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Abstract

The Hanwoo traceability system currently utilizes 11 dinucleotide repeat microsatellite (MS) markers. However, dinucleotide repeat markers are known to have a high incidence of PCR artifacts, such as stutter bands, which can complicate the accurate reading of alleles. In this study, we examined the polymorphisms of the 11 dinucleotide repeat MS markers currently employed in traceability systems. Additionally, we explored four trinucleotide repeat MS markers and one tetranucleotide repeat MS marker in a sample of 1,106 Hanwoo cattle. We also assessed the potential utility of the tri- and tetranucleotide repeat MS markers. The polymorphic information content (PIC) of the five tri- and tetranucleotide repeat markers ranged from 0.663 to 0.767 (mean: 0.722), sufficiently polymorphic and slightly higher than the mean (0.716) of the current 11 dinucleotide repeat markers. Using all 16 markers, the mean PIC was 0.718. The estimated probability of identity (PI) was 3.13×10^{-12} using the 11 dinucleotide repeat markers, 7.03×10^{-6} using the five tri- and tetranucleotide repeat markers, and 2.39×10^{-17} using all 16 markers; the respective $PI_{half\text{-}sibs}$ values were 2.69×10^{-9}, 1.29×10^{-4}, and 3.42×10^{-13}; and the respective PI_{sibs} values were 3.89×10^{-5}, 9.6×10^{-3}, and 3.69×10^{-7}. The probability of exclusion$_1$ (PE_1) was 0.999864 for the 11 dinucleotide repeat markers, 0.981141 for five of the tri- and tetranucleotide repeat markers, and > 0.99 for all 16 markers; the respective PE_2 values were 0.994632, 0.901369, and > 0.99; and the respective PE_3 values were 0.998702, > 0.99, and > 0.99. The five investigated tri- and tetranucleotide repeat MS markers can be used in combination with the 11 existing MS markers to improve the accuracy of individual identification and paternity testing in Hanwoo.

Keywords: Hanwoo, microsatellite, probability of exclusion, probability of identification

Introduction

Hanwoo cattle are an indigenous Korean livestock recognized for their unique genetic characteristics and pure bloodline distinguishable from exotic beef species. Hanwoo are being improved at the national level; excellent Korean-proven bulls (KPNs) are selected through the Hanwoo National Genetic Evaluation Program, and their semen is distributed to farms [1, 2].

Hanwoo meat is managed through a traceability system, and consumers are provided historical farm-to-table information [3]. Korean traceability began with a pilot project in 2004, was promoted in 2008, and enacted and implemented as the Cattle and Beef Traceability Act in 2010. In 2014, it was revised to the Livestock and Livestock Products Traceability Act. The administrative rules of this act include the DNA Identification Methods for Livestock and Livestock Product Identification, which defines 11 dinucleotide repeat microsatellite (MS) markers used in DNA identity testing.

MS markers are short sequence repeats of 1–6 bp, which have proven valuable for studying variation within and between breeds. The Food and Agriculture Organization of the United Nations (FAO) and the International Society for Animal Genetics (ISAG)–FAO Advisory Group proposed 30 MS markers for each of the nine major livestock species, including cattle, and recommended their use in genetic diversity studies [4].

While the continued development and commercialization of genetic analysis methods using high-density DNA microarrays has highlighted the accuracy and importance of studying paternity and genetic diversity using single-nucleotide polymorphisms (SNPs), MS markers are the most efficient means of identifying individuals and analyzing paternity and population relationships. In Hanwoo, MS markers are used mainly to improve the accuracy of pedigree through paternity testing. Currently, the Hanwoo Improvement Center provides MS marker information for paternity verification of KPNs, and the Korea Animal Improvement Association uses MS markers to mark individuals whose paternity testing has been completed. Securing and managing accurate pedigrees enables accurate evaluation of the genetic performance of individuals.

