- Note -

Development of Microsatellite Markers for Discriminating Native Korean and Imported Cattle Breeds

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Three Korean native cattle (KNC) and seven exotic breeds (Chikso, Hanwoo, Jeju black, Holstein, Japanese black, Charolais, Angus, Hereford, Simmental, and Cross breed) were characterized by using five microsatellite (MS) markers (INRA30, TGLA325, UMN0803, UMN0905, and UMN0929) from the sex chromosome. Genetic diversity was evaluated across the 10 breeds by using the number of alleles per locus, allele frequency, heterozygosity, and polymorphism information content (PIC) to search for locus and/or breed specific alleles, allowing a rapid and cost-effective identification of cattle samples, avoiding mislabeling of commercial beef. It was divided into two main groups from STRUCTURE analysis, one corresponding to KNC and the other to exotic cattle breeds. These results also showed specific genetic differences between KNC and exotic breeds. Nei's standard genetic distance was calculated and used in the construction of a neighbor-joining tree. Results evidenced a correspondence between genetic distance, breeds' history, and their geographic origin, and a clear separation between KNC and exotic breeds. Overall, this study evidenced that DNA markers can discriminate between domestic and imported beef, contributing to the knowledge on cattle breeds' genetic diversity and relationships by using MS markers of the sex chromosome. These markers would be useful for inhibitory effect about false sales and for building an effective tracking system.

Key words : Heterozygosity, Korean native cattle, microsatellite markers, polymorphism information content, sex chromosome

Introduction

Korean domestic cattle, in particular, is also facing extinction risk due to the policies prevailing during the period of Japanese occupation to uniformize cattle-coat colors, import exotic breeds, and ensure animal improvement practices. Hanwoo (HW) has been the subject of livestock improvement projects for enhancing its genetic ability and performance since the 1960's [15]. However, other breeds have not received such protection from research projects and their existence has been threatened [19]. Recently, Chikso (CS), Heugu (HU), and Jeju Black (JJ) have been preserved through embryo transfer and *in vitro* fertilization techniques. An increased interest in threatened Korea domestic cattles has motivated studies on coat-color expression, intramuscular fat synthesis, and phylogeny by using molecular

markers [16, 18, 28].

In Korea, HW is the main source of Korean native beef whereas Holstein Friesian (HL) is the main dairy source. Holstein females are famous for their excellent milk production capability and males are used as a source of domestic beef. Although HL meat quality is lower than that of HW, it is popular as a domestic beef source due to its low price. However, Many consumers prefer Korean Native cattle (KNC) because of meat quality. Recently, many problems associated with meat consumption have occurred. Imported beef might have been mislabeled as domestic beef, motivated by consumer's increasing concerns with the safety of imported beef because of bovine spongiform encephalopathy outbreak. Therefore, the demand for an identification system to trace cattle from birth to market has gradually increased, as it is necessary to prevent false sales and guarantee beef quality and safety. Besides accurately discriminating between domestic and imported beef, this should also be an effective traceability system.

Along with significant progress in molecular technology, DNA markers have been used for population discrimination in livestock animals [3, 11], and might also be powerful tools for ensuring correct food labeling [1]. In addition, mitochon-

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drial DNA polymorphisms and microsatellite (MS) markers have been used in the evaluation of genetic diversity within and between breeds [12], and have provided improved genetic information on several regions and breeds [4, 6, 9, 20, 25, 30]. Especially, MS maker are suitable markers for genetic structure studies, because they are distributed throughout the genome, are co-dominantly inherited, neutral with respect to selection, exist in large numbers, and present high levels of polymorphism [29].

This study aimed to evaluate the relationships among 10 cattle breeds by using MS markers in sex chromosomes and to develop effective DNA markers to discriminate between domestic and imported beef, reducing incorrect food labeling.

