Fast Identification of Korean Cattle Based on Simultaneous Detection of Various Single Nucleotide Polymorphisms Markers by Capillary Electrophoresis

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Due to the establishment of the free trade agreement (FTA) and the various changes that have occurred in international trade, fast and accurate identification methods for the species-specific genes of various cattle are increasingly needed. Most cattle breeds have a specific coat color pattern that distinguishes them. Korean cattle called Hanwoo also have a unique coat color ranging from yellowish brown to dark brown, including a red coat color, which allows them to be distinguished.[1,2] However, processed meat derived from Hanwoo cannot easily be identified in this way. Therefore, the development of fast and accurate analytical methods of identifying the species-specific genes of various cattle based on a combination of scientific analysis and the field inspection of the unique coat color and shape is increasingly needed.

The Melanocortin-1-receptor (MC1R) gene encodes a protein with key regulatory functions in the synthesis of melanin.[3] The MC1R associated with the coat color information is located in the melanocytes on the extension locus of chromosome 18. The MC1R gene has two types of mutations, in which either the C base is substituted for the T base in the 296th open reading frame (ORF) or the G base is deleted in the 310th ORF.[4,5] Based on gene analysis, these mutations can distinguish Korean cattle and non-Korean cattle according to their coat colors.

In this study, a simple allele-specific polymerase chain reaction (AS-PCR) was co-amplified for various different specific DNA fragments of Hanwoo AS-PCR primers (*i.e.* MC1R 310G del, MC1R 296Y SNP, LM215-1,2 C and LM215-1,2 D). The amplified AS-PCR products have conventionally been detected by electrophoresis on agarose gels.[6] Even though traditional slab gel electrophoresis enables parallel analysis, its throughput is limited by the slow speed of electrophoresis, approximately 1 - 2 h for a single run. Moreover, the sample and reagents, such as the agarose gel, are largely consumed in the process. Guttman *et al.* used ultra-thin-layer agarose gel electrophoresis in an attempt to overcome these weak points.[7] However, their method still has the disadvantages of low sensitivity and inaccurate detection of the PCR product size. Based on voltage programming, the four different specific single nucleotide polymorphism (SNP) markers of Korean cattle were simultaneously determined by capillary electrophoresis (CE) for fast and accurate identification. The CE with laser-induced fluorescence detection (LIF) system has the advantages of a short analysis time and small sample volume (< nL).[8-10] Furthermore, the high voltage applied in the capillary causes the heat to effectively diffuse outside of it, because the ratio of the surface area to the volume is very large. Although too high voltage causes Joule heating, which lowers the reproducibility and decomposes the biosamples, the voltage programming method can efficiently reduce the Joule heating and analysis time.[11-14]

The eighteen genomic DNA samples (nine Korean cattle and nine non-Korean cattle samples) obtained from Chungbuk National University were amplified using the designed primers (The Solgent Co., Ltd., MC1R 310G del, MC1R 296Y SNP, LM215-1,2 C and LM215-1,2 D) for the AS-PCR of Hanwoo, which showed DNA products with various lengths, viz. 165, 170, 207, 225, 240, 308, 422 and 647 bp (Table 1). The developed AS-PCR primers are formed with mutant DNA sequences to identify the SNPs of the specific cattle breeds by PCR. The MC1R gene has a mutation which converts leucine into proline. This conversion of the amino acid is due to the replacement of the T base (E^D) with the C base (E^+) at the 296th ORF. The other mutation has a frame shift which is caused by the deletion of the G base at the 310th ORF.[4,5] At the extension locus, three major alleles were previously reported (E^D: dominant, dominant black; E^+: intermediate, recessive black; e: recessive, red).[15] In this study, Korean cattle having a yellowish brown color were confirmed to have gene frequencies of E^+/e (0.05) and e/e (0.95),[15,16] which showed different values to other cattle breeds.[17] On the basis of this finding, primers were designed for the Korean cattle and non-Korean cattle.

