zinc finger gene is present in both X- and Y-chromosome (Page et al., 1987; Schneider-Gadicke et al., 1989). Aasen and Medrano (1990) described a quick and efficient method of determining the sex of DNA samples from humans, cattle, sheep, and goats. Using universal primers, 447/445 bp fragments from male or female genomic DNA corresponding to the *ZFX/ZFY* genes were amplified. Restriction fragment length polymorphism (RFLP) analysis of the fragments yielded specific banding patterns between the two sexes in these species. Pollevick et al. (1992) designed nested primers for sex determination of bovine embryos by RFLPs of PCR-amplified ZFX/ZFY loci. Schmoll and Schellander (1996) reported a method for sex determination of porcine embryos by RFLP of PCR-amplified ZFX/ZFY loci. Senese et al. (1999) documented the detectable dimorphic patterns in horses in the *ZFX* and *ZFY* genes using HaeIII PCR–RFLP and subsequent DNA sequencing. Ortega et al. (2004) described a reliable, noninvasive method for sex determination in the endangered San Joaquin kit fox (*Vulpes macrotis mutica)* and other canids by amplification of zinc finger (*ZFX* and *ZFY*) protein genes. He designed a primer set that amplifies a short (195 bp) fragment of the zinc finger (*ZFX* and *ZFY*) protein genes. This fragment contains a *Taql* digestion site unique to the *ZFY* gene, and successful PCR products showed a clear single band for females and a double band for males in 3% agarose gels. As RFLP analysis requires an additional restriction enzyme digestion step of the PCR product, thus increasing the risks in contamination and misdiagnosis.

Kirkpatrick and Monson (1993) developed a rapid, sensitive method for sex determination of bovine embryos which uses nested, allele-specific amplification of bovine *ZFX* and *ZFY* genes. Upon PCR amplification, male samples yielded both X- and Y-specific bands (247 bp, 167 bp) and the female samples yielded only one X-specific band (247 bp). Similarly, Zinovieva et al. (1995) also reported a rapid, sensitive method for sex

determination of bovine blastomeres using allele-specific amplification of bovine *ZFX* and *ZFY* genes in capillary PCR. Here, upon PCR amplification, male samples yielded both X-, Y-specific bands (282 bp, 132 bp) and the female samples only one X-specific band (282 bp). It was also reported that the total time required for the sex determination can be reduced to less than two hours by using capillary PCR.

Vidya et al. (2003) demonstrated that molecular sexing from dung-extracted DNA, based on ZFX–ZFY fragment amplification and ZFY-specific *Bam*HI site restriction can be applied to estimate sex ratios of free ranging Asian elephants (*Elephas maximus*) populations in southern India. Qiao et al. (2007) described a rapid sex identification method for the forest musk deer (*Moschus berezovskii*) using PCR based on *ZFX/ZFY* located on the X- and Y-chromosomes. Upon PCR amplification, both male and female animals showed amplification bands of 447 bp and 212 bp separated in agarose gel. A sex-specific 278 bp band was amplified only from males. Durnin et al. (2007) developed a simple and reliable PCR-based sexing method aimed at degraded, low yield DNA extractions from the giant panda (*Ailuropoda melanoleuca*) by amplification of *ZFX/ZFY* gene. Comparisons of this new primer set with others showed that the reliability of sex determination from low yield, degraded DNA extractions was improved if; amplification products were short (<170 bp) and the Y-chromosome amplification product was shorter than the X-chromosome amplification product.

Wei et al. (2008) developed an inexpensive, time saving, and reliable method using PCR with confronting two-pair primers (PCR-CTPP), for sex identification in tiger (*Panthera tigris*) based on zinc finger alleles (ZFX/ZFY). Zeng et al. (2009) developed a triple-primer PCR method for sexing endangered caprine species based on the last introns of the ZFX/Y alleles. Upon PCR amplification, the male samples generated a ∼230 bp ZFX-specific fragment and a ∼140 bp ZFY-specific fragment, and the female samples only generated the ∼230 bp fragment. Fontanesi et al. (2010) reported a new molecular sexing method for three leporid species (*Oryctolagus cuniculus*, *Lepus europaeus* and *Lepus timidus*) based on the analysis by PCR–RFLP of point mutations that differentiate the *ZFX* and *ZFY* gene sequences.