Materials and Methods

Blood samples collection and DNA extraction

Blood samples of CS, HW, and JJ individuals were randomly collected, although avoiding parent-offspring or siblings based on pedigree information available at the institutes from where samples were obtained. Blood samples were obtained from the Gangwon Province Livestock Research Center, Jeonbuk Livestock Experiment Station, and Chungbuk Veterinary Service Center (CS individuals) and from the Jeju Special Self-Governing Provincial Livestock Institute (JJ individuals). Blood samples from HW individuals were collected from Animal Genetic Resources Station and additionally sampled from 11 farms in Yeongju City. Animals received care in accordance with the standard guideline for the Care and Use of Laboratory Animals provided by the National Institute of Animal Science Animal Care Committee, and the experiment was conducted with approval from the animal ethics committee and Operation rule of animal experiment ethics in the National Institute of Animal Science (approval number: NIAS-2015159).

Genomic DNA was extracted from blood samples using the DNeasy® Blood Kit (Qiagen, Germany) following the manufacturer's procedure. Genomic DNA of individuals belonging to the exotic breeds HL, Japanese black (JB), Charolais, Angus, Hereford, and Simmental and from HW-CR (HC) cross breed individuals (HC) was obtained from the Animal Genetic Resources Station, National Institute of Animal Science, Rural Development Administration .

Polymerase chain reaction (PCR) amplification and microsatellite genotyping

MS markers located on sex chromosomes were analyzed using five primer set (INRA30, UMN0803, UMN0905, UMN 0920 and UMN0929) with FAM dye (Table 1). Amplification was performed in a 20 μl reaction mixture, containing 1.5 mM $MgCl_2$, 1 U AmpliTaq Gold (Applied Biosystems, Foster, CA, USA), 3-10 pmol of each forward (labeled with a fluorescent-colored dye) and reverse primer, and 10 ng genomic DNA as template. PCR took place in a GeneAmp PCR System 9700 (Applied Biosystems) by using the following profile: initial denaturation at 95°C for 15 min; 35 cycles of denaturation at 95°C for 60 s, annealing at 55°C to 63°C (according to primer optimal annealing temperature) for 45 s, extension at 72°C for 60 s; and a final extension at 72°C for 30 min. Electrophoresis was carried out in an ABI 3130xl Genetic Analyzer (Applied Biosystems) and allele sizes for each microsatellite were determined using GeneMapper ver. 4.0 (Applied Biosystems). Allele data were then used in statistical analyses.

Table 1. Information of five microsatellite markers

Locus	Accession No.	Primer sequences Forward / Reverse	Type of repeat	Size of loci (bp)	Anneal Tm (℃)
INRA30	X67822	F:5′-ATGCAAATGTGCTACATCACCTAT-3′ R:5′-TGGCCCAACTCTCACATCCAGATC-3′	$(TG)_{13}$	163-169	60
TGLA325	-	F:5′-GGGCACTTTACTCTCTGAACAAATC-3′ R:5′-GCTGACAGTCTATTTCCAGAAGGTA-3′	$(CA)_{17}$	98-122	56
UMN0803	AF483745	F:5′-GATCCACATCCCCCTCAC-3′ R:5′-CTGCTTCTCTTGTCCGCTAA-3′	$(CA)_{4-6}$ $CCCTCACACAA)_6$	264-282	61
UMN0905	AF483748	F:5′-ATCAACCGTGGTAGCTCTAA-3′ R:5′-CTAGAATGTAAACCAGCTGC-3′	$(CA)_{16}$	159-169	61
UMN0929	AF483749	F:5′-ACCAGCTGATACACAAGTGC-3′ R:5′-GGTCAGAGAATGAAACAGAG-3′	$(CA)_{19}$	175-201	61

Statistical analyses

Using Microsatellite Toolkit software [23], alleles were organized individually and in groups. Cervus ver. 3.0.7[14] was used to estimate allele frequencies, total number of alleles, mean observed (HObs) and expected (HExp) heterozygosities, and mean polymorphism information content (PIC) per locus and breed. The D_A genetic distance was calculated in MSA and used to construct neighbor-joining tree in DISPAN software [7, 21, 22]. Genetic structure and the degree of admixture among the 10 breeds were evaluated using the Bayesian clustering procedure in STRUCTURE ver. 2.3 [26]. To identify the number of groups (K) that best fit the data, we used STRUCTURE HARVESTER [8], which implements the Evanno method [10]. Thirty independent runs were performed for K ranging from 2 to 10, with a 20,000 iterations burn-in period followed by 100,000 Markov chain Monte Carlo iterations. The program CLUMPP ver. 1.1[13] was used to align the 30 repetitions of each K. CLUMPP out files were visualized using DISTRUCT ver. 1.1[27].

