ORIGINAL ARTICLE

Allele frequencies of the extension locus encoding the melanocortin-1 receptor in Japanese and Korean cattle

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ABSTRACT

In order to estimate the influence of the Extension (E) locus in cattle coat color, the melanocortin-1 receptor (MC1R) gene in Japanese Black, Japanese Brown and Korean (Hanwoo) cattle were sequenced. The sequences of the coding region revealed three alleles (E^D, E^+ and *e*), which were previously reported. Polymerase chain reaction-restriction fragment length polymorphism was performed to investigate the gene frequencies of the three breeds. Japanese Black was almost composed of E^D and E^+ individuals, $E^D = 0.481$ and $E^+ = 0.514$, and no homozygous e/e, therefore that is consistent with the hypothesis that E^D and E^+ induce black pigment synthesis. Allele frequencies between Japanese Brown and Hanwoo were obviously different; however, recessive red *e* allele frequency was 0.038 for Japanese Brown and 0.948 for Hanwoo, even though both breeds have quite similar coat colors (ranging from yellowish brown to dark brown including a red coat color). This result suggested that other genes are also associated with a coat color of red and brown in cattle.

KEYWORDS: coat color, Japanese Black, Japanese Brown, Korean cattle, MC1R.

INTRODUCTION

Most cattle breeds have a coat color pattern that is characteristic for that breed. Several studies for coat color in cattle have been reported (Joerg *et al*. 1996; Berryere *et al*. 2003). The melanocortin-1 receptor gene (MC1R), encoded by the Extension (E) locus that corresponds to the melanocyte-stimulating hormone receptor gene, was recently studied at the molecular level in black, brown and red (Klungland *et al*. 1995). At the *E*-locus, three major alleles were previously reported: E^D, dominant, producing dominant black; E^+, intermediate, producing recessive black; and *e*, recessive, producing red when homozygous. The wild type E^+ allele basically produces a black color, however, that black pigment is modified by the allele A^+ at the agouti locus to produce the brown color of many breeds (Adalsteinsson *et al*. 1995). The dominant E^D allele gives black color, whereas a frameshift mutation producing a prematurely terminated receptor in homozygous *e*/*e* animals induce a red coat color (Rouzaud *et al*. 2000).

There are four kinds of beef cattle (Japanese Black, Japanese Brown, Japanese Polled and Japanese Shorthorn) in Japan. Japanese Black has a black coat color and is the main breed in Japan. It is famous for its ability to produce high-quality meat. Japanese Brown was created by the Korean native cattle (Hanwoo) bulls when they were mated to Japanese native cattle dams approximately 100 hundred years ago. Hanwoo has been bred as a beef cattle throughout the Korean Peninsula. Japanese Brown and Hanwoo have similar coat colors ranging from yellowish brown to dark brown including a red coat color.

In the present study we investigated the gene frequencies at the *E*-locus for the three cattle breeds (Japanese Black, Japanese Brown and Hanwoo). The current study is the first investigation of coat color gene in Japanese and Korean cattle as far as we know.

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MATERIALS AND METHODS

Breeds

Animals belonging to three different breeds (Japanese Black, Japanese Brown and Hanwoo) were examined in the present study. The numbers of animals studied are 215 in Japanese Black, 144 in Japanese Brown and 77 in Hanwoo. Genomic DNA was extracted from blood samples according to standard protocols (Mannen *et al*. 1993).

MC1R gene coding sequence

The MC1R coding region was amplified using F202 and R697 primers (Table 1) designed according to bovine sequence (GenBank accession no. AF445642). The amplified polymerase chain reaction (PCR) products were cloned by pGEM-T Easy Vector System II (Promega, WI, USA) according to the manufacturer's instruction. After checking the product size of DNA fragment, white positive colonies were isolated and cultured overnight in Luria-Bertani medium. The plasmids were then isolated. The sequence of the plasmid was determined by Sequi Therm EXCEL II DNA sequencing Kits-LC (EPICENTRE Technologies, WI, USA) with IRD800 labeled M13 forward (5′-CAC GAC GTT GTA AAA CGA C) and reverse primers (5′-GGA TAA CAA TTT CAC ACA GG).

