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Discrimination of Korean Cattle (Hanwoo) Using DNA Markers Derived from SNPs in Bovine Mitochondrial and SRY Genes

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ABSTRACT : In order to distinguish Korean cattle (Hanwoo) beef from the imported beef from Australia in Korean markets, DNA markers based on PCR-RFLP from mitochondrial genes and SRY gene were applied. A total of 2,826 beef samples comprising 1,495 Hanwoo and 1,331 foreign cattle breeds were obtained in Korea. An 801 bp fragment of the *SRY* gene on the bovine Y chromosome, a 343 bp fragment of *ND4* gene and a 528 bp fragment of *ND5* gene in the bovine mtDNA were amplified by PCR and digested with three restriction enzymes, *Mse*I, *Hpy*CH_4III and *Tsp*509I, respectively. The results showed that *Bos taurus* (T) type was the majority in Hanwoo by combining three markers (99.5%). However, 78.2% of *Bos indicus* (I) type was observed in the imported beef samples. These results indicated that three markers used in this study will be used as valuable markers for discriminating imported beef against Hanwoo. (**Key Words :** Hanwoo, *Bos taurus*, *Bos indicus*, PCR-RFLP, *SRY*, *ND4*, *ND5*)

INTRODUCTION

Korean cattle, also called Hanwoo, is a well-known domestic cattle breed in Korea and the recorded population of this breed was about 1.84 million heads in 2006 (Statistics in the Ministry of Agriculture and Forestry in Korea, 2006). Because of their high meat quality and the breed reputation, Korean consumers are eager to buy Hanwoo meat rather than imported beefs, even though the price of Hanwoo meat is higher.

Since 1990, large amount of beef was imported from outside of Korea and their market share in 2005 was about 70% of the beef consumed in Korea. Because of the high price of the Hanwoo beef compared with the imported beef, some of the mislabeled beef has arisen, resulting illegal false sales. Also because of the outbreak of Bovine spongiform encephalopathy (BSE) in the United States in 2003, consumers prefer to know the exact origin of the beef that they have. In this point of view, the Korean Ministry of Agriculture and Forestry is planning to launch traceability system for the Hanwoo beef in order to trace beef from farm to table. However, settlement of this system takes couple of years from now and discrimination of Hanwoo beef from other imported beef is urgently needed in order to satisfy consumers and protect false sales of the beef.

Until recently, only 33 DNA markers have been developed using the causative mutations for disease or production traits in cattle (http://omia.angis.org.au/) and the number of markers will be rapidly growing. In Korea, a breed discrimination marker based on the coat color related MC1R (melanocortin 1 receptor) gene between Hanwoo and Holstein was developed (Kim et al., 2000; Lee et al., 2000) and the impact of this marker to the society is enormous in relation to give trust to the consumers and decrease the mislabeled false sales. In China, two domestic Buffalo populations were differentiated with the SNPs in Y-linked SRY gene and microsatellite markers were also used for population genetic studies (Hai-Guo et al., 2005; Zhang et al., 2006). Very recently, Sasazaki et al. (2006) developed AFLP (Amplified Fragment Length Polymorphism) oriented DNA markers in order to discriminate between Japanese Black cattle and F1 (Japanese Black cattle×Holstein) breeds. They showed that the probability of identifying F1 was 0.9168 and the probability of misjudgment was 0.0066 using only four selected markers.

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Table 1. Primer information for amplifying SRY, ND4 and ND5 genes and restriction enzymes used to distinguish *Bos taurus* and *Bos indicus* alleles

Genes	PCR product sizes	Primer sequence	Restriction enzymes used
SRY	801 bp	F 5′-TTAGAACGCTTACACCGCATATTACTTC-3′	*Mse*I
		R 5′-TAGTAAAATTGAGATAAAGAGCGCCTTTGTTAG-3′	
ND4	343 bp	F 5′-CGCCGGACTCTATTTCCTATTCT-3′	*Hpy*CH_4III
		R 5′-TGCTATAAAGTCGGTCATAGGGT-3′	
ND5	528 bp	F 5′-CGCAAACAACCTCTTCCAGCTATTC-3′	*Tsp*509I
		R 5′-TGACTGGATGTGGAGAAGGCGATGA-3′	

