Capillary Gel Electrophoretic Analysis of Cattle Breeds Based on Difference of DNA Mobility of Microsatellite Markers

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Received June 17, 2009, Accepted September 17, 2009

A breed of cattle, i.e., Korean cattle (Hanwoo), was identified based on the DNA mobilities of their microsatellites (MSs) by capillary gel electrophoresis (CGE) with a laser-induced fluorescence (LIF) detector. The MS markers were used for the accurate identification of species-specific genes. The DNA mobilities of the MS markers of Hanwoo and Holstein were measured using a CGE system with a fused-silica capillary (inner diameter of 75 μm, outer diameter of 365 μm, and total length of 50 cm). The capillary was dynamically coated with 1.0% (w/v) polyvinylpyrrolidone (M_r = 1,000,000) and then filled with a mixture of 1.3% (w/v) poly(ethylene oxide) (M_r = 600,000) and 1.9% (w/v) poly(ethylene oxide) (M_r = 8,000,000) as a sieving gel matrix. The species-specific genes of Hanwoo and Holstein were clearly distinguished within 33 min. This CGE assay technique is expected to be a useful analytical method for the fast and accurate identification of breeds of cattle.

Key Words: Cattle breed, Microsatellite, Capillary gel electrophoresis, DNA mobility

Introduction

Bovine spongiform encephalopathy (BSE) is a kind of transmissible spongiform encephalopathy (TSE) in cattle. A considerable amount of research is being conducted into this disease in a variety of bovines, because BSE can be transmitted to human beings in the form of its variant, Creutzfeldt-Jakob disease (vCJD).[1,2] BSE has arisen in many countries, including France (number of cases of BSE, 993), Germany (415), Japan (34), the United Kingdom (181 591), the United States of America (2) and many others according to the Office International des Epizooties (OIE). Due to the impact of the free trade agreement (FTA) and various changes in international trade, the import and export of bovine exposed to BSE has become a major risk. Therefore, the development of fast and accurate identification methods for species-specific genes of cattle is increasing.

According to the existing theory, the breed of Korean cattle called Hanwoo was formed by the hybridization of Bos Taurus (ethnic European) and Bos Zebu (ethnic Indian) about four thousand years ago.[3] It was introduced into Korea from Manchuria after hybridization in Outer Mongolia, a region of northern China. However, there is also an alternative theory based on microsatellite marker analysis, suggesting that it originated only from Bos Taurus.[4,5] Until now, breeds of Korean cattle have been distinguished based on their appearance, because it is not easy to establish a scientific detection method.[6]

Recently, the identification of cattle species has been accomplished using various methods, including restriction fragment length polymorphisms (RFLPs),[7,8] random amplified polymorphic DNAs (RAPDs),[9,10] amplified fragment length polymorphisms (AFLPs)[11,12] and simple sequence repeat polymorphisms (SSRs) or microsatellites (MSs).[13-15] These methods detect polymorphisms by assaying subsets of the total amount of DNA sequence variation in a genome. For example, RFLPs can be readily and directly detected as the differences in the length of DNA fragments after digestion with DNA sequence specific restriction endonucleases. However, RFLPs reveal the lack of polymorphisms for some species.[16,17] RAPDs use markers formed from 10 mer oligonucleotides of arbitrary sequences for the amplification of DNA fragments.[17,18] Oh *et al.* showed accuracy of RAPDs method that the specific band is absent in 644 of 673 Hanwoo (96%), but present in 245 of 256 Holstein (95%).[10] Although the accuracy of RAPDs is high, it shows low reproducibility on account of the sensitivity of PCR amplification.[11] AFLPs use restriction enzymes to cut genomic DNA, followed by the ligation of the adaptors to the sticky ends of the restriction fragments.[19] AFLPs are highly sensitive tool, and besides the reproducibility of AFLPs is higher than that of RAPDs. However, both of these techniques are dominant and low informative.[12,17] MSs are polymorphic loci present in nuclear and organellar DNA that consist of repeating units consisting of 1-6 base pairs.[13-15] MS markers are particularly useful due to their high diversity and ease of detection by polymerase chain reaction (PCR). They are most abundant in eukaryotic genomes and uniformly distributed along a genome. SSRs or MSs are highly variable and codominant, therefore their information value is higher than that of other markers such as RFLPs, RAPDs and AFLPs.[20,21] Also, a in previous investigation we identified Honwoo within 4 min using the single nucleotide polymorphism (SNP) markers.[22] Although the SNP method showed a fast analysis time, its accuracy was low comparable to that obtained using MS markers (~99%).[23]

