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PCR-RFLP for the Identification of Mammalian Livestock Animal Species

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ABSTRACT

Precise, rapid and simple methods for species identification in animals are among the most important techniques in the livestock industry and research fields including meat classification. In this study, polymerase chain reaction (PCR) based molecular identification using inter species polymorphisms were examined by PCR-restriction fragment length polymorphism (RFLP) analysis for mitochondrial DNA (mtDNA) *cytochrome b* (*CYTB*) gene sequences among four mammalian livestock animals (cattle, horse, goat and pig). The results from PCR-RFLP analysis using the *Alu*I restriction enzyme were also provided for the species-specific band patterns among *CYTB* gene sequences in these four species. The *Alu*I-digestion for *CYTB* genes provided interesting migration patterns differentially displayed according to each species. Cattle and horse had one *Alu*I-recognition site at different nucleotide positions and their *Alu*I-digested fragments showed different band patterns on the gels. Pig had two *Alu*I-recognition sites within the amplified *CYTB* sequences and produced three bands on the gels. Goat had no *Alu*I-recognition site and was located at the same position as the uncut PCR product. The results showed the species-specific band patterns on a single gel among the four livestock animal species by *Alu*I-RFLP. In addition, the results from blind tests for the meat samples collected from providers without any records showed the identical information on the species recorded by observing their phenotypes before slaughter. The application of this PCR-RFLP method can be useful and provide rapid, simple, and clear information regarding species identification for various tissue samples originating from tested livestock species.

(Key words : species identification, PCR-RFLP, restriction digestion, *CYTB*, livestock animals)

INTRODUCTION

Species identification in meat and processed foods is important to verify the source animal for consumers. Recently, the use of horse meat instead of beef for fast foods has led to social problems worldwide. In addition, the authentication of the animal's country of origin is a topic in international trade. Species identification is very important in providing the clear evidence for food safety and insurance purposes and ensures compliance with food labeling laws. Molecular methods for species identification using DNA samples extracted from various sample sources are usually deemed credible for economic, public health, forensic, scientific, or other industrial purposes (Zehner *et al*., 1998; Bataille *et al*., 1999; Kusama *et al*., 2004; Khairalla *et al*., 2007; Jonker *et al*., 2008; El-Sayed *et al*., 2010; Ali *et al*., 2012; Santos *et al*., 2012).

Recent reports have described new methods developed for species identification based on genetic differences between species using PCR techniques. The proteins in meat, dairy, and processed products have been analyzed for species identification using electrophoresis, immunological methods, and so on. But, the protein methods are not suitable because the proteins can be easily lost or degraded by heating, high pressure, and other operations during food processing (Bottero *et al*., 2003; Rodríguez *et al*., 2004; Rao and Hsieh, 2007; Yarmand and Homayouni, 2010; Şakalar *et al*., 2012). Some reports tried to identify the species using the differences of fatty acid composition between species, but scientists have concluded that the differences in composition of various fats may result from differential rates of growth of fatty tissues and other animal conditions (Shorland, 1953; Marchello and Cramer, 1963; Payne, 1971). DNA is more stable than proteins during heat proce-

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ssing and recent DNA techniques based on PCR allow the identification of DNA from different species present in a sample. To date, PCR-based molecular identification has been used for tissues from living animals, carcasses, embryo-derived cells, carcass remains and meat-derived products (Zehner *et al*., 1998; Marfa *et al*., 2004; López-Andreo *et al*., 2005; Zhang *et al*., 2007; Jung *et al*., 2011; Koh *et al*., 2011, 2012).

In order to develop a precise, rapid, and simple method of species identification among mammalian livestock animals, a molecular method was examined by PCR-RFLP for mtDNA *CYTB* gene sequences among cattle, horse, goat, and pig.

MATERIALS AND METHODS

1. Animal Samples and DNA Extraction

A total of 502 DNA samples from four livestock animal species (cattle, horse, pig and goat) were used in this study. Among those, meat samples (n=276) with species information were randomly collected from retail markets in the Seoul, Gyeonggi-do, and the Jeju-do provinces in South Korea. DNA samples (n=80), species certified, were kindly provided by researchers with the Subtropical Animal Experiment Station, National Institute of Animal Science. For the blind test, the meat pieces (n=146) isolated from carcasses and species information were separately provided from professional meat-quality graders at the Animal Products Grading Service in a slaughterhouse in the Jeju-do province, South Korea. DNA was extracted from the meat using standard techniques (Sambrook *et al*., 1989) with slight modification. Isolated DNA was diluted to approximately 100 ng/ul in TE buffer and used for PCR analysis as a template.

