SHORT COMMUNICATION

Genetic polymorphism of bovine beta-casein gene in Japanese dairy farm herds

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Abstract
The aim of this study was to investigate beta-casein polymorphism among 320 Japanese cows sampled from eight dairy farms. We used a newly-developed genotyping method that involved collecting DNA from hairs and a Cycleave polymerase chain reaction (PCR) assay to detect the A1, A2, and B variants. Results revealed the presence of five genotypes (A1A1, A2A2, A1A2, A1B, and A2B). We found that the most common genotype was A2A2 (0.42), followed by A1A2 (0.39) and A1A1 (0.11). The A1B and A2B genotypes were less frequent (<0.05). The frequencies of alleles A1, A2, and B were calculated to be 0.32, 0.64, and 0.04, respectively. Our study is the first to show the current status of beta-casein polymorphisms in Japanese dairy farms. Given the adverse effects of A1 beta-casein on human health, attempts have been made to develop herds consisting solely of A2A2 cows. Our study provides a reference for improving cow populations in Japanese dairy farms. The Cycleave PCR-based assay we developed here can be used for rapid and reliable genotyping of bovine beta-casein.

KEYWORDS
A2 milk, allele frequency, beta-casein, Cycleave PCR, genotyping

1 | INTRODUCTION

Casein is a major bovine milk protein, comprising about 75%–80% of total milk protein. Mammalian milk is a complex fluid mixture whose properties are influenced greatly by the tendency of casein to form protein complexes. The casein protein complex is composed of four caseins, namely, alpha S1, alpha S2, beta, and kappa caseins. These proteins differ in amino acid sequence and are encoded by four casein genes (CSN1S1, CSN1S2, CSN2, and CSN3) locating on a cluster on chromosome 6 (Rijnkels, 2002).

Beta-casein contains 209 amino acid residues and the CSN2 gene has 12 reported variants (Farrell et al., 2004). Among dairy cattle breeds, the most common CSN2 variants are A1 and A2. These variants result from a single nucleotide polymorphism (SNP) at codon 67 within exon 7, which changes CCT (A2, proline) to CAT (A1, histidine). Domesticated non-bovine species, such as water buffalo and goat, only express beta-casein A2 (Oliveira et al., 2021). The A2 variant is therefore thought to be encoded by the wild-type allele. Variant B is less common and results from a SNP at codon 122 in exon 7, which changes the codon from AGC (serine) to AGG (B, arginine). This arginine codon always coincides with a histidine-coding codon 67. Thus, variant B shares the His 67 residue with variant A1.

Consumption of milk from cows expressing beta-casein A1 may be associated with the etiology of various chronic diseases, including type 1 diabetes, ischemic heart disease, autism, and schizophrenia (Elliott et al., 1999; McLachlan, 2001). The mechanism of action involves the A1 variant, which can produce a bioactive opioid peptide, beta-casomorphin-7 (BCM7) during gastrointestinal digestion.
BCM7 is an exogenous agonist of μ-opioid receptor (MOR), which modulates a large number of physiological functions, including endocrine and autonomic nervous system functions, emotions and cognitive ability, and gastrointestinal functions (Ribeiro et al., 2005; Sternini et al., 2004; Vuong et al., 2010). MORs are highly expressed in the central and peripheral nervous systems and, interestingly, in the small intestine (Duraffourd et al., 2012; Le Merrer et al., 2009). Thus, BCM7 appears to activate MORs and may thereby influence endocrine, cognitive, and gastrointestinal functions.

Furthermore, A1-free milk, which contains only the A2 variant of beta-casein, causes fewer symptoms of lactose intolerance in patients suffering from lactose malabsorption than conventional milk, which contains both A1 and A2 beta-caseins (Ramakrishnan et al., 2020).

A1-free milk is therefore thought to have the potential to prevent several chronic diseases and digestive discomfort from dairy products. This has led to attempts to select dairy cows based on their beta-casein polymorphism. Indeed, selection programs have been started to develop dairy cattle herds that produce milk containing only beta-casein A2 in different countries, including New Zealand (Kaminski et al., 2007) and Mexico (Duarte-Vazquez et al., 2017). To achieve this, cattle of the herds have been genotyped for the beta-casein. To date, several genotyping methods have been developed that included direct sequencing (Massella et al., 2017) and polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) (Cieslinska et al., 2019), but these methods needed downstream sample processing after PCR.