Han et al. (2010) developed a method for sex determination of equine by simultaneous amplification of the intron 9 flanking region of the *ZFX–ZFY* and *SRY* genes. The amplified PCR product for *ZFX–ZFY* genes showed two distinct band patterns. Those from some foals had heteroduplex bands (553 and 604 bp), while those from mares had a single 604 bp band, respectively. The band that foals shared with mares is likely that amplified from the X-chromosome, while the foal-specific band could be amplified from the Y-chromosome and also reported that foals had an SRY band, while the mares did not. Peppin et al. (2010) developed a PCR-based method for sex identification of African Rhinoceros (*Ceratotherium simum* and *Diceros bicornis*) that could easily be incorporated into fluorescent short-tandem repeat (STR) profiling. A single primer pair, consisting of a fluorescently labeled forward primer and an unlabeled reverse primer, is used to co-amplify homologous fragments of a ZF protein intron which exhibits size polymorphism between the X- and Y-chromosomes. In both species, the amplified ZFX and ZFY amplicons differ in size by 7 bp and can thus be differentiated by capillary electrophoresis. Blood, tissue, horn, and fecal samples were correctly sexed using this method. Cross species testing also demonstrated that this method could be used to sex Indian rhinoceros (*Rhinoceros unicornis*) samples.

### *Testis-specific Protein, Y-encoded (TSPY) Gene*

The Y-encoded, *TSPY* (Arnemann et al., 1991) is a Y-specific gene (Affara et al., 1996). TSPY homolog exists in several mammalian species, including humans, horses, and cattle (Jakubiczka et al., 1993; Schempp et al., 1995; Vogel et al., 1997). The *TSPY* genes are arranged in clusters on the Y-chromosome of many mammalian species and form part of a super family, *TTSN* (*TSPY–TSPYL–SET–NAP1L1* genes), with highly conserved autosomal representatives (Vogel and Schmidtke, 1998). In humans and cattle, TSPY expression is apparently restricted to male germ cells and their precursors, and begins during fetal development. The cellular site of expression suggests a function in spermatogonial proliferation (Vogel et al., 1997). Bovine TSPY consists of 7 exons that are separated by 6 introns. The genomic organization of the bovine and human genes is highly conserved and both are part of a Y-specific gene family (Vogel et al., 1997). The copy numbers range from 20 to 60 in men and up to 200 in bulls (Manz et al., 1998).

Lemos et al. (2005) designed the primers for sex determination in cattle based on PCR amplification of *TSPY* gene. Upon PCR amplification, the male animals showed a single 260 bp band, whereas in female animals no band was observed. Also he reported that this assay is highly specific, and positive results can be obtained at low DNA concentrations (less than 1 pg/$\mu$L). These results showed that TSPY is a good male-specific marker, the usefulness of which gets enhanced by the high copy number of the gene in cattle. Manz et al. (1998) demonstrated the potential usefulness of TSPY for sex diagnosis in equine preimplantation. Pierce et al. (2000) described a highly accurate method for determining the sex of human embryos by the real-time PCR of *TSPY* gene. Tan et al. (2010) reported *TSPY* gene as a genetic marker for sex determination of cattle spermatozoa.

### *Sex Determination Region of the Y-chromosome (SRY) Gene*

The *SRY* gene also known as the testis determining factor (Sultan et al., 1991) was discovered by analysis of the small fragments of the Y-chromosome that had translocated to the X-chromosome in the genomes of XX males and true hermaphrodites (Sinclair et al., 1990). *SRY* being the gene which controls differentiation into the male (Goodfellow and Lovell-Badge, 1993) and the studies in humans and mice proved that

SRY was indeed the long-sought after *TDF* gene (Berta et al., 1990; Gubbay et al., 1990; Koopman et al., 1990). The protein encoded by SRY showed sequence-specific DNA binding activity, which was absent or reduced in SRY from certain XY females with gonadal dysgenesis (Nasrin et al., 1991; Harley et al., 1992; Tohonen et al., 2005). The demonstration that XX mice transgenic for *SRY* gene develop as males with normal testis and testicular cords indicated that *SRY* gene was the only Y-encoded gene necessary for testes formation and the consequent male phenotype (Koopman et al., 1991, 2001; Parma et al., 2004; Takada et al., 2006).