Results and Discussion

The five MS markers (INRA30, TGLA325, UMN0803, UMN0905, and UMN0929) analyzed to estimate the several parameters of genetic diversity in the 10 cattle breeds, presented total 41 alleles (Table 2). Because species-specific alleles can be used as a measure of genetic distinction within and between species, alleles within each MS marker that were specific to a locus and/or breed group were identified. Several breeds evidenced polymorphism loci: INRA30 showed a 159 bp allele specific to HL; TGLA325 presented 106 bp and 112 bp alleles specific to CS and a 118 bp allele specific to JJ; UMN0803 had a 266 bp allele specific to KNC breeds; a UMN0905 163 bp allele was specific to HW and a 169 bp allele was specific to Asian breeds (KNC + JB); and UMN0929 showed a 201 bp allele specific to CS and HW. Therefore, the combination of these alleles can be used to distinguish the 10 cattle breeds.

The genetic diversity of the 10 breeds was calculated in Table 3. The number of alleles per locus ranged from 3 (UMN0803) to 14 (UMN0929) with a mean of 8.2±2.083 alleles. The mean HObs across loci was 0.464±0.09, with estimation per locus ranging from 0.274 (INRA30) to 0.726 (UMN0929). The mean HExp across loci was 0.607±0.085, ranging from 0.312 (INRA30) to 0.779 (TGLA325). The PIC ranged from 0.274 (INRA30) to 0.748 (TGLA325) with a mean of 0.556±0.089, meaning most MS markers were highly informative (PIC > 0.5) not only for chromosomal mapping

Table 2. Alleles (in bp) identified within each of the five polymorphic microsatellites found for the 10 cattle breeds

Breed	INRA30	TGLA325	UMN0803	UMN0905	UMN0929
Chikso (CS)	163, 165, 167	98, 100, 104, 106, 112, 116, 120, 122	264, 266, 282	161, 165, 167, 169	175, 177, 181, 187, 189, 191, 193, 197, 201
Jeju Black (JJ)	165, 167, 169	98, 100, 102, 104, 116, 118, 120	264, 266, 282	161, 165, 167, 169	175, 177, 187, 189, 193, 197, 201
Hanwoo (HW)	163, 165, 167	98, 100, 104, 110, 120	264, 266, 282	159, 161, 163, 165, 167, 169	175, 177, 181, 187, 193, 195, 197
Holstein (HL)	159, 165, 167	98, 100, 102, 104, 110, 114, 116, 120, 122	264, 282	161, 165, 167	175, 177, 179, 181, 185, 187, 189, 191, 193, 197, 199
Japanese Black (JB)	165, 167	98, 100, 102, 104, 114, 116, 120	264, 282	165, 169	175, 177, 181, 187, 193, 195
Charolais (CR)	165, 167, 169	98, 100, 102, 116, 120	264, 282	165, 167	175, 177, 181, 185, 193
Cross breed (HC)	165, 167, 169	98, 104, 120	264, 282	165, 167	177, 181, 189, 193
Angus (AA)	165, 167	98, 114	264, 282	167	177, 179
Hereford (HF)	165, 167	98, 100, 104, 116	282	159, 165	177, 189
Simmental (SM)	165	98	264, 282	159, 165	177, 195

Table 3. Microsatellite Loci polymorphism in the ten cattle breeds

Locus	Allele Number	HObs	HExp	PIC	N. typed individuals	HW	F(Null)
INRA30 (DXS8)	5	0.274	0.312	0.274	208	NS	0.063
TGLA325 (DXYS3)	12	0.630	0.779	0.748	208	***	0.110
UMN0803	3	0.375	0.533	0.431	208	***	0.173
UMN0905	7	0.317	0.673	0.617	208	***	0.351
UMN0929	14	0.726	0.740	0.708	208	NS	0.006
Mean	8.2	0.464	0.607	0.556			
SE	2.083	0.090	0.085	0.089			

