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Allele Distributions and Frequencies of the Six Prion Protein Gene (*PRNP*) Polymorphisms in Asian Native Cattle, Japanese Breeds, and Mythun (*Bos frontalis*)

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Abstract Six polymorphic sites of the bovine prion protein gene (*PRNP*) were genotyped in 569 animals of Asian native cattle, Japanese breeds, purebred mythun (*Bos frontalis*), and mythun × cattle composite animals. At the 23-bp indel site, a deletion (23−) allele was a major allele in all populations except mythun. At the 12-bp indel site, an insertion (12+) allele was a major allele in all populations. The 14-bp indel site was polymorphic in all Asian native cattle. In the octapeptide repeat

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region, a six-repeat allele was a major allele in all populations, and 5/5 and 4/6 genotypes were detected in Japanese Black and Mongolian cattle and in mythun, respectively. Two nonsynonymous single nucleotide polymorphisms (SNPs) (K3T and S154N) were detected in Asian native cattle and mythun. Haplotype analysis using the genotypes of the six sites estimated 33 different haplotypes. The haplotype 23− 12− K 6 S 14+ was found in all populations.

Keywords Asian native cattle · Mythun · Polymorphism · *PRNP* · Variability

Introduction

Prion protein (PrP) plays a central role in the pathogenesis of neurodegenerative diseases such as the bovine spongiform encephalopathies (BSE) in cattle, scrapie in sheep, and Creutzfeldt–Jakob disease in humans (Prusiner 1998). Therefore, the PrP gene (*PRNP*) has been suggested as a candidate gene for disease susceptibility. DNA polymorphisms within *PRNP* correlate with the susceptibility to and incubation time of such diseases in humans and sheep (Shibuya et al. 1998; Goldmann et al. 1994). In cattle, two insertion/deletion (indel) polymorphisms within the bovine *PRNP* are tentatively associated with BSE susceptibility (Sander et al. 2004, 2005; Seabury et al. 2004b). A 23-bp indel polymorphism within the promoter and a 12-bp indel polymorphism within intron 1 seem to affect the binding sites for the transcriptional factors RP58 and SP1, respectively, and might influence *PRNP* expression (Sander et al. 2005). In addition, transgenic mice expressing the bovine PrP suggested that the PrP with lower numbers of octa/nona-peptide (Pro-His/Gln-Gly-(Gly)-Gly-Gly-Trp-Gly-Gln) repeats reduced BSE susceptibility (Brun et al. 2007). This octapeptide region selectively binds copper ion (Cu^{2+}) in vitro (Aronoff-Spencer et al. 2000).

In this study, we provide a detailed comparative analysis of the 23-bp promoter region, the 12-bp intron region, the octapeptide region, and relevant *PRNP* polymorphisms for Asian native cattle populations, Japanese breeds, purebred mythun (*Bos frontalis*), and a composite population of mythun × cattle.

Materials and Methods

DNA Samples

Genomic DNA samples were collected from 569 animals representing three Japanese breeds (Black, Polled, and Shorthorn) and six Asian native populations (Vietnam, Laos, Myanmar, Mongolia, and Bangladesh), purebred mythun (*Bos frontalis*), and a mythun × cattle composite population (MCC).

DNA Sequencing

We sequenced the PrP coding region using six cattle genomic DNAs (one Japanese Black, two Laos native, two Myanmar native, and one purebred mythun). Primer

sets Bov_prnp_Ex3F and Bov_prnp_Ex3R were used for polymerase chain reaction (PCR) amplification of the region (Table 1). Standard double-strand DNA sequencing was performed by the primer walking method. The BigDye Terminator Version 3.1 Cycle Sequencing Kit and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Tokyo) were used for sequencing, according to the manufacturer's instructions. Nucleotide sequences were aligned using Clustal W (http://align.genome.jp/) to identify polymorphic sites.

Genotyping

We examined six polymorphic sites for the bovine *PRNP*: a 23-bp indel within the promoter, a 12-bp indel within intron 1, two nonsynonymous single nucleotide polymorphisms (SNPs) with one converting lysine to threonine at codon 3 (K3T) and the other converting serine to asparagine at codon 154 (S154N), the octapeptide repeats within the coding region, and a 14-bp indel within the 3′ untranslated region (3′ UTR). Four bovine *PRNP* regions with known polymorphism lengths were amplified by PCR: the promoter (23-bp indel, Sander et al. 2004); intron 1 (12-bp indel, Hills et al. 2001), the open reading frame (octapeptide repeat unit, Goldmann et al. 1991; Premzl et al. 2000), and the 3′ UTR (14-bp indel, Hills et al. 2003). The allelic PCR products were visualized by 10% polyacrylamide gel electrophoresis. Cattle *PRNP* variants were detected by the conventional analysis of length polymorphism for the indels (23, 12, and 14 bp) and for the octapeptide repeat numbers and were genotyped as described by Sander et al. (2004), Hills et al. (2001, 2003), and Premzl et al. (2000). Primers used in this study are from earlier reports (Table 1).