2. Universal Primer Designation

Universal primers were designed to amplify the mtDNA *CYTB* gene sequences of the animals used in this study. Standard *CYTB* sequences used for primer designation were obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) database. After multiple alignments using the CLUSTAL W program (Thompson *et al*., 1994), highly conserved sequence regions were selected to design the primers from the sequences. The information for the primers and standard sequences is shown in Table 1.

3. PCR Amplification and DNA Sequencing

PCR was performed using 25 ul of reaction mixture including 100 ng of DNA, 1.0 nmole of each primer, and 2.0 units of i-*Taq* DNA polymerase (Intron Biotechnology, Korea). PCR conditions included initial heating at 95℃ for 3 min, 40 cycles of 45 s for denaturation at 94℃, 45 s for annealing at 55℃, and 60 s for extension at 72℃, followed by a 5 min extension at 72℃. PCR reaction was carried out in a PTC-200 thermal cycler (Bio-Rad, USA). The PCR products were separated on agarose gels containing ethidium bromide (EtBr) and visualized by UV-illumination. The PCR products were purified with a QIAEX II Gel Extraction Kit (Qiagen, USA), and directly sequenced with a MegaBase1,000 automated sequencer (Amersham-Pharmacia, USA). Using a BLAST search, we compared the sequences with those previously reported in the NCBI database.

4. PCR-RFLP Analysis

The amplified *CYTB* fragments were digested in independent reactions using the different restriction enzymes *Alu*I, *Hae*III, *Msp*I, *Rsa*I and *Tsp*509I. RFLP was performed using 20 ul of reaction mixture including 5 ul of PCR product, and 5.0 units of each restriction enzyme (New England BioLabs, USA). The reaction solution were incubated at 37℃ (*Alu*I, *Hae*III, *Msp*I and *Rsa*I) or 65℃ (*Tsp*509I) for two hours. These enzymes were chosen from the results of preliminary recognition site analysis using the Webcutter 2.0 (http://users.unimi.it/~camelot/tools/cut2.html) and the NEBcutter v.2.0 (http://tools.neb.com/NEBcutter2/index.php). Two-replicate PCR-RFLP tests for all

Table 1. Primers used for PCR amplification of mtDNA *CYTB* gene sequences

Gene	Primer name	Nucleotide sequence (5’ → 3’)	Standard sequence	
			Acc. No.	Reference
CYTB	BESH_F	CTGTTACTCACATCTGCCGAGACG	DQ186238	Cai *et al*. (2007)
	BESH_F	AATCGGGTAAGGGTTGCTTTGTC	AJ002189	Ursing and Arnason (1998)
			AY522328	Han *et al*. (2004)
			DQ089475	Chen *et al*. (2005)

samples were separated on agarose gels and visualized. The digests were separated on 2.5% agarose gels containing EtBr and visualized by UV-illumination.

RESULTS

1. PCR-RFLP Polymorphisms of *CYTB* Gene

The PCR products were amplified as a single band from DNA samples of the meats from four animal species using a pair of universal primers designed for the study. Among the five restriction enzymes, all except the *Alu*I produced polymorphic patterns within populations of the species (data not shown). The other four enzymes (*Hae*III, *Msp*I, *Rsa*I, and *Tsp*509I) were excluded for the final test. *CYTB* fragments digested by *Alu*I have not shown polymorphism within intra-species levels and, furthermore, provided distinguishable band patterns among the four animal species. In addition, pigs originated from the two maternal lineages, Asian origin and European origin previously documented by Han *et al*. (2011), were also tested, but the *Alu*I-digested fragments from both lineages did not show different band pattern (Fig. 1).