Table 2. The PCR conditions for amplifying SRY, ND4 and ND5 genes

	SRY		*ND4*		*ND5*	
Initial incubation step	94°C 10 min (1 cycle)					
Denaturation	94°C 30 sec	38 cycles	94°C 30 sec	35 cycles	94°C 30 sec	35 cycles
Annealing	60°C 30 sec		65°C 30 sec		63°C 30 sec	
Extension	72°C 45 sec		72°C 30 sec		72°C 35 sec	
Final extension	72°C 10 min (1 cycle)					
Final step	4°C ∞					

Subsequently, in order to distinguish between Japanese domestic and Australian imported beef in Japan, Sasazaki et al. (2007) developed DNA markers derived from mtDNA, SRY gene, MC1R gene and AFLP.

The aim of this study was to develop DNA markers for discrimination of Hanwoo beef compared with the imported beef. Since the outbreak of BSE in USA in 2003, most of the imported beef came from Australia and large number of cattle was crossbred with *Bos indicus* breeds in order to overcome hot and arid environments in Australia. Take this point account, previously reported DNA mutations for *Bos indicus* breeds in two mitochondrial genes (ND4 and ND5) and SRY gene (Sasazaki et al., 2007) were investigated as possible DNA markers for discrimination between Hanwoo and imported cattle breeds in Korea.

MATERIALS AND METHODS

Genomic DNA preparations

Genomic DNA samples were extracted from blood or meat samples in three Universities (Gyeongsang National Univ., Chungbuk National Univ. and Konkook Univ.) and National Institute of Animal Science (NIAS) in Korea using commercially available several DNA extraction kits. Total 2,826 DNA samples, composed of 1,495 samples from Hanwoo (Korean cattle) and 1,331 DNA samples from imported cattle, mainly from Australia, were investigated.

Designing of primers

Primers for PCR amplification of the SRY, ND4 and ND5 genes were designed basically according to Sasazaki et al. (2007). In case of SRY gene, *Bos taurus* SRY gene sequence (AB039748) was aligned with that of *Bos indicus* sequence (AY079145) and identified the nucleotide mismatches, which were used for the breed differentiation marker based on the PCR-RFLP using *Mse*I restriction enzyme. Therefore, the PCR primers were designed to span this mutation and the size of PCR products was decided for giving optimum results obtained from PCR-RFLP. Similarly, nucleotide mutations between *Bos taurus* and *Bos indicus* sequences in ND4 and ND5 genes were also investigated. Primers were designed to amplify a 343 bp PCR fragment corresponding to nucleotides 10,960-11,302 in ND4 gene and a 528 bp PCR fragment corresponding to nucleotides 12,507-13,034 in ND5 gene from the *Bos taurus* mtDNA sequence (GenBank accession number V00654) (Anderson et al., 1982). Nucleotides 10,963-12,305 in ND4 gene and nucleotides 12,510-13,037 in ND5 gene from the *Bos indicus* mtDNA sequence (GenBank accession number NC_005971) were also amplified using these primers. The adenine in nucleotide position at 11,071 of ND4 gene in *Bos indicus* mitochondrial sequence was not recognized by the restriction enzyme *Hpy*CH_4III and showed the different PCR-RFLP patterns between *Bos taurus* and *Bos indicus* breeds. Also, the thymine in nucleotide position at 12,923 of ND5 gene in *Bos taurus* mitochondrial sequence was not recognized by the restriction enzyme *Tsp*509I. The primer information for SRY, ND4 and ND5 genes was shown in Table 1.