Parentage testing using genotypes such as MS presupposes that the data an individual possesses comes from its sire and dam. However, if an error occurs in genotyping, the actual paternity may be incorrectly excluded. Genotyping errors can occur due to stutter, null alleles, contamination, human error, among other factors. In fact, increasing the number of markers used for paternity determination without accommodating such errors may increase false exclusion [5].

Research on genetic diversity using MS markers in various livestock breeds and populations is ongoing [6-

9]. In Hanwoo cattle, MS markers with three or more sequence repeats have been developed to improve the reliability and accuracy of individual identification and paternity testing [10, 11]. Simple sequence repeats (SSRs), including MSs, are subject to PCR artifacts, such as stutter bands and differential amplification, which can confound estimates of allele frequency. Stutter is prevalent with dinucleotide repeats, but less in tri- and tetranucleotide repeats [12, 13].

The three or more nucleotide repeat markers studied in previous research have low discriminatory power due to a limited number of multiplex loci and are not configured for multiplex PCR with the dinucleotide markers currently used in the traceability system. Therefore, we investigated both the existing 11 dinucleotide repeat markers and new tri- and tetranucleotide repeat markers, which are enable for multiplex PCR, assessing their utility for individual identification and paternity testing in Hanwoo.

Materials and methods

Animals

The 1,106 Hanwoo cattle utilized in this study were bred at the Hanwoo Research Institute of the National Institute of Animal Science, comprising 367 females and 739 males, all born between 2006 and 2022. DNA analysis was conducted on blood or ear tissue samples collected from each individual.

MS marker information

This study investigated 11 dinucleotide repeat markers currently employed in the Hanwoo traceability system, along with four trinucleotide repeat markers and one tetranucleotide repeat marker previously investigated by Sim [14]. The selection of the five new markers was based on Sim's research [14], specifically focusing on markers with a Power of Discrimination (PD) value exceeding 0.76 that can be multiplexed with the existing 11 dinucleotide repeat markers. For primer information, refer to the studies by Seilsuth et al. [15] and Sim [14]. Additional details are provided in Table 1.

DNA extraction

First, 10 mg of tissue sample was placed in a 96-deep-well plate and lysed with 400 μL of lysis buffer (20 mM Tris-HCl, pH 8.0; 50 mM NaCl; 10 mM EDTA, pH 8.0; and 0.2% sodium dodecyl sulfate) with 20 μL of proteinase K (20 mg/mL) for 6 hours at 55°C. Then, 800 μL of binding buffer (6M GuHCl; 10 mM Tris-HCl, pH 6.1; and 20 mM EDTA, pH 6.1) was added to each sample. Finally, 100 μL of silica-coated magnetic beads was added and mixed. The magnetic beads in each well were washed twice with 800 μL of 80% ethanol. DNA was eluted in 110 μL of TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). The purified DNA was stored at −20°C.

PCR amplification

Multiplex amplification was carried out in a final volume of 15 μL containing 20 ng of template DNA, 2 units of hot-start Taq polymerase (GenetBio, Daejeon, Korea), 1.5 μL of 10× Reaction buffer (with 20 mM $MgCl_2$), 200 μM of each dNTP, 8.25 μL of 11 dinucleotide repeat markers fluorescence-labeled primer, and 0.2 μL (10 pM/μL) each tri- and tetranucleotide repeat marker fluorescence-labeled primer. The PCR steps included: initial denaturation at 94°C for 10 minutes; nine cycles of 60 seconds at 94°C, 75 seconds at 60°C, and 60 seconds at 72°C; 5 cycles of 60 seconds at 94°C, 75 seconds at 57°C, and 60 seconds at 72°C; 25 cycles of 60 seconds at 94°C, 75 seconds at 55°C, and 60 seconds at 72°C; and final extension for 30 minutes at 65°C. The DNA was amplified in a ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in 96-well PCR plates.