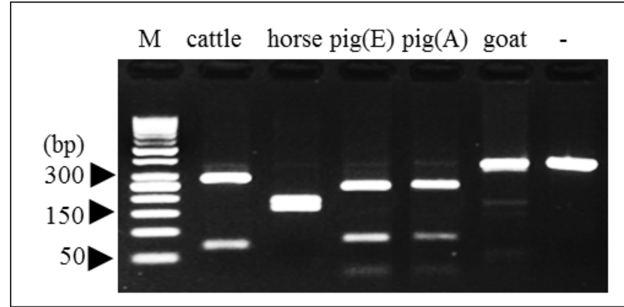

Fig. 1. *Alu*I-RFLP patterns detected in four livestock animal species (cattle, horse, pig, and goat). Pig(E) and Pig(A) represent the pigs from European origin and Asian origin postulated by Han *et al*. (2011), respectively. The dash on the top of last column indicates the negative control uncut PCR product. M is the DNA size marker (50-bp DNA Ladder).

2. Comparison of CYTB Sequences

Fig. 2 shows the results of multiple alignments among the *CYTB* sequences obtained from DNA sequencing. The sequences had different numbers or different nucleotide positions of *Alu*I-recognition sequence according to the species. The *CYTB* sequences from cattle and horse had one *Alu*I-recognition site at different nucleotide positions, and *Alu*I-digested fragments separated into two bands showing different lengths on the gels. The *Alu*I-digested fragments showed 66-bp and 274-bp bands in cattle, and 160-bp and 180-bp bands in horse. The goat *CYTB* sequence, which showed no *Alu*I recognition site, did not produce a digested band, and the *CYTB* fragment located at the same position (340-bp) as the negative control uncut PCR product. The pig *CYTB* sequence had two *Alu*I-recognition sites within 340-bp products and the *Alu*I-digested fragments showed three bands (27-bp, 79-bp, and 234-bp) on the

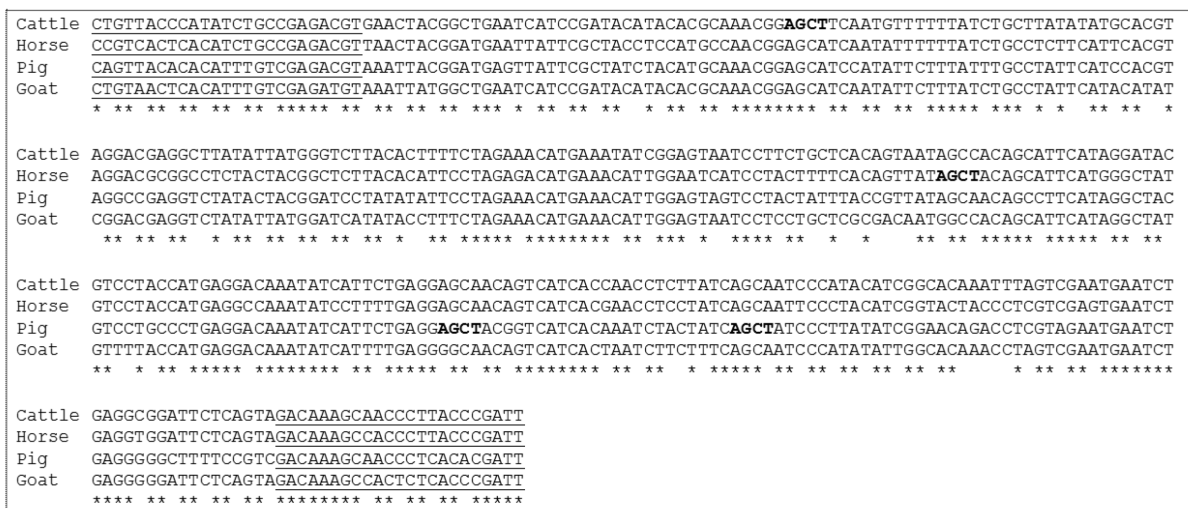

Fig. 2. Multiple alignments for *CYTB* gene sequences in the four livestock animals (cattle, horse, pig, and goat) tested in this study. Underlined and bold sequences indicate the primer-binding sites and the recognition sites against *Alu*I restriction enzyme, respectively. Asterisks are the consensus sequences found among the four animal species.

gels. The results from *Alu*I-RFLP for *CYTB* sequences were identical to those from sequence comparison in multiple alignments among the sequences. The number of *Alu*I-recognition sites and fragment length digested by the enzyme are shown in Table 2.

