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Molecular Sexing and Species Identification of the Processed Meat and Sausages of Horse, Cattle and Pig

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ABSTRACT

We developed a polymerase chain reaction (PCR)-based molecular method for sexing and identification using sexual dimorphism between the Zinc Finger-X and -Y (*ZFX-ZFY*) gene and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for mitochondrial DNA (mtDNA) *cytochrome B* (*CYTB*) gene in meat pieces and commercial sausages from animals of different origins. Sexual dimorphism based on the presence or absence of SINE-like sequence between *ZFX* and *ZFY* genes showed distinguishable band patterns between male and female DNA samples and were easily detected by PCR analyses. Male DNA had two PCR products appearing as distinct two bands (*ZFX* and *ZFY*), and female DNA had a single band (*ZFX*). Molecular identification was carried out using PCR-RFLP of *CYTB* gene, and showed clear species classification results. The results yielded identical information on the sexes and the species of the meat samples collected from providers without any records. The analyses for DNA isolated from commercial sausage showed that pig was the major source but several sausages originated from chicken and Atlantic cod. Applying this PCR-based molecular method was useful and yielded clear sex information and identified the species of various tissue samples originating from livestock.

(Key words: species identification, molecular sexing, horse, cattle, pig)

INTRODUCTION

Because some retail sellers and local animal meat brands adopted only male or female-derived meat for marketing, species and sex information of animal source used for meat production and processed foods is important to ensure the identity of the originating animals for consumers. Specific sex and species information of livestock animals is legally prevented for religious reasons or customs in several countries. This information is needed to provide clear evidence for tracing and safety of food as well as food labeling required by law. Moreover, animal sex is a grading system meat quality parameter in most countries. Molecular methods using DNA extracted from various sources have been developed for economic, religious, forensic, scientific, or industrial purposes (Matsunaga *et al.*, 1999; Girish *et al.*, 2005; Jonker *et al.*, 2008; Ali *et al.*, 2012; Han *et al.*, 2013).

PCR-based DNA detection methods have been developed to identify the animal species from various sample sources using PCR-RFLP, species-specific PCR, real-time PCR, and DNA sequencing (Girish *et al.*, 2005; López-Andreo *et al.*, 2005; Jonker *et al.*, 2008; Ali *et al.*, 2012; Koh *et al.*, 2012). Mitochondrial DNA (mtDNA) is very abundant in all cells. More than 100 molecules are generally found in all animal cells. Diversities in mtDNA sequences among species supply enough information to discriminate species revealed until now (Matsu-

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naga *et al.*, 1999; Chen *et al.*, 2005; Cai *et al.*, 2007; Han *et al.*, 2013). Reports of molecular sexing have been documented by PCR based analyses amplifying the Y-chromosome-specific *SRY* gene and the sexually dimorphic sequences of homologous genes on the X- and Y-chromosomes, such as the *ZFX*/*ZFY* and *AMELX/AMELY* genes (Sinclair *et al.*, 1990; Pomp *et al.*, 1995; Poloumienko, 2004; Han *et al.*, 2010).

A two-step molecular approach of *Alu*I-RFLP for the mt-DNA *CYTB* gene sequences and sex chromosome-based sexual dimorphism between the *ZFX* and *ZFY* genes was used on meat pieces and processed food samples from cattle, horses, and pigs to develop a precise, rapid, and simple method to identify the species and determine the sex among livestock.

MATERIALS AND METHODS

1. Samples and DNA Extraction

A total of 411 DNA samples from cattle, horses, and pigs were used in this study. Genomic DNA samples of species- and sex-certified cattle (n=96) and pigs (n=103) were kindly provided by researchers at the Subtropical Livestock Research Institute, National Institute of Animal Science, South Korea. Hair from horses (n=40) was collected from horse farms in Jeju-do province, South Korea. Additionally, meat pieces (n= 142) isolated from carcasses and the species and sex information were provided separately by official veterinarians and professional meat-quality graders of the Animal Products Grading Service of Korea at a slaughterhouse in Jeju-do province, South Korea. Commercial sausage (n=30) was purchased from retail markets and used for the test. The study was conducted in accordance (approval number 2015-0023) with recommendations described in “The Guide for the Care and Use of Laboratory Animals” published by the Institutional Animal Care and Use Committee of the Jeju National University, Republic of Korea. DNA was extracted from animal tissue, meat, and sausage using Sambrook *et al.* (1989) with slight modification, and those from hair roots were extracted with 5% Chelix 100 resin (Bio-Rad,USA) using boiling methods.