In this study, as a model case, we analyzed the genetic differences between Hanwoo and Holstein by the gene analysis

Table 1. Information of the MS markers analyzed

Locus	Number of Alleles (Hanwoo)	Size range (bp)	Primer Sequence (5'-3')
ETH3	6	103-131	F GAA CCT GCC TCT CCT GCA TTG G R ACT CTG CCT GTG GCC AAG TAG G
ETH225	6	138-168	F GAT CAC CTT GCC ACT ATT TCC T R ACA TGA CAG CCA GCT GCT ACT
BM1824	6	178-194	F GAG CAA GGT GTT TTT CCA ATC R CAT TCT CCA ACT GCT TCC TTG
TGLA126	7	111-129	F CTA ATT TAG AAT GAG AGA GGC TTC T R TTG GTC TCT ATT CTC TGA ATA TTC C
TGLA122	8	134-190	F CCC TCC TCC AGG TAA ATC AGC R AAT CAC ATG GCA AAT AAG TAC ATA C
TGLA227	4	78-106	F CGA ATT CCA AAT CTG TTA ATT TGC T R ACA GAC AGA AAC TCA ATG AAA GCA
BM2113	7	116-150	F GCT GCC TTC TAC CAA ATA CCC R CTT CCT GAG AGA AGC AAC ACC
ETH10	6	210-226	F GTT CAG GAC TGG CCC TGC TAA CA R CCT CCA GCC CAC TTT CTC TTC TC
SPS115	7	245-261	F AAA GTG ACA CAA CAG CTT CTC CAG R AAC GAG TGT CCT AGT TTG GCT GTG

F = forward primer. R = reverse primer.

technique using MS markers. A 2-bp DNA allelic ladder was amplified by five MS markers (TGLA227, BM2113, TGLA53, ETH10, and SPS115). Both Hanwoo and Holstein were amplified by the nine MS markers shown in Table 1. These were selected as the best out of the many MS markers which are recommended for bovine paternity tests by the International Society for Animal Genetics (ISAG).[24,25] The selected MS markers gave rise to high polymorphism and species-specific bands for Hanwoo and Holstein.[23] After the PCR amplification of the MS markers, CGE analysis based on DNA mobility using an LIF detector was performed for the highly sensitive and selective detection of Hanwoo genes.

Experimental Section

Chemicals and reagents. The single strength (1×) TBE buffer solution was prepared by dissolving a pre-mixed powder (Amresco, Solon, OH, USA) containing 0.089 M Tris, 0.089 M borate and 0.002 M EDTA (pH 8.45) in deionized water. The dynamic coating matrix for the gel electrophoresis was made by dissolving 1.0% (w/v) of polyvinylpyrrolidone (PVP, M_t = 1,000,000) (Polyscience, Warrington, England) in the 1× TBE buffer along with 0.5 μg/mL ethidium bromide (EtBr, Molecular Probes, Eugene, Oregon, USA). The solution was subjected to rocking until the bubbles disappeared. The sieving matrices were made by dissolving 1.3% (w/v) of poly(ethylene oxide) (PEO, M_t = 600,000) and 1.9% (w/v) of PEO (M_t = 8,000,000) (Sigma, St. Louis, MO, USA) in the 1× TBE buffer along with 0.5 μg/mL EtBr. The mixed polymer was stirred vigorously at first and then slowly overnight to obtain a homogeneous mixture.