Table 1 Nucleotide primers used in this study

Primer	Nucleotide sequence	Mer	Source
PRNP 47784-F	5′ GTGCCAGCCATGTAAGTG 3′	18	Sander et al. (2004)
PRNP 47883-R	5′ TGGACAGGCACAATGGG 3′	17	Sander et al. (2004)
Bov PRNP 1	5′ CTCGGTTTTACCCTCCTGGT 3′	20	Hills et al. (2001)
Bov PRNP 2	5′ CTCGCCCTTGTTCTTCTGAG 3′	20	Hills et al. (2001)
PRNP 67911-F	5′ TGGCTTGCACTTTGTGGTAT 3′	20	Hills et al. (2001)
PRNP 68060-R	5′ CCCACGTCTCCTTAGTACCTT 3′	21	Hills et al. (2001)
Bov_prnp_octa1	5′ ACGTGGGCCTCTGCAAGAAGCGAC 3′	24	Premzl et al. (2000)
Bov_prnp_octa2	5′ GCACTTCCCAGCATGTAGCCACCA 3′	24	Premzl et al. (2000)
Bov_prnp_RFLP_F2	5′ TGCTGGCATTCTACATTTATCAA 3′	23	K3T and RFLP
Bov_prnp_R71	5′ TGCTGGCATTCTACATTTATCAA 3′	23	K3T and RFLP
Bov_prnp_F382	5′ GCAGCTGGAGCAGTGGTAGG 3′	20	S154N and RFLP
Bov_prnp_Ex3R2	5′ ATGTCAGTTTCGGTGAAGTTCT 3′	22	S154N and RFLP
Bov_prnp_Ex3F	5′ CTTTAAGTGATTTTTACATGGGCAT 3′	25	PCR and sequencing
Bov_prnp_Ex3R	5′ GTAGATACTCCCTCCCCCAACCTG 3′	24	PCR and sequencing

PCR–Restriction Fragment Length Polymorphism (RFLP)

PCR–RFLP for the two SNP sites of *PRNP* was performed using primer pairs Bov_prnp_RFLP_F2 and Bov_prnp_R71 for K3T and Bov_prnp_F382 and Bov_prnp_Ex3R2 for S154N (Table 1). The amplified DNA fragments were 336 bp (K3T) and 260 bp (S154N). Each fragment was digested with 1 U restriction endonuclease *Nmu*CI (MBI Fermentas, Lithuania), followed by 2% agarose gel electrophoresis.

Statistical Analysis

Allele frequencies were calculated by direct counting. Haplotypes and their frequencies were inferred from the genotypes at the six polymorphic sites using Arlequin Version 3.11 software online (Excoffier et al. 2007).

Results

Sequence Comparison of the PrP Encoding Region

We sequenced the PrP encoding region among six animals. These nucleotide sequences have been submitted to GenBank (acc. nos. AB534900-AB534906). The comparison of these sequences shows that four nonsynonymous SNPs causing amino acid substitutions, K3T (AAA to ACA nt 65586 in the reference sequence AJ298878), G49S (GGC to AGC nt 65723), S154N (AGT to AAT nt 66039), and I252 M (ATC to ATG nt 66334), were found in our samples (data not shown). Two amino acid substitutions, K3T and S154N, were detected in Laos and Myanmar native cattle, and all four amino acid substitutions were found in a mythun animal. In addition, we found four-, five- and six-repeat alleles at the octapeptide repeat site.

Genotyping of Six Polymorphic Sites of the Bovine *PRNP*

We estimated the allele distribution of the six *PRNP* polymorphic sites and their frequency in cattle populations examined here. The six *PRNP* polymorphisms include three indels in the promoter, intron 1, and 3′ UTR; a variable number of 24-bp octapeptide repeat units; and the two SNPs K3T and S154N.

A 23-bp indel site in the promoter was polymorphic in all populations except Japanese Shorthorn. The deletion allele (23−) was a major allele, with frequencies of 0.63–1.00 in all populations except mythun (0.18; Table 2). A 12-bp indel site in intron 1 was polymorphic in all populations. The insertion allele (12+) was a major allele, with frequencies of 0.55–0.93 in all populations except the Mongolian native cattle (0.49; Table 2). A 14-bp indel site in the 3′ UTR was polymorphic in all Asian native cattle, but not in all Japanese breeds, mythun, and MCC. The insertion allele (14+) was a major allele in all populations (Table 2).

