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ORIGINAL ARTICLE

Gene and haplotype polymorphisms of the Prion gene (*PRNP*) in Japanese Brown, Japanese native and Holstein cattle

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ABSTRACT

Polymorphisms in the prion protein gene (*PRNP*) are known to be associated with transmissible spongiform encephalopathies in human, sheep and goats. There is tentative association between *PRNP* promoter polymorphism and bovine spongiform encephalopathy (BSE) susceptibility in cattle. In this study, we genotyped for six bovine *PRNP* polymorphic sites including a 23-bp indel in the promoter, a 12-bp indel in the intron 1, two nonsynonymous single nucleotide polymorphisms (SNPs), octapeptide repeats in the coding region and a 14-bp indel in the 3′-untranslated region in 178 animals representing Japanese Brown, Kuchinoshima feral, Mishima, Japanese Shorthorn and Holstein. In 64 Japanese Brown cattle, three indel sites were polymorphic. All of the six sites were monomorphic in Kuchinoshima. The 23-bp and 12-bp indel sites were polymorphic in Mishima cattle. The 23-bp and 14-bp indel sites were polymorphic in Japanese Shorthorn cattle. Both SNP sites were monomorphic in all cattle examined in this study. At the 23-bp indel site, the genotype frequencies of Japanese Brown and Holstein breeds were similar to that of BSE affected cattle. We estimated 12 different haplotypes from these genotypic data. A '23-12-K6S14+' haplotype was the major haplotype in all populations, whose frequencies ranged from 0.50 to 1.00.

Key words: *Japanese Brown cattle, Kuchinoshima feral cattle, Mishima cattle, polymorphisms, Prion, PRNP.*

INTRODUCTION

Prion diseases are fatal neurodegenerative diseases of human and animals. They result from the accumulation of a modified isoform (PrP^{Sc}) of the normal prion protein (PrP^{c}) in the central nervous system (Prusiner 1998). PrP^{c} is encoded by the prion protein gene (*PRNP*). Polymorphisms of the *PRNP* have been shown to influence the susceptibility of these diseases and the incubation period in humans (Shibuya *et al.* 1998), sheep (Goldmann *et al.* 1994; Westaway *et al.* 1994), goats (Goldmann *et al.* 1996) and mice (Moore *et al.* 1998). In cattle, various polymorphisms were reported in the *PRNP*: a 23-bp insertion/deletion (indel) in the promoter region (Sander *et al.* 2004), a 12-bp indel in intron 1, a 14-bp indel in the 3′-untranslated region (3′UTR) (Hills *et al.* 2001), the number of octapeptide repeats in the open reading frame (ORF) (Goldmann *et al.* 1991) and single nucleotide polymorphisms (SNPs) (Sander *et al.* 2004; Abe *et al.* 2006; Kues *et al.* 2006). Among these polymorphisms, the 23-bp indel and the number of octapeptide repeats were supposed to be associated with the susceptibility of bovine

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spongiform encephalopathy (BSE) (Sander *et al.* 2004; Brun *et al.* 2007), but it remains unclear whether the polymorphisms correlate with BSE. It was also reported that the 23-bp indel in the promoter and the 12-bp indel in intron 1 regions contain repressor protein (RP 58) and transcription factor SP1, respectively, responsible for BSE resistance (Brunelle *et al.* 2008).

There are four populations of beef cattle in Japan: Japanese Black (JB), Japanese Brown (JBr), Japanese Polled (JP) and Japanese Shorthorn (JS). JBr is the second largest beef cattle population in Japan and was introduced in 1800s from Korea. JBr was approved as a breed after successful crossbreeding with Simmental and Devon breeds (Nishida 1973). It is however facing a reduction in numbers because of competition from profitable breeds, especially the famous JB cattle (Honda *et al.* 2006). Mishima and Kuchinoshima feral cattle are the typical native cattle of Japan., They are now endangered, and there are deliberate efforts to conserve these populations (Minezawa 2005).