HObs = observed heterozygosity.
HExp = expected heterozygosity.
PIC = Polymorphism Information Content.
N. typed individuals = number of individual samples genotyped.
HW = Hardy‑Weinberg equilibrium; NS = not significant; *** = $p<0.001$.
F(Null) = Null allele frequency estimate

and genetic diversity [2] but also for breed discrimination. Exceptions were INRA30 and UMN0803 (0.431). The genetic diversity of a population provides important information about its structure, subdivision, and evolution. Comparing the frequency of allele expression within each genetic marker allows detecting its specificity for each breed. MSs are the most suitable markers to obtain refined pictures of the biodiversity of species [24], as they are hypervariable, uniformly distributed in the genome, and quite easy to analyze. The use of automatic analyzers limits tests' cost and time and improves the reliability of the results reducing the variability in scoring. The results obtained here by using MS markers support their use to rapidly and effectively distinguish cattle breeds at a low cost.

STRUCTURE software was used to determine the unbiased structure without prior knowledge regarding the number of breeds. To evaluate whether data could be classified into several groups, samples were repeatedly analyzed (30 times) by setting K from 2 to 10 (Table 4). The optimum delta K ($\Delta K = 269.8468$), calculated as previously described [10], was obtained at $K = 2$, and two main groups were formed: one corresponding to KNC and the other to exotic cattle breeds. As K increased, the contributions of the assumed populations resulted in the progressively complete separation of breeds. These results also showed specific genetic differences between KNC and exotic breeds.

Table 5 shows the estimation of genetic uniformity when the 208 individuals were analyzed assuming two clusters. JB showed 56.4% of genetic uniformity in cluster 1, together with the KNC breeds CS, JJ, and HW. Exotic breeds had more than 80% of genetic uniformity in cluster 2, and the crossbreed HC presented only about 71% uniformity in this cluster. The low genetic uniformity of HW and JB breeds might be a result of gene inflow from exotic breeds for genetic improvement. Although the levels of genetic diversity among the 10 breeds differed according to their population

Table 4. Estimated delta K values by Evanno method

K	Repeats	Mean LnP(K)	SD LnP(K)	Ln'(K)	|Ln''(K)|	ΔK
1	30	-2515.3933	0.2959	-	-	-
2	30	-2295.0300	0.7278	220.3633	196.4067	269.8468
3	30	-2271.0733	17.2247	23.9567	8.9400	0.5190
4	30	-2238.1767	6.2703	32.8967	140.2500	22.3672
5	30	-2345.5300	44.2667	-107.3533	94.9433	2.1448
6	30	-2357.9400	27.8366	-12.4100	61.1000	2.1950
7	30	-2431.4500	77.5857	-73.5100	51.2967	0.6612
8	30	-2453.6633	63.0426	-22.2133	34.0567	0.5402
9	30	-2509.9333	68.2517	-56.2700	67.5967	0.9904
10	30	-2633.8000	108.6032	-123.8667	-	-

Bold indicates the largest value of ΔK [ΔK = Mean(\|Ln''(K)\|)/S ΔK D(LnP(K))].

Table 5. Proportional contribution of the inferred clusters derived from STRUCTURE analysis (K = 2) of 10 breeds

Breed	Inferred clusters		Population size
	1	2	
Chikso	0.838	0.162	60
Jeju Black	0.880	0.120	47
Hanwoo	0.567	0.433	25
Japanese Black	0.564	0.436	12
Simmental	0.071	0.929	2
Charolais	0.185	0.815	18
Cross breed	0.292	0.708	5
Holstein	0.098	0.902	30
Hereford	0.050	0.950	4
Angus	0.064	0.936	5

size, they were higher than those obtained for several native cattle breeds in other countries.

