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ARTICLE

Comparison of the Microsatellite and Single Nucleotide Polymorphism Methods for Discriminating among Hanwoo (Korean Native Cattle), Imported, and Crossbred Beef in Korea

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Abstract

The identity of 45 Hanwo and 47 imported beef (non-Hanwoo) samples from USA and Australia were verified using the microsatellite (MS) marker and single nucleotide polymorphism (SNP) methods. Samples were collected from 19 supermarkets located in the city of Seoul and Gyeonggi province, South Korea, from 2009 to 2011. As a result, we obtained a 100% concordance rate between the MS and SNP methods for identifying Hanwoo and non-Hanwoo beef. The MS method presented a 95% higher individual discriminating value for Hanwoo (97.8%) than for non-Hanwoo (61.7%) beef. For further comparison of the MS and SNP methods, blood samples were collected and tested from 54 Hanwoo × Holstein crossbred cattle (first, second, and third generations). By using the SNP and MS methods, we correctly identified all of the first-generation crossbred cattle as non-Hanwoo; in addition, among the second and third generation crossbreds, the ratio identified as Hanwoo was 20% and 10%, respectively. The MS method used in our study provides more information, but requires sophisticated techniques during each experimental process. By contrast, the SNP method is simple and has a lower error rate. Our results suggest that the MS and SNP methods are useful for discriminating Hanwoo from non-Hanwoo breeds.

Keywords: Hanwoo, genetic identification, microsatellite method, single nucleotide polymorphism method

Introduction

Korean native cattle (Hanwoo) have been raised in the Korean Peninsula since 2000 B.C. The specific traits of Hanwoo have been maintained through careful breeding, and therefore the current blood lineage is very valuable and pure (Kim and Lee, 2000; Lim *et al.,* 2014). In 2011, approximately 3 million heads of beef cattle were raised in Korea. In the same year, the total number of slaughtered cattle was 851,000, including 720,000 Korean native cattle and 131,000 domestic Holsteins. Nevertheless, Korea is only a 42.8% self-sufficient nation in the beef market; the country imports the rest of Korean beef from Australia, the USA, New Zealand, Mexico, and Canada, making Korea the fourth-largest global importer of beef (Korea Meat Trade Association, 2011).

Domestic meat consumption is increasing in Korea, because of a steady expansion in the proportion of Koreans eating out, and this is leading to a rise in imports of livestock products (Min *et al.,* 1995). The Uruguay Round, in particular, has accelerated the opening of markets to imports of livestock products; further, the signing of the Free Trade Agreement (FTA) has fully opened doors to large quantities of imported beef. Meanwhile, consumer anxiety regarding imported livestock products is rising because of the occurrences of bovine spongiform encephalopathy (BSE) in countries such as the USA, Canada, Israel, and Japan (Hwang, 2010). Imported beef may be mislabeled as Hanwoo, based on the higher price it commands, and unscrupulous companies are taking advantage of consumers' increasing concerns regarding the safety of imported beef (Cheong *et al*., 2013).

Korean consumers' preference for Hanwoo is increasing; however, there has been a rise in the number of cases of restaurants selling imported beef and domestic Holstein beef, which are relatively low in cost, as Hanwoo (Hong *et al*., 2010). Hence, in August 2008, the Korean

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government implemented a system for labeling beef by country of origin, as part of safety measures designed to distinguish Hanwoo from imported beef. On 22 December 2008, the Animal and Meat Traceability System for Hanwoo put these rules into practice. Additionally, the Law of Labeling the Origin of Meat in Restaurants was implemented to protect the rights of consumers by providing accurate information on the origins of meat products (Hwang, 2010).

Given these circumstances, breed determination based on scientific methods is crucial, in order to provide consumers with accurate information about beef products. Conventionally, cattle breeds are identified by means of physical attributes, such as horns, coat color, and frame. However, in the case of seasoned or processed beef, it is difficult to distinguish products by means of visual inspection; hence, the need for scientific methods such as gene analysis has been raised (Chung and Chung, 2004; Hong *et al*., 2010). To fulfill the need for a scientific breed identification method based on gene analysis, PCR-single-strand conformation polymorphism by using the melanocortin 1 receptor (MC 1R) gene, which determines cattle coat color, was developed in 2000 (Berryers *et al*., 2003; Chung *et al*., 2000; Chung and Chung, 2004; Cone *et al*., 1996; Do *et al*., 2007). Subsequently, the MC1R PCR-RFLP (restriction fragment length polymorphism) marker was used for discrimination of beef origin (Min *et al*., 1995).

