

Coat Color Patterns and Genotypes of *Extension* and *Agouti* in Hanwoo and Jeju Black Cattle

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Received February 17, 2011 / Accepted April 26, 2011

To understand the relationship between coat color inheritance patterns and genotypes of *Extension* (*E*) and *Agouti* (*A*) loci in cattle, the genotypes for *melanocortin-1 receptor* (*MC1R*) and *agouti signaling protein* (*ASIP*) were analyzed in Hanwoo, Jeju black cattle (JBC), and their crossbred progeny. Three *MC1R* alleles (E^D , E^e , and e) were found in the black-colored JBC population. JBC had no recessive homozygotes (ee), but this genotype was predominant in the Hanwoo breed. However, *MC1R* E^e/e Hanwoo did not produce a black coat color as they appeared either as brown or solid red. For *ASIP*, three genotypes (A/A , A/A^{Br} , and A^{Br}/A^{Br}) were determined by insertion/deletion of an L1-BT element in Hanwoo. The *ASIP* A^{Br} allele was rarely observed, and no *ASIP* A^{Br}/A^{Br} homozygotes were detected in the JBC population. Cattle carrying *ASIP* A^{Br} did not show any agouti-like brindle pigmentation patterns in either breed or their progeny. The coat colors of the crossbred progeny were discriminated by two colors, yellowish-brown versus dark-brown or black, and their coat colors were directly related to the genotypes of the *Extension* locus, yellowish-brown (ee) and dark-brown or black (E^e/e), but not to the *Agouti* locus. *ASIP* genotypes probably did not affect coat color development in the Hanwoo or crossbred progeny. Our results suggest that the *ASIP* genotypes do not play key roles in coat color variation, but the *MC1R* genotypes do direct the phenotypes of Hanwoo, JBC, and their progeny.

Key words : Coat color, *melanocortin-1 receptor* (*MC1R*), *agouti signaling protein* (*ASIP*), Hanwoo, Jeju black cattle

Introduction

In Korea, there are 4 indigenous cattle breeds distinguished by 3 coat color patterns: a brown or red cattle breed (Hanwoo), a black-striped brown cattle breed (Korean brindle cattle, also called Chik-so) and 2 black cattle breeds. The 2 indigenous black cattle breeds can be separated by their localities: mainland (Korean black cattle) and Jeju Island (Jeju black cattle, JBC). The popular cattle breed Hanwoo has the largest population estimated at about 1.8 million-head, while the other 3 breeds have small populations (total number animals of each breed are fewer than 500 head). In particular, Jeju black cattle (JBC) that are reared only on Jeju Island are regarded as an endangered breed, which fostered the JBC Conservation Program in the early

1990s. After its political protection for maintenance and promotion, the JBC population has increased to about 300 head at present. JBC cattle are typically characterized as black cattle with/without abdominal white spots. Some other animals show partially diluted, light-yellow or colorless.

In mammals, coat color inheritance is regulated by more than 120 genes, and is mainly determined by the combinations of genotypes from several distinct loci, e.g. *agouti* (*A*), *extension* (*E*), *spotting* (*S*), *dilution* (*D*) and *dun* and *albino* (*C*) [9,20,23]. The *melanocortin-1 receptor* (*MC1R*) expressed by the *E* locus plays a primary role in signal transduction for the melanocyte-stimulating hormone α (MSHa) in melanocytes, and was recently studied at the molecular level for black, brown and red coat colors in various cattle breeds [10,16,24,27]. At the *E* locus, 3 major alleles, E^D for dominant black, E^e for intermediate/wild type and e for recessive red/yellowish-brown, were previously described. The dominant E^D allele produces a completely