We separated the standard 100-bp DNA ladder between 100 bp and 700 bp, because the amplified AS-PCR products have specific ranges of DNA lengths, viz. 165, 170, 207, 225, 240, 308, 422 and 647 bp (Table 1). In general, increasing the voltage increases the electroosmotic flow (EOF) and reduces the migration times, leading to shorter analysis times and increasing the efficiency of resolution.[18,19] However, higher voltages lead to higher currents and increased Joule heating that may lead to broader peaks, non-reproducible migration times, sample decomposition or denaturation.[18] Figure 1 shows the electropherograms obtained using the low constant field strength (LCFS), high constant field strength (HCFS) and voltage pro-

Table 1. Characteristics of Korean cattle (Hanwoo) and non-Korean cattle SNP marker primers used for allele-specific PCR

SNP marker	Product size (bp)	Primer sequence
MC1R 310G del	K[a] = 170, 308 NK[b] = 207, 308	F = CCATTGCCAAGAACCGCAACCT R = GATCCTCCACGCTCGGGGCAG aF = GCAGTCATGCCGCTGCTGGAGGCCAGT aR = ACGGCCGCCTGGGTGGCCAGGACAAG
MC1R 296Y SNP	K[a] = 225, 308 NK[b] = 165, 308	F = CCATTGCCAAGAACCGCAACCT R = GATCCTCCACGCTCGGGGCAG aF = AGCAACGTGCTGGAGACGGCAGTCATGAT aR = GGTGGCCAGGACACCGGCCTCCAGACATCG
LM215-1,2 C	K[a] = No* NK[b] = 422, 647	LM215-1 F = AGAAGATGTGGGTTCAATCACC LM215-1 R = CTCTTCCGGACACTGGGCTCTT LM215-2 F = GATGTGGGTTCAATCCCYGTGCC LM215-2 R = GCTTCAGCCTTTACTCTGTGAACC
LM215-1,2 D	K[a] = 240, 647 NK[b] = No*	LM215-1 F = CTATAGAAGATGTGGGTTCAATCACT LM215-1 R = TCCACACAATTCTTACCTGGTGG LM215-2 F = GATGTGGGTTCAATCCCYGTGCT LM215-2 R = GCTTCAGCCTTTACTCTGTGAACC

[a]K = Korean cattle (Hanwoo), [b]NK = non-Korean cattle, F = Forward primer, R = Reverse primer, aF = Allele forward primer, aR = Allele reverse primer. *No indicates no detection in CE-LIF.

gramming methods with respect to the migration time and resolution of the selected DNAs. Figure 1A demonstrates that the DNAs up to the 700 bp DNA were completely separated after 7.0 min using LCFS. Figure 1B shows that when HCFS was used, a short migration time of 2.1 min was obtained, but

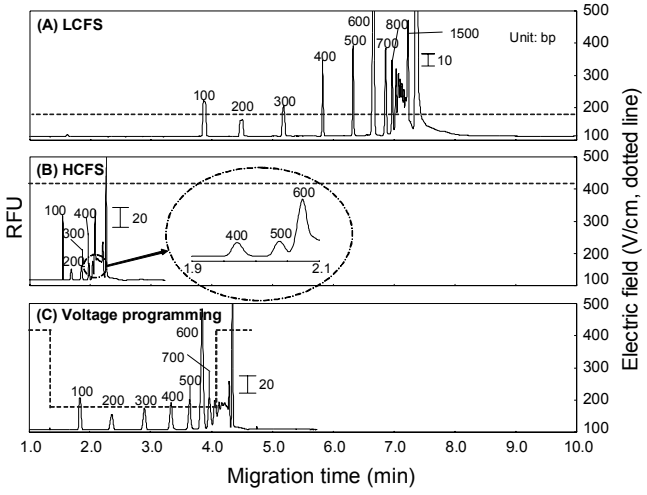

Figure 1. Comparisons of CE-LIF electropherograms for the 100-bp DNA ladder obtained using (A) LCFS, (B) HCHS, and (C) voltage programming. CE separation condition: Capillary, 75 μm I.D. fused-silica capillary; total length, 25 cm; effective length, 10 cm; excitation source, 5 mW He-Ne laser (543 nm); running buffer, 0.5 ppm EtBr in 1× TBE buffer (pH 8.45); coating gel, 1.0% PVP (M_r 1,000,000); sieving matrix, 0.8% PEO (M_r 8,000,000). 100-bp DNA ladder (Invitrogen) concentration, 25 ng/μL. Sample injection, electrokinetic injection at electric field strength of 120 V/cm for 10 s. Sample separation electric field: LCFS = 180 V/cm. HCFS = 420 V/cm. Voltage programming = 420 V/cm for 1.3 min, 180 V/cm for 2.9 min and 420 V/cm for 0.5 min. Indications: LCFS, low constant field strength; HCFS, high constant field strength. The blue dotted line represents the applied electric field. *RFU = relative fluorescence unit.