The open reading frame (ORF) of human *SRY* gene is contained within a single exon and encodes a 204-amino-acid protein. The central 79 amino acids encode the high motility group (HMG) box, which functions as a DNA-bending domain and also contains two nuclear localization signals. Surprisingly, comparison of mammalian SRY sequences and amino acid sequences reveals that sequence conservation is largely confined to the HMG box, suggesting its rapid evolution (Wright and Dixon, 1988; Kageyama et al., 1992; Payen and Cotinot, 1993; Tucker and Lundrigan, 1993; Whitfield et al., 1993). However, there is no sequence conservation outside the HMG box. The HMG box of the *SRY* gene is a conserved motif for minor groove DNA recognition, which has the ability to recognize the DNA binding motif A/TAACAAT/A with highest affinity (Harley et al., 1994; Katoh, 2002; Kelly et al., 2003). These characteristics of the SRY-HMG box region make it an ideal target for development of DNA-based sex determination tests. Mutations of the *SRY* gene that gives rise to male-to-female sex reversal almost always occur in the HMG box (Hawkins et al., 1992; McElreavy et al., 1992).

Utsumi and Iritani (1993), Zeng et al. (1994) applied *SRY* gene-specific primers for sexing of bovine embryos. Upon PCR amplification, agarose gel electrophoresis reveals a 300 bp band for males, whereas no band was observed in female samples. Taberlet et al. (1993) developed a method for sexing free ranging brown bears *Ursus arctos* using hairs found in the field. The two-step PCR amplification of *SRY* and mitochondrial gene as control, a male-specific band of the *SRY* (92 bp) in males, and a common band of mitochondrial gene (175 bp) was observed in both males and females. The sex can reliably be identified using about 50 pg of DNA extract as template by using this method. Sathasivam et al. (1995) developed a duplex PCR-based method for the sexing of pig embryos by amplifying HMG box of *SRY* gene and porcine Home box gene. Upon PCR amplification, a male-specific band of the SRY (146 bp) in males and a common band of porcine Home box gene (246 bp) observed in both males and females.

Pomp et al. (1995) developed a duplex PCR based sex determination in mammals like pigs, cattle, sheep, goats, llamas, horses, humans, Baboons, dogs, cats, rats, and mice by amplification of the HMG box of *SRY* gene and *ZFX*/*ZFY* (Aasen and Medrano, 1990) gene primers. The presence or absence of a region of the *SRY* (sex determining region Y) gene determines sex, and amplification of the *ZFY* (male) or *ZFX* (female) genes acts as a positive control for PCR. Upon PCR amplification, a male-specific band of the SRY (163 bp) in males and a common band of *ZFX*/*ZFY* gene (455 bp) was observed in both males and females. Meer and Eddinger (1996) developed *SRY* gene primers for detection of male tissue in hot-boned pork and fresh, cooked sausage products using PCR. Upon PCR amplification, male tissue yielded one band (158 bp). Male tissue can be detected down to at least 1:10,000 parts of sow tissue. There was no effect of the addition of spices to the sample (fresh bratwurst) or cooking (50, 70, and 100°C for 15 minutes) on the efficacy of the assay.

Ng et al. (1996) developed a rapid, reliable method for the sexing of the domestic sheep (*Ovis aries*) by amplification of Y-chromosome-specific sequences in male genomic DNA using the PCR. Oligo nucleotide primers were selected from a conserved sequence, the HMG box, in the sequence of ovine SRY, permitting amplification of a defined 161 bp fragment only from male-specific genomic DNA. As a control, micro satellite primers also were used in PCR reactions, recognizing a sequence that is amplifiable in genomic DNA from both males and females. Gutierrezl et al. (1996) developed a nested PCR amplification of the HMG box of *SRY* for sex determination across a range of mammals like mice, rats, horses, sheep, goats, pigs, cows, llamas, rabbits, humans, macaques, dogs, and cats. All male samples showed 162 bp bands, whereas no band was observed in female samples. Bryja and Konecny (2003) developed a duplex PCR-based sex determination in wild mammals by amplification of the HMG box of SRY (Sanchez et al., 1996) and *ZFX*/*ZFY* (Aasen and Medrano, 1990) gene primers. Upon PCR amplification, a male-specific band of the SRY (202 bp) in males and a common band of ZFX/ZFY (445/447 bp) was observed in both males and females.