Preparation of samples. Hanwoo and Holstein DNA samples were received from the National Institute of Animal Science in Korea. They consisted of genomic DNA extracted from the muscles using a wizard genomic DNA purification kit (Promega, Madison, Wisconsin, USA) and amplified by PCR. The PCR reaction mixture consisted of a total volume of 15 μL with 1.5 μL of 10× reaction buffer, 1.5 μL of dNTPs mixture containing a concentration of 2.5 mM, 8.25 μL of forward and reverse primer mix, 0.375 μL of HotStart DNA Polymerase (Bioneer, Daejeon, Korea), 1.175 μL of distilled water, and 1.95 μL of the purified DNA sample. The PCR profile was as follows: one cycle of incubation at 95 °C for 15 min and 5 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 75 s, extension at 72 °C for 60 s and 5 cycles of denaturation at 94 °C for 60 s, annealing at 57 °C for 75 s, extension at 72 °C for 60 s and 25 cycles of denaturation at 94 °C for 60 s, annealing at 56 °C for 75 s, extension at 72 °C for 60 s and extension at 65 °C for 30 min. The resulting solution was kept at 8 °C.

Capillary gel electrophoretic identification. The experimental home-made CGE system with LIF detector was similar to that described previously.[22] Briefly, a diode-pumped solid-state laser (λ_{ex} = 532 nm; Power Technology Inc., Alexander, AR, USA) was used as the light source in the CGE system, which was coupled to an LIF detector. A Bertan ARB 30 high-voltage power supply (Bertan High Voltage Inc., New York, USA) was used to drive the electrophoresis. A 50 cm total length (30 cm effective length), 75 μm inner diameter, and 365 μm outer diameter bare fused-silica capillary (Polymicro Technologies, LLC, Phoenix, AZ, USA) was used as a separation capillary. The

running buffer was made up of 1× TBE buffer along with 0.5 μg/mL EtBr. Both the coating and sieving matrix were hydrodynamically injected at one end of the capillary through a syringe. After the sample was injected electrokinetically at 60 V/cm for 20 s, sample separation was performed in an electric field of 120 V/cm. After each run, the capillary was reconditioned before the next analysis by rinsing it successively in water, 0.1 M NaOH, water and the running buffer. The data was recorded as a function of time during the CGE, and saved on a Samsung computer (1.50-GHz Mobile Pentium M) at 5 Hz. Data output and analysis were performed using Lab view (version 6.1, National Instruments, Austin, USA).

Results and Discussion

We carried out the separation of a 2-bp DNA allelic ladder with a size range of 82 - 257 bp as the standard for the accurate identification of Hanwoo. The separation conditions such as the capillary effective length, electric field, composition and concentration of the separation media were varied to determine the optimum conditions. Increasing the capillary effective length was found to increase the resolution, but gave rise to broader peaks and an increase of the migration time (Fig. 1). The resolution can be enhanced by optimizing the capillary effective length (i.e., R_s of peaks 23 and 24 = 1.45 and R_s of peaks 24 and 25 = 1.77 with an effective length of 30 cm). Increasing the electric field strength increased the velocity of the DNA fragments and reduced the migration times, leading to shorter analysis times (Fig. 2). However, higher voltages led to higher currents and increased the Joule heating. Increased heat in the capillary may lead to broader peaks, non-reproducible migration times, sample decomposition or even the boiling of the buffer, which can cause electrical discontinuity throughout the capillary, thereby shutting down the CGE system. 251, 253 and 257 bp DNAs (i.e., peaks 53, 54 and 55) were not separated when using electric fields of over 120 V/cm. By using an electric field of 120 V/cm, however, the DNA molecules can be separated into three peaks within a relatively short time.