To date, gene polymorphisms and their frequencies of *PRNP* have been reported in JB (Abe *et al.* 2006; Nakamitsu *et al.* 2006), other cattle elsewhere and BSE affected cattle (Hunter *et al.* 1994; Sander *et al.* 2004; Kues *et al.* 2006; Czarnik *et al.* 2007). However, there is no report of polymorphisms in JBr, JP, JS, or Mishima and Kuchinoshima feral cattle. In order to analyze the *PRNP* variability in JBr, Kuchinoshima, Mishima and Holstein, we genotyped for the six polymorphic sites of the bovine *PRNP* and estimated the *PRNP* haplotypes and their frequencies in these populations. The six polymorphic sites were the 23-bp indel within the promoter, the 12-bp indel within intron 1, two nonsynonymous SNPs converting lysine to threonine at codon 3 (K3T) and serine to asparagine at codon 154 (S154N), and the number of the octapeptide repeats within the ORF and a 14-bp indel within the 3′UTR. The 12-bp indel was shown to make a regulatory contribution to promoter activity (Inoue *et al.* 1996; Sander *et al.* 2004), and the 14-bp indel might affect *PRNP* expression and hence BSE susceptibility due to its location and quality of polymorphisms (Sander *et al.* 2004, 2005).

MATERIALS AND METHODS

In this study, blood or semen samples were obtained from 58 JBr cattle, 52 Kuchinoshima feral cattle, two Mishima cattle, 65 Holstein cattle and one JS cattle (Table 2). Genomic DNA was extracted from these samples using a conventional method.

Polymerase chain reaction (PCR) was carried out in a final volume of 20 μL containing 0.5 units of *Ex Taq* (TaKaRa, Otsu, Shiga, Japan), 40 ng of genomic DNA, 200 μmol/L each of four deoxynucleoside triphosphates (dNTPs), 10 pmoles of each primer and 2.0 μL of 10× *Ex Taq* buffer (TaKaRa). Primer sets used in this study are listed in Table 1.

The mixture was first treated at 94°C for 2 min, then with 28–35 cycles of denaturation at 94°C for 30 s, annealing at an appropriate temperature indicated in Table 1 for 30 s, and extension at 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. All PCRs were carried out using the GeneAmp PCR System 9700 (Applied Biosystems). For the analysis of three indel sites and octapeptide repeats, the PCR products were separated on 10% polyacrylamide gels and visualized with ethidium bromide staining under UV light. For the analysis of the two SNPs, the PCR products were digested with one unit of restriction endonuclease Tsp45I (NEB), followed by 10% polyacrylamide gel electrophoresis.

Genotype and allele frequencies were computed according to the Hardy-Weinberg principle to reveal gene and genotype frequencies. Frequency results of Japanese cattle analyzed in this study were compared to German's BSE-affected cattle by Fisher's exact test using SAS software. Haplotypes and their frequencies were inferred using the expectation-maximization (EM) algorithm implemented in arlequin v.3.11 software (Excoffier *et al.* 2007).

RESULTS

In this study, we analyzed the bovine *PRNP* polymorphisms for three indels (23-bp, 12-bp and 14-bp), the number of the octapeptide repeats and two SNPs (K3T

Table 1 Primers used in the study

Site	Forward primer	Reverse primer	AT °C
23-bp	PRNP 47784-F GTGCCAGCCATGTAAGTG	47883-R TGGACAGGCACAATGGG	56
12-bp	Bov PRNP1 CTCGGTTTTACCCTCCTGGT	BovPRNP2 CACTTCCCAGCATGTAGCCACCA	62
14-bp	PRNP 676911-F TGGCTTGCACTTTGTGGTAT	68060-R CCCACGTCTCCTTAGTACCTT	63
Octa	prnp octa 1 ACGTGGGCCTCTGCAAGAAGCGAC	prnp octa 2 CTCGCCCTTGTTCTTCTGAG	65
K3T	BprnpRFLPF2STGCTGGCATTCTACATTTATCAA	BprnpR71A TGCTGGCATTCTACATTTATCAA	63
S154N	BprnpRFLPF382SGCAGCTGGAGCAGTGGTAGG	BprnpEx3R2A TGTCAGTTTCGGTGAAGTTCT	66

AT, Annealing temperature.