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black color in cattle breeds, such as Holstein, Angus and others, whereas the recessive *e* allele has a frameshift mutation that causes a prematurely terminated protein in homozygous *ee* individuals corresponding to yellow or red colors. Animals with the intermediate wild type *E^f*/- chiefly show black color, but their coat color can be modified to a brown coat color by the allelic presence of *A⁺* at the *A* locus [1,19,22]. Wild-type *agouti* hair found in rodents is characterized by a sub-terminal or terminal yellow band with the hair containing black or brown eumelanin pigment. Recently, a bovine *agouti* allele for brindle coat color was described in the Normande cattle breed by an L1-BT insertion between the skin-specific promoter and the first coding exon for the *ASIP* gene [6,7]. Previous *MC1R* studies for 2 indigenous Korean cattle breeds, Hanwoo and JBC, documented interesting genetic aspects for the relationship between their genotypes and coat color phenotypes. In Hanwoo, 2 *MC1R* alleles (predominant *e* and rare *E^f*) were identified and their coat colors ranged from yellowish- to reddish-brown [2,3,14,17,18,27]. In contrast, the predominant *MC1R E^f* was responsible for black coat color in the JBC population. However, there is currently no information on the genetic relationship for coat-color dilution and *agouti*-like pigmentation in either breed.

In this study, the genotypes of both major coat-color controlling genes, *MC1R* and *ASIP*, were investigated to determine the relationship between phenotypic traits, including coat color, and the genetic details for 2 indigenous Korean cattle breeds, Hanwoo and JBC, and their progeny.

Materials and Methods

Animals and DNA extraction

A total of 618 animals representing 2 native Korean breeds (JBC and Hanwoo), 4 foreign breeds (Black Holstein, Red Holstein, Angus and Hereford) and JBC×Hanwoo

crossbred calves were analyzed. Blood samples from JBC were collected at the Subtropical Animal Experiment Station (SAES), National Institute of Animal Science, RDA, Institute for Livestock Promotion (ILP) of Cheju-special Self-governing Province, and at licensed JBC-rearing farms on Jeju Island, South Korea. Samples from Hanwoo cattle were collected at 10 different farms on the Korean mainland and Jeju Island. JBC×Hanwoo crossbred calves were collected by SAES. Foreign cattle breeds were sampled on farms. Genomic DNA was extracted from blood samples using standard methods [26] with slight modifications.

Genotyping of *MC1R* gene

Bovine *MC1R* genotypes were determined using a polymerase chain reaction mediated restriction fragment length polymorphisms (PCR-RFLP) technique. Partial fragments of bovine *MC1R* genes containing the *E^D* determining substitution, g.296T>C (p.99Leu>Pro), and a recessive *e* allele determining deletion G (delG) mutation at g.310th position were amplified using bMC1RmisF and bMC1RmisR primers (Table 1) designed by Han et al. [8]. Amplified products were enzymatically digested with 2 restriction enzymes (*AluI* for g.296T>C; *MspI* for g.310delG). Digests were separated on 3% agarose gels and the genotypes were determined using combinations of the enzyme digestion patterns for each animal.

Genotyping of *ASIP* gene

To analyze *ASIP* genotypes for the presence/absence of an 1.2 kb L1-BT retroposon, the *ASIP* gene was amplified using specific primers (Table 1) designed by Girardot et al. [6]. The presence of an L1-BT element in *ASIP* was detected on 1.5% agarose gels.

Pedigree analysis and microsatellite genotyping

To elucidate phenotypic inheritance patterns for coat

Table 1. Primers for genotyping used in this study

Gene	Primer name	Nucleotide sequence	Reference
<i>MC1R</i>	MC1RmisF	5'-TGGTGAGCGTCAGCAACGTGCTGGAGACGGCAGTCAAG-3'	Han et al. (2008)
	MC1RmisR	5'-TGGATCCGCACATGAGCACGTGATGACATTGTCCAAC-3'	Han et al. (2008)
<i>ASIP</i>	ASIP_II4s	5'-ATAACTTGCTCCCTATTTTTCA-3'	Girardot et al. (2006)
	ASIP_III4as	5'-CTGTTTCAAATAAGGACCAGAT-3'	Girardot et al. (2006)
	ASIP_L1-BT1as	5'-GATGCTGCAAACAAATAACA-3'	Girardot et al. (2006)

The underlined nucleotide sequences of MC1RmisF and MC1RmisR were modified to replace the *AcI* recognition site at *MC1R* g.296T>C with that of *AluI* for genotyping because components of the PCR reaction buffer usually inhibit the digestion activity of the *AcI* restriction enzyme [27].