PCR-RFLP

Primers F202 and R356M and primers F228 and R447 were used to distinguish the E^D and E^+ alleles and the *E* and *e* alleles, respectively (Table 1). The PCR reactions were performed in a volume of 10 μL, containing 20 ng genomic DNA as a template, 2.0 μL reaction buffer (100 mM Tris-HCl, 15 mM MgCl, 500 mM KCl), 1.6 μL deoxyribonucleoside triphosphate (dNTP) mix (2.5 mM), 0.5 μL of each primer (10 nmol/mL) and 1.0 U of EX Taq polymerase (Takara Shuzo, Tokyo, Japan). The PCR reactions were carried out using a standard PCR program with 5 min denaturation at 94°C, 30 cycles for 1 min at 94°C, 1 min annealing at 65°C, 1 min extension at 72°C, and final extension for 7 min at 72°C. The PCR products were digested with restriction endonuclease *Msp*A1 ($C^{A/C}GC^{G/T}G$) and *Msp*I (CCGG), respectively, and detected in a 2% agarose gel electrophoresis. We used different restriction endonuclease to classify each allele easily and swiftly from the separated fragments in the current study. The E^D allele identification resulted from the substitution of T by C and appearance of a new restriction site for *Msp*A1 (Fig. 1). Consequently, a purposeful mismatch primer (R356M) induced the removal of the *Msp*A1 restriction site (CAGCTG) in position 346. These alleles could be distinguished by digestion with the restriction endonuclease *Aci*I (Rouzaud *et al*. 2000), however, this enzyme is inhibited by PCR reagents which require purification prior to digestion. The deletion of G at position 310 in the *e* allele induced the removal of *Msp*I restriction endonuclease site (Fig. 1).

Table 1 Sequence of melanocortin-1 receptor primers

Name	Sequence (5′→3′)
F202	AACCTGCACTCCCCCATGTACTACT
R356M†	ACATTGTCCAACTGCTGCACCACGG
F228	ATCTGCTGCCTGGCTGTGTCTGACT
R447	GGCGTAGAAGATGGAGATGTAGCGG
R697	GATGAATGGGGCGCTGCCTCTTCTG

†A mismatch primer induced the removal of the *Msp*AI restriction site. The position of mismatch was underlined.

RESULTS

MC1R alleles

We sequenced the MC1R gene using genomic DNA from the Japanese Black, Japanese Brown and Hanwoo cattle. In order to amplify the MC1R cording region, we used primers of F202 and R697 (Table 1). Three alleles, which were previously reported in Norwegian and Icelandic cattle (Adalsteinsson *et al*. 1995), have been identified. Any other nucleotide substitutions were not observed in the present study. The wild type *E* allele coding sequence is 954 bp long and encodes a full length MC1R of 317 amino acids. The length of protein-encoding sequence of MC1R was 954 bp and a 317 amino acid protein was coded. The E^D allele results from one single base substitution (T296C) leading to an amino acid change from leucine

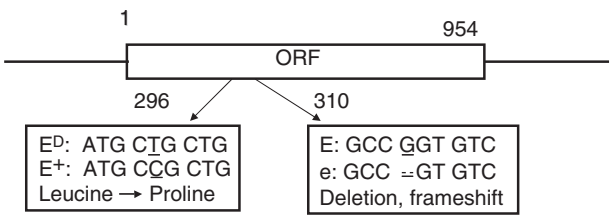

Fig. 1 Schematic illustration of full length bovine MC1R cording region. The boxes indicate melanocortin-1 receptor alleles and amino acid replacement. Underlines show the nucleotide substitution. ORF, open reading frame.

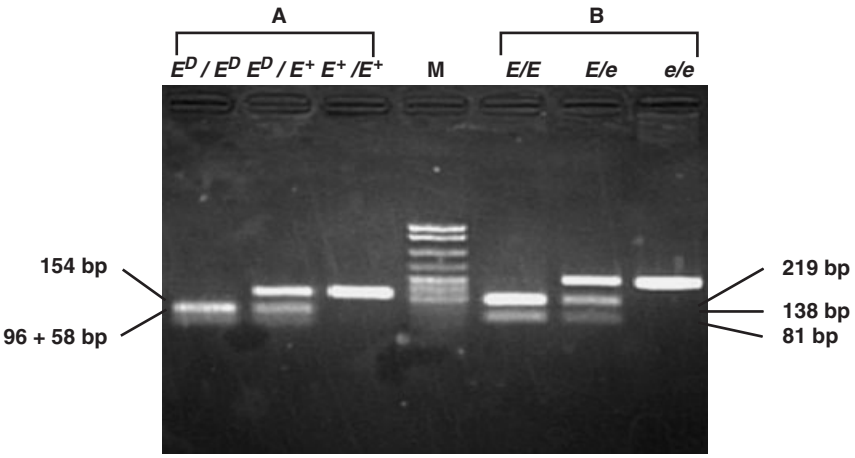

Fig. 2 Genotyping of three alleles for the melanocortin-1 receptor gene. Polymerase chain reaction products were digested by restriction enzyme MspAI (a) or MspI (b). These fragments were detected in 2% agarose gel electrophoresis.