Jayasankar et al. (2008) developed a duplex PCR-based sex determination in skin samples of cetaceans and dugong from the Indian seas by amplification of the *SRY* and *ZFX*/*ZFY* (Aasen and Medrano, 1990) gene primers. Upon PCR amplification, a male-specific band of the SRY (210–224 bp) in males and a common band of ZFX/ZFY (442–445 bp) was observed in both males and females. The techniques, based on the PCR amplification of the SRY and the sex-specific regions of the zinc finger sex chromosome-linked *ZFX* and *ZFY* genes, have proved to be fast, cheap, and reliable for sex determination (Palsboll et al., 1992; Berube and Palsboll, 1996; Rosel, 2003; Cunha and Solecava, 2007). Moreover, PCR techniques work well with degraded DNA, which is typical of samples from stranded carcasses, a major source of cetacean samples.

Lindsay and Belant (2008) developed a multiplex PCR-based sex determination in white-tailed deer (*Odocoileus virginianus*) by amplification of the *SRY* and *ZFX*/*ZFY* genes. Upon PCR amplification with subsequent gel electrophoresis, females showed a single band and males showed three bands. Amplifications with the SRY primers alone showed no amplification products from female samples but obvious products from the male samples (∼350 bp in length). Amplifications from the zinc finger primers alone showed dimorphic results for male and female

samples. Both sexes showed a single band representing a product ~220 bp in length and males showed an additional band representing a product ~410 bp in length. Mara et al. (2004) developed a fast and easy duplex PCR method, for sex determination of ovine in vitro-produced embryos by amplifying *SRY* gene and bovine 1.715 satellite DNA (SAT). The PCR utilized two different sets of primers: the first pair recognized a bovine Y-chromosome-specific sequence (SRY) that showed 100% homology with the corresponding sequence of the ovine Y-chromosome and was amplified in males only. The second pair recognized the bovine 1.715 satellite DNA (SAT) which was amplified in all ovine samples.

Phua et al. (2003) reported a PCR-based sex determination method for possible application in caprine gender selection by simultaneous amplification of the *SRY* and *AMELX* genes. He designed the two sets of primer to amplify a portion of AMELX on the X-chromosome to give a 300 bp product and *SRY* gene on the Y-chromosome to give a 116 bp product. In duplex reaction, it was possible to identify the sex by amplifying both male- and female-specific genes simultaneously with males yielding two bands and females yielding one band. The AMELX primer set, which served as an internal control primer, did not interfere with amplification of the Y-specific sequence even a low amount of DNA was used. In a similar report Hasegawa et al. (2000) documented a method for sex determination of horses by simultaneous amplification of equine *SRY* and amelogenin genes. Stallions have a Y-specific SRY band and AMELX–AMELY heteroduplex bands, while mares only have homoduplex AMELX. Fiore (2005) developed a multiplex PCR-based sex determination in nonhuman primates by simultaneous amplification of the *SRY* and *AMELX* genes. Upon PCR amplification, the female animals showed a single 200 bp band (*AMELX*), whereas in male animals an additional band of 165 bp (SRY) was observed.

Lu et al. (2007) developed a fast and reliable method for bovine sexing based on amplification of the bovine HMG box of the *SRY*. Upon PCR amplification, a normal bull showed 1 SRY band (237 bp), and a normal cow showed no SRY band. This method differs from that of Thomsen and Poulsen (1993), working with pigs, in that here the target sequences of the PCR primers are within the HMG box of the sex-determining gene itself, rather than in DNA from the heterochromatic long arm of the Y-chromosome. This method also differs from that of Sathasivam et al. (1995), working with domestic pigs, which did not result in a good rate of prediction of sex. Fu et al. (2007) developed an accurate and reliable method for sexing swamp buffalo (*Bubalus bubalis*) embryos. In this method, two pairs of nested primers are targeted to amplify the *SRY* conserved region and *G3PDH* gene (to serve as an internal control).