Polymerase chain reaction

The Polymerase Chain Reaction (PCR) was conducted in 25 μl volumes, each containing 10× PCR Gold buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer, 1 unit of *Taq* DNA polymerase (Ampli Taq Gold, USA) and 50 ng of template DNA. The PCR amplification reaction was carried out using a GeneAmp® PCR System 2700 (Applied Biosystems, USA). The PCR conditions for the three genes investigated are represented in Table 2.

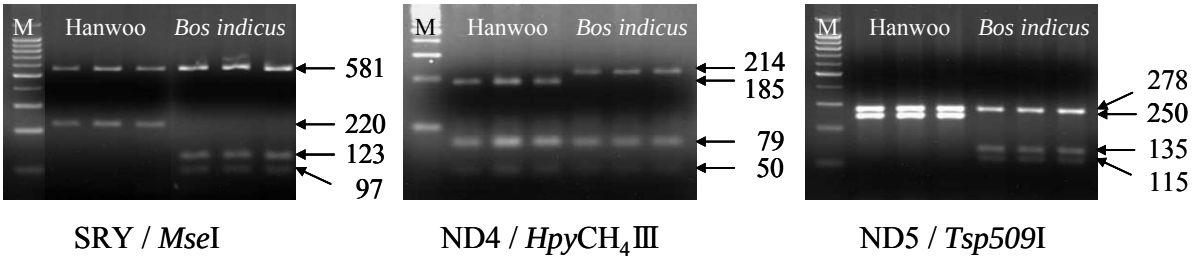

Figure 1. PCR-RFLP patterns for SRY, ND4 and ND5 genes showing *Bos taurus* (Hanwoo) and *Bos indicus* alleles. Restriction enzyme fragments were obtained by digestion of the SRY (801 bp), ND4 (343 bp) and ND5 (528 bp) gene fragments with *Mse*I, *Hpy*CH$_4$III and *Tsp*509I restriction enzymes, respectively. M : 100 bp DNA ladder (Sun Genetics, Korea).

Table 3. The number of animals showing *Bos taurus* (T) and *Bos indicus* (I) alleles in SRY, ND4 and ND5 genes in imported beef and Hanwoo samples

Gene	Type	Imported beef	Hanwoo beef
SRY (%)	T	342 (25.7)	1,445 (96.7)
	I	775 (58.2)	0 (0.0)
	NA	214 (16.1)[1]	50 (3.3)[2]
ND4 (%)	T	671 (50.4)	1,487 (99.5)
	I	594 (44.6)	8 (0.5)
	NA	66 (5.0)[3]	0 (0.0)
ND5 (%)	T	661 (49.7)	1,487 (99.5)
	I	573 (43.0)	8 (0.5)
	NA	97 (7.3)[4]	0 (0.0)
Occurrence of *Bos indicus* alleles (%)		1,041/1,331	8/1,495
		(78.21)	(0.54)

[1] Unable to amplify PCR products. [2] Indicates female beef samples.
[3] 58 were not amplified by PCR and 8 were identified as mixture type.
[4] 91 were not amplified by PCR and 6 were identified as mixture type.

Restriction enzyme digestion and gel electrophoresis

Three restriction enzymes, namely *Mse*I, *Hpy*CH$_4$III, *Tsp*509I (New England Biolabs, USA), were used for PCR-RFLP tests in SRY, ND4 and ND5 genes, respectively. The PCR products were digested in a total volume of 20 μl including 15 μl of the PCR products, 2 μl of 10× reaction buffer, 5 units each of restriction enzymes and the reaction was carried out overnight at 37°C for *Mse*I and *Hpy*CH$_4$III and at 65°C for *Tsp*509I restriction enzymes. The digested products were electrophoresed in 3% (SRY, ND5) or 5% (ND4) agarose gels and visualized on the UV light for identifying different restriction enzyme digestion patterns between *Bos taurus* (Hanwoo) and *Bos indicus* alleles.