Genotyping

The alleles were genotyped on a 3730xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) using POP-7™ Polymer (Thermo Fisher Scientific, Waltham, MA, USA) and 36-cm capillaries. Next, 1/20 of the amplified PCR product and 0.05 µL of GeneScan™ LIZ™ 500 size standard was prepared in 10 µL of Hi-Di™ formamide (Thermo Fisher Scientific, Waltham, MA, USA). The samples were denatured for 2 minutes at 96°C, followed by rapid cooling on ice. The alleles were resolved using GeneMapper™ Software 5.0 (Thermo Fisher Scientific, Waltham, MA, USA).

Data analysis

Cervus version 3.0.7 [16, 17] and GenAlEx version 6.4 [18, 19] were used to calculate allele counts and frequencies, observed (H_{obs}) and expected (H_{exp}) heterozygosity, and F-values (fixation index, inbreeding coefficient) for the markers. The polymorphic information content (PIC) and Hardy-Weinberg equilibrium tests for the markers were calculated using Cervus version 3.0.7 [16, 17]. The probability of identity (PI) of the markers was calculated using API-CALC version 1.0 [20] and the probability of exclusion (PE) was calculated using GenAlEx version 6.4 [18, 19]. F-statistics for the PI value estimation were calculated using GENEPOP version 4.7.3 [21, 22], and scored genetic data used in GENEPOP version 4.7.3 [21, 22] and GenAlEx version 6.4 [18, 19] were converted to Microsatellite analyzer (MSA) version 4.05 [23].

Results and discussion

Polymorphism analysis of MS markers

Table 2 shows the results of the polymorphism analysis of 16 MS markers in 1,106 Hanwoo. The number of alleles for the 16 markers ranged from 5 to 14 (mean: 9.438). The 11 dinucleotide repeat markers currently used for DNA identity testing ranged from 5 (*ETH3*) to 14 allels (*TGLA227* and *TGLA53*) (mean: 9.182). The number of alleles for the five tri- and tetranucleotide repeats markers ranged from 8 (*B9S5866*) to 13 (*B8S7996*) (mean: 10).

The 16 markers had H_{obs} values of 0.662–0.863 (mean: 0.759) and H_{exp} values of 0.66–0.843 (mean: 0.754). *ETH225* had the lowest H_{obs} and H_{exp} values, and *TGLA122* the highest, both dinucleotide repeat markers. The 11 dinucleotide repeat markers had mean H_{obs} and H_{exp} values of 0.753 and 0.752, respectively. The five tri- and tetranucleotide repeats markers had H_{obs} values of 0.737 (*B12S5209*) to 0.810 (*B3S0990*) (mean: 0.77), and H_{exp} values of 0.714 (*B9S5866*) to 0.794 (*B3S0990*) (mean: 0.759).

For the PIC, the dinucleotide repeat markers had values of 0.611 (*ETH225*) to 0.823 (*TGLA122*) (mean: 0.716). The tri- and tetranucleotide repeat markers had PIC values of 0.663 (*B9S5866*) to 0.767 (*B3S0990*) (mean: 0.722). The PIC values were slightly higher for the tri- and tetranucleotide repeat markers than the dinucleotide repeat markers, but all were above 0.5. PIC is calculated as the number and frequency of alleles, and lies within the range of 0–1. PIC values are indicative of more informative markers [24], where markers with values above 0.5 are classified as very informative [25]. Therefore, all 16 MS markers used in this study had sufficient polymorphism and were suitable for analyzing the genetic diversity of Hanwoo. The frequency of each allele is presented in the Supplementary table.

Probability of identity and probability of exclusion

Table 3 lists the PI and PE values calculated using combinations of the 11 dinucleotide repeat markers, of the five tri- and tetranucleotide repeat markers, and of all 16 markers. PI is the probability that the genotypes of two unrelated individuals in a randomly mated population are the same. $PI_{half\text{-}sibs}$ and PI_{sibs} are the probabilities that two individuals have the same genotype in the half-sib and full-sib groups, respectively. If these values are high, there is a high probability that the genotypes of the markers used to distinguish the individuals are the same; this means that the usability as an entity identification marker is low. As the number of markers used increases, the genotype difference between the two individuals to be distinguished increases, so the probability of identity decreases; as a result, the ability to distinguish individual increases. Therefore, it is necessary to find an appropriate number of genetic marker combinations with high discrimination power and use them for individual identification [26].