that not all of the DNAs up to 700 bp were completely separated. So, we attempted to find the optimum voltage programming condition for the baseline separation of all specific DNAs (*i.e.* 100 - 700 bp) in the 100-bp DNA ladder as follows: Before the first peak (100 bp DNA) appeared, we applied an HCFS of 420 V/cm to reduce the separation time to 1.3 min. Then, we applied an LCFS of 180 V/cm for 2.9 min for the base separation of the 100 - 700 bp DNAs. After the separation of the 700 bp DNA, the electric field strength was increased again to 420 V/cm for fast separation. Figure 1C indicates that all of the DNAs up to 700 bp were separated after 4.0 min using voltage programming, which was 3 min faster than LCFS. The DNAs over 800 bp exhibited poor resolution, but these resolutions were not important in this study, because the AS-PCR products of the SNP markers of Korean cattle have specific and distinct lengths within the range of 100 - 700 bp (Table 1).

When the nine Korean cattle samples were amplified using AS-PCR, the MC1R 310G del appeared at 170 and 308 bp, the MC1R 296Y SNP appeared at 225 and 308 bp, LM215-1,2 C gave no peak and LM215-1,2 D appeared at 240 and 647 bp. When the nine non-Korean cattle were amplified, the MC1R 310G del appeared at 207 and 308 bp, the MC1R 296Y SNP appeared at 165 and 308 bp, LM215-1,2 C appeared at 422 and 647 bp and LM215-1,2 D gave no peak. Figure 2 shows that all of the AS-PCR products with different DNA lengths (*i.e.* 165 - 647 bp) appeared in the electropherograms within 4 min under the optimum voltage programming conditions as follows: 420 V/cm for 1.3 min, 180 V/cm for 2.9 min and 420 V/cm for 0.5 min. The PCR products of Hanwoo were analyzed at least 15 - 30 times faster than that achieved using the conventional slab gel electrophoresis method. After the successful detection of the Korean and non-Korean cattle samples under the optimum separation conditions, the CE results showed 100% correlation for the cattle samples. Excellent reproducibility

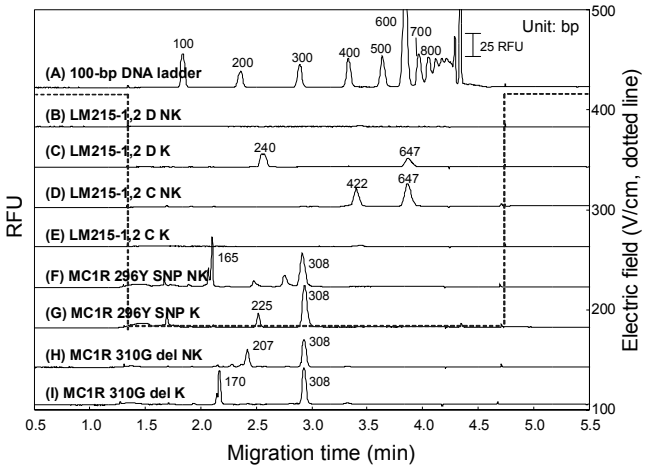

Figure 2. Representative electropherograms based on the voltage programming by CE-LIF of the various Korean cattle and non-Korean cattle SNP markers under the optimum separation conditions. CE separation conditions: sample separation electric field, 420 V/cm for 1.3 min, 180 V/cm for 2.9 min and 420 V/cm for 0.5 min. Indications: (A) = Standard 100-bp DNA ladder. (B) = LM215-1,2 D non-Korean cattle. (C) = LM215-1,2 D Korean cattle. (D) = LM215- 1,2 C non-Korean cattle. (E) = LM215-1,2 C Korean cattle. (F) = MC1R 296Y SNP non-Korean cattle. (G) = MC1R 296Y SNP Korean cattle. (H) = MC1R 310G del non-Korean cattle. (I) = MC1R 310G del Korean cattle. The other CE conditions are the same as those shown in Figure 1.