Table 2 *PRNP* Allele and genotype frequencies in Japanese cattle populations

Locus	*n*	Genotype frequency			Allele frequency		Reference
Promoter 23-bp indel		++	+−	−−	+	−	
Japanese Holstein	65	12	34	19	0.45	0.55	
Japanese Short horn	1	0	1	0	0.50	0.50	
Japanese Brown	58	7	20	31	0.29	0.71	
Mishima	2	0	0	2	0.00	1.00	
Kuchinoshima	52	0	0	52	0.00	1.00	
BSE affected cattle	43	0.05	0.44	0.51	0.27	0.73	Sander *et al.* (2004)
Intron 12-bp indel		++	+−	−−	+	−	
Japanese Holstein	65	27	22	16	0.58	0.42	
Japanese Short horn	1	0	1	0	0.50	0.50	
Japanese Brown	58	9	20	29	0.33	0.67	
Mishima	2	1	0	1	0.50	0.50	
Kuchinoshima	52	0	0	52	0.00	1.00	
BSE affected cattle	43	0.15	0.52	0.33	0.41	0.59	Sander *et al.* (2004)
3′UTR 14-bp indel		++	+−	−−	+	−	
Japanese Holstein	65	40	23	2	0.79	0.21	
Japanese Short horn	1	1	0	0	1.00	0.00	
Japanese Brown	64	28	18	18	0.58	0.42	
Mishima	2	1	0	1	0.50	0.50	
Kuchinoshima	52	52	0	0	1.00	0.00	
BSE affected cattle	43	0.88	0.11	0.01	0.93	0.07	Sander *et al.* (2004)
Octarepeat units		**66**	**65**	**55**	**6**	**5**	
Japanese Holstein	65	57	8	0	0.94	0.06	
Japanese Short horn	1	1	0	0	1.00	0.00	
Japanese Brown	58	58	0	0	1.00	0.00	
Mishima	2	2	0	0	1.00	0.00	
Kuchinoshima	52	52	0	0	1.00	0.00	
Japanese Black bulls	75	69	6	0	0.96	0.04	Abe *et al.* (2006)
Holstein bulls	147	141	6	0	0.98	0.02	Abe *et al.* (2006)
BSE affected cattle	43	0.90	0.10	0.00	0.95	0.05	Sander *et al.* (2004)
K3T		**KK**	**KT**	**TT**	**K**	**T**	
Japanese Holstein	65	65	0	0	1.00	0.00	
Japanese Short horn	1	1	0	0	1.00	0.00	
Japanese Brown	58	58	0	0	1.00	0.00	
Mishima	2	2	0	0	1.00	0.00	
Kuchinoshima	52	52	0	0	1.00	0.00	
Japanese Black	153	153	0	0	1.00	0.00	
S154N		**SS**	**SN**	**NN**	**S**	**N**	
Japanese Holstein	65	65	0	0	1.00	0.00	
Japanese Short horn	1	1	0	0	1.00	0.00	
Japanese Brown	58	58	0	0	1.00	0.00	
Mishima	2	2	0	0	1.00	0.00	
Kuchinoshima	52	52	0	0	1.00	0.00	
Japanese Black	152	153	0	0	1.00	0.00	

and S154N) in Japanese cattle population (Fig. 1). These results showed that the four sites, namely 23-bp, 12-bp, 14-bp indels and the number of octapeptide repeats were polymorphic, while the K3T and S154N sites were monomorphic in all populations (Table 2). Three sites were polymorphic in JBr, four sites in Holstein, two sites in Mishima cattle, while all sites were monomorphic in Kuchinoshima cattle.