Prashant et al. (2008) developed a simple and accurate PCR-based sex determination protocol, which can be applicable to six major domesticated species of the family Bovidae, *viz. Bos frontalis*, *B. grunniens*, *B. indicus*, *Bubalus bubalis*, *Capra hircus*, and *Ovis aries*. In silico, analysis was done to identify conserved DNA sequence in the HMG box region of the sex-determining region of the Y-chromosome (*SRY* gene) across the Bovids. He reported the possibility 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 (SRY-122 bp, GAPDH-218 bp) and the female only one band (GAPDH-218 bp). *GAPDH* gene primers serve as a PCR-positive control, and did not interfere with amplification of the Y-specific target sequence. Similarly Shi et al. (2008) developed a duplex PCR-based method for the sexing of goat by amplifying HMG box of *SRY* gene and Beta-actin. Upon PCR amplification, a male-specific band of the SRY (162 bp) in males and a common band of Beta actin (298 bp) was observed in both males and females. Beta-actin gene primers serve as a PCR-positive control, did not interfere with amplification of the Y-specific target sequence.

Choi et al. (2009) developed a duplex PCR-based sex determination in pig by amplification of conserved porcine SRY HMG box sequence motif. He reported that *SRY* gene primer designed in his study was 1.46 times more specific than previously reported primers. Bai et al. (2010) developed a multiplex PCR-based method for the sexing of yak meat by amplifying the target sequences of male-specific *SRY* gene and *12S rRNA*. Upon PCR amplification with subsequent gel electrophoresis, two amplification fragments of 290 bp (common to both male and female yak) and 121 bp (male specific in yak) detected simultaneously in a single reaction set without further analysis of requiring RFLP or sequencing. The method proved to be reliable, cheap, and applicable to raw and heat-treated yak meat samples.

Currently, the focus seems to have shifted from traditional PCR toward the real-time PCR. In the real-time PCR, TaqMan® & SYBR® Green technology are mostly used. TaqMan® technology utilizes in addition to the two primers a TaqMan® hybridization probe. TaqMan® probes are designed to bind between the forward and reverse primer, and contain a fluorophore attached to the 5′-end and a quencher attached to the 3′-end. During PCR, 5′-exonuclease activity of the Taq polymerase degrades the probe and the close proximity between the fluorophore and the quencher no longer exists, which allows fluorescence and subsequently detection. SYBR® Green is a dye that binds to the minor groove of double-stranded DNA, SYBR® Green is highly fluorescent, but unbound is not. The intensity of fluorescence is proportional to the amount of double-stranded DNA. One advantage of SYBR® Green is that a melting curve can be generated after PCR, and single mutations (Lopez-Andreo et al., 2006) and deletions (Ballin and Madsen, 2007) can be detected.

Ballin and Madsen (2007) developed the real-time PCR-based SYBR® Green technology combined with the melting curve analysis of amplified AMELX and AMELY genes for sex determination in beef samples. The PCR amplicons of 130/130 and 130/67 base pairs produced from female and male beef, respectively, are easily distinguished by both melting curve analysis and gel electrophoresis. Results from the melting curve analysis of amplicons were ready in less than three minutes, and requires no additional work in addition to the PCR setup. Chang et al. (2008) developed the real-time PCR-based SYBR® Green technology combined with the melting curve analysis that

amplifies genes encoding chromodomain-helicase-DNA binding protein (CHD) for avian sexing. Parati et al. (2006) developed the real-time PCR-based TaqMan® technology directed toward the specific Y-chromosomal SRY and the X-chromosomal proteolipid protein gene for sex determination in cattle.