Because adsorption may cause broad and tailing peaks,[26] the capillary inner wall was treated to eliminate electroosmotic flow and minimize adsorption by dynamically coating it with 1.0% (w/v) PVP (M_r = 1,000,000). In a CGE system, the composition and concentration of the separation media have an effect on the separation of the DNA molecules. There are pores within these gels and, as the charged solutes migrate through the gel-filled capillary, they are separated by a molecular sieving mechanism on the basis of their sizes.[27] Firstly, we investigated the molecular sieving mechanism using a gel polymer with a single molecular weight. However, we could not obtain good separation results (data not shown). Therefore, mixed sieving gel polymers with different molecular weights were used to separate the DNA molecules with a size range of 82 - 257 bp. This is because short-chain polymers interact preferentially with small DNA molecules, while long-chain polymers interact preferentially with large DNA molecules.[28] In the mixed gel polymer system, increasing the molecular weight of the polymer gave rise to an increase in the resolution (Figs. 3 and 4). The

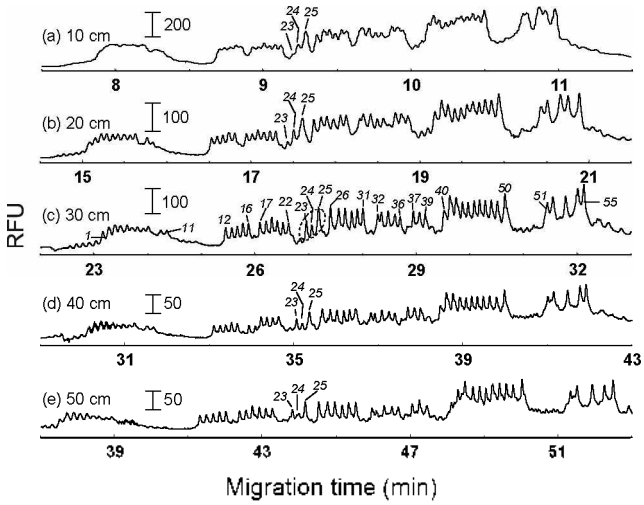

Figure 1. Representative CGE electropherograms of the PCR products of the 2-bp DNA allelic ladder with various DNA sequences of TGLA227, BM2113, TGLA53, ETH10 and SPS115. CGE separation conditions: fused-silica capillary, 75 μm I.D. × 365 μm O.D.; total lengths, 30, 40, 50, 60 and 70 cm; effective lengths, 10, 20, 30, 40 and 50 cm; running buffer, 1× TBE buffer (pH 8.45) with 0.5 ppm EtBr; coating gel matrix, 1.0% PVP (M_r = 1,000,000) in running buffer; sieving gel matrix, a mixture of 1.3% PEO (M_r = 600,000) and 1.9% PEO (M_r = 8,000,000) in running buffer; sample injection, electrokinetic injection for 20 s at 60 V/cm; applied separation electric field, 120 V/cm. RFU: relative fluorescence unit. *Indicate: 1 = 82 bp, 11 = 105 bp, 12 = 125 bp, 23 = 154 bp, 24 = 156 bp, 25 = 158 bp, 39 = 192 bp, 40 = 209 bp, 50 = 228 bp, 51 = 246 bp, and 55 = 257 bp.

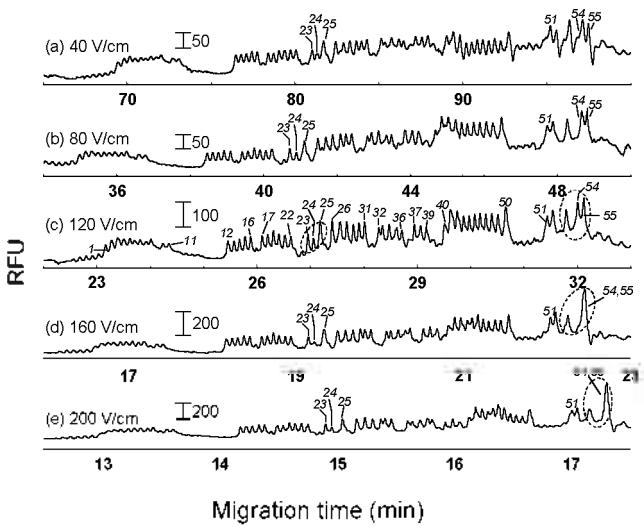

Figure 2. CGE electropherograms describing the effect of the electric field on the separation. CGE separation conditions: fused-silica capillary, 75 μm I.D. × 365 μm O.D., total length 50 cm, effective length 30 cm; applied separation electric fields, 40, 80, 120, 160 and 200 V/cm. The other conditions used were the same as those shown in Fig. 1.