However, the MC1R method alone cannot easily discriminate between domestic Hanwoo beef and imported beef from animals with a genealogy of light brown coat color (Cheong *et al*., 2013; Mohanty *et al*., 2008). As such, methods based on other DNA markers such as microsatellite (MS) markers - recommended by the International Society of Animal Genetics (ISAG) for analysis of polymorphism in cattle - are widely used in Hanwoo traceability research (Lee *et al.*, 2004; Lee *et al.*, 2008; Lim *et al*., 2005; Yoon *et al*., 2005). Currently, single nucleotide polymorphism (SNP) markers, which were found in large numbers through decoding of the entire nucleotide sequence of the bovine chromosome, are distributed in a readily usable DNA chip form (Ball *et al.,* 2010; Sasazaki *et al*., 2011; Van Eenennaam *et al*., 2007).

In 2008 and 2010, respectively, the MS (Lim *et al*., 2009) and SNP (Cheong *et al*., 2013) methods were developed domestically as certified testing methods for verifying Hanwoo. However, the efficacies and accuracies of these two methods have not previously been evaluated objectively. For example, the MS method's standard probability is currently set at 0.5 for identifying Hanwoo and non-Hanwoo beef (MFDS, 2013); this value is too low, which increases the chance of mislabeling crossbred cattle as Hanwoo. In addition, the tests require to be validated, including a comparison in terms of accuracy. In the present study, we verified the identity of 45 native beef samples (Hanwoo) and 47 imported beef samples (non-Hanwoo), by using the MS and SNP methods. In addition, we conducted a comparison analysis of the agreement rates between the two methods for meat and blood samples from Hanwoo, imported beef, and Hanwoo-Holstein crossbred animals in Korea.

Materials and Methods

Samples

We collected 45 beef samples labeled as Hanwoo and 47 beef samples labeled as non-Hanwoo (including domestic Holstein and imported beef from the USA and Australia) of beef portion such as tender loin, sirloin, chuck roll and loin from 19 major supermarket chains and department stores located in Seoul city and Gyeonggi province, South Korea, from 2009 to 2011. We applied the MS and SNP methods to all of the samples, to discriminate their origin. In order to evaluate the accuracy of identifying crossbred cattle by using the MS and SNP methods, we tested blood samples collected from 54 Hanwoo × Holstein crossbred cattle (44 first-generation, and 10 second- and third-generation) on individual cow farms in Korea.

DNA extraction from samples

DNA extraction was conducted in accordance with the Hanwoo verification test method specified by the Standards for Processing and Ingredient Specifications of Livestock Products of the Ministry of Food and Drug Safety (MFDS, 2013). Briefly, 1 g of a test sample was diced into fine pieces and added to 5 mL of SPK buffer (50 of 54% sucrose, 2 mL of 50 mM ethylene diamine tetraacetic acid [EDTA], 10 mL of 10% SDS, 5 mL of 20× SSC, and 33 mL of distilled water), 2 μL of RNaseA (Qiagen, USA), and 5 μL of Proteinase K (Qiagen, USA), and then inverted. The sample was then placed in a 55°C water bath overnight, mixed with 3 M NaCl solution (500 μL), inverted, and then centrifuged for 15 min at 3000 rpm. The DNA pellet was transferred to a 1.1-mL Eppendorf (EP) tube, mixed with 70% EtOH (1 mL), inverted, and then centrifuged for 1 min at 13000 rpm. After removing the supernatant, the DNA in pellet form was collected

and dried in an oven. Finally, TE buffer (150 μL) was added to the dried DNA pellet to produce the DNA test sample.

Microsatellite method

We used the MS method with 48 markers for identifying Hanwoo and non- Hanwoo cattle, in accordance with the Standards for Processing and Ingredient Specifications of Livestock Products (Kim *et al*., 2009; MFDS, 2013). Briefly, the PCR reaction mixture contained 2 mL of genomic DNA (25-50 ng) test sample, PCR primer mix (8.25 μL), 10× reaction buffer (1.5 μL), dNTPs (1.2 μL), $MgCl_2$ (1.1 μL), 0.4 μL of Taq DNA polymerase (1-2 U), DMSO (0.25 μL), and deionized water (0.3 μL). We used triplicate (15-μL) sets of primer mix (SET 0, SET 1, SET 2) for each reaction. For PCR analysis, denaturation was conducted for 25 repeating cycles. The first 5 cycles each consisted of 4 min at 95°C, 60 s at 94°C, 75 s at 58°C, and 60 s at 72°C. The next 5 cycles each consisted of 60 s at 94°C, 75 s at 57°C, and 60 s at 72°C. The final 5 cycles each consisted of 60 s at 94°C, 75 s at 56°C, and 60 s at 72°C. Finally, a 30-min extension step was performed at 65°C, to conclude the cycle at 8°C. The PCR products (1 μL) were diluted with distilled water (SET 0, 60 times; SET 1, 40 times; SET 2, 60 times) and used for allelic analysis. To 1 μL of the diluted PCR product sample, 10 μL of Hi-Di Formamide™ (Applied Biosystems, UK), 1 μL of size marker (GeneScan™-500 LIZ® size standard, Applied Biosystems, UK) were added, and the mixture was then divided into 96-well plates. These 96-well plate mixtures were allowed to react at 95°C for 3 min, and were then immediately cooled to a temperature of 4°C. The PCR products were analyzed by using an ABI 3130xl Genetic Analyzer (Applied Biosystems), and the exact size of alleles was determined by using GeneMapper Software v.4.0 (Applied Biosystems). The identification decision was made by applying the breed assignment method of Rannala and Mountain (1997), based on the relative probability of a sample's assignment to the 2 groups, namely, Hanwoo or non-Hanwoo. The sample was identified as belonging to 1 of the 2 groups when the probability was ≥ 0.5.