In this study, the estimated average PI values were 3.13×10^{-12} using the existing 11 dinucleotide repeat markers, 7.03×10^{-6} using the five tri- and tetranucleotide repeat markers, and 2.39×10^{-17} using all 16 markers; the respective $PI_{half\text{-}sibs}$ values were 2.69×10^{-9}, 1.29×10^{-4}, and 3.42×10^{-13}; and the respective PI_{sibs} values were 3.89×10^{-5}, 9.6×10^{-3}, and 3.69×10^{-7}. The cumulative PI was estimated to be 4.81×10^{-12} when using 11 markers, 9.43×10^{-6} when using five markers, and 4.15×10^{-17} when using all 16 markers.

PE aids in establishing the requisite number of loci for paternity tests. Within a population, a higher concordance percentage of markers between a sire (or dam) and offspring increases the confidence that they are related. A discrepancy in the genetic makeup between an individual and its purported parents amplifies PE. PE_1, PE_2, and PE_3 are specific metrics that gauge the likelihood of excluding a certain parentage type. Pedigrees usually come from both the sire and dam. The rejection chance of MS markers for sire is used to challenge a sire's claim by comparing the dam-offspring genotypes and a potential sire (PE_1). When the genetic information of one parent isn't available, PE_2 represents the exclusion chance. If an offspring's origin is wrongly linked to two parents and their genetic data is examined, the likelihood of denying their relationship can be estimated using PE_3 [18, 19, 27, 28].

In this study, PE_1 was 0.999864 when using the 11 dinucleotide repeat marker combination, 0.981141 for the five tri- and tetranucleotide repeat marker combination, and > 0.99 for all 16 markers; the respective PE_2 values were 0.994632, 0.901369, and > 0.99; and the respective PE_3 values were 0.998702, > 0.99, and > 0.99.

In 163 Hanwoo, Lim et al. [29] reported PI and $PI_{half\text{-}sibs}$ values of 1.55×10^{-14} and 4.10×10^{-10} calculated from 11 MS markers and 1.09×10^{-17} and 1.42×10^{-10} from nine MS markers, respectively. Furthermore, in 480 Hanwoo, Lim et al. [30] reported PI, $PI_{half\text{-}sibs}$, and PI_{sibs} values of 3.43×10^{-27}, 4.18×10^{-19}, and 3.98×10^{-8} calculated from 14 MS markers and 2.09×10^{-24}, 4.69×10^{-20}, and 8.02×10^{-12} from 60 SNP markers. All PE values exceeded 0.99, except for the case using a combination of nine marker sets (PE_{PU} = 0.981904). Based on these results, Lim et al. [29, 30] reported that the individual identification and paternity of the investigated marker combinations were sufficient when considering the total number of herds in Korea at the time and assuming a large half-sib population of Hanwoo.

As of March 2023, the number of Hanwoo raised nationwide was reported to be 3,470,499 heads [31]. When using only the five trinucleotide repeat marker combination investigated in this study, the individual discrimination ($PI_{half\text{-}sibs} = 1.29 \times 10^{-4}$) and paternity rate ($PE_1$ = 0.981141) were low level. However, the use of the five tri- and tetranucleotide repeat markers along with the 11 dinucleotide repeat markers increased the rate of individual identification and paternity ($PI_{half\text{-}sibs} = 3.42 \times 10^{-13}$, $PE_1 \geq 0.99$). The five markers are useful because they

all have adequate polymorphism (PIC > 0.5) and are compatible multiplex PCR with the 11 dinucleotide repeat markers. Sim et al. [10] confirmed that the stutter appearance ratio of four trinucleotide repeats, including *B8S7996*, in 105 Hanwoo was lower than those for the dinucleotide loci recommended by ISAG.