Table 2. The migration times and resolutions of the SNP markers of the Korean cattle (Hanwoo) and non-Korean cattle under optimum conditions based on voltage programming by CE

SNP marker	Length (bp)	Migration time (min)[a]	R_s[b]
MC1R 310G del Korean cattle	170	2.19 ± 0.02	4.19 ± 0.30
	308	2.92 ± 0.03	
MC1R 310G del non-Korean cattle	207	2.43 ± 0.01	3.01 ± 0.15
	308	2.93 ± 0.02	
MC1R 296Y SNP Korean cattle	225	2.50 ± 0.03	2.37 ± 0.28
	308	2.94 ± 0.03	
MC1R 296Y SNP non-Korean cattle	165	2.16 ± 0.02	3.97 ± 0.27
	308	2.98 ± 0.02	
LM215-1,2 C non-Korean cattle	422	3.40 ± 0.02	1.81 ± 0.23
	647	3.84 ± 0.02	
LM215-1,2 D Korean cattle	240	2.56 ± 0.03	5.89 ± 0.29
	647	3.82 ± 0.07	

[a]Migration time indicates the mean ± standard deviation (n = 5), [b]$R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1, t_2, w_1 and w_2 are the migration times and peak widths of the adjacent DNA peaks, respectively (n = 5).

and accuracy of the amplified AS-PCR products were obtained without any loss of resolution (R_s) for the different SNP markers of the Korean and non-Korean cattle (Table 2). In conclusion, we successfully developed a CE-LIF with voltage programming method for the determination of the SNP markers of Korean and non-Korean cattle, which correspond to the MC1R gene associated with the coat color. The unique SNP markers of closely related Hanwoo MC1R genes (*i.e.* MC1R 310G del, MC1R 296Y SNP, LM215-1,2 C and LM215-1,2 D) were obtained on a fused-silica capillary under voltage programming CE separation conditions within only 4 min. Based on the simultaneous detection of various AS-PCR products, this method can provide the key to the more accurate identification of Korean cattle within several minutes. This assay technique has the potential to be a powerful tool for the fast and simultaneous determination of SNP markers of various cattle breeds with high accuracy.

Experimental Section

Chemical and reagents. 1× TBE buffer (0.089 M Tris, 0.089 M borate, 0.002 M EDTA, pH 8.45) was prepared by dissolving a pre-mixed powder (Amresco®, Solon, OH, USA) in deionized water and further filtered with a 0.2 μm membrane-filter (Whatman®, Maidstone, England). Then, the buffer solution was photobleached for one day. A dynamic coating gel was made with 1.0% (w/v) polyvinylpyrrolidone (PVP, M_r 1,000,000) (Polyscience, Warrington, England) in 1× TBE buffer containing 0.5 μg/mL ethidium bromide (EtBr, Molecular Probes, Eugene, Oregon, USA). The mixture was shaken for 1 min and left to stand for 2 h to remove the bubbles. The sieving matrix was prepared by dissolving 0.8% (w/v) of PEO (M_r 8,000,000) (Sigma, St. Louis, MO, USA) in 1× TBE buffer together with 0.5 μg/mL EtBr with slow stirring overnight. The Korean cattle (Jeongeup, Korea) and non-Korean cattle (Michigan, USA) genomic DNA were provided by the Department of Animal Science, Chungbuk National University. The genomic DNA was extracted from the tissue using a QIAamp® DNA Micro Kit (Qiagen, Seoul, Korea). The 2× Multiplex PCR Premix and designed primers used for the amplification of the SNP markers of the Korean and non-Korean cattle were purchased from Solgent (Daejeon, Korea). A 100-bp DNA ladder (25 ng/μL) (Invitrogen, CA, Korea) was used to confirm the length of the PCR products.