Gokulakrishnan et al. (2012c) developed a duplex PCR-based sex determination protocol which can be applicable to beef, carabeef, mutton, and chevon by simultaneous amplification of the *SRY* and autosomal (*GAPDH*) genes in a single reaction. Upon PCR amplification, a male-specific band of the SRY (119 bp) in males and a common band of GAPDH (332 bp) was observed in both males and females. Gokulakrishnan et al. (2012a) reported a highly reliable and fast PCR-based sex determination method for cattle meat by simultaneous amplification of the *SRY*, *AMELX* and *AMELY* genes. Upon PCR amplification with subsequent gel electrophoresis, females showed a single band and males showed three bands.

### *Amelogenin X-linked/Y-linked (AMELX/AMELY) Gene*

The amelogenin (*AMEL*) gene encodes an important protein in the developing mammalian tooth enamel which belongs to the family of extracellular matrix proteins (Akane et al., 1992; Mannucci et al., 1994; Sasaki and Shimokawa, 1995; Fincham et al., 1999; Gibson, 1999; Hoang et al., 2002), and has been conserved during the evolution of vertebrates (Lyngstadaas et al., 1990). The amelogenin gene was localized on both sex chromosomes in cattle (Gibson et al., 1992), humans (Nakahori et al., 1991; Levinson et al., 1992; Gibbon et al., 2009), sheep, deer (Pfeiffer and Brenig, 2005), goat (Phua et al., 2003), horse (Hasegawa et al., 2000), and bear (Yamamoto et al., 2002), but was found only on the X-chromosome in mice (Chapman et al., 1991). Iwase et al. (2003) reported that the amelogenin loci span an ancient pseudo autosomal boundary in diverse mammalian species. Differences in the sequence length have been observed between X- and Y-specific *AMEL* genes in a variety of species. Therefore, the X–Y homologs of the amelogenin gene (*AMELX* and *AMELY*) may be suitable for sex determination on the molecular level. Following a single PCR with one pair of primers, male- and female-specific amplification products with different sizes are detectable, simultaneously. In cattle, Gibson et al. (1991) detected a 63 bp deletion in exon 5 of the *AMELY* gene when compared to the *AMELX* homolog. In humans, AMELX in intron 1 has a characteristic deletion of six base pairs relative to AMELY (Sullivan et al., 1993).

Ennis and Gallagher (1994) designed primers for exon 5 (SE47/SE48) for sex determination in bovine embryos based on PCR amplification of amelogenin gene. Upon PCR amplification, the female animals showed a single 280 bp band (*AMELX*), whereas in male animals an additional band of 217 bp (AMELY) was observed. The method is rapid, does not require prior purification of DNA and contains an internal control which detects failure. Chen et al. (1999) reported a rapid, highly reproducible and sensitive technique for sexing of cow embryos. Based on the low homology (45.1%) between the fifth intron of the *AMELX* and *AMELY* gene with multiple deletions, gender-specific primers were utilized to amplify a 467 bp fragment from the female bovine X-chromosome, and a 467 bp fragment from X-chromosome and a 341 bp fragment from the Y-chromosome in male bovine, respectively. Zeleny et al. (2002) evaluated the two PCR-based beef sexing methods and reported that the amelogenin-type method revealed excellent accuracy and robustness, whereas the bovine satellite/Y-chromosome duplex PCR procedure showed more ambiguous results.

Quilter et al. (2002) studied the arrangement of porcine sex chromosome genes and found copies of amelogenin on the X- and Y-chromosome. Based on these findings Fontanesi et al. (2008) reported that sequence analysis of about 400 bp of intron 3 of the porcine amelogenin genes showed the presence of a 9–10 bp deletion in *AMELY* gene compared to *AMELX* sequences. Moreover, one single nucleotide polymorphism (SNP) was detected for the AMELY sequence. Four other SNPs and 1 bp insertion differentiated three AMELX haplotypes indicating an unexpected quite high nucleotide diversity for a chromosome X region. Two sex determination assays targeting the 9–10 bp difference between AMELX and AMELY were developed. No amplification was obtained in human, cattle, goat, sheep, and horse genomic DNA. Sex determination can be performed with at least 20 pg of genomic DNA, enables the investigation of small amounts of DNA from meat, hair, bones, and embryo biopsies. Checa et al. (2002) reported a PCR amplification of a segment of the X–Y homologous amelogenin gene in order to estimate the X- and Y-chromosome frequencies in bovine sperm by capillary electrophoresis. Yamauchi et al. (2000) reported a sex determination based on fecal DNA analysis of the amelogenin gene in sika deer (*Cervus nippon*). Pfeiffer and Brenig (2005) applied amelogenin gene primer pair for exon 5 (SE47/SE48) for sex determination in sheep (*Ovis aries*) and European red deer (*Cervus elaphus*). The results show that the amelogenin gene of sheep and red deer is located on both sex chromosomes and there are two diagnostic insertion/deletion of 55 bp together within the Y-specific gene in the region amplified.