The neighbor-joining tree obtained for the genetic divergence among the 10 cattle breeds (Fig. 1) is largely divided in two groups as STRUCTURE analysis results, one comprising three KNC breeds and JB, and another comprising the remaining exotic cattle breeds, although genetic dis-
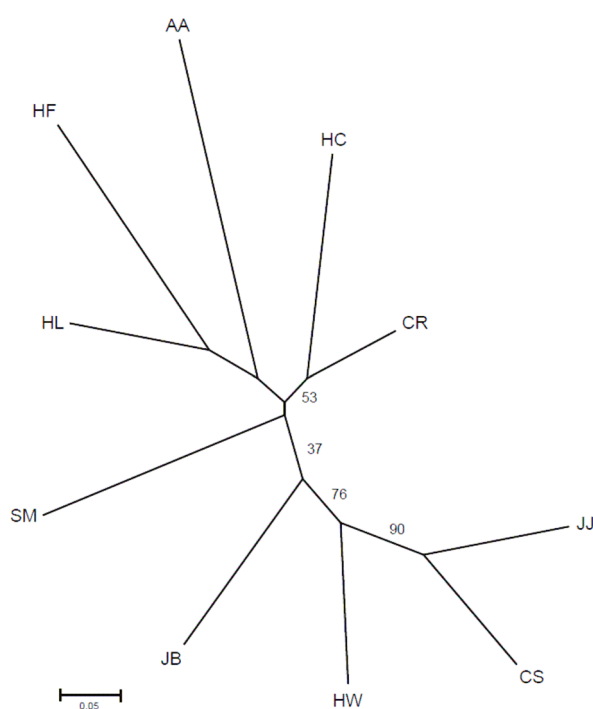

Fig. 1. Neighbor-joining tree showing the genetic divergence among the 10 cattle breeds, by using Nei's D_A genetic distance, calculated from allele frequencies in the five microsatellites from the sex chromosome. The number in the branch indicates the percentage occurrence in 1,000 bootstrap replicates. Abbreviations are described in Table 2.

tance was relatively high among all breeds. KNC and European cattle breeds have been suggested to be different lineages at *Bos taurus*. The results of NJ tree and structure suggested that the breeds analyzed are consistent with their modern geographical locations similar to the results of other markers systems [5, 17]. Therefore, genetic distances appear to reflect geographic distances. Thus, genetic distance can also be used for breed discrimination.

This study indicated that DNA markers could be developed to discriminate between domestic and imported beef, contributing to the knowledge of genetic diversity and relationships among cattle breeds by using MS markers of the sex chromosome. These markers would be useful for inhibitory effect about false sales and for building an effective tracking system. Also, this genetic information is also useful for the conservation, improvement, management, and utilization of KNC breeds as animal genetic resources.

Acknowledgments

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초록 : 한국 재래품종과 외래품종의 구별을 위한 초위성체 마커의 개발

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　　성염색체에 위치하는 5개의 초위성체 마커(INRA30, TGLA325, UMN0803, UMN0905, UMN0929) 를 이용하여 재래소 3품종과 외래소 7품종(칡소, 한우, 제주흑우, 홀스타인, 일본화우, 샤롤레, 앵거스, 헤어포드, 시멘탈, 한우X 샤롤레 교잡종)의 유전적 특징을 확인하였다. 상업적으로 판매되는 소고기의 잘못된 원산지 표기를 통해 부당한 경제적 이득을 취하고자 하는 문제를 해결하기 위한 방법으로 소고기 샘플을 빠르고 저비용으로 확인 하기 위한 방법으로 사용하기 위해 좌위 또는 품종 특이적 대립유전자를 탐색하고 좌위별 대립유전자수, 대립유전자빈도, 이형접합도 그리고 다형정보량(PIC)을 구하여 이들 10품종의 유전적 다양성을 평가하였다. STRUCTURE 분석을 통한 군락의 분류 및 유전적 균일성 분석에서 재래소 품종과 외래소 품종으로 두개의 주요 그룹으로 나뉘어진다. 이러한 결과들은 재래소와 외래소 품종의 특이적인 유전적 차이를 나타낸다. 또한 Nei's 표준 유전적 거리로 나타 난neighbor-joining tree에서도 독립적인 계통유전학적인 위치를 보여주었다. 이러한 결과는 국내 재래종과 외래 품종 사이의 유전적 거리, 품종의 역사 및 그들의 지리적 기원 사이에 명백한 차이를 나타내는 증거로 사료된다. 이러한 결과들로 이들 성염색체의 초위성체 마커들에 의해 소 품종들의 유전적 다양성과 연관성은 과학적인 기초 자료로 활용되고 재래소와 외래품종 소고기를 구별할 수 있는 DNA 마커들로 이용될 수 있을 것으로 사료된다. 그러므로 이러한 마커들은 효율적인 이력추적 시스템을 만드는데 사용되어 원산지 표시 위반을 억제하는데 유용 할 것이다.