SHORT COMMUNICATION

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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

(Bell et al., 2006). 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 maldigestion and intolerance 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 has 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 $1 \times$ 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

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

Name	SNP position[a]	Nucleotide	Codon	Amino acid	Sequence[b]	Size (bp)
For detecting A and C of the 8101 SNP						
bCSN2_SNP_CA-F					CAGACACAGTCTCTAGTCTATCC	113
bCSN2_SNP_CA-R					TCAGGCTGAAGGAAAGG	
bCSN2_Probe_C_A2_FAM	8101	C	CCT	Pro	CTGTTAGrGGAT-FAM	
bCSN2_Probe_A_A1_ROX	8101	A	CAT	His	CTGTTATrGGAT-ROX	
For detecting C of the 8267 SNP						
CSN2-8267-C-Primer-F					GAGGCTATGGCTCCTAAG	131
CSN2-8267-C-Primer-R					CAAGACTGGAGCAGAGG	
CSN2-8267-C-Probe C-FAM	8267	C	AGC	Ser	CTGrGCTTTCA-FAM	
For detecting G of the 8267 SNP						
CSN2-8267-G-Primer-F					AATGGGAGTCTCCAAAGTGAA	157
CSN2-8267-G-Primer-R					CATCCAAGACTGGAGCAGAG	
CSN2-8267-G-Probe G-FAM	8267	G	AGG	Arg	CTGAAAGrGCA-FAM	

[a]Position refers to bovine *CSN2* gene (GenBank accession number: M55158).
[b]RNA base of the probe was marked by "r".
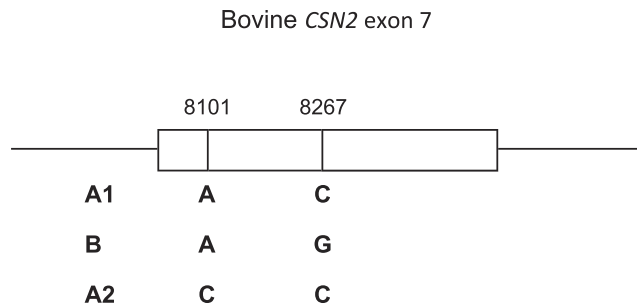
Bovine *CSN2* exon 7

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 bovine *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)

detection were performed with a Thermal Cycler Dice Real Time
System (TP970; Takara Bio Inc.). The allele type was determined by
the haplotype consisting of the genotypes of positions 8101 and
8267 within *CSN2* exon 7 (Figure 1). The A-C haplotype was desig-
nated the A1 allele, the A-G haplotype was the B allele, and the C-C
was the A2 allele.

3 | 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 sam-
pling 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 heterozy-
gous (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

TABLE 2 Genotype and allele frequency of the beta-casein gene
in populations examined

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

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 exam-
ined results from Holstein cows ($n = 311$) and regarded the B allele as
allele A1 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 gastro-
intestinal 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 frequen-
cies 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.

CONFLICT OF INTEREST

Authors declare no conflict of interests for this article.

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