3. Blind Test for Unidentified Carcass Samples

To evaluate the confidence of this molecular method, the species information was compared with two different species records obtained separately from the phenotype observation and the blind test for the unrecorded carcass samples. The carcass samples were collected with only the carcass number during the slaughtering period and the species information was provided only after the molecular tests. The results from *Alu*I-RFLP showed that a total of 146 unidentified samples were identified as pig (105), cattle (23), horse (14), and goat (4) (Table 3). The species information obtained from this molecular approach was identical to that provided by professional meat-quality graders.

DISCUSSION

A molecular method was developed and tested to identify the species of mammalian livestock animals including cattle, horse, goat, and pig. A total of five restriction enzymes were selected from the preliminary sequence analysis, but only the results from *Alu*I digestion fragments were valid in species identification. *Alu*I-RFLP allows the fragments to divide into four distinguishable band patterns on a single gel without any polymorphisms within intra-species levels. The results from *Alu*I-RFLP for the species-certified DNA samples showed identical species information to that from phenotypic observation. Interestingly, our previous report (Han *et al*., 2011) described the *Alu*I-RFLP polymorphisms within the complete sequences of *CYTB* gene in the pig population. That result suggested that pigs may be divided into two different maternal lineages, Asian origin and European origin. Here, however, we have used only 340-bp partial region of *CYTB* gene for species identification, and there were no polymorphisms within the amplified PCR products between both lineages in the pig population (Fig. 1). Consequently, *Alu*I-RFLP for 340-bp fragments of mtDNA *CYTB* gene is a valid molecular method for identifying the species among cattle, horse, goat, and pig.

Species identification in animal products and related industries is important to verify the source animals and to provide evidence for food safety and insurance. Many molecular methods have been developed for species identification using protein, lipid and DNA samples extracted from various sample sources (Shorland, 1953; Marchello and Cramer, 1963; Payne, 1971; Zehner *et al*., 1998; Montiel-Soa *et al*., 2000; Girish *et al*., 2005; Martín *et al*., 2007). Due to stability during heat treatment, DNA samples are the most powerful marker for species verification and various DNA techniques have been developed for most animal-derived tissues including meat, food, blood, ancient remains, processed meat products and even an embryo-derived single cell (Zehner *et al*., 1998; Kusama *et al*., 2004; Jonker *et al*., 2008; El-Sayed *et al*., 2010; Farjado *et al*., 2010; Koh *et al*., 2011, 2012; Santos *et al*., 2012). Among the methods regarded as useful tools are species-specific PCR (SS-PCR), multiplex PCR, real-time PCR and PCR-RFLP. SS-PCR analysis is powerful and valid in identification of two or three species, but requires specific primer set for each of the species tested. Multiplex PCR and real-time PCR are also powerful and sensitive techniques for species identification. However, for multiple species, some reports have described the unexpected cross reactions and non-specific PCR products which might result in false positive information (Klein, 2002; Jung *et al*., 2011). Se-

Table 2. *Alu*I-recognition sites and fragment sizes for *CYTB* gene sequences in four species

Species	*Alu*I-recognition site (nt)	Fragment size (bp)
Cattle	66	66, 274
Horse	180	160, 180
Goat	n.d.	340
Pig	234, 261	27, 79, 234

n.d., not detected and had no *Alu*I-recognition site.

Table 3. Blind test results by *Alu*I-RFLP for unidentified carcass samples

Sample	No. of animals identified			
	Cattle	Horse	Pig	Goat
Carcass samples unidentified (n=132)	23	14	105	4

veral molecular techniques using fluorescent dye labeled primers or probes, such as real-time PCR and multiplex PCR, recommended for special instructions and professional analysts for the analyses. PCR-RFLP supplies rapid, simple and precise information for the species using PCR subsequent restriction digestion. Compared to the molecular techniques, PCR-RFLP also has the benefit of being an economical experiment.

A highly distinguishable PCR-RFLP system for the species identification in meats of animal origins has been presented. The use of a pair of universal primers makes 340-bp PCR products of *CYTB* gene sequences, and *Alu*I-digestion yields differentially-displayed band patterns on a single gel among the four species tested. Moreover, the results of blind testing using *Alu*I-RFLP provides clear proof of the accuracy of the information compared to phenotype observation. Because it is simple, distinguishable, precise and rapid (less than one working day), the species-differential PCR-RFLP method developed in this work could potentially be used as a routine monitoring assay to screen the identification of the mammalian livestock animal species. This DNA analysis represents a powerful tool for species discrimination and identification of raw materials, foods and processed meat products.

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