At the 23-bp indel site, the frequency of the deletion (–) allele ranged from 0.50 in JS to 1.00 in Kuchinoshima and Mishima. This was higher than that of the insertion (+) allele in almost all populations in this study. JBr cattle showed a slightly higher frequency of the deletion allele than the Holstein population. While JS was heterozygous at the site, the deletion allele was fixed in Kuchinoshima and Mishima populations.

At the 12-bp indel site, frequencies of the – allele ranged from 0.42 in Holstein to 1.00 in Kuchinoshima. The site was polymorphic in all populations except Kuchinoshima cattle in which the allele was fixed. JBr

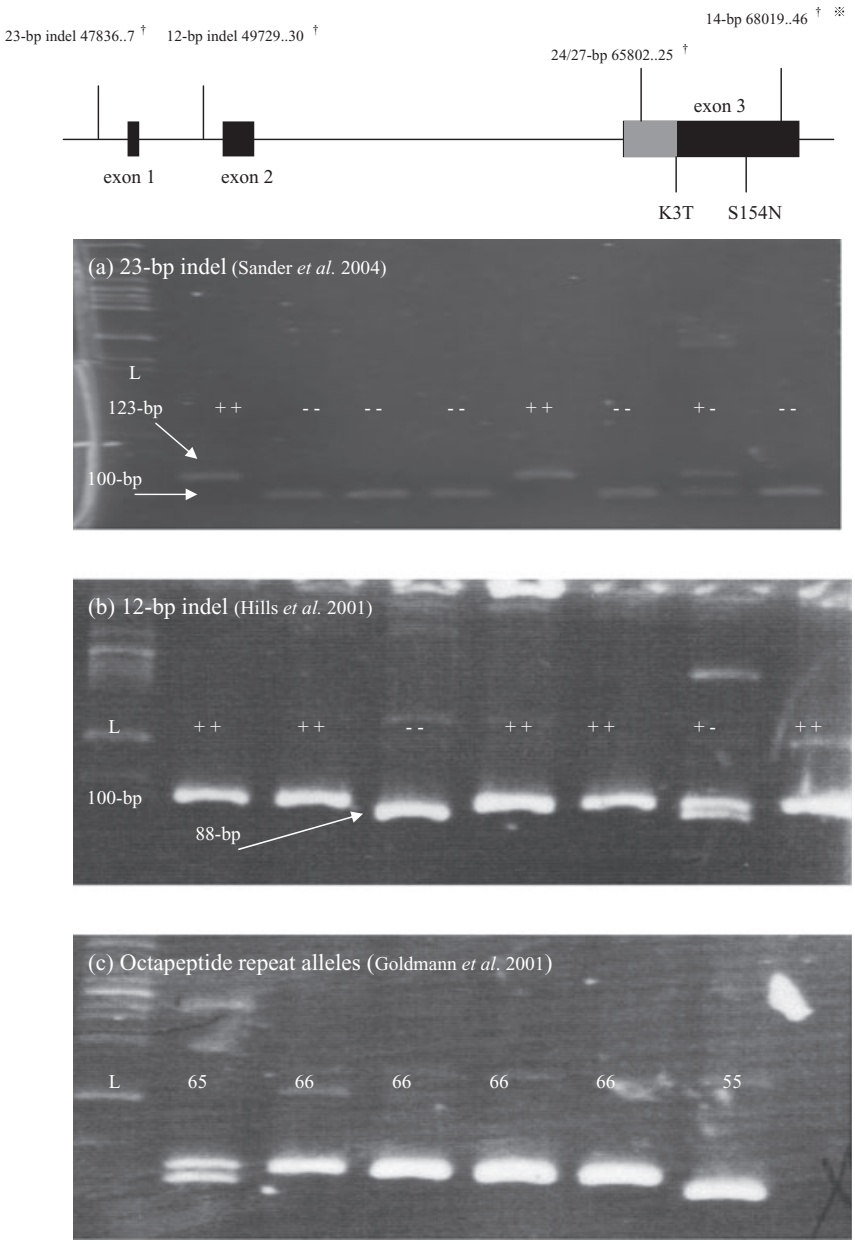

Figure 1 Bovine *PRNP* structure and nature of PCR products of loci analyzed. †indicate positions in the bovine *PRNP* (Hills *et al.* 2001; Sander *et al.* 2004) (※EMBL/GenBank Ac. number AJ 298878).

showed a higher frequency of the – allele than JB (Nakamitsu *et al.* 2006) and cattle with BSE (Table 2) while it was vice versa in Holstein (Abe *et al.* 2006; Nakamitsu *et al.* 2006).