Table 2 Genotype and allele frequencies of the extension locus for three breeds

Breed	Σ	Genotype frequencies					Allele frequencies		
		E^D/E^D	E^D/E^+	E^+/E^+	E^+/e	e/e	E^D	E^+	e
Japanese Black	215	55	97	61	2	0	0.481	0.514	0.005
Japanese Brown	145	0	0	135	9	1	0.000	0.962	0.038
Korean	77	0	0	0	8	69	0.000	0.052	0.948

to proline. The *e* allele has a deletion of guanine in position 310, leading frameshift and a predicted protein of 155 amino acids (Fig. 1).

PCR-RFLP

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was carried out in order to detect these three alleles. The F202 and R356M primers allowed the amplification of 154 bp fragment for E^D and E^+ alleles. Digestion with *Msp*A1 separated the E^D allele, which are digested into fragments of 96 and 58 bp, and E^+ allele, which is not digested owing to a nucleotide substitution (Fig. 2a). The F228 and R447 produced 219 and 218 bp PCR products for the *E* and *e* allele, respectively. Digestion with *Msp*I separated between the *E* alleles, which are digested into fragments of 138 and 81 bp, and the *e* allele, which is not digested owing to a missing G (Fig. 2b).

Allele frequencies

Table 2 shows the genotype frequencies and allele frequencies of the extension locus for the three breeds. Five genotypes were observed, corresponding to E^D/E^D, E^D/E^+, E^+/E^+, E^+/e and e/e. The genotyping assay revealed their characteristic gene frequencies for each breed. Japanese Black was almost composed of E^D and E^+ individuals, $E^D = 0.481$ and $E^+ = 0.514$. Japanese Brown and Hanwoo were either homozygous E^+/E^+ or e/e and heterozygous E^+/e. However, their allele frequencies differed considerably between the two breeds. The wild type E^+ allele frequency was 0.962 for Japanese Brown and 0.052 for Hanwoo.

DISCUSSION

We investigated the allele frequencies of the E locus in Japanese and Korean beef cattle. Japanese Black has no homozygous *e/e*, therefore that is consistent with their black coat color as E^D and E^+ alleles allow α-melanocyte-stimulating hormone to bind to receptor and that leads to a black pigment. As expected, most of Hanwoo were homozygous *e/e*. Klungland *et al.* (1995) reported that all *e/e* animals had a red coat color in Norwegian cattle. However, in the current study, E^+/e animals with a red coat color were found in Hanwoo and Japanese Brown. In addition, Japanese Brown showed homozygous E^+/E^+ and heterozygous E^+/e except one homozygous *e/e* animal. Significant differences in MC1R allele frequencies between the Japanese Brown and Korean cattle were observed. The

results suggest that the other genes are associated with a coat color of red and brown in cattle.

Japanese Brown had been improved by adoption of Korean cattle bulls in the past. Therefore, both breeds have quite similar phenotype on body shape and coat color, and Japanese Brown had been called as 'modified Korean cattle breed' for a period of time. However, it is reported that Simmental bulls with a brown coat color had been introduced to improve Japanese Brown for several years since 1906 (Mizuma *et al*. 1982). That could affect the allele frequencies in Japanese Brown. In addition, Japanese Brown has been selected by a particular shade of brown coat color with black eyelashes, hoofs, horns and tails. The selection based on the appearance trait is likely to cause the accumulation in a specific allele. Finally, most Japanese Brown cattle have a dark-pink skin color and Hanwoo cattle has skin color of creamy-white or pinkish white. Therefore, skin colors of Japanese and Korean cattle would be strongly controlled by the E locus, while coat colors by multigenes.

It can be hypothesized that products of agouti could modify the cattle coat color. The interaction between MC1R and agouti is likely to have an effect on the phenotypes. Agouti interacts with MC1R receptor as a competitive antagonist (Lu *et al*. 1994). However, Virador *et al*. (2000) suggested that agouti might act as an inverse agonist rather than as an antagonist of the MC1R as agouti is able to elicit changes in cell shape and in the number and type of melanosomes produced. Therefore, agouti is probably the best candidate gene for that explanation about their hair color differences. Based on our findings, additional investigation of coat color gene, especially agouti gene, will be required to identify the coat color mechanism in Japanese and Korean cattle.

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