Single nucleotide polymorphism method

We conducted the SNP method, which uses 90 markers for identifying Hanwoo/crossbred/non-Hanwoo cattle, in accordance with the Standards for Processing and Ingredient Specifications of Livestock Products (Cheong *et al*., 2013; MFDS, 2013). DNA samples were used at a concentration of 50 ng/μL. For discrimination marker genotyping, 5 μL of DNA plate production reagent were added to DNA plates. The DNA samples were transferred to the plates, and were allowed to react according to the manufacturer's instructions. Next, 10 μL of the DNA reaction mixture were added to genotype-specific extension reaction plates, and mixed until the beads were completely dispersed. Pooled-sampling lengthening and pairing reactions of beads with 90 markers were then conducted. For PCR analysis, 64 μL of DNA polymerase and 50 μL of uracil DNA glucosylase were added to the PCR mixture solution tube and thoroughly mixed. Next, 30 μL aliquots of the mixture were placed on PCR plates, and 35 μL of DNA were added to each genotype-specific extension reaction containing beads. PCR analysis was conducted over 34 cycles, consisting of 10 min of denaturation at 37°C and 3 min of denaturation at 95°C, followed by 35 s at 95°C, 35 s at 56°C, and 2 min at 72°C, and a final extension step of 10 min at 72°C. The VeraCode bead plates completed through PCR reactant combination process and VeraCode bead plates compound the reaction, were scanned by using BeadXpress reader (Illumina, USA). On completion of scanning, genotyping was determined by using bead studio software. The final genotype file was analyzed automatically by using the Hanwoo discrimination formula of MFDS. The object was determined as Hanwoo if the estimated value was ≤ 0.45 and as non-Hanwoo if the estimated value was > 0.45.

Results and Discussion

Monitoring the labeling of Hanwoo beef and imported beef by using the MS and SNP methods

On December 22, 2008, the Korean government implemented laws governing cattle and beef traceability, as part of safety measures designed to distinguish Hanwoo from imported beef (Hwang, 2010). Currently, the MS and SNP methods are used domestically as certified testing methods for verifying Hanwoo (MFDS, 2013). In the present study, we verified the identity of 45 native beef samples (Hanwoo) and 47 imported beef samples (non-Hanwoo; domestic Holstein meat and imports from the USA and Australia), by using the MS and SNP methods. We obtained a 100% concordance rate for beef labeling between the 2 methods for all of the 92 beef samples (Table 1). In the case of the MS method for Hanwoo beef, 44 samples (97.8%) had a probability value of ≥ 95.0%, and only 1 sample (2.2%) showed a probability value of 85.0-94.9% (Table 2). On the other hand, for non-Hanwoo beef, 29

Table 1. Comparison between microsatellite (MS) and single nucleotide polymorphism (SNP) methods for discriminating Hanwoo and non-Hanwoo-labeled beef obtained from supermarkets in Korea

Classification		No. of positive result			
		MS method		SNP method	
		Hanwoo	Non-Hanwoo	Hanwoo	Non-Hanwoo
No. of samples tested (n=92)	Hanwoo (n=45)	45	0	45	0
	Non-Hanwoo (n=47)	0	47	0	47
Positive ratio for label		100%	100%	100%	100%

Table 2. Analysis of discriminating values for Hanwoo and non-Hanwoo-labeled beef by using the microsatellite (MS) method