At the 14-bp indel site, the frequency of the + allele ranged from 0.50–1.00, and was higher than that of the – allele in all populations with the exception of the Mishima population. The alleles were shared equally in Mishima. The frequency of the – allele was higher in JBr than Holstein, JB and BSE affected cattle (Table 2).

The octapeptide repeat site was polymorphic only in the Holstein population. The 6 alleles were predominant in all populations examined in this study. At the K3T and S154N sites, genotypes KK and SS, respectively, were observed in all cattle examined in this study.

We inferred from the genotypes of the six polymorphic sites that 12 haplotypes were present in our samples using the EM algorithm (Table 3). A 23- 12- K 6 S 14+ haplotype was common and the most frequent

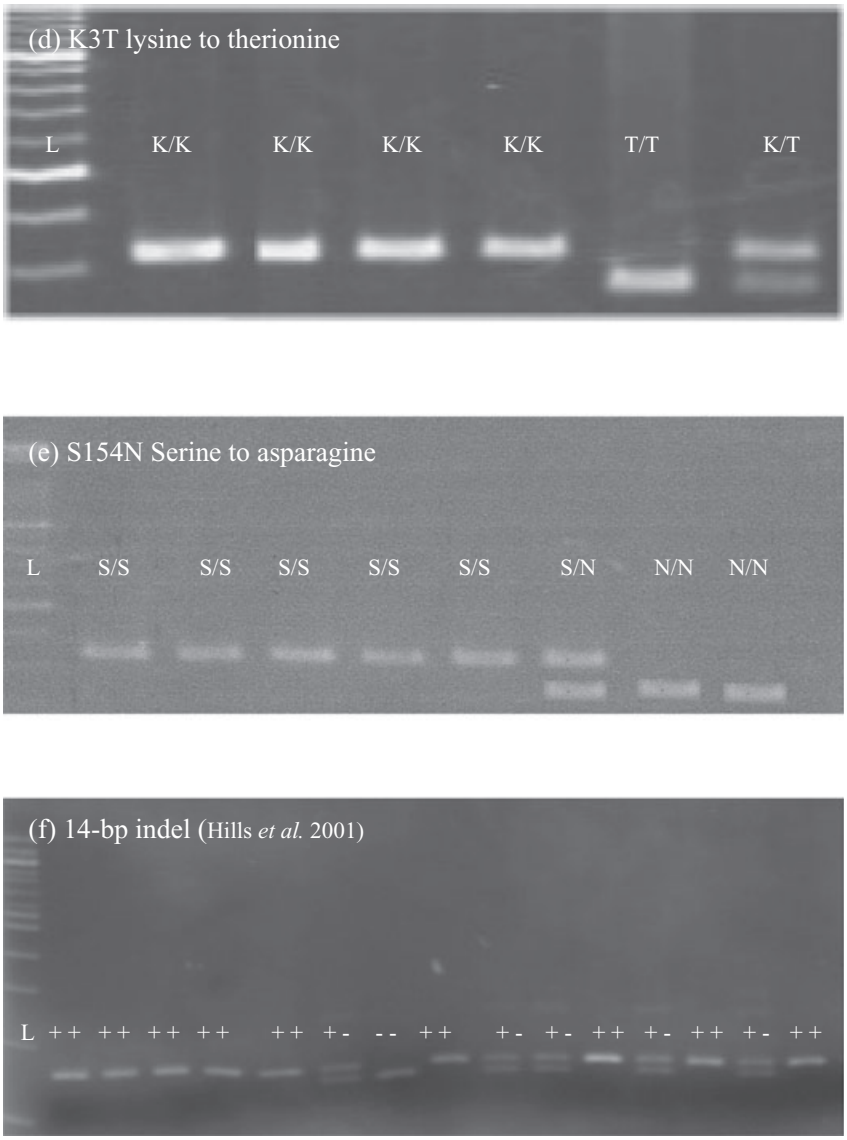

Figure 1 *Continued*

in all populations except Mishima, indicating that the 23^{-} and 12^{-} types which have been associated with BSE exist in our cattle groups. Eight haplotypes were inferred in JBr, 11 in Holstein, two in Mishima, two in JS and one in Kuchinoshima cattle. Kuchinoshima cattle are fixed in haplotype 23- 12- K 6 S 14+.

DISCUSSION

We confirmed the presence of polymorphisms at four of the six sites of the bovine *PRNP* gene in beef, dairy and native cattle breeds in Japan. Because of difficulties in obtaining samples from Mishima and JS cattle, we analyzed only two samples of Mishima and one sample of JS cattle from the National Institute of Agrobiological Sciences.

The frequency of the − allele has been reported to be higher than that of the + frequency at the 23-bp site in JB cattle (Nakamitsu *et al.* 2006) and in other cattle populations outside Japan (Seabury *et al.* 2004; Czarnik *et al.* 2007). We confirmed a similar tendency in this study. The gene frequency of JBr was similar to that reported earlier for BSE affected cattle but different from Korean native cattle (Sander *et al.* 2004; Jeong *et al.* 2006). There was no difference in the 23-bp gene frequency between JBr in the present