The Cycleave PCR assay (Takara Bio Inc., Kusatsu, Shiga, Japan) can detect a single polymorphism nucleotide in real time. This assay utilizes a chimeric RNA–DNA probe and RNase H to detect the target nucleotide. The chimeric probe contains an RNA base which can be cut by RNase H when the probe forms a hybrid with a complementary DNA sequence. This cleavage results in the emission of strong fluorescence. If there is a mismatch within the probe-binding region, the probe will not be cut by RNase H, which leads no emission. The assay can therefore detect the beta-casein alleles in real time.

In Japan, although commercial services of the beta-casein genotyping have been available, there have been no reports describing the current status of beta-casein variants in dairy farms. In this study, we applied the Cycleave PCR method to determine CSN2 genotypes and measured both genotype and allele frequencies for the beta-casein gene in cows raised on several dairy farms in Japan.

2 | MATERIALS AND METHODS

2.1 | Hair sampling and DNA isolation

Hairs samples were collected from 320 cows, namely, Holstein \( n = 311 \), Jersey \( n = 7 \), Brown Swiss \( n = 1 \), and Ayrshire \( n = 1 \). The cows belonged to eight dairy farms in either the Kanto or Tohoku regions of Japan. The hairs were plucked from either the root or distal part of the tail and washed with ethanol to remove fine debris. DNA was isolated from 10 to 20 hair samples using ISOHAIR (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Isolated DNA was diluted 20 times with Tris-EDTA buffer and stored at 4°C.

2.2 | Genotyping by Cycleave PCR assay

The PCR reaction mixture contained 1 × Cycleave PCR reaction mix (Takara Bio Inc.), 0.1 μM each of forward and reverse primer, 0.2-μM RNA–DNA chimeric probe, and 1-μl diluted DNA. The reaction volume was made up to 25 μl with sterile distilled water. Primers and probes for the Cycleave PCR were designed with the Cycleave PCR Assay Designer (Takara Bio Inc.) (Table 1). PCR and fluorescence

| Name | SNP position* | Nucleotide | Codon | Amino acid | Sequenceb | Size (bp) |
|------|---------------|------------|-------|------------|-----------|-----------|
| For detecting A and C of the 8101 SNP | | | | | CAGACACAGTCTCTAGTCTATCC | 113 |
| bCSN2_SNAP_CA-F | | | | | TCAAGCTGAGGAAGG | |
| bCSN2_SNAP_CA-R | | | | | | |
| bCSN2_Probe_C_A2_FAM | 8101 | C | CCT | Pro | CTGTAGGGAT-FAM | |
| bCSN2_Probe_A_A1_ROX | 8101 | A | CAT | His | CTGTTATGGAAT-ROX | |
| For detecting C of the 8267 SNP | | | | | | |
| CSN2-8267-C-Primer-F | | | | | GAGGCTATGGCTCTGTAAG | 131 |
| CSN2-8267-C-Primer-R | | | | | CAAGACTGGAGCAGAG | |
| CSN2-8267-C-Probe C-FAM | 8267 | C | AGC | Ser | CGTTCATGGAT-ROX | |
| For detecting G of the 8267 SNP | | | | | | |
| CSN2-8267-G-Primer-F | | | | | CATCGAGGTCTGCAAATGAA | 157 |
| CSN2-8267-G-Primer-R | | | | | CATTCAAGACTGGAGCAGAG | |
| CSN2-8267-G-Probe G-FAM | 8267 | G | AGG | Arg | CTGAAAGGCA-FAM | |

*Position refers to bovine CSN2 gene (GenBank accession number: M55158).

**RNA base of the probe was marked by “r”.

TABLE 1 Primers and probes used for Cycleave polymerase chain reaction (PCR) assay in this study
Bovine CSN2 exon 7

| 8101 | 8267 |
|------|------|
| A1   | A    |
| B    | C    |
| A2   | C    |

FIGURE 1  A1, A2, and B alleles of the bovine CSN2 gene
Two SNPs in exon 7 of the bovine CSN2 gene were genotyped in this study. The first, at position 8101 within CSN2 (GenBank, accession number: M55158), is characterized by an A (adenine) for the A1 and B alleles and by a C (cytosine) for the A2 allele. The other, at position 8267, is characterized by a C for the A1 and A2 alleles, and by a G (guanine) for the B allele. The 8101 SNP alters codon CAT (His) to CCT (Pro) in position 67 of β-casein protein (H67P). The 8267 SNP alters codon AGC (Ser) to AGG (Arg) in position 122 β-casein protein (S122R)

| Genotype frequency | Allele frequency |
|--------------------|-----------------|
| A1A1               | 0.11            |
| A1A2               | 0.39            |
| A1B                | 0.03            |
| A2A2               | 0.42            |
| A2B                | 0.05            |
| A1                 | 0.32            |
| A2                 | 0.64            |
| B                  | 0.04            |