Brenig and Schütz [32] examined 12 MS markers selected by ISAG in the Holstein Friesian cattle population from 2004 to 2014 and found that most of the markers were associated with genes affecting economically important traits and reproduction. Therefore, they reported that the allele frequencies of some markers were increased or decreased significantly by selective breeding for these traits, reducing the overall informativeness and exclusion power of the marker panel, which could be addressed by adding markers. Hanwoo has also been improved by focusing on carcass traits, the markers investigated in this study can be considered for introduction as additional markers in the future.

Since the introduction of the Hanwoo traceability system, it has been possible to verify the pedigree information of individuals. Accurate pedigree management is an important factor in the production of superior individuals. Paternity testing can improve the accuracy and reliability of pedigree information; as the effect of improvement increases, the importance of pedigree information for predicting the genetic performance of an individual increases [33, 34, 35].

The tri- and tetranucleotide repeat microsatellite markers investigated in this study offer the potential to diminish genotyping errors, such as stutter, and proactively address potential changes in the existing dinucleotide repeat marker set. Rather than exclusively utilizing the five tri- and tetra nucleotide repeat markers as a set, they could be considered for integration with the current set of 11 dinucleotide repeat markers used in the traceability system or for substitution of some of the existing 11 markers. Ultimately, the tri- and tetranucleotide repeat microsatellite markers examined in this study have the capability to enhance individual identification and paternity testing rates in Hanwoo, contributing to the precise assessment of genetic performance.

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Table 1. Information on the 16 microsatellite markers examined in this study

Marker	Chromosome	Repeat motif	Label	Size range (bp)
BM1824	23	$(TG)_n$	NED	181–201
BM2113	2	$(CA)_n$	FAM	125–157
ETH10	5	$(AC)_n$	FAM	209–232
ETH225	9	$(TG)_4CG(TG)(CA)_n$	NED	143–164
ETH3	19	$(GT)_nAC(GT)_6$	NED	106–136
INRA23	3	$(AC)_n$	VIC	118–226
SPS115	15	$(CA)_nTA(CA)_6$	FAM	241–271
TGLA122	21	$(AC)_n(AT)_n$	VIC	138–196
TGLA126	20	$(TG)_n$	VIC	119–136
TGLA227	18	$(TG)_n$	FAM	77–115
TGLA53	16	$(TG)_6CG(TG)_4(TA)_n$	FAM	159–200
**B28S3299*	28	$(TTA)_n$	FAM	294–325
**B3S0990*	3	$(GCT)_n$	VIC	281–324
**B12S5209*	12	$(AGC)_n$	NED	258–298
**B9S5866*	9	$(ATAG)_n$	NED	304–348
**B8S7996*	8	$(AGC)_n$	PET	253–318

Markers marked with * are tri- and tetra nucleotide repeat microsatellite (MS).

Table 2. The number of alleles, observed and expected heterozygosity, P-value of Hardy-Weinberg equilibrium test, fixed index, and polymorphic information content of 16 microsatellite markers in 1,106 Hanwoo

Marker	N	H_{obs}	H_{exp}	HWE(P-value)	F	PIC
BM1824	6	0.752	0.751	0.8339	-0.002	0.708
BM2113	10	0.756	0.740	0.5490	-0.022	0.698
ETH10	9	0.773	0.766	0.2018	-0.010	0.74
ETH225	6	0.662	0.660	0.9707	-0.003	0.611
ETH3	5	0.774	0.775	0.3126	0.001	0.737
INRA23	11	0.716	0.707	0.8178	-0.013	0.661
SPS115	6	0.685	0.673	0.9816	-0.019	0.626
TGLA122	13	0.863	0.843	0.0153	-0.024	0.823
TGLA126	7	0.667	0.689	0.0430	0.031	0.648
TGLA227	14	0.834	0.836	0.1883	0.003	0.816
TGLA53	14	0.807	0.830	0.0059	0.028	0.813
B28S3299	9	0.770	0.772	0.7934	0.002	0.74
B3S0990	10	0.810	0.794	0.2994	-0.021	0.767
B12S5209	10	0.737	0.731	0.2739	-0.009	0.686
B9S5866	8	0.748	0.714	0.1332	-0.048	0.663
B8S7996	13	0.783	0.785	0.2595	0.002	0.754
Average	9.438	0.759	0.754	0.8339	-0.010	0.718