color in JBC×Hanwoo crossbred calves, 3 pedigrees were constructed based on information from breeding records and our previous parentage analysis using microsatellite (MS) genotyping with two MS marker sets: International Society of Animal Genetics (ISAG) marker set and SAES marker set. The ISAG marker set comprised 11 MS markers fluorescently labeled with 5'-FAM, HEX, and NED (BM1824, BM2113, ETH3, ETH10, ETH225, INRA23, SPS115, TGLA53, TGLA122, TGLA126 and TGLA127), which were recommended for cattle population diversity studies by the MoDAD program of US FAO for Management of Farm Animal Genetic Resources. The SAES marker set also comprised 11 MS markers (BL1134, BMS1580, BMS1907, BMS2060, BMS4028, DIK3027, DIK4224, DIK4591, DIK4460, IDVGA-37 and MNS-2), and was selected based upon the authors' preliminary study on parentage tests for the JBC population. References and primer sequences for the MS used are available in the Cattle Diversity Database. PCR for MS genotyping was performed using a multiplex PCR procedure described by Yoon et al. [28] with slight modifications. Amplified PCR products were separated on a MegaBACE1000 DNA Sequencer (Amersham Biotechnologies, USA) and allele data were collected using Genetic Analyzer 2.0 and Fragment Analyzer 3.3.3 (Amersham Biotechnologies, USA). Combining the data for genotypes of the *MC1R* and *ASIP* analyses and those from MS analysis, parentage tests for JBC×Hanwoo crossbred calves were carried out using the CERVUS 3.0.3 program [11].

Results and Discussion

To determine the relationship between coat colors and the genotypes of the *Extension* and *Agouti* loci for the Korean

cattle breeds, we investigated the genotypes of both genes in Hanwoo, JBC and their F₁ calves, as well as in other non-domestic cattle breeds (Table 2). To analyze the *MC1R* genotypes, g.T296C (p.99Leu>Pro) and a recessive *e* allele caused by a g.310delG frame-shift mutation for *MC1R* gene expression were tested. PCR-RFLP patterns using *AluI* and *MspI* restriction enzymes showed polymorphisms in the *MC1R* gene for JBC and Hanwoo, respectively (Fig. 2A). Combining each fragment pattern, 3 alleles (*E^D*, *E^{*}*, and *e*) were detected in JBC, but only 2 alleles (*E^{*}* and *e*) were observed in Hanwoo. JBC showed a higher *E^{*}* allele frequency than those of *E^D* and *e* but Hanwoo did not demonstrate the *E^D* allele. The black coat-colored JBC derived from 3 genotypes (*E^DE^{*}*, *E^{*}E^{*}*, and *E^{*}e*), except for 2 homozygotes, *E^DE^D* and *e^e*. This is similar to results for genotype distributions of Hanwoo and JBC previously reported [4,14,18,27].

The Hanwoo were either brown or red coat color cattle, and they had only 2 *MC1R* genotypes (*e^e* and *E^{*}e*). Of these, the recessive *MC1R* gene homozygote *e^e* was predominant (91.4%) in this population, but *E^{*}e* was rare. Other reports have also described that the *MC1R* *E^{*}* allele frequency was rare in the Hanwoo population [2,14,18,27]. Scientists have argued if the *MC1R* functions in coat color development of Hanwoo, as the coat color of *MC1R* *E^{*}e* Hanwoo cattle is not clearly discriminated from that of *e^e* Hanwoo cattle. However, the *MC1R* *E^{*}* allele was also found in other red or yellowish-brown cattle breeds, including Japanese Brown [14,18,19,27] and Chinese brown cattle [5]. The *MC1R* *E^{*}* allele is also found in the Hereford and Red Holstein red or yellowish-brown cattle breeds. There are no *E^{*}E^{*}* homozygote cattle found in Hereford, Red Holstein or Hanwoo (Table 2). Thus, additional molecular studies will be needed in order to determine why the red cattle's *MC1R*

Table 2. Coat color patterns and genotypes of *MC1R* and *ASIP* genes in cattle breeds