2. Molecular Sexing

Amplification of *ZFX-ZFY* gene intron 9 in the DNA samples was carried out using *ZFX-ZFY* specific primers (ZF9iF, ATCAAAACCTTCATGCCAAAGT; ZF9iR, CCGGTTTTCA-ATTCCATCAGAA). The PCR products were separated on agarose gels containing ethidium bromide and visualized by UV-illumination. Molecular sexing was carried out based on the presence/absence of male-specific band on the gels. The sexes of the samples were identified as male if two distinct bands appeared or as female if a single band appeared as a PCR product of the *ZFX-ZFY* gene on the gels.

3. DNA Sequencing

The PCR products were purified each band using a QIAEX II Gel Extraction Kit (Qiagen, USA). After purification of PCR products, for each sex of animals three individuals were selected and sequenced using ET Dye-Terminator Sequencing kit (Amersham Pharmacia, USA). The nucleotide sequences for intron 9 and flanking regions newly obtained in this study were deposited in NCBI database under accession numbers DQ-179231 (pig *ZFY*), DQ179232 (pig *ZFX*), DQ415953 (cattle *ZFX*), DQ415954 (horse *ZFX*), and compared with those (DQ-179227-DQ179230) previously reported (Han *et al.*, 2010).

4. Species Identification

The originating species of the samples was identified using the method of Han *et al.* (2013). Briefly, the *CYTB* fragment was PCR amplified using universal primers for the three animal species, digested using the *Alu*I restriction enzyme, and the reactions were incubated at 37℃ for at least 2 hr. All digests were separated on 2.5% agarose gels containing ethidium bromide and visualized by UV-illumination.

RESULTS AND DISCUSSION

Molecular sex was determined using the sexually dimorphic patterns of the PCR products in the three animal samples. The amplified PCR product for the *ZFX-ZFY* gene displayed species-specific band patterns (Fig. 1). All males had two different sized PCR bands, whereas those from females had a single PCR band. The PCR amplification results of the *ZFX-ZFY* gene intron 9 flanking region were identical to those based on a phenotypic investigation of sex. DNA samples of the three livestock species were discriminated using *Alu*I-RFLP to detect the *CYTB* sequence PCR products and showed identical results to our previous report (Han *et al.*, 2013) (Table 1).

The *Alu*I-RFLP analysis for mitochondrial *CYTB* gene PCR products provided species information for the meat pieces and carcass meat through species-specific band patterns on agarose

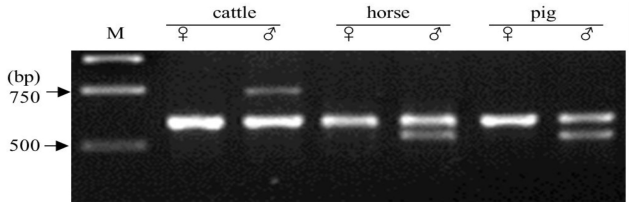

Fig. 1. Polymerase chain reaction amplification patterns of the intron 9 flanking regions of the *ZFX* and *ZFY* genes in cattle, horses, and pigs. M, DNA size marker (1-kb DNA Ladder).

Table 1. Sexing and identifying livestock species in unidentified samples.

Sample	No. of samples detected					
	Cattle		Horse		Pig	
	Male	Female	Male	Female	Male	Female
Cattle (n=96)	70	26	-	-	-	-
Horse (n=40)	-	-	23	17	-	-
Pig (n=103)	-	-	-	-	42	61
Meat piece (n=142)	20	3	8	6	61	44
Sausage (n=30)[a]	-	-	-	-	21	8

[a] Chicken-derived and Atlantic cod-derived DNA were found in eight and 12 sausage samples, respectively. Especially among all sausage DNA samples, one had no DNA derived from livestock and had that derived from Atlantic cod.

gels. The *ZFX-ZFY* gene amplification patterns provided accurate sex information (Table 1), showing two distinguishable bands for males from the *ZFX* and *ZFY* genes and a single female band from *ZFX* in all species of meat samples (Fig. 1).