RESULTS AND DISCUSSION

To validate our Cycleave PCR assay, we genotyped cows (n = 61) whose CSN2 genotypes had previously been determined by DNA sequencing. The genotyping data obtained by Cycleave PCR were identical to those derived by sequencing; it could correctly detect the A1A1, A1A2, and A2A2 genotypes. Thus, we determined that our Cycleave PCR assay could be used for beta-casein genotyping. As Cycleave PCR allowed us to detect the target allele in real time, we could genotype the beta-casein locus more easily and quickly than conventional methods such as sequencing (Massella et al., 2017) and PCR-RFLP (Cieslinska et al., 2019). Hair sampling was easier and safer than the blood sampling usually performed by veterinarians requiring specialized medical equipment. Hair sampling also considered animal welfare more than blood sampling. Furthermore, this method has been thought to be applicable to any tissue samples. For example, we can use a piece of ear tissue collected when a cow is ear-tagged as a source sample. Therefore, the genotyping method with the hair sampling and the Cycleave PCR was suitable for routine determination of beta-casein genotype.

We detected five genotypes in the cohorts that we examined: two were homozygous (A1A1 and A2A2) while three were heterozygous (A1A2, A1B, and A2B) (Table 2). The most common genotype was A2A2, which occurred at a frequency of 0.42, while the second was A1A2 (0.39), followed by A1A1 (0.11). The A1B and A2B genotypes were less common with frequencies below 0.05, which agrees with previous data (Massella et al., 2017).

The most common beta-casein allele was A2, followed by A1, with frequencies of 0.64 and 0.32, respectively (Table 2). The allele B was less common with a frequency of 0.04.

To compare our results with previously published data, we examined results from Holstein cows (n = 311) and regarded the B allele as allele A2 because the B allele could be classified as A1 if the identity of nucleotide 8267, which is specific to the B allele, is not determined. We calculated the A1 and A2 allele frequencies to be 0.36 and 0.64, respectively, similar to the allele frequencies observed among the Holstein populations of Denmark, the Netherlands, Poland, and Italy; however, in China, Turkey, and Iran, the A1 and A2 allele frequencies are dissimilar, having been reported as between 0.43–0.50 and 0.46–0.50, respectively (Cieslinska et al., 2019; Kaminski et al., 2007).

Our present study is the first survey of beta-casein variants in Japanese dairy cattle herds. The adverse effects of BCM7, a derivative of A1 beta-casein following gastrointestinal digestion, on human health have been widely suggested. For example, BCM7 triggers in vitro histamine release from human peripheral blood leukocytes (Kurek et al., 1992). When injected into animals, BCM7 slows gastrointestinal motility in a similar manner to morphine (Shah, 2000), and this finding is reproduced in humans (Ho et al., 2014). Furthermore, BCM7 is strongly associated with clinical severity of autism spectrum disorders in children (Sokolov et al., 2014).

Thus, it is very likely that dairy farms from which the A1 variant has been eliminated may have an advantage when it comes to offering new, high-value products. Indeed, New Zealand was the first country to have eliminated the A1 allele from its dairy cattle population, with no negative effect on either milk yield or composition. In addition, an infant formula suitable for healthy full-term infants up to six months of age has been developed based on A1-free milk (Duarte-Vazquez et al., 2017). To develop A2A2 cow populations, dairy farms should monitor the beta-casein genotypes of their cows to select for A2A2 cows and use A2A2 semen to breed an A2A2 population.

In summary, we developed a new, fast, and reliable genotyping method for the bovine beta-casein gene and determined the frequencies of the A1, A2, and B alleles in Japanese dairy cattle herds. This study can therefore contribute to genetic improvement of dairy farm herds and the development of A2A2 cow populations in Japan.

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CONFLICT OF INTEREST
Authors declare no conflict of interests for this article.

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