Breed	Characteristic coat color pattern	<i>MC1R</i>						<i>ASIP</i>		
		<i>E^DE^D</i>	<i>E^DE[*]</i>	<i>E^De</i>	<i>E[*]E[*]</i>	<i>E[*]e</i>	<i>e^e</i>	<i>A/A</i>	<i>A/A^{Br}</i>	<i>A^{Br}/A^{Br}</i>
Black Holstein	black pied	0.935	0.043	0.022	0	0	0	1.000	0	0
Red Holstein	red pied	0	0	0	0	0.333	0.667	1.000	0	0
Angus	black solid	0.743	0.057	0.171	0	0	0	0.200	0.400	0.400
Hereford	brown/red/pied ¹	0	0	0	0	0.385	0.615	0.384	0.308	0.308
Jeju black cattle	black solid	0	0.184	0.058	0.408	0.350	0	0.937	0.063	0
Hanwoo	brown or red solid	0	0	0	0	0.086	0.914	0.479	0.381	0.140
Crossbred ²	brown or red/black solid	0	0	0.023	0	0.698	0.279	0.860	0.140	0

¹pied region mainly appeared on the head.
²produced between Hanwoo and Jeju black cattle.

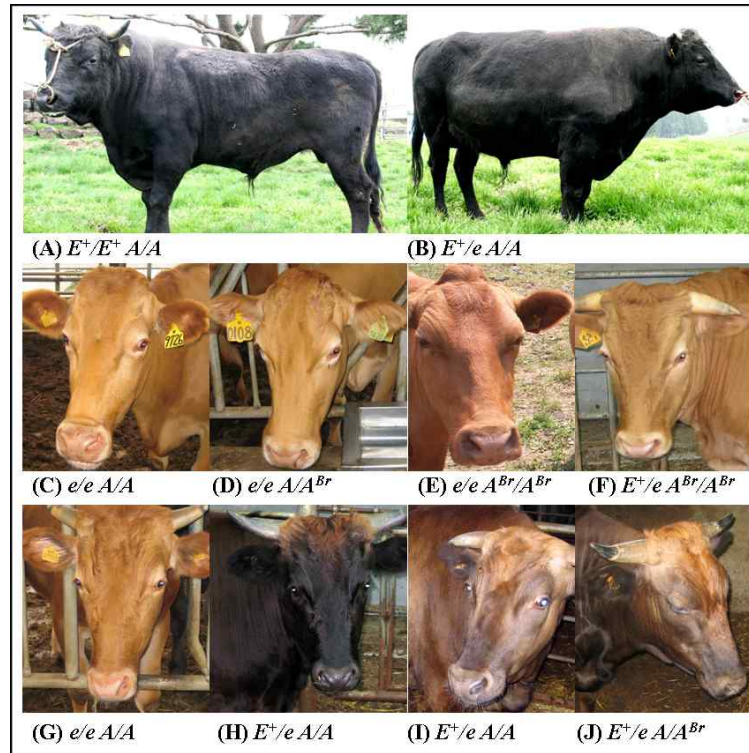


Fig. 1. Coat color patterns and combining haplotypes for *MC1R* and *ASIP* genes of JBC sires (A-B), Hanwoo dams (C-F) and their crossbred progenies (G-J).

E^+ does not produce the black coat color in several cattle breeds.

ASIP genotypes were also investigated. PCR genotyping

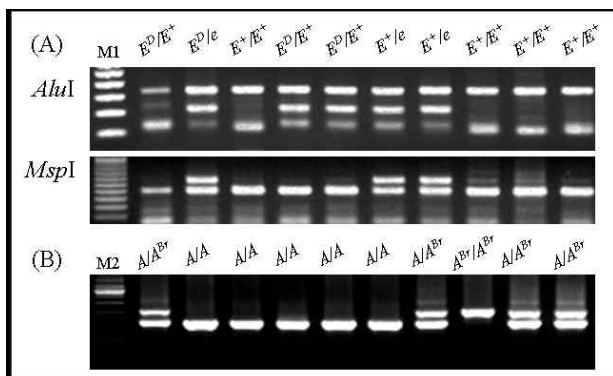


Fig. 2. Genotyping for *MC1R* (A) and *ASIP* (B) in cattle. *MC1R* genotypes (A) were determined by combining the RFLP patterns obtained from digests using *AluI* for E^D and others and *MspI* for e and others. *ASIP* genotypes (B) were determined by the presence/absence of a 1.2-kb L1-BT retroposon; lower and upper bands indicate the absence of L1-BT (allele A) and the presence of L1-BT (A^{Br}), respectively.