Weikard et al. (2006) reported the amelogenin cross amplification in the family Bovidae and its application for sex determination. He found an identical length pattern in the species from genera *Bos* and *Bison*. The female animals showed a single 280 bp band (AMELX), whereas in male animals an additional band of 217 bp (AMELY) was observed. In African Buffalo (*Syncerus caffer*), water buffalo (*Bubalus arnae*) and anoa (*Bubalus depressicornis*), AMELX amplicon contained 289 bp. In contrast, amplification of AMELX and AMELY sequences in sheep and goat revealed shorter amplicons, 262 and 202 bp, respectively. Sex determination can be performed with at least 40 pg of genomic DNA, enables the investigation of small amounts of DNA from meat, hair, bones, and embryo biopsies. Chen et al. (2007) applied amelogenin gene primers for exon 5 (SE47/SE48) for sex determination in goat embryos. Upon PCR amplification, the female animals showed a single 262 bp band (AMELX), whereas in male animals an additional band of 202 bp (AMELY) was observed. Pajares et al. (2007) applied

amelogenin gene primers for exon 5 (SE47/SE48) for sex determination in wild ruminants (roe deer, Spanish ibex, chamois, fallow deer, red deer, and mouflon). Upon PCR amplification, the female animals showed a single 255 bp band (AMELX), whereas in male animals an additional band of 211 bp (except for red deer- 205 bp) (AMELY) was observed.

Sembon et al. (2008) designed amelogenin gene primers for sex determination in pigs and porcine embryos. Upon PCR amplification, the female animals showed a single 520 bp band (AMELX), whereas in male animals an additional band of 350 bp (AMELY) was observed. Colley et al. (2008) reported a simple, reliable, and cost effective method for single bovine sperm sex typing using nested PCR, based on the amelogenin gene. Xu et al. (2008) designed amelogenin gene primers for sex determination in giant panda (*Ailuropoda melanoleuca*). Upon PCR amplification, the female animals showed a single 237 bp band (AMELX), whereas in male animals an additional band of 174 bp (AMELY) was observed. Dervishi et al. (2008) studied the reliability of sex determination in ovine embryos using amelogenin gene (*AMEL*) and reported that the amelogenin gene can be used for rapid sex determination in ovine embryos, with a high efficiency and accuracy (100%). Khaledi et al. (2009) applied amelogenin gene primers for exon 5 (SE47/SE48) to investigate reliability of sex differentiation in *Bos indicus* cattle. Upon PCR amplification, the female animals showed a single 280 bp band (AMELX), whereas in male animals an additional band of 213 bp (AMELY) was observed. Thus the SE47/48 primer pair was not only reliable for sex differentiation in *Bos indicus*, but may also be used to differentiate the *Bos indicus* and *Bos taurus* males.

Langen et al. (2010) reported the semiquantitative detection of male pork tissue in meat and meat products by PCR using amelogenin gene primers. Upon PCR amplification, the female animals showed a single 741 bp band (AMELX), whereas in male animals an additional band of 561 bp (AMELY) was observed. Male and female meat samples could be correctly identified, and the mixtures with as little as 0.1% male meat content could be detected. Gurgul et al. (2010) designed amelogenin gene primers for sex determination in red deer (*Cervus elaphus*). Upon PCR amplification, the female animals showed a single 282 bp band (AMELX), whereas in male animals an additional band of 225 bp (AMELY) was observed. Gokulakrishnan (2011) developed a protocol for sexing of cattle meat based on the amelogenin gene (*AMELX/AMELY*) using PCR technique which is superior to earlier work in terms of band patterns.