Table 3 Estimated haplotypes and frequencies in Japanese cattle populations

Haplotype	Haplotype frequency				
	JH (65)	JS (1)	JBr (58)	M (2)	KF (52)
23+ 12+ K 6 S 14+	0.28	0.50	0.12	–	–
23+ 12+ K 6 S 14–	0.10	–	0.03	–	–
23+ 12+ K 5 S 14+	0.03	–	–	–	–
23+ 12– K 6 S 14+	0.02	–	0.05	–	–
23+ 12– K 6 S 14–	0.02	–	0.13	–	–
23+ 12+ K 5 S 14–	0.01	–	–	–	–
23– 12+ K 6 S 14+	0.10	–	0.11	0.50	–
23– 12+ K 6 S 14–	0.04	–	0.09	–	–
23– 12+ K 5 S 14+	0.03	–	–	–	–
23– 12– K 6 S 14+	0.32	0.50	0.24	–	1.00
23– 12– K 5 S 14+	–	–	–	–	–
23– 12– K 6 S 14–	0.06	–	0.23	0.50	–
Total	11	2	8	2	1

JH, Holstein; M, Mishima; JS, Japanese Short horn; JBr, Japanese Brown; KF, Kuchinoshima; JB, Japanese Black; ( ) Sample size.

study and JB analyzed earlier in our laboratory (T. Shimogiri *et al.* 2008 unpubl. data). Notably, the 23-bp gene frequency for Holstein differed from that of the other cattle populations in this study and other studies, and also differed from BSE affected cattle. Holstein are said to be the most prone to BSE (Bradley & Wilesmith 1993; Kimura *et al.* 2002). It was also noted that the 23-bp gene frequency for Holstein cattle used in the study differed from those of other Holstein cattle in Japan (Nakamitsu *et al.* 2006), South Korea (Jeong *et al.* 2006), Germany (Juling *et al.* 2006) and the United Kingdom (Juling *et al.* 2006), but were similar to those of US Holstein cattle and the Holstein archive in the United states (Brunelle *et al.* 2008). There was no statistically significant difference between Japanese brown cattle and BSE affected cattle ($P = 0.3756$), and thus the former may not be excepted from prion disease susceptibility. Holstein and Kuchinoshima cattle differed significantly from BSE affected cattle ($P < 0.05$).