based on the presence or absence of an L1-BT retroposon in the *ASIP* gene also showed polymorphisms in the tested cattle breeds (Fig. 2B). However, the *ASIP* brindle allele (A^{Br}) was not found, and there was no A^{Br}/A^{Br} homozygote among JBC cattle. In contrast, Hanwoo had all three genotypes (A/A , A/A^{Br} , and A^{Br}/A^{Br}), but their coat colors were not discriminated according to these genotypes (Table 2). In the analysis of Hanwoo×JBC progeny, 2 *MC1R* genotypes (E^+/e and eA) were found, and both *MC1R* genotypes produced black (E^+/A) and yellowish-brown (eA). However, two *ASIP* genotypes (A/A and A/A^{Br}) were also found, but both *ASIP* genotypes did not produce different coat colors (non-brindle and brindle).

Pedigrees constructed from mating records and genotype data from MS analyses of crossbred families between Hanwoo and JBC (Fig. 3) provided important information on coat color inheritance patterns and the relationship between coat color phenotypes and genotypes for the *extension* and *agouti* loci. All F_1 calves produced from a JBC E^+/E^+ sire (an arrow in Fig. 3) and Hanwoo e/e dams showed black coat colors and had *MC1R* E^+/e . That is, the coat colors of F_1 calves with *MC1R* E^+/e did not become red or brown.

Table 3. Combining haplotypes for *MC1R* and *ASIP* genes in cattle breeds

Genotype		Cattle breed						
<i>MC1R</i>	<i>ASIP</i>	Balck Holstein (n=46)	Red Holstein (n=3)	Angus (n=35)	Hereford (n=11)	JBC (n=223)	Hanwoo (n=257)	Crossbred ¹ (n=43)
E^D/E^D	A/A	0.935	0	0.143	0	0	0	0
E^D/E^D	A/A^{Br}	0	0	0.257	0	0	0	0
E^D/E^D	A^{Br}/A^{Br}	0	0	0.343	0	0	0	0
E^D/E^+	A/A	0.043	0	0	0	0.180	0	0
E^D/E^+	A/A^{Br}	0	0	0.029	0	0.013	0	0
E^D/E^+	A^{Br}/A^{Br}	0	0	0.029	0	0	0	0
E^D/e	A/A	0	0	0.057	0	0.045	0	0
E^D/e	A/A^{Br}	0.022	0	0.114	0	0.009	0	0
E^D/e	A^{Br}/A^{Br}	0	0	0.029	0	0	0	0.023
E^+/E^+	A/A	0	0	0	0	0.386	0	0
E^+/E^+	A/A^{Br}	0	0	0	0	0.018	0	0
E^+/E^+	A^{Br}/A^{Br}	0	0	0	0	0	0	0
E^+/e	A/A	0	0.333	0	0.076	0.327	0.023	0.558
E^+/e	A/A^{Br}	0	0	0	0.154	0.022	0.039	0.14
E^+/e	A^{Br}/A^{Br}	0	0	0	0.154	0	0.023	0
e/e	A/A	0	0.667	0	0.308	0	0.455	0.279
e/e	A/A^{Br}	0	0	0	0.154	0	0.343	0
e/e	A^{Br}/A^{Br}	0	0	0	0.154	0	0.117	0

¹produced between Hanwoo and Jeju black cattle.