Genomic DNA preparation. A total of eighteen Korean and non-Korean cattle DNA samples (each nine samples) were obtained from the Chungbuk National University in Korea. The genomic DNA was isolated from the tissues using a QIAamp® DNA Micro Kit, according to the manufacturer's instructions. Briefly, 180 μL of Buffer ATL was placed in a 1.5 mL microcentrifuge tube containing less than 10 mg of the tissue sample and equilibrated at room temperature (15 - 25 °C). 20 μL of Proteinase K was added, mixed by vortexing for 15 s and incubated at 56 °C overnight. Subsequently, 200 μL of Buffer AL and 200 μL of ethanol (96 - 100%) were added and incubated for 5 min at room temperature. The entire lysate was centrifuged at 8,000 rpm for 1 min and the flow-through discarded. Next, 500 μL of Buffer AW1 was added and centrifuged at 8,000 rpm for 1 min and the flow-through discarded. This step was performed one more time and the mixture centrifuged at 14,000 rpm for 3 min to dry the membrane to the center of which 20 - 100 μL Buffer AE or distilled water was applied. Finally, the 1.5 mL-microcentrifuge tube of lysate was incubated at room temperature for 1 min and centrifuged at 14,000 rpm for 1 min.

Allele-specific PCR sample preparation. The Korean and non-Korean cattle SNP markers were amplified by allele-specific PCR. MC1R 310G del, MC1R 296Y SNP, LM215-1,2 C and LM215-1,2 D primers were designed by Solgent (Deajeon, Korea) (Table 1). The total volume of the PCR reaction was 40 μL. The PCR mixture contained 3 μL of 50 - 100 ng template DNA, 20 μL of 2× Multiples PCR Pre-Mix, 3 μL of each of the MC1R 310G del, MC1R 296Y SNP, LM215-1,2 C and LM215-1,2 D primers (10 pmole/μL). The PCR mixture was used for the co-amplification of both specific target DNAs under the same PCR conditions. The PCR was performed in a MyGenie96 Thermal Block (Deajeon, Korea) using the following two temperature protocol: firstly, the temperature protocol of the MC1R 310G del and MC1R 296Y SNP primers PCR mixture: 15 min pre-denaturation at 95 °C, 30 cycles of denaturing at 95 °C for 20 s, annealing at 60 °C for 40 s, 72 °C for 1 min and then 1 cycle of extension at 72 °C for 1 min. Secondly, the temperature protocol of the LM215-1,2 C and LM215-1,2 D primers PCR mixture: 15 min pre-denaturation at 95 °C, 35 cycles of denaturing at 95 °C for 20 s, annealing at 68 °C for 1 min and then 1 cycle of extension at 72 °C for 3 min. The PCR products were analyzed using the CE-LIF system.

Capillary electrophoresis system with LIF detector. The laboratory-assembled CE setup with an LIF detector was described previously.[8,9,20,21] Briefly, a 5 mW He-Ne laser (λ_{ex} = 543.5 nm, λ_{em} = 610 nm; Melles Griot 05-LGR-193, CA, USA) coupled to a laser-induced fluorescence detector was used as the light source in this work. A Spellman 1000R high-voltage power supply (Spellman®, CZE1000R, NY, USA) was used for sample injection and separation in the electrophoresis. A 25 cm total length (10 cm effective length) and 75 μm I.D. bare fused-silica capillary (Polymicro Technologies Inc., AZ, USA) was utilized as the separation capillary. The running buffer was composed of 1× TBE buffer (pH 8.45) with 0.5 μg/mL EtBr. The dynamic coating gel and sieving matrix were sequentially injected hydrodynamically at one end of the capillary using a syringe for 5 min and 2.5 min, respectively. The sample was injected electrokinetically at 120 V/cm for 10 s. The applied electric field strength was in the range of 180 - 420 V/cm. After each run, the capillary was reconditioned before subsequent analysis by refilling it with sieving matrix for 2.5 min. The fluorescence signal was collected with a 10× objective lens (Nikon, Japan) into the photomultiplier module (H7732-10, Hamamatsu, Japan) and transferred to an Autochro data module. Data collection and treatment were performed using an Autochro® data system (Young Lin Instrument Co., Anyang, Korea) at 5 Hz.

Voltage programming for fast DNA separation. The voltage programming technique is used as an efficient separation method with a short separation time while maintaining reasonable resolution.[11-14] Firstly, before the first peak (100 bp DNA) appeared, we applied HCFS of 420 V/cm for 1.3 min. Then, for the separation of the 100 to 700 bp DNA fragments, we applied LCFS for 2.9 min. Finally, since the non target DNA fragments do not need to be separated, we applied HCFS for 0.5 min. The voltage programming method was designed in our lab using Labview™ (version 6.1, National instruments Co., Austin, USA).

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