differences in the resolutions of the 154, 156 and 158 bp DNAs (i.e., peaks 23, 24 and 25) are shown in Table 2. However, using too high a molecular weight led to broader peaks and non-reproducible migration times. As a result, we carried out the experiments with a gel mixture consisting of 1.3% (w/v) PEO

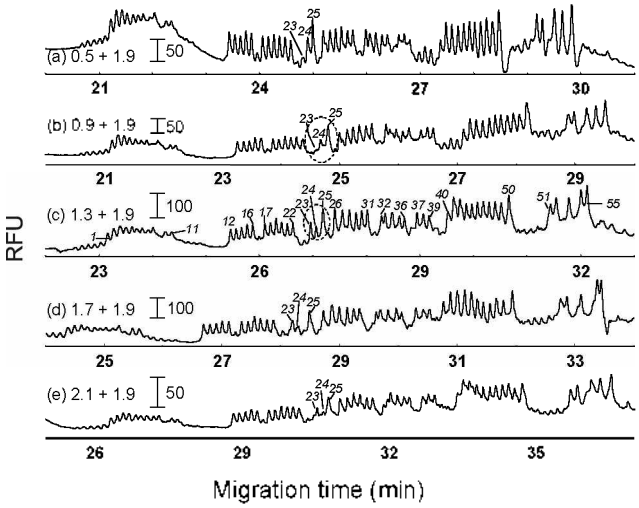

Figure 3. CGE electropherograms describing the effect of the PEO (M_r = 600,000) concentration on the separation. CGE separation conditions: applied separation electric field, 120 V/cm, sieving gel matrix concentrations, 0.5%, 0.9%, 1.3%, 1.7% and 2.1% PEO (M_r = 600,000) + 1.9% PEO (M_r = 8,000,000) in running buffer. The other conditions used were the same as those shown in Fig. 2.

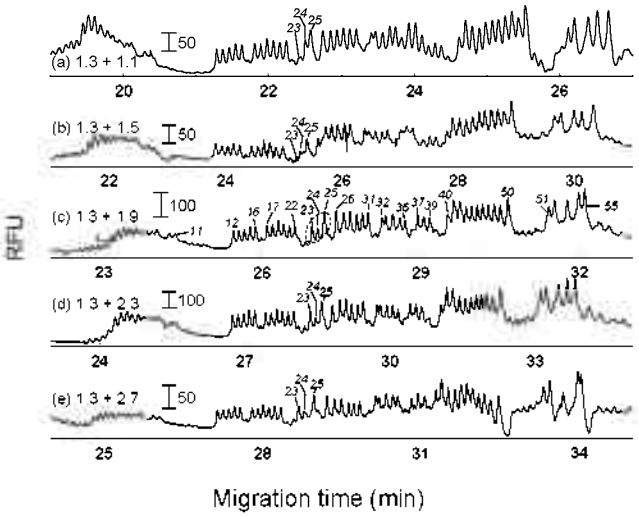

Figure 4. CGE electropherograms describing the effect of the PEO (M_r = 8,000,000) concentration on the separation. CGE separation conditions: sieving gel matrix concentrations, 1.3% PEO (M_r = 600,000) + 1.1%, 1.5%, 1.9%, 2.3% and 2.7% PEO (M_r = 8,000,000) in running buffer. The other conditions used were the same as those shown in Fig. 3.

(M_r = 600,000) and 1.9% (w/v) PEO (M_r = 8,000,000) as the optimum sieving gel matrix.