We identified and sexed commercial sausage purchased from a retail market. As results, some of the sausage samples had undigested PCR fragments after using the *Alu*I enzyme, and two PCR products yielded no digested bands on the gel using this enzyme. We attempted to sequence these undigested fragments. After DNA sequencing, similarity search results in the NCBI database showed that these undigested fragments were derived from chicken and cod (data not shown). We found chicken- and cod-derived DNA in eight and 12 sausage samples, respectively. One of the sausage DNA samples had no livestock animal DNA including chicken and only had DNA derived from Atlantic cod. Chicken and Atlantic cod are generally used to produce commercial sausages in South Korea, and they were confirmed as components on the product label. We determine the sexes of the source meat for sausage production using the *ZFX-ZFY* gene PCR amplification patterns, except in one sausage prepared only with cod. The sexing results showed that the amplification patterns for these 29 samples were identical to those of the pig and support the species identification results using PCR-RFLP of *CYTB* gene mtDNA. These results suggest that the *ZFX-ZFY* gene PCR amplification patterns may be useful to identify species as well as the sex.

Many molecular methods using biomolecules have been developed from various sample sources to collect data for many purposes and social reasons (Matsunaga *et al.*, 1999; Girish *et al.*, 2005; Jonker *et al.*, 2008; López-Andreo *et al.*, 2005; Ali *et al.*, 2012). Information on source animals used in meat and related processed foods is important to identify the originating animal to consumers. We obtained species and sex information for cattle, horses, and pigs from various sources of samples. Our molecular sexing and species identification results suggest that these molecular approaches are useful analytical technique for species identification and molecular sexing of livestock, their carcasses, and processed products. Moreover, these methods were simple and precise.

REFERENCES

Ali ME, Hashim U, Kashif M, Mustafa S, Che Man YB and Abd Hamid SB. 2012. Development of swine-specific DNA markers for biosensor-based halal authentication. Genet. Mol. Res. 11:1762-1772.

Cai X, Chen H, Lei C, Wang S, Xue K and Zhang B. 2007. mtDNA diversity and genetic lineages of eighteen cattle breeds from *Bos taurus* and *Bos indicus* in China. Genetica 131:175-183.

Chen SY, Su YH, Wu SF, Sha T and Zhang YP. 2005. Mitochondrial diversity and phylogeographic structure of Chinese domestic goats. Mol. Phylogenet. Evol. 37:804-814.

Girish PS, Anjaneyulu AS, Viswas KN, Shivakumar BM, Anand M, Patel M and Sharma B. 2005. Meat species identification by polymerase chain reaction-restriction fragment length

polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Sci. 70:107-112.

Han SH, Park S, Oh HS, Kang G, Park BY, Ko MS, Cho SR, Kang YJ, Kim SG and Cho IC. 2013. PCR-RFLP for the identification of mammalian livestock animal species. J. Emb. Trans. 28:355-360.

Han SH, Yang BC, Ko MS, Oh HS and Lee SS. 2010. Length difference between equine *ZFX* and *ZFY* genes and its application for molecular sex determination. J. Assist. Reprod. Genet. 27:725-728.

Jonker KM, Tilburg JJ, Hagele GH and de Boer E. 2008. Species identification in meat products using real-time PCR. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 25:527-533.

Koh BRD, Kim JY, Na HM, Park SD and Kim YH. 2012. Development of TaqMan probe-based real-time PCR for rapid identification of beef, pork and poultry meat. Korean J. Vet. Sci. 35:215-222.

López-Andreo M, Lugo L, Garrido-Pertierra A, Prieto MI and Puyet A. 2005. Identification and quantitation of species in complex DNA mixtures by real-time polymerase chain reaction. Anal. Biochem. 339:73-82.

Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J and Shinmura Y. 1999. A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci. 51:143-148.

Pomp D, Good BA, Geisert RD, Corbin CJ and Conley AJ. 1995. Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or -11 pig embryos. J. Anim. Sci. 73:1408-1415.

Poloumienko A. 2004. Cloning and comparative analysis of the bovine, porcine, and equine sex chromosome genes *ZFX* and *ZFY*. Genome 47:74-83.

Sambrook J, Fritsch EF and Manniatis T. 1989. Isolation of high molecular weight DNA from mammalian cells. In: Molecular Cloning: A Laboratory Mannual. 2nd Ed. Cold Spring Harbor Laboratory Press. New York. pp. 9.14-9.23.

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R and Goodfellow PN. 1990. A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. Nature 346:240-244.1

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