The 12-bp indel site behaved in the same way as the 23-bp site in three cattle groups by showing a higher frequency of the – allele than of the + allele, which agrees with previous reports (Sander *et al.* 2004; Czarnik *et al.* 2007). Thus an association between the *PRNP* promoter and the intron 1 region is shown, as reported previously (Inoue *et al.* 1996). There was a difference in gene frequency between Holstein cattle in this study and Korean Holstein cattle at the 12-bp indel site. The gene frequencies reported for Holstein in South Korea were 0.30 and 0.70 for + and – alleles, respectively. When the gene frequencies at the 12-bp indel are compared between JBr and the native cattle of Korea (Korean Hanwoo), we observe that the former differed from the latter (Jeong *et al.* 2006). Korea is believed to be the origin of JBr. Despite the low sample numbers, this site was polymorphic in JS and Mishima cattle, and the + allele frequency in Mishima cattle resembled that of JS cattle.

Conversely, there was a higher frequency of 14^+ than 14^-, as reported in other *PRNP* studies (Sander *et al.* 2004; Seabury *et al.* 2004). The 14-bp site was polymorphic in JBr, thus distinguishing JBr from other Japanese cattle in which the site was monomorphic (Shimogiri T. *et al.* 2008, unpublished data). Despite the low number of samples, polymorphisms at the 12-bp and 14-bp indels in the native Mishima cattle indicate some degree of *PRNP* polymorphism in this population, and thus a difference between them and the native Kuchinoshima cattle.

A genotype of consisting of a 66 octapeptide repeats allele dominated our cattle, but a few Holstein cattle showed 65 repeats. A 55-repeat genotype was not detected in this study, as its frequency is usually very low (Brown *et al.* 1993). We found similarities between Japanese Holstein and Korean Holstein, and between JBr and Korean native Hanwoo for octapeptide repeat frequency (Jeong *et al.* 2005).

Unlike a previous Brazilian study in which polymorphisms at codon 154 was reported (Kues et al. 2006), variations at these sites are rare in Japanese cattle (Shimogiri T. *et al.* 2008, unpublished data), Holstein-Friesian and beef cattle (Sander *et al.* 2004). In another bovine *PRNP* analysis in our laboratory, the two SNP sites were polymorphic in genomic DNA samples from Myanmar, Vietnam and Laos native cattle (T. Shimogiri *et al.* 2008, unpubl. data).

Haplotypes involving 23del/12del are always higher, not only in healthy cattle (Czarnik *et al.* 2007), but also in BSE affected cattle (Sander *et al.* 2005). Haplotype results in the present study indicate a deletion of the important RP 58 and SP 1 binding sites, thus making the cattle prone to BSE (Brunelle *et al.* 2008).

In contrast to most Japanese cattle, potentially high polymorphisms in JBr cattle may be due to their origin and management in Japan (Nishida 1973; Honda *et al.* 2006). JBr genotype frequencies found in this study resemble those of native cattle of Asia (Shimogiri T. *et al.* 2008, unpublished data). Polymorphisms at two sites (12-bp and 14-bp indels) distinguish Mishima from Kuchinoshima, despite the fact that they are both

native cattle of Japan. Non polymorphism at all *PRNP* sites in Kuchinoshima may be due to the founder effect or some bottleneck effects. It is an endangered native population, so maybe ongoing efforts to conserve the breed by controlled mating and migration (Minezawa 2005) have resulted in gene fixation due to inbreeding (Falconer 1961).

In conclusion, we note that polymorphism frequency results in JBr did not differ with those of BSE affected cattle in Germany (Sander *et al.* 2004). Uneven polymorphisms in JBr cattle may be interesting, so future analysis within the population is required. Our results in Mishima and JS indicate some degree of polymorphism in bovine *PRNP*, and therefore more analyses are needed. Holstein analyzed in the present study showed interesting results and more analyses may be worth being carried out to reveal the real frequency situation in Holstein.

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