### *DEAD Box Polypeptide 3 X-linked/Y-linked (DDX3X/DDX3Y) Gene*

DEAD box polypeptide 3 (DDX3) is a subfamily of the DEAD (for the sequence Asp-Glu-Ala-Asp) box RNA helicases, a family of proteins characterized by the conserved DEAD box motif (Abdelhaleem, 2005; Rosner and Rinkevich, 2007). The DEAD box proteins are ATP dependent, and involved in unwinding double-stranded RNA structures and remodeling RNA protein interactions. Thus, they are crucial for RNA metabolism, including transcription, splicing, RNA export, ribosome biogenesis, RNA degradation, and translation initiation (Benz et al., 1999; Rosner and Rinkevich, 2007). In the human genome, there are two functional *DDX3* genes on the sex chromosome (DDX3X and DDX3Y) and one pseudoautosomal (Kim et al., 2001). The Y-chromosome homolog DDX3Y (also known as DBY) is located in the AZFa (azoospermia factor a) interval of the male-specific region (MSY) (Vogt et al., 1996; Lahn and Page, 1997). Deletion of the AZFa region has been shown to disrupt spermatogenesis, causing subfertility and infertility in otherwise healthy men (Foresta et al., 2000). Liu et al. (2009) characterized the genomic structures of the bovine DDX3Y (bDDX3Y) and its homologs (bDDX3X and bPL10), and studied the expression patterns of these genes in gonads and different somatic tissues. The results revealed that, all three members of bDDX3 are active at the transcription level with predominant expression observed in the testis and brain. The bDDX3Y is composed of 17 exons. The homologous gene on the X-chromosome, bDDX3X, is highly conserved to the Y-copy at mRNA (83%) and protein (88%) levels as well as in the genomic structure.

Villesen and Fredsted (2006) developed a method for sex determination in ape and monkey by amplification of *DEAD box* gene. Upon PCR amplification, a male-specific band of the *DBY* gene (209 bp) in males and a common band of DBX (180 bp) observed in both males and females. Barbosa et al. (2009) developed a method for sex determination in red deer (*Cervus elaphus*) by amplification of *DBY* gene. Upon PCR amplification, a male-specific band of the *DBY* gene (200 bp) in males and no band were observed in females. Decrease in PCR stringency caused a second clearly larger fragment (~350 bp) to be amplified, this one appearing also, and with higher yield, in females. Vidal et al. (2010) developed a method for sex determination in Iberian desman (*Galemys pyrenaicus*) based on a three primer amplification of DBX and DBY fragments with noninvasive samples. Katsushima et al. (2010) developed a multiplex PCR assay for molecular sexing of the naked mole rat (*Heterocephalus glaber*) by amplification of *DBY* and *16S rRNA* gene (as control). Upon PCR amplification, a male-specific band of the *DBY* gene (163 bp) in males and a common band of 16S rDNA (446 bp) observed in both males and females. Gokulakrishnan et al. (2012b) reported a fast and reliable method for sex determination of cattle meat by amplification of the *DDX3X/DDX3Y* gene. Upon PCR amplification, a male-specific band of the *DDX3Y* gene (208 bp) in males and a common band of *DDX3X* (184 bp) was observed in both males and females.

## *CONCLUSIONS*

PCR-based methods with subsequent gel electrophoresis proved to be reliable tools for gender determination of meat

and meat products. Accurate results can be obtained in a short time, usually a few hours. Furthermore, additional means are available to underpin the obtained results, such as sequencing, hybridization, RFLP analysis, and a second PCR method with other targets and primers. These techniques are not required for routine analyses, but can serve as complementary methods in ambiguous cases. Methods such as hybridization assays, PCR with subsequent CE analysis, or real-time PCR are also applicable for meat sexing, but are more costly than the above technique. Hormone analysis by GC-MS represents a valuable tool for sex determination of meat, but is not competitive with PCR-based methods in terms of low cost and rapid availability of results.

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