Breeds	No. of beef samples (%)	Individual discriminating value (%)				
		< 50	50-74.9	75-84.9	85-94.9	≥ 95
Hanwoo	45(100)	0(0)	0(0)	0(0)	1(2.2)	44(97.8)
Non-Hanwoo	47(100)	1(2.1)	8(17.0)	4(8.5)	5(10.6)	29(61.7)
Total	92 (100)	1(1.1)	8(8.7)	4(4.4)	6(6.5)	73(79.4)

samples (61.7%) had a probability value of ≥ 95.0%, 5 samples (11.6%) showed probability values of 85.0-94.9%, 4 samples (9.3%) showed probability values of 75-84.9%, 8 samples (18.6%) showed probability values of 50-74.9%, and only 1 sample (2.3%) showed a probability value of < 50%. In the case of MS method, Hanwoo respresented higher overall probability rates compared to non-Hanwoo with all samples showing probability rates of 85% and higher. This result could be due to difference in sample that Hanwoo was relatively purebred Korean native cattle in contrast with non-Hanwo (imported beef). However, despite the small differences in the probability values between Hanwoo and non-Hanwoo beef, our results showed a 100% concordance rate for labeled beef. We subsequently identified the test samples by comparing the results of our present analysis with the reference genotype database of samples that had previously been identified as Hanwoo or non-Hanwoo by using the SNP method (Cheong *et al*., 2013; MFDS, 2013). The results of previous analyses of test samples (Hanwoo, crossbred, and pure non-Hanwoo beef) by using the SNP method showed that the SNP values ranged 0.30-0.45 for Hanwoo beef, 0.80-1.34 for crossbred beef, and 1.60-2.24 for pure non-Hanwoo beef (Cheong *et al*., 2013; MFDS, 2013). Accordingly, the results of our present study identified samples with discrimination values of 0.45 as Hanwoo, and samples with discrimination values of > 0.45 as non-Hanwoo beef (data not shown). The results of the SNP method showed a 100% concordance rate for country of origin of beef labeling for all the 92 beef samples. Thus, we obtained a 100% concordance rate between the MS and SNP methods for Hanwoo and non-Hanwoo-labeled beef.

Concordance rate between the MS and SNP methods for crossbred Hanwoo cattle and Holstein cattle

In Korea, crossbred cattle are limited to Hanwoo and Holstein crossbreeds. The reason is the specificity of the country's livestock industry in Korea, with the Korean government allowing only Hanwoo and Holstein sires to be used in breeding projects or in the domestic beef industry (Cheong *et al*., 2013). Therefore, in our present comparison study, we used only Hanwoo and Holstein crossbreeds.

We investigated the concordance rate between the MS and SNP methods conducted on blood samples of 54 crossbred cattle, consisting of first-, second-, and third-generation Hanwoo × Holstein crossbred cattle (Table 3). Our results showed that the two methods correctly identified first-generation crossbred cattle as non-Hanwoo with 100% accuracy; however, both methods were less likely correctly to identify second- and third-generation crossbred cattle - showing the appearance of Hanwoo - as non-Hanwoo (accuracies of 80.0% and 90.0% for the MS and SNP methods, respectively). All of the samples that were incorrectly identified as Hanwoo by using the two methods from second- and third-generation crossbred cattle.

According to the current Hanwoo verification test procedure of Standards for Processing and Ingredient Specifications of Livestock Products, a probability value of ≥ 50% determined by using the MS method is sufficient to classify a sample as Hanwoo (MFDS, 2013). However, in our present study, a probability value of ≥ 85% showed a higher accuracy for Hanwoo discrimination. Thus, in order to increase accuracy of Hanwoo discrimination, the probability value criterion should be adjusted to ≥ 75%. In fact, a large number of third-generation Holstein × Hanwoo crossbreds are phenotypically identifiable as Hanwoo.

Table 3. Concordance rate between microsatellite (MS) and single nucleotide polymorphism (SNP) methods used for analysis of blood samples obtained from crossbred Hanwoo and Holstein cattle

Crossbred (Hanwoo × Holstein)	No. of blood samples	MS method			SNP method		
		Hanwoo	Non-Hanwoo	accuracy	Hanwoo	Non-Hanwoo	accuracy
F1	44	0	44	100%	0	44	100%
F2 & F3	10	2	8	80.0%	1	9	90.0%
Total	54	2	52	96.3%	1	53	98.1%

Therefore, it is likely that third and higher generations of crossbred cattle have a higher probability of genetically being identified as Hanwoo. Further studies to discriminate Hanwoo in the next generation of various crossbred cattle are required.

Microsatellites have been widely used for genetic characterization of beef origin and the study quantitative trait loci (Chung and chung 2004; Oh *et al*., 2008**).** However, they may provide misleading results with higher error rates than those of SNPs (Ball *et al.,* 2010; Cheong *et al*., 2013; Van Eenennaam *et al.,* 2007). In comparison with the SNP methods, the MS method is more informative. However, the SNP method is simple and has a lower error rate (Cheong *et al*., 2013). Our present results indicate that, if the discrimination value of the MS method is corrected, the SNP and MS methods may be usefully combined with specific Hanwoo DNA markers, to detect violations of the country of origin labeling law. Given the increased imports of beef in Korea, more intensive evaluation of Hanwoo discriminating methods, and also the construction of a national beef DNA database are required.

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