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Haplotype analysis revealed candidate region for black/brown coat color gene in cattle

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ABSTRACT

MC1R gene is known to be the main regulator of the switch between black and red coat color pigments in cattle. However, another gene would be associated with black and brown coat colors of cattle because our previous study reported that there were different coat color animals with the same *MC1R* genotype. The aim of this study was to identify the candidate region related to black/brown coat color in cattle. For this purpose, we genotyped 313 microsatellite markers evenly distributed across all cattle autosomes in the F₂ Japanese Black × Limousin resource family. In addition, 36 microsatellite markers located within candidate regions were developed in order to narrow down the candidate region. Our results revealed that the responsible gene for black/brown coat color would be included in the region from 16.1Mb to 26.5Mb on BTA 16.

INTRODUCTION

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 coat color (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 in cattle (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

induces a red coat color (Rouzaud *et al.* 2000).

In cattle, *MC1R* gene is known to be the main regulator of the switch between the two coat color pigments. However, the coat exhibits a wide range of colors and many breeds have a characteristic and specific color pattern. For example, our previous study reported that *E⁺/e* animals with red coat color were found in Korean cattle (Sasazaki *et al.* 2005). In addition, Japanese Brown, with brown coat color, showed homozygous *E⁺/E⁺* and heterozygous *E⁺/e* genotypes except one homozygous *e/e* animal. The results suggested that another gene would be associated with red and brown coat colors of cattle in addition to the *MC1R* gene.

Several genes associated with black/brown coat color have already been reported. Agouti protein, which acts as an antagonistic ligand for *MC1R*, causes hair follicle melanocytes to synthesize reddish yellow pheomelanin instead of black or brown eumelanin (Bultman *et al.* 1992; Miller *et al.* 1993; Barsh *et al.* 2000). Mutations in the ASIP gene, which are associated with black/brown coat color, have been reported in mouse, dog and rat (Kerns *et al.* 2004; Kuramoto *et al.* 2001; Miltenberger *et al.* 2002). Tyrosinase related protein 1 (*TYRP1*) appears in the melanin synthesis pathway after the branch point between eumelanin and pheomelanin synthesis (Kobayashi *et al.* 1998). In mouse, three mutations of *TYRP1*, which result in brown eumelanin pigmentation instead of black, have been described (Zdarsky *et al.* 1990). Of them, a mutant with a substitution from a conserved cysteine to a tyrosine in exon 2 is thought to cause

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the brown phenotype because it encodes a non-functional protein. However no causative genes associated with black/brown coat color have been identified yet in cattle.

The aim of this study was to identify the candidate region related to brown coat color in cattle. For this purpose, the F₂ Japanese Black × Limousin resource family was used for haplotype analysis with microsatellite markers.

MATERIALS AND METHODS

Resource family

A F₂ resource population was generated at National Livestock Breeding Center in Japan. The animals used as parents were two Japanese Black sires (JB-A and JB-B) and two Limousin dams (L-A and L-B). F₁ animals were obtained by crossing JB-A with L-A (family A) and JB-B with L-B (family B). Family A consisted of two F₁ males and 17 F₁ females, and family B consisted of two F₁ males and 15 F₁ females. To avoid obtaining progeny homozygous for latent recessive hereditary disease loci that may present in the two JB sires, F₂ animals were obtained by crossing F₁ males and their non-sibling F₁ females (between family A and B) by using embryo transfer techniques. A total of 198 F₂ animals were produced from July 1999 to October 2001.

MC1R genotyping

PCR-RFLP for *MC1R* genotyping was carried out according to Sasazaki *et al.* (2005).

Microsatellite markers

At first step, 313 microsatellite markers evenly distributed across all cattle autosomes were included in an initial whole genome scan. In addition, new microsatellite markers located within candidate regions were developed in this study (Table 1). Primers were designed according to bovine genomic sequence based on the Bovine Genome Resources at NCBI (<http://www.ncbi.nlm.nih.gov/genome/guide/cow/>).

Genotyping

The PCR reactions were performed using 10 µl reaction volumes with 20ng genomic DNA as a template, 2.0 µl reaction buffer (100mM Tris-HCl, 15mM MgCl₂, 500mM KCl, pH8.6), 1.6 µl dNTP Mix (2.5mM), 0.13 µl of each primer (20nmol/ml) and 1.0U of Ex Taq polymerase Hot Start Version (Takara Shuzo Co., Tokyo,

Japan). Amplification of PCR products was 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 55-65°C, 1-min extension at 72°C, and final extension for 7-min at 72°C. Annealing temperatures of each marker are shown in Table 1. After PCR amplification, reaction products were fractionated on ABI377 DNA sequencer (Applied Biosystems, Foster City, CA), and fragment analysis was performed with GeneScan and Genotyper software (Applied Biosystems, Foster City, CA).

Linkage and Haplotype Analysis

Linkage maps for the 29 bovine autosomes were constructed by using CRI-MAP software (Green *et al.*, 1990), and the constructed map was used for the whole-genome scan to find the candidate regions. In second analysis using new developed markers, haplotypes were constructed manually, by minimizing the number of recombinants and assuming no mutation of marker alleles. The candidate region was defined on the basis of observed ancestral recombination events.

RESULTS AND DISCUSSION

At first, genotypes of the *MC1R* gene were investigated in the F₂ resource family (Table 2). In parental generation, two Japanese Black cattle with black coat color have *E^D/E^D* and *E^D/E⁺*, respectively, and two Limousin cattle with red coat color had *e/e* genotype. In 36 F₁ animals, 29 and seven, all of which showed black coat color, had *E^D/e* and *E⁺/e* genotypes, respectively. In 198 F₂ animals, 138 with black coat color had one of *E^D/E^D*, *E^D/E⁺*, *E^D/e* or *E⁺/e* genotypes. 56 animals, with brown coat color, had *e/e* genotype. The remaining four animals with dark brown coat color had *E⁺/e* genotype. Therefore, seven animals, with *E⁺/e* genotype, showed different coat colors of black and dark brown, although F₁ individuals having *E⁺/e* genotype revealed black color (Table 2). As well as our previous study for Korean and Japanese Brown cattle (Sasazaki *et al.* 2005), current result suggested that there was another gene associated with coat color, which interacts with the *MC1R* gene. Since all Japanese Black (Sasazaki *et al.* 2005) and F₁ animals with *E⁺/e* genotype have black coat color, we assumed that Japanese Black had dominant allele for black coat color and Limousin had recessive one in parental generation.

Table 1. Microsatellite markers developed in this study

Marker	BTA	Location (Mb)	Tm (°C)	Forward primer (5' → 3')	Reverse primer (5' → 3')
<i>KboMS001</i>	14	20.7	63	ACGGTCAACTCAGAGGCGGGT	AACCCACTCCAGTATTCTTGCCT
<i>KboMS002</i>	14	21.1	60	ACATCCACCTTACAAACCAACCT	CAGTCCATAGCGTCGCAAAGA
<i>KboMS003</i>	14	23.1	60	GGATTGCGCTTCTCTTCTGACAT	AGCAACCTAAGTGCCATCAACGA
<i>KboMS004</i>	14	23.0	63	TAGAAATCCACCCCTGCTGTCACT	CTGGGGTGGAAGGAGCAATGT
<i>KboMS005</i>	14	21.9	60	ATGTCTTGAAATTGATGGAGTTAC	GCCTCTCCTCTTCCCACTGATA
<i>KboMS006</i>	14	22.1	60	AATCTGGGTCTCTTGCGTTGC	CCCAGTGACAAATAGACAGCCAAT
<i>KboMS007</i>	14	18.9	65	GGCTGTCATAGGCATCTGTCATT	GTTCCCTTGGTGGTCTGTGAGT
<i>KboMS008</i>	14	18.9	60	GACTACAGGGAGGTTATATTTCAAT	GTAACAGAGCAATGAAAGCCAAT
<i>KboMS009</i>	14	20.0	65	ACCATCTCTACCGCTGCTACGAAC	TTTTTGTGTCCAGCCAGAGAACCT
<i>KboMS010</i>	14	20.1	60	TTTTTATTGGAGTAGAGTTGCTTTA	GTGACCTAAATGGGAAAGAAATC
<i>KboMS011</i>	14	19.3	60	AGTGAGGTTGGCGAGTTGAGTAT	AATCCACCTGCCATACGTGCACT
<i>KboMS012</i>	14	19.9	61	CCTCCAGTGTCATAGCAGCA	TTAGGTCCATTGTTGCTGCA
<i>KboMS013</i>	14	19.7	60	ACCTAAATACTCTGTGTCCCTACCT	GACGACTGCCATTTTTTACCACAT
<i>KboMS014</i>	14	19.3	60	ATTGTTGTGTTGGTTTCTGCTCTG	GTGAAACAGATAGCTAGTGGCAAG
<i>KboMS015</i>	14	19.6	58	CAAAAGCTCCTCAGGTGATACT	GTTTCTGAGTTAATACAAGGCTGA
<i>KboMS016</i>	14	20.4	58	GATTTTTGGGGGAAGCAGAGAT	TTGATACATTGTTCTGAGCATTGA
<i>KboMS017</i>	14	20.4	58	GCACTAACCTCCCCAACAAAGA	AGGTTAGGACACATCTTTGGTTAT
<i>KboMS018</i>	14	23.7	60	AGAATGGGTAAACAAAAGGTCCT	CTGGTGGGCTACAATTCATGG
<i>KboMS019</i>	14	22.5	60	CATGGGGTCACAAAAGATCAGA	AGGCTTAGAAAACCCAGAAAACCAT
<i>KboMS020</i>	14	21.8	60	TATTATTTTTCTGGTTTGGATTGCA	TGGAGAAGGGAATGGCTACC
<i>KboMS021</i>	14	23.7	60	CAATGACAATAGCAACAAAACTG	AGTGCTCCTTCTAAATTCAGTGTG
<i>KboMS022</i>	14	22.4	60	TCCACAAATCTCTCAAAAGTATCT	ATGTATGCAATTCAGTGAAACCTC
<i>KboMS023</i>	14	21.7	60	AGAGCAGCCAAAGGTAAATAAGTAA	AATCTGACCAAAACAAAGCCACC
<i>KboMS024</i>	14	23.5	60	GATGTTCTCTCTTTTCCCTACTTTA	CCACCCAGTATTCTTGCCT
<i>KboMS025</i>	14	23.1	60	CCCTTTGCTGAACCTGTTGCTA	GTGTTTGATAGCGTGTGGTTAGTA
<i>KboMS026</i>	14	21.9	60	GCAGGCTACAGTCTATGGGGT	GTCTTTTCATTGTTCTCTTGGT
<i>KboMS027</i>	14	21.0	60	TGTTAGGCAGGGTGGTACTTGAG	ACCCCCAGGAGCAAAAGATCAG
<i>KboMS028</i>	14	24.1	60	GAAGGCAGATAACGGGGCTAC	CTATTTGAATTATTGGGCAGAAGA
<i>KboMS029</i>	14	21.5	65	GTGCCCCTGCCTGGAGA	ACGATACACAAGTTGGAATGCCG
<i>KboMS030</i>	16	17.2	60	ATAAGCTAACCTCTGACATTGAC	CGGTTTCTTGTTACTTATCTAGG
<i>KboMS031</i>	16	18.4	60	GGAAGCAGGATTGGCATAGTGA	TCCCTTCCAATGACTGTTCCGT
<i>KboMS032</i>	16	18.4	60	CTTTCTCTCTCATAACCTTGCC	TGTAGAATCATCAGGGCAGAGTG
<i>KboMS033</i>	16	20.9	60	TACTGTATAAATGAGCTTGACG	TGGTAATTCTGTATCTGAGGCA
<i>KboMS034</i>	16	17.3	60	GTGGGAGCTGATGAGTTTCTGA	TGGGAGGCTATTACTTTTCATTC
<i>KboMS035</i>	16	18.6	65	GGAGGAGGGAATGGCTACCGACT	AGGGACCCATGACAAGATTACAG
<i>KboMS036</i>	16	20.9	58	CATAGAAGTGTAGTTGCTGAGTC	ATAAGAGCAATGTAAGTGAAAC
<i>KboMS037</i>	16	18.2	60	TAAAGTAAGGTGCTCAATAAAGGTT	TGAGAGGCGTGAGGAGACTTTAA
<i>KboMS038</i>	16	19.3	60	GAGAATACAGAGGTGGACATGAC	GGGTTGAAAAGAGTTGGATACGA
<i>KboMS039</i>	16	19.4	60	GCTTTGCCCTTTTTCAGAATGTAATA	GGATTAAAAGAAGACTGAGACATGA
<i>KboMS040</i>	16	20.9	60	AGTCCATCTACCTTTCAAGTGCA	TGAAATTACTTATCTACCTCTCCA
<i>KboMS041</i>	16	21.7	60	CAGAGACCAGGGGGATAAGAACT	CTATGTGGTCTGATGAAACTTA
<i>KboMS042</i>	16	21.6	60	GAAAAAGACAGTCCCCCTACCTA	TGGAGCGTCAGACCTATTTTGGGA
<i>KboMS043</i>	16	24.3	60	AACGTTGAATTCTGGTATACATATC	CCTTGGTAAATGTCTGGTATGTT
<i>KboMS044</i>	16	25.2	60	TGACAACCACAGAGCAAATAGGA	GAGCCTGGGCAACTAAGCACATT
<i>KboMS045</i>	16	24.8	60	CTACATACAGTCCTTGGGGTCAC	ATTTTTTCCTCCAAGCTCTCAG
<i>KboMS046</i>	16	26.1	60	GGAAATGAGATAGCAGTGAATGA	CTACCCGCCAGGCACAAAATAAA

Table 2. Genotypes of the extension locus in Japanese Black × Limousin F2 resource family

Generation (Breed)	color	n	Genotype frequencies				
			E^D/E^D	E^D/E^+	E^D/e	E^+/e	e/e
P (Japanese Black)	Black	2	1	1	-	-	-
(Limousin)	Red	2	-	-	-	-	2
F ₁	Black	36	-	-	29	7	-
F ₂	Black	138	51	9	75	3	-
	Brown	56	-	-	-	-	56
	Dark brown	4	-	-	-	4	-

Consequently we performed haplotype analysis for 16 animals including four parents, five F₁ and seven F₂ animals, with E^+/e genotype, to identify candidate region where responsible gene for black/brown coat color might be located on. 313 microsatellite markers evenly distributed across all cattle autosomes were subjected to an initial whole genome scan. According to the hypothesis described above, candidate regions are the regions where in seven F₂ animals with E^+/e genotypes, three black animals are homozygous or heterozygous for the allele of Japanese Black while four brown are homozygous for that of Limousin. This result revealed two candidate regions ranged from 18.6 Mb to 27.3Mb on cattle chromosome (BTA) 14 and from 8.8Mb to 26.5Mb on BTA 16.

Subsequently, we tried to confirm and narrow down the candidate region using additional microsatellite markers. A total of 46 markers, 29 on BTA 14 and 17 on BTA 16 in candidate regions, were developed using information of cattle genome sequences and their polymorphisms were tested on parents of resource family. Twenty-two of the 46 markers were polymorphic between the parents and 10 markers were haplotype analysis capable. The genotypes of four parents, four F₁ and seven F₂ animals were analyzed using 10 markers in the region, and then their haplotypes were constructed based on the physical map of the markers deduced from the draft whole-genome bovine sequence (based on Btau_3.1). Figure 1 showed haplotypes in seven F₂ animals observed recombination events in candidate region.

In candidate region ranged from 18.6 Mb to 27.3Mb on BTA 14 (Fig. 1A), three black F₂ animals (OU-012, -066 and -084) had homozygous allele derived from Japanese Black. Of the four brown F₂ animals, two (OU-054 and -144) were homozygous for the Limousin allele. In the other two animals (OU-025 and -157), Japanese Black

alleles were overlapped between *MS001* (20.7Mb) and *MS027* (21.0Mb). Therefore we concluded that this would not be candidate region.

In the candidate region ranged from 8.8Mb to 26.5Mb on BTA 16 (Fig. 1B), the three F₂ black animals were homozygous or heterozygous for the Japanese Black allele. The four brown F₂ animals were homozygous for the Limousin allele at the region from *MS008* (18.2Mb) to *MS014* (24.3Mb). Therefore, these results suggested that the responsible gene for black/brown coat color would be included in this region from 16.1Mb to 26.5Mb on BTA 16.

We investigated functional genes in the candidate region on BTA 16, and then 84 genes were assumed to be in this region, ranged from 16.1Mb to 26.5Mb on BTA 16. Out of those, 16 genes have functions that are already known. However no genes related to pigment function were included in those genes. Sasazaki *et al.* (2005) suggested that *ASIP* is probably the best candidate gene for the explanation concerning black/brown coat color differences in cattle, but this gene is located on BTA 13, not within candidate region on BTA 16.

Comparison of the order of these genes of the cattle genomic sequence with mouse and human revealed that this candidate region showed homology with regions ranged from 165.2Mb to 166.8Mb and 177.1Mb to 184.3Mb on MMU1 and 146.7Mb to 147.9Mb on MMU4 in mouse, 10.5Mb to 12.0Mb, 166.8Mb to 168.9Mb, 221.0Mb to 225.0Mb, 239.1Mb to 244.9Mb on HSA1 in human. These homologous regions on MMU and HSA contain 54 and 68 known genes, respectively. However no one of these genes is obviously involved in coat color function.

In cattle, most of the genes related to coat color have not been identified yet. In mouse, more than 100 genes related to coat color have already been identified. However Bennett *et al.* (2003) suggested that it seems likely

that the number of distinct genes will rise to at least 150. Therefore the causable genes for brown coat color, which have not been identified to date, might be located within this candidate region. In order to pursue candidate genes for brown coat color, it will be essential to narrow down the candidate region interval.

In this study, we localized the candidate gene region

for bovine brown coat color to approximately 10Mbp region on BTA 16. In order to identify a responsible gene for cattle coat color, further genetic and biochemical research for genes located in this region would be required. Identification of the responsible gene will contribute to the study of pigment mechanisms of coat color.

A: BTA14

B: BTA16

Fig. 1. Haplotype analysis of seven F2 animals in candidate region. (A):BTA14 (B): BTA16. Black boxes indicate alleles derived from Japanese Black. Grey boxes indicate alleles derived from Limousin. Open boxes indicate alleles whose sources are unclear.

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