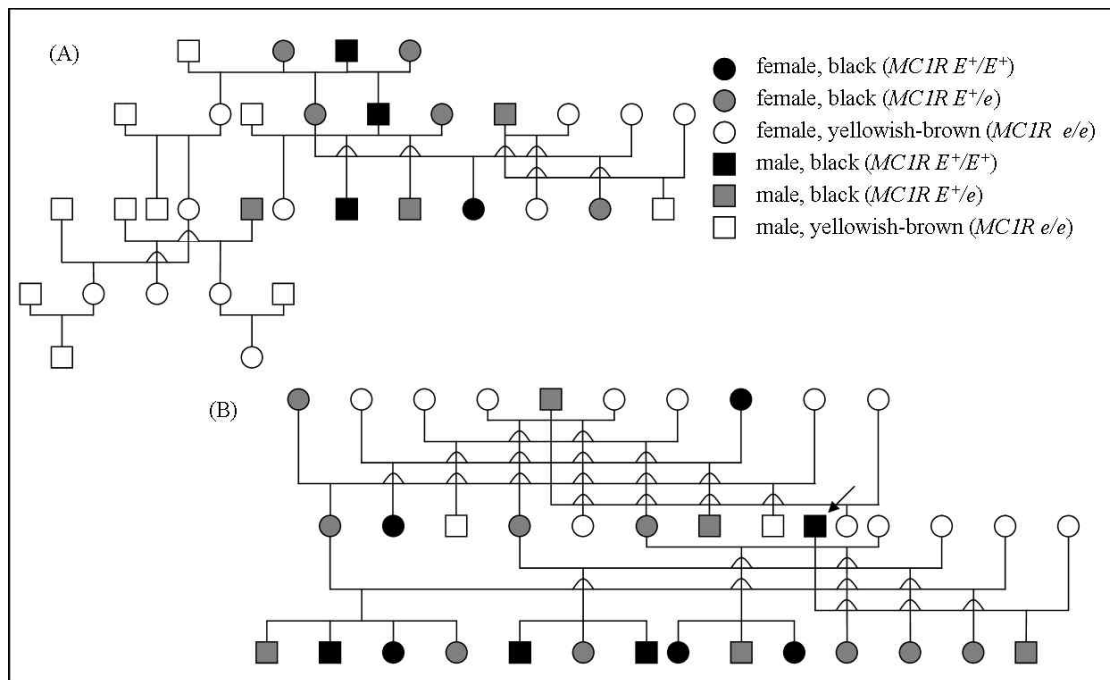


Fig. 3. Pedigrees showing the relation of coat color inheritance patterns to *MC1R* genotypes in two family groups constructed by crossbreeding between Hanwoo and JBC. Black squares and circles represent black colored E^+/E^+ males and females, respectively. Gray squares and circles represent black colored E^+/e males and females, respectively. Open squares and circles represent reddish-brown colored e/e males and females, respectively. An arrow indicates a JBC E^+/E^+ sire.

This indicates that the wild type E^+ allele of JBC is dominant to the recessive e allele. However, after mating a JBC E^+/e sire with Hanwoo e/e dams, calves appeared with 2 distinct coat colors: brown/red (e/e) or black (E^+/e). Coat color phenotypes of crossbred calves between the 2 breeds showed Mendelian dominance and segregation of genes. However, the *ASIP* genotypes did not directly affect the coat color phenotypes of the progeny. For example, brown or red colored Hanwoo with the *ASIP* A^{Br} allele did not show brindle- or agouti-like pigmentation patterns (Fig. 1D~F). Among crossbred calves, an $E^+/e/A^{Br}$ haplotype was also detected in 6 animals, although a brindle-like pigment was not found.

These results suggest that the coat color patterns of offspring may be directed by their *MC1R* genotypes rather than their *ASIP* genotypes. Royo et al. [25] suggested that the *ASIP* coding region does not play a central role in coat color variation in cattle, as there was no mutation found in the coding region of the *ASIP* gene in 9 Spanish and French cattle breeds. Do et al. [4] described two SNPs in the intronic region of *ASIP*, but not in coding sequences, and suggested that the *ASIP* intron 2 g.-157A>T showed polymorphisms in Hanwoo and Hereford, although there was no relationship to coat color development in either breed. However, Girardot et al. [6,7] found that the bovine *Agouti* allele for brindle coat color (A^{Br}) was described in Normande cattle breed and other breeds by an L1-BT insertion between the skin-specific promoter and the first coding exon of the *ASIP* gene, and suggested that the A^{Br} allele is responsible for the brindle phenotype in the presence of a functional E allele. Yet, brindle-like pigmentation patterns were not found in JBC, Hanwoo and their crossbred calves suggesting that the *ASIP* L1-BT polymorphism may not related to coat color variation in both Korean cattle breeds.

In Korea, the yellowish-brown Hanwoo is the most abundant cattle breed. The black JBC, considered as an endangered breed until the early 1990s, are totally isolated either in two separate institutions (SAES and ILP) or several restricted JBC-rearing farms on Jeju Island under the political control of a pure breeding program. Indeed, the Hanwoo population is abundant and widespread over the mainland (>2.2 million head) and Jeju Island (>1.8 thousand head), South Korea. Before political protection for maintenance and promotion, the JBC population was not distinguished from other cattle and were reared together with Hanwoo and other breeds, and regarded as a mixed breed. Even after the

start of the JBC pure breeding program, some progeny born with brown-red coat color (*MC1R* e/e) were actually excluded from the JBC pure breeding population.