The optimum conditions of the sieving gel matrix for the 2-bp DNA allelic ladder were found to be a total length of 50 cm, electric field of 120 V/cm, and a mixture of 1.3% (w/v) PEO and 1.9% (w/v) PEO. Table 3 shows the migration times (mean ± standard deviation, n = 3) of the 2-bp DNA ladder in the experiment conducted to confirm the reproducibility, for which the standard deviations were less than 0.04. A serial gap of 2-bp DNA allelic ladder was separated with a migration time interval of 8 s. Under the optimum separation conditions,

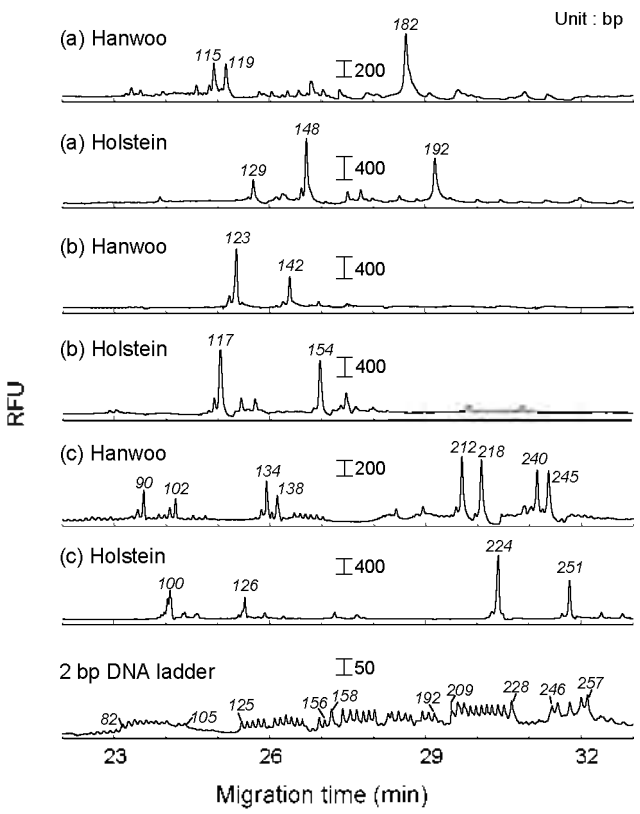

Figure 5. Representative CGE electropherograms of the PCR products of Hanwoo and Holstein. CGE separation conditions: sieving matrix, a mixture of 1.3% PEO (M_r = 600,000) and 1.9% PEO (M_r = 8,000,000) in running buffer. The other conditions used were the same as those shown in Fig. 4. MS markers (a) ETH3, ETH225, BM1824; (b) TGLA126, TGLA122; (C) TGLA227, BM2113, ETH10, SPS115.

Table 2. Resolutions (R_s) of 154, 156 and 158 bp DNAs (i.e., peaks 23, 24 and 25) at various sieving gel concentrations of the mixture polymer of PEO

PEO (%)[a]	R_s of peaks 23 and 24	R_s of peaks 24 and 25
0.5/1.9	-	1.26
0.9/1.9	-	1.63
1.3/1.9	1.52	1.77
1.7/1.9	1.14	1.98
2.1/1.9	2.25	1.35
1.3/1.1	-	0.95
1.3/1.5	0.96	1.51
1.3/1.9	1.52	1.77
1.3/2.3	1.54	1.65
1.3/2.7	0.94	1.64

[a]The PEO (%) indicates the ratio of PEO (M_r = 600,000) and PEO (M_r = 8,000,000).

the DNA molecules of Hanwoo and Holstein amplified with their corresponding MS markers (i.e., a = 3, b = 2 and c = 4) were separated within 33 min through the CGE-LIF system (Fig. 5). A breed of Hanwoo was identified based on Table 4 which shows a database of the gene frequencies of Hanwoo

Table 3. The migration times of the 2-bp DNA ladder in the sieving gel matrix containing a mixture of 1.3% PEO (M_r = 600,000) and 1.9% PEO (M_r = 8,000,000)

Locus	Size (bp)	Migration time (min)[a]	Size (bp)	Migration time (min)[a]
TGLA227	82	25.43 ± 0.04	105	26.81 ± 0.03
BM2113	125	28.00 ± 0.04	156	29.77 ± 0.05
TGLA53	158	29.88 ± 0.03	192	31.93 ± 0.09
ETH10	209	32.71 ± 0.03	228	34.03 ± 0.02
SPS115	246	35.05 ± 0.02	257	35.86 ± 0.02

[a]Migration time shows the mean ± standard deviation (n = 3).