Regarding the variable number of 24-bp octapeptide repeats, four-, five-, and six-repeat alleles were found in their *PRNP*s (Table 2). The seven-repeat allele,

Table 2 Observed *PRNP* genotypes and allele frequencies in cattle populations

Locus	Population	*n*	Observed genotype			Allele frequency	
		567	+/+	+/−	−/−	23+	23−
Promoter, 23-bp indel	Vietnam native	100	9	50	41	0.34	0.66
	Laos native	71	7	39	25	0.37	0.63
	Myanmar native	110	5	33	72	0.20	0.80
	Mongolia native	44	2	22	20	0.30	0.70
	Bangladesh native	30	1	8	21	0.17	0.83
	Mythun (*Bos frontalis*)	11	7	4	0	0.82	0.18
	Mythun × cattle	6	1	1	4	0.25	0.75
	Japanese Black	153	7	53	93	0.22	0.78
	Japanese Polled	16	2	8	6	0.06	0.94
	Japanese Shorthorn	26	0	0	26	0.00	1.00
Locus	Population	*n*	Observed genotype			Allele frequency	
		569	+/+	+/−	−/−	12+	12−
Intron 1, 12-bp indel	Vietnam native	100	87	12	1	0.93	0.07
	Laos native	72	63	7	2	0.92	0.47
	Myanmar native	110	74	31	5	0.81	0.29
	Mongolia native	44	6	31	7	0.49	0.51
	Bangladesh native	30	20	5	5	0.75	0.25
	Mythun (*Bos frontalis*)	11	7	3	1	0.77	0.33
	Mythun × cattle	6	4	1	1	0.75	0.25
	Japanese Black	153	45	77	31	0.55	0.45
	Japanese Polled	16	4	10	2	0.56	0.44
	Japanese Shorthorn	27	23	3	1	0.93	0.07
Locus	Population	*n*	Observed genotype			Allele frequency	
		569	+/+	+/−	−/−	14+	14−
3′ UTR, 14-bp indel	Vietnam native	100	68	28	4	0.82	0.18
	Laos native	72	43	27	2	0.78	0.22
	Myanmar native	110	75	31	4	0.82	0.18
	Mongolia native	44	27	14	3	0.77	0.23
	Bangladesh native	30	26	4	0	0.93	0.07
	Mythun (*Bos frontalis*)	11	11	0	0	1.00	0.00
	Mythun × cattle	6	6	0	0	1.00	0.00
	Japanese Black	154	154	0	0	1.00	0.00
	Japanese Polled	16	16	0	0	1.00	0.00
	Japanese Shorthorn	26	26	0	0	1.00	0.00

Table 2 continued

Locus	Population	*n*	Observed genotype				Allele frequency		
		569	6/6	6/5	5/5	6/4	6	5	4
Octapeptide repeat units	Vietnam native	100	100	0	0	0	1.00	0.00	0.00
	Laos native	72	72	0	0	0	1.00	0.00	0.00
	Myanmar native	110	107	3	0	0	0.99	0.01	0.00
	Mongolia native	44	30	13	1	0	0.83	0.17	0.00
	Bangladesh native	30	25	5	0	0	0.92	0.08	0.00
	Mythun (*Bos frontalis*)	11	6	3	0	2	0.77	0.14	0.09
	Mythun × cattle	6	4	1	1	0	0.75	0.25	0.00
	Japanese Black	154	90	56	8	0	0.77	0.23	0.00
	Japanese Polled	16	14	2	0	0	0.94	0.06	0.00
	Japanese Shorthorn	26	23	3	0	0	0.94	0.06	0.00

Locus	Population	*n*	Observed genotype			Allele frequency	
		561	K/K	K/T	T/T	K	T
K3T	Vietnam native	100	92	8	0	0.96	0.04
	Laos native	67	59	7	1	0.93	0.07
	Myanmar native	109	103	6	0	0.97	0.03
	Mongolia native	44	44	0	0	1.00	0.00
	Bangladesh native	30	30	0	0	1.00	0.00
	Mythun (*Bos frontalis*)	11	4	6	1	0.64	0.36
	Mythun × cattle	6	3	3	0	0.75	0.25
	Japanese Black	153	153	0	0	1.00	0.00
	Japanese Polled	16	16	0	0	1.00	0.00
	Japanese Shorthorn	25	25	0	0	1.00	0.00

Locus	Population	*n*	Observed genotype			Allele frequency	
		539	S/S	S/N	N/N	S	N
S154N	Vietnam native	100	62	36	2	0.80	0.20
	Laos native	71	27	38	6	0.65	0.35
	Myanmar native	109	69	34	6	0.79	0.21
	Mongolia native	44	37	7	0	0.92	0.08
	Bangladesh native	30	27	1	2	0.92	0.08
	Mythun (*Bos frontalis*)	11	6	4	1	0.73	0.27
	Mythun × cattle	6	3	1	2	0.58	0.42
	Japanese Black	127	127	0	0	1.00	0.00
	Japanese Polled	16	16	0	0	1.00	0.00
	Japanese Shorthorn	25	25	0	0	1.00	0.00

previously reported in European cattle (Schlapfer et al. 1999), was not observed in this study. Of these alleles, the six-repeat allele (6) was a major allele in all populations. The 5/5 genotype was detected in nine animals from the Japanese

Black and Mongolian cattle groups. The four-repeat allