

Identification of mitochondrial DNA substitutions related to meat quality in Japanese Black cattle^{1,2}

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ABSTRACT: Complete sequences of mitochondrial (mt) genomes of eight Japanese Black cattle were determined to investigate the relationships between mt deoxyribonucleic acid (DNA) displacement loop (D-loop) types and other mtDNA regions and to identify the variation in the coding region that may influence the economic traits. The survey of mitochondrial sequences in the encoding region revealed 14 substitutions including six antonymous substitutions and one in 16S ribosomal ribonucleic acid (rRNA). Three methods of polymorphic DNA analyses (polymerase chain reaction [PCR]-restriction fragment length polymorphism [RFLP], mismatch PCR-RFLP, PCR-single-strand conformation polymorphism [SSCP]) were performed on these seven candidate substitutions (base pair [bp] 2,232, 12,158, 12,908, 13,310, 14,122, 14,140, and 14,565) for 202 Japanese Black cattle. The substitution

of bp 13,310 was observed in all samples, but not in the reference sequence, indicating that this is a minor substitution or a sequencing mistake in the reference sequence. The substitutions at bp 14,122, 14,140, and 14,565 were observed in only a few samples, suggesting that these were also minor substitutions. The substitutions at bp 2,232 (16S rRNA), 12,158, and 12,908 (reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase chain-5) were closely related to mitochondrial D-loop types that have previously been related to differences in the carcass traits of Japanese Black cattle. Evaluation of the effects on six carcass traits with mixed model procedures suggests that the bp 2,232 substitution affects longissimus muscle area and beef marbling score. The substitution at bp 2,232 is a strong candidate for the mitochondrial effect on meat quality.

Key Words: Beef Cattle, Carcass Quality, Cytoplasmic Inheritance, Mitochondrial DNA, Nucleotide Sequence, Ribosomal RNA

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Introduction

The presence of maternal genetic effects has been hypothesized about and discussed by several authors (Venge, 1953; Wagner, 1972). Mitochondria are a likely source since they contain their own DNA and are maternally inherited (Brown et al., 1989). The cytoplasmic effects on yield traits in dairy cattle have been pre-

viously detected using field data sets (Bell et al., 1985; Kennedy, 1986; Boettcher et al., 1996b), but by performance traits in beef cattle (Tess and Robison, 1990; Northcutt et al., 1991; Tess and MacNeil, 1994). Recent studies showed the relationships between economic traits and mitochondrial DNA (**mtDNA**) sequence variations in dairy and beef cattle (Schutz et al., 1994; Boettcher et al., 1996a; Mannen et al., 1998a).

A previous study determined the sequences of the displacement loop (**D-loop**) region of mtDNA in order to detect the effects of mtDNA variations on carcass traits of Japanese Black cattle (Mannen et al., 1998a). These results showed significant differences between the mitochondrial haplotypes for two carcass traits of Japanese Black cattle. The D-loop region does not code any gene products, so variations of this region were used as genetic markers for the remaining regions of mtDNA.

In this study, we determined the complete mtDNA sequences from eight Japanese Black cattle in order to

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identify the variation of the transcribed mtDNA product that may have an influence on carcass traits.

Materials and Methods

Animals

This study used DNA from 202 fattening Japanese Black steers. These were the same samples used in previous works, and the mitochondrial D-loop sequences from these samples were already determined (GenBank accession numbers U87633–U87650, U87893–U87905) (Mannen et al., 1998a,b). These mtDNA sequences showed 26 haplotypes and were classified into five major mitochondrial types (mtDNA D-loop types 1 to 5) (Mannen et al., 1998a). In order to determine complete mtDNA sequences, we used eight samples (JBC1 to 8), which provided two samples from each of four representative haplotypes (type 1 to 4). A complete sequence of bovine mtDNA sequence determined by Anderson et al. (1982) classified the additional mitochondrial D-loop type 5 (Mannen et al., 1998a).

Sequencing

Fifty-four primers were designed and used for PCR amplification and sequencing of mtDNA based on published bovine gene sequences (accession number V00654; Anderson et al., 1982). The PCR reactions were done with 50 ng of genomic DNA as a template in a volume of 50 μ L of 1 \times reaction buffer; 25 μ M of dNTPs; 0.5 μ M of each primer; and 2.5 U of EX *Taq* polymerase (Takara Shuzo Co., Tokyo, Japan). Amplification of PCR products was carried out using a standard PCR program with 2-min denaturation at 94°C, 30 cycles for 1 min at 94°C, 1-min annealing at 65°C, 2-min extension at 72°C, and a final extension for 7 min at 72°C. After purification of the PCR product using a GENE-Mate DNA purification kit (ISC BioExpress, Kaysville, UT), standard double-strand DNA cycle sequencing was performed with approximately 200 to 500 ng of amplified product using SequiTherm EXCEL II DNA sequencing kits (Epicentre Technologies, Madison, WI) and was analyzed on a LI-COR automatic sequencer (model 4200L, Lincoln, NE). Variations in complete mitochondrial genome were defined by comparison with the bovine mtDNA reference sequence published by Anderson et al. (1982).

Detection of Polymorphisms

In order to detect mtDNA candidate substitutions for the 202 Japanese Black cattle, PCR-RFLP, mismatch PCR-RFLP (Haliassos et al., 1989a,b), and PCR-single-strand conformation polymorphism (SSCP) were used depending on the mtDNA substitution. The primer sequences used to amplify the mtDNA segment that contained substitution loci are shown in Table 1.

The PCR-RFLP was performed to detect the polymorphisms as substitutions at bp 2,232 and 14,565. The

PCR reactions used 50 ng of genomic DNA as a template in a volume of 20 μ l of 1 \times reaction buffer; 25 μ M of dNTPs; 0.5 μ M of each primer; and 1 U of EX *Taq* polymerase. Amplification of the PCR products were carried out using a standard PCR program with 2-min denaturation at 94°C, 30 cycles for 1 min at 94°C, 1-min annealing at 63°C, 1-min extension at 72°C, and a final extension for 7 min at 72°C. To detect the substitutions at bp 2,232 and 14,565, amplified PCR products were digested with *Eco*T22I and followed by electrophoresis in a 1% agarose gel.

Mismatch PCR-RFLP was performed to detect polymorphisms at bp 13,310 and 14,122. Primers BMT13334R and BMT14123R contained a purposeful mismatch sequence, so that when incorporated into the PCR products, they create a *Bbr*PI restriction site with the allele from the reference sequence but not with a mutation allele, and a *Nla*III restriction site with a mutation allele, but not with the reference allele, respectively. The PCR reactions were done with 50 ng of genomic DNA in a volume of 5 μ L of 1 \times reaction buffer; 25 μ M dNTPs; 1 μ M of each primers; and 0.5 U of EX *Taq* polymerase. Cycling conditions were 1 min at 94°C, 30 cycles for 30 s at 94°C, 30 s at 53°C (bp 13,310) or 65°C (bp 14,122), 1 min at 72°C, and final extension for 7 min at 72°C. The PCR products were digested with *Bbr*PI (bp 13,310) or *Nla*III (bp 14,122), followed by electrophoresis in a 3% MetaPhor agarose gel (BioWhittaker Molecular Applications, Rockland, ME).

The PCR-SSCP was performed to detect the polymorphisms at bp 12,158, 12,908 and 14,140 using a modification of the method described previously (Mannen et al., 2000). Briefly, forward PCR primers (BMT12074F, BMT12849F, BMT14047F) were labeled with [γ -³²P] ATP (6,000 Ci/mmol) and T4 polynucleotide kinase (Takara Shuzo Co., Tokyo, Japan). The PCR reactions were done with 10 ng of genomic DNA in a volume of 5 μ L of 1 \times reaction buffer; 25 μ M of dNTPs; 1 μ M of each primer; and 0.5 U of EX *Taq* polymerase. Cycling conditions were 1 min at 94°C, 20 cycles for 30 s at 94°C, 30 s at 66°C, 1 min at 72°C, and a final extension for 4 min at 72°C. The products were mixed with 245 μ L of deionized formamide solution including 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The samples were analyzed by 5% polyacrylamide gel electrophoresis under controlled gel temperature at 6°C (bp 12,158) or 12°C (bp 12,908 and 14,140).

Statistical Analysis

Six carcass traits—carcass weight, longissimus muscle area (**LMA**), rib thickness, subcutaneous fat thickness, yield estimate, and beef marbling score (**BMS**)—of the animals were analyzed by a mixed linear model similar to that of a previous study (Mannen et al., 1998a). The model included herd year as a fixed effect, slaughter age and inbreeding coefficient as covariates, and both nuclear additive and maternal genetic effects and residuals as random effects. In addition, the ab-

Table 1. Seven candidate substitutions of bovine mitochondrial genome observed in this study and primers used for PCR amplification in PCR-RFLP, mismatch PCR-RFLP, and SSCP analyses^a

Locus ^b	Primer name	Gene ^c	Sequence (5' to 3') ^d
G2232A (PCR-RFLP)	BMT1513F BMT2541R	16S rRNA	CAACCAAGCTAGAATAAAACAAAACA GGTTAAATCTATTCTCTTTGGGTTGG
T12158C (SSCP)	BMT12074F BMT12226R	ND5	GTTTATCCGTTGGTCTTAGG GTAATGAAGGCGTATGAGAT
C12908T (SSCP)	BMT12849F BMT13007R	ND5	CTCCCGTCTCAGCACTACTC TGTGGAGAAGGCGATGATTT
A13310C (mis-PCR-RFLP)	BMT13243F BMT13334R	ND5	CCTCATTGTTGGCAGTCTCG AGGGCTCAGGCGTTGGTATAACAC
C14122T (mis-PCR-RFLP)	BMT14080F BMT14123R	ND6	TATCATAAATCACCCAATCCCCTAAACC GTTGATGGAGTCTTTATGGTCTATCAT
A14140G (SSCP)	BMT14047F BMT14206R	ND6	TCACTAAAGAACCAGAATC GGCTATGGCTACAGAACAGT
T14565C (PCR-RFLP)	BMT14177F BMT14752R	CytB	CAAGCCACTAACAAATGCCCTAAAACA ATTGAAGCTCCGTTTGGTGTATGTATC

^aSSCP = single-strand conformation polymorphism.

^brRNA = ribosomal RNA; ND5 = NADH-ubiquinone oxidoreductase chain-5; ND6 = NADH-ubiquinone oxidoreductase chain-6; CytB = cytochrome *b*.

^cG2232A indicates G to A substitution on base pair 2,232. Parentheses indicate analysis methods applied to each locus. mis-PCR-RFLP = mismatch PCR-RFLP.

^dUnderline in the primer indicates purposeful mismatch sequence to create restriction site of endonuclease.

sence or presence for each of the four substitutions at a protein-coding region was considered to be a fixed effect to detect the influence of each substitution on the traits. Pedigrees of the steers were traced back to individuals born in 1975, and the additive relationship matrix of steers and their ancestors included 1,722 animals. The variance components assumed were shown in a previous study (Mannen et al., 1998a). The general linear hypothesis tests were conducted for each substitution using best linear unbiased estimators (**BLUE**).

Results

The complete sequence of bovine mtDNA genome has been determined by Anderson et al. (1982). Here, we have determined the complete mtDNA sequences of eight Japanese Black cattle (DDBJ accession numbers AB074962 to AB074968). The positions of nucleotide substitutions are given in Table 2. The alignment of eight Japanese Black steers and the reference sequence identified eight Bos haplotypes of complete mtDNA genomes. The sequences of JBC3 and JBC4 showed a completely identical sequence, and JB7 and JB8 sequences were identical when the D-loop region was excluded. A total of 13 substitutions among eight haplotypes in D-loop region were observed, along with 14 substitutions in the rest of the mitochondrial genome. The mean sequence divergence of the D-loop regions among haplotypes was 0.47%, whereas that of the gene coding regions was 0.032%.

Of the 14 substitutions, one was in the 16S ribosomal RNA (**rRNA**) (bp 2,232) and six of the 13 substitutions in protein-coding genes were antonymous substitutions and would cause amino acid replacements. Three of the substitutions (bp 12,158, 12,908, and 13,310) were in

the NADH-ubiquinone oxidoreductase chain-5 (**ND5**) region, two (bp 14,122 and 14,140) were in the NADH-ubiquinone oxidoreductase chain-6 (ND6) region, and one (bp 14,565) was in the cytochrome *b* region. These seven nucleotide substitutions are candidates as markers for the effects of mtDNA variation on carcass traits in Japanese Black cattle. The remaining seven substitutions (bp 4,301, 6,178, 6,424, 8,400, 10,889, 11,174, and 15,510) were all synonymous substitutions.

In the seven candidate substitutions, the substitution at bp 2,232 was observed in JBC7 and JBC8 (Table 2), the mitochondrial D-loop type 4 as described previously (Mannen et al., 1998a). The substitution at bp 12,158 was observed in JBC1, 2, 3, and 4, which are in both D-loop types 1 and 2. The substitution at bp 12,908 was observed in JBC 5 and 6, which are in the D-loop type 3. The substitution of bp 13,310 was observed in all samples used in this study, but not in reference sequence (EU1, Table 2). The other three substitutions (bp 14,122, 14,140, and 14,565) were detected in only one sample from eight animals.

In order to assess the relationships between the candidate substitutions and the mitochondrial D-loop types defined, we subsequently performed polymorphic DNA analyses using PCR-RFLP, mismatch PCR-RFLP, and PCR-SSCP methods for 202 Japanese Black cattle whose mitochondrial D-loop sequences had been determined previously (Mannen et al., 1998a,b). These results were summarized in Table 3.

The substitution at bp 2,232 was detected in only mtDNA D-loop type 4. Only one sample of D-loop type 4 did not have this substitution. The substitution at bp 12,158 was detected in D-loop types 1, 2, and 5. However, most samples of D-loop types 1 (99.2%) and 2 (100%) contained this substitution, whereas it was ob-

Table 4. Difference of the effect (BLUE) with nucleotide substitution from the effect without substitution on six carcass traits of Japanese Black cattle^a

Substitution	CW, kg	LMA, cm ²	RT, cm	SFT, cm	YE, %	BMS, unit
G2232A ^a	9.00	5.33**	0.22	0.05	0.66	0.53*
T12158C	3.90	2.28	-0.08	-0.07	0.24	0.15
C12908T	-6.54	-1.43	-0.24	-0.26	-0.05	-0.32
T14565C	-4.47	0.27	-0.07	-0.10	0.15	0.04

^aBLUE = best linear unbiased estimator. CW = carcass weight; LMA = longissimus muscle area; RT = rib thickness; SFT = subcutaneous fat thickness; YE = yield estimate; BMS = beef marbling score.

* $P < 0.05$.

** $P < 0.01$.

population, polymorphic DNA analyses were performed for the substitutions in 202 Japanese Black cattle whose D-loop sequence had been previously determined (Mannen et al., 1998a,b).

Of the seven mtDNA substitutions, one was in the 16S rRNA (bp 2,232). This was a consistent substitution throughout mtDNA D-loop type 4 and the substitution was observed only in this D-loop type (Table 3). The cattle with D-loop type 4 mtDNA tended to have better meat quality than the cattle with other D-loop types (Mannen et al., 1998a). The substitution of bp 12,158 was observed in most of D-loop type 1 animals (99.2%), all of type 2 (100%) and a few of type 5 (7.3%) (Table 3). The D-loop types 1 and 2 have been detected exclusively in Japanese Black cattle and are closely related (Mannen et al., 1998a,b). This substitution causes the amino acid replacement of Met to Thr in the ND5 protein at AA 17. The substitution of bp 12,908 was observed in all samples of D-loop type 3 (100%) and in one animal of D-loop type 5 (2.4%). This seems to be a common polymorphism in D-loop type 3 cattle, although the number of samples with the D-loop type 3 analyzed was small. This substitution causes the amino acid replacement of Thr to Met in the ND5 protein at AA 207. These amino acids differ by having polar uncharged side chains and a nonpolar side, respectively. These substitutions are likely candidate gene polymorphisms associated with the traits of meat quality.

The substitution of bp 13,310 was observed in all samples examined in this study, but was not observed in the reference sequence (Anderson et al., 1982). The substitution causes the amino acid replacement of Lys to Thr in the ND5 protein at AA 341. A search of gene databases determined that usual amino acids at this position are Thr, Ser, Asp, or Met for most vertebrates. While Met is the most frequent amino acid in primates, Thr is most common for other vertebrates at this amino acid position. Also, the amino acids have similar properties, even though Thr, Ser, and Asp are polar uncharged side-chain amino acids, and Met has nonpolar side chains. The replacement of Lys, which has basic side chains, may be a crucial AA substitution as it has different properties to the other amino acids. Further, no vertebrate species with Lys at this position of the ND5 protein has been identified apart from the reference sequence (data not shown). This shows a limitation of

the reference sequence and the lack of the substitution in the reference may be a sequencing artifact or reading mistake. Our results suggest that an amino acid at position 341 in ND5 should be Thr in cattle.

The substitutions at bp 14,122, 14,140, and 14,565 were observed in only a few samples (Table 3). These substitutions cause the amino acid replacements of Val to Met at bp 14,122 and Phe to Leu at bp 14,140 in the ND6 protein and Phe to Leu at bp 14,565 in the cytochrome *b* protein. In addition, these are all nonpolar side-chain amino acids, so that the protein character would not be changed by these replacements.

Our results also revealed intraspecific comparisons to assess variability in coding regions of complete mtDNA in cattle. Interestingly, the six substitutions causing amino acid replacement observed in this study were limited to regions from bp 12,158 to 14,565 that encode ND5, ND6, and cytochrome *b* proteins. In addition, the rate of amino acid replacement in this study (6/13, 46.2%) is higher than that of human (12/35, 34.3%), mouse (9/47, 19.1%), and sheep (2/31, 6.5%) (Hienleder, 1998). This is useful information as it suggests that the genetic diversity of mtDNA in cattle is greater than for other species. The reasons for these differences among species remain unclear.

Differences in BLUE were examined for the substitutions at bp 2,232, 12,158, 12,908, and 14,565, and were significant at the bp 2,232 substitution in LMA and BMS (Table 4). There were differences of 5.33 cm² ($P < 0.01$) in LMA and 0.53 units ($P < 0.05$) in BMS. The BMS is the most important economic trait in Japanese beef markets. Given that BMS ranges from null (0) to very abundant (5) with the average of 1.60, a difference of 0.53 units is substantially large. This substitution corresponds with mtDNA D-loop type 4 mtDNA; cattle with this D-loop type had better meat quality than the cattle with other D-loop types (Mannen et al., 1998a). These results were consistent with the findings in the previous study. This substitution may alter the function of the mitochondrial ribosome and the rate of mitochondrial protein synthesis. This substitution can be regarded as a strong candidate in the mitochondrial genome for the expression of BMS.

In conclusion, we suggest one candidate gene polymorphism from mtDNA substitutions at bp 2,232 that is related to the carcass traits of LMA and BMS in

Japanese Black cattle. This finding results from the effect of mtDNA sequence and not nuclear effects from dams and sires because additive nuclear genetics effects and nuclear maternal genetic effects were considered in the mixed linear model. The representative mtDNA haplotypes from European cattle breeds were also observed in Japanese Black cattle (Mannen et al., 1988b). This suggests that these polymorphisms might be used to detect genetic diversity for carcass traits in both Japanese and European breeds.

Implications

One strong candidate gene substitution associated with longissimus muscle area and beef marbling score was identified in 16S ribosomal ribonucleic acid of mitochondrial deoxyribonucleic acid. The polymorphism may be utilized to detect the maternal effect of multifactorial genetic variation in cattle. Further analysis of the mitochondrial deoxyribonucleic acid variation in the gene would be important to determine the mechanism and magnitude of the mitochondrial deoxyribonucleic acid polymorphism as a new method of selection for the carcass trait in Japanese Black cattle.

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