The *MC1R* genotype is broadly used as one of the molecular markers for breed-discriminating in the cattle industry of Korea, for example for the identification of Hanwoo versus imported beef cattle breeds. In addition, the F_1 calves produced by crossbreeding between Hanwoo×Holstein or Hanwoo×Angus show solid black coat colors (data not shown), and they are not completely distinguishable from the JBC in coat color patterns. Because these F_1 calves sometimes engendered social problems regarding breed identification in Korea, previous studies were carried out to develop a precise, simple method for distinguishing exotic cattle breeds with *MC1R* E^D/e and *MC1R* e/e Hanwoo in Korea [2,3,13,15,21].

Historically, the dominant black cattle breeds with the *MC1R* E^D allele, including Holstein and Angus, and other coat colored cattle breeds have been imported since the 1960s for improving milk and beef productivity and for developing a new mixed breed by intercrosses among these and Korean cattle. The *MC1R* genotypes of the Black Holstein and Angus predominantly showed the E^D allele in this study, as well as other reports [2,3,14,18]. No JBC cattle showed the E^D/E^D homozygous *MC1R* genotype, and no Hanwoo cattle had the E^D allele, supporting that the dominant black allele E^D found in the JBC population was introduced from foreign cattle breeds, probably Holstein or Angus. During JBC breeding, the candidate sires collected from farms with E^D/e had been excluded, because the Korean cattle breeds did not possess this *MC1R* allele. However, because *MC1R* E^D/e cattle are present in the JBC population, JBC breeding researchers plan to remove them in the immediate future. From this view, we suggest that this study will not only contribute to the breeding of the endangered cattle breed, JBC, by providing critical information on a genetic marker for breed-identification to prohibit the introduction of gene pools of foreign black-colored cattle breeds for rearing on Jeju Island, but also to understand coat-color inheritance patterns in cattle.

Acknowledgement

This study was supported by Korea Rural Development Administration Fund No. 2009OFT072149135 and a grant (308008-5) of Technology Development Program for

Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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초록 : 제주흑우와 한우에서 *Extension*, *Agouti* 유전자형과 모색 출현 양상

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소의 *Extension* (*E*), *Agouti* (*A*) 유전자형과 모색유전자형의 연관관계를 이해하기 위해, 한우와 제주흑우(JBC), 이들 사이에서 생산된 교배후손에서 *melanocortin-1 receptor* (*MC1R*)과 *agouti signaling protein* (*ASIP*)의 유전자형을 분석하였다. 흑모색의 JBC에서는 세 가지 *MC1R* 대립인자들(E^D , E^F , e)이 모두 발견되었다. JBC 집단은 한우에서 우점적으로 나타나는 열성동형접합자(ee)가 발견되지 않았다. 반면, *MC1R* E^F/e 한우는 흑모색이 아닌 갈색이나 적색의 모색을 나타내었다. *ASIP* 유전자에서, 한우에서는 L1-BT 전위인자의 삽입/결실에 의해 세 가지 유전자형(A/A , A/A^{Br} , A^{Br}/A^{Br})이 모두 관찰되었다. JBC 집단에서는 *ASIP* A^{Br} 대립인자가 드물게 관찰되고, *ASIP* A^{Br}/A^{Br} 동형접합인 개체들은 발견되지 않았다. *ASIP* A^{Br} 대립인자를 보유하더라도 한우와 JBC, 이들의 교배후손에서도 agouti-유사 얼룩무늬 모색은 발견되지 않았다. 교배후손의 모색은 *Agouti* 유전자형과는 무관하고, *Extension* 유전자형과 직접적으로 연관되어 황-갈색(ee)과 암-갈/흑색(E^F/e)으로만 구분되었다. *ASIP* 유전자형은 아마도 한우 또는 교배 후손에서 모색의 발달에 영향이 없는 것으로 추정된다. 본 연구 결과는 한우, 제주흑우, 이들의 교배후손에서 *MC1R* 유전자형이 모색 표현형을 결정하며, *ASIP* 유전자형은 모색의 변이에는 핵심적인 역할을 수행하지 않는다는 것을 시사한다.