Table 4. Gene frequencies of Hanwoo and Holstein

Locus	Size (bp)	Hanwoo	Holstein
ETH3	115	0.0185	
	117	0.3056	0.5750
	119	0.2778	
	121	0.0833	
	125	0.1389	0.0750
	127	0.1759	0.2000
	129		0.1500
ETH225	140		0.0833
	142	0.0833	0.0278
	144	0.4352	0.0556
	146	0.1481	0.0833
	148	0.1944	0.4167
	150	0.1296	0.2778
	152		0.0556
	158	0.0093	
BM1824	180	0.0093	0.1500
	182	0.4167	0.2250
	184	0.4074	0.2500
	186	0.0278	
	190	0.1296	0.3500
	192	0.0093	0.0250
TGLA126	111	0.0172	
	115	0.2759	0.4500
	117	0.1724	0.4250
	119	0.0172	
	121	0.2069	0.1250
	123	0.2069	
	125	0.1034	
TGLA122	134	0.0921	
	138	0.0263	
	140	0.0789	
	142	0.1842	
	144	0.3553	0.2750
	150	0.1974	0.1000
	152	0.0526	0.1000
TGLA122	154		0.0250
	160	0.0132	
	164		0.0250
	166		0.3000
	172		0.0250
	174		0.0500
	184		0.1000
TGLA227	78	0.2791	
	86	0.1512	0.1000
	90	0.3488	0.0250
	94	0.2209	0.2500
	100		0.6250
BM2113	126	0.0093	0.2750
	128	0.0833	0.1500
	134	0.0556	
	136	0.2130	0.4000
	138	0.2500	0.1000
	140	0.3796	0.0750
	142	0.0093	
ETH10	212	0.0278	
	214		0.0250
	218	0.2870	0.1500
	220	0.2500	0.6250
	222	0.1759	0.0250
	224	0.0556	0.1250
	226	0.2037	0.0500
SPS115	245	0.0093	
	247	0.4259	0.5625
	249	0.0833	
	251	0.1204	0.2188
	253	0.0741	
	255	0.1574	0.1250
	257		0.0938
	259	0.1296	

and Holstein for each MS marker.[23] Because breeds of cattle have variable polymorphic loci, Hanwoo was able to be distinguished from the other breed through the CGE-LIF assay method based on the DNA mobilities of the MSs.

Conclusions

The genetic differences between two cattle breeds, Korean cattle (Hanwoo) and Holstein, were identified by a gene analysis technique using MSs based on their capillary gel electrophoretic DNA mobilities determined by CGE-LIF detection. The species-specific genes were separated within 33 min using the CGE-LIF detector under the optimum conditions, viz. an electric field of 120 V/cm; fused-silica capillary with an inner diameter of 75 μm, outer diameter of 365 μm and total length of 50 cm dynamically coated with 1.0% (w/v) PVP (M_r = 1,000,000); sieving gel matrix consisting of a mixture of 1.3% (w/v) PEO (M_r = 600,000) and 1.9% (w/v) PEO (M_r = 8,000,000). Although MSs are useful in the identification of species-specific genes, it is not easy to achieve the baseline separation of DNA molecules with a serial gap of 2-bp DNA. However, the use of mixed sieving gel polymers with different molecular weights showed good separation results, which enabled us to identify the species-specific genes of Hanwoo and Holstein. The CGE-LIF assay method based on the DNA mobilities of the MSs can be used for a wide range of applications such as the diagnosis of diseases and the differentiation of genetic polymorphisms of species, as well as the identification of cattle breeds.

Acknowledgments. This work was supported by a grant (Code 20070501034006) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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