RESULTS AND DISCUSSION

The partial fragments of SRY, ND4, ND5 genes were successfully amplified by PCR and the product sizes were 801 bp, 343 bp and 528 bp, respectively, as expected. These PCR fragments were subjected to three restriction enzyme digestions in order to identify *Bos indicus* and *Bos taurus* alleles, which were used for breed differentiation between imported and Hanwoo beefs. Specifically, *Mse*I was used for SRY gene and two mitochondrial gene fragments, ND4 and ND5, were digested by *Hpy*CH$_4$III and *Tsp*509I restriction enzymes, respectively. The PCR-RFLP patterns in *Bos taurus* and *Bos indicus* breeds were shown in Figure 1. For the ND4 restriction enzyme digestion fragments, one (29 bp) of the four fragments (185, 79, 50 and 29 bp) was not shown in *Bos taurus* because of the low resolution of the pictures for the small fragments on the agarose gel.

The identified *Bos indicus* and *Bos taurus* specific alleles were investigated whether they can use for the discrimination of DNA markers between Hanwoo and imported beef samples in Korea. The PCR-RFLP results from 1,495 Hanwoo meat samples showed that 1,445 (96.7%) Hanwoo samples had *Bos taurus* alleles, indicating the high possibility for using this SRY marker as breed discrimination. However, the SRY gene is located only in Y chromosome and because of this, the SRY marker can not be used for beef samples from cows. In Table 3, 50 beef samples (3.3%) are females. The PCR-RFLP results for the SRY gene in imported beef samples from Australia indicated that 58.2% of the samples had *Bos indicus* alleles. This result suggested that large number of Australian cattle was mixed with *Bos indicus* cattle breeds, mainly Brahman, in order to overcome arid environmental conditions. In imported beef samples, the PCR amplification was not

succeed for 214 samples (16.1%). Most of these are assumed to be the female samples, but unfortunately we do not have any gender information for the imported beef samples. The results of the SRY marker indicated that this marker is very powerful for discrimination of the imported beef samples. However, there is a limitation for using this marker for breed discrimination in female beef samples.

In order to identify female origin of the beef samples, two mitochondrial genes, ND4 and ND5, were investigated. Both markers were almost same results for *Bos taurus* and *Bos indicus* allele frequencies. In imported beef samples, the observed *Bos indicus* allele frequencies were very similar that 44.6% of *Bos indicus* allele frequency was observed in ND4 gene and 43% was observed in ND5 gene. This is not surprising that these two genes have similar gene frequencies because these two genes located on the mitochondrial genome with no homologous recombination and will be inherited in a maternal lineage. However, small portion of the samples were identified as neither *Bos taurus* nor *Bos indicus* alleles. They are actually mixed type of the alleles due to the heteroplasmy of the mitochondrial genome. Therefore more investigation should be carried out for the mixed type of the beef samples that they are really came from the heteroplasmy of the mitochondrial genome, not from the experimental errors. When these two mitochondrial genes applied to Hanwoo beef samples, 1,487 beef samples (99.5%) showed *Bos taurus* allele, indicating our assumption was true that the Hanwoo is *Bos taurus* breed. However, we also observed small portion of *Bos indicus* allele frequency (0.5%) in Hanwoo population. These results may support the hypothesis that Hanwoo breed had not been originated from a crossbred between *Bos primigenius* in Europe and *Bos indicus* in India, however, North Eastern Asian cattle breeds might be had separate domestication from the European and African *Bos taurus* followed by the small extents of *Bos indicus* influx (Mannen et al., 2004; Yoon et al., 2005).

Using the three markers together, the discrimination rate from imported beef samples was calculated. If the animal has at least one *Bos indicus* allele, this can be specified as *Bos indicus* origin and decided that this animal is different from Hanwoo. Using this process, the observed frequencies of *Bos indicus* origin were 78.21% and 0.54% for imported and Hanwoo beef samples, respectively. The huge differences of these allele frequencies indicate that these markers will be used as breed differentiation, especially imported beef from Australia against Hanwoo samples. In order to achieve 100% discrimination for breed identification, autosomal DNA markers are ultimately needed in the future. The current progress of molecular biology and recent success of bovine genome sequencing will make this possible in a short period of time.

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