N, number of alleles; H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity; HWE(P-value), P-value of Hardy-Weinberg equilibrium test; F, fixed index (inbreeding coefficient); PIC, polymorphic information content

Table 3. The probability identification and probability of exclusion for 5, 11, and 16 microsatellite marker combinations

Marker set	PI	$PI_{half\text{-}sibs}$	PI_{sibs}	PE_1	PE_2	PE_3
5 MSs	7.03×10^{-6}	1.29×10^{-4}	9.60×10^{-3}	0.9811412141	0.9013686772	0.9987024033
11 MSs	3.13×10^{-12}	2.69×10^{-9}	3.89×10^{-5}	0.9998643997	0.9946317194	0.9999997071
16 MSs	2.39×10^{-17}	3.42×10^{-13}	3.69×10^{-7}	0.9999974427	0.9994705194	0.9999999996

PI, Probability that the genotypes of two unrelated individuals in a randomly mated population are the same;
$PI_{half\text{-}sibs}$, Probability that two individuals have the same genotype in the half-sib group;
PI_{sibs}, Probability that two individuals have the same genotype in the half-sib group;
PE_1, probability of exclusion of one putative parent when the other parent's genotype is known;
PE_2, probability of exclusion of one putative parent when the genotype of the other parent is missing;
PE_3, probability of excluding a putative parent pair

Supplementary Table. The allele frequency of 16 microsatellite markers in 1,106 Hanwoo.

Allele	*BM1824*	*BM2113*	*ETH10*	*ETH225*	*ETH3*	*INRA23*	*SPS115*	*TGLA122*
1	0.0158	0.0014	0.0375	0.0267	0.2351	0.0009	0.4860	0.0479
2	0.2749	0.0113	0.0660	0.5014	0.2758	0.0710	0.0054	0.1392
3	0.3300	0.0574	0.0298	0.1478	0.0886	0.0145	0.1026	0.2373
4	0.1261	0.0027	0.1700	0.2486	0.2554	0.0041	0.1004	0.1334
5	0.2184	0.0859	0.4091	0.0683	0.1451	0.0023	0.2622	0.0077
6	0.0348	0.2939	0.0800	0.0072		0.4218	0.0434	0.2102
7		0.1763	0.0511			0.2993		0.0751
8		0.3635	0.1496			0.0054		0.0090
9		0.0023	0.0068			0.0448		0.0072
10		0.0054				0.1347		0.0104
11						0.0014		0.0018
12								0.1081
13								0.0127
Allele	***TGLA12***	***TGLA227***	***TGLA53***	***B28S3299***	***B3S0990***	***B12S5209***	***B9S5866***	***B8S7996***
1	0.0063	0.0443	0.0005	0.0145	0.1234	0.0534	0.0059	0.1524
2	0.4765	0.0018	0.3305	0.0710	0.0253	0.0009	0.1763	0.3273
3	0.0832	0.2333	0.0036	0.0231	0.0637	0.2459	0.3617	0.0032
4	0.0127	0.0633	0.0005	0.3590	0.0262	0.0014	0.0081	0.0032
5	0.0859	0.0402	0.0036	0.2071	0.1184	0.0005	0.0036	0.0231
6	0.2496	0.0045	0.1130	0.1356	0.0384	0.0538	0.0827	0.0326
7	0.0859	0.0023	0.0380	0.1786	0.0253	0.2419	0.3427	0.1722
8		0.1985	0.0317	0.0104	0.2378	0.0077	0.0190	0.0113
9		0.1912	0.1008	0.0009	0.3364	0.3802		0.2301
10		0.0244	0.0674		0.0050	0.0145		0.0276
11		0.1542	0.0321					0.0086
12		0.0005	0.1316					0.0009
13		0.0009	0.1049					0.0077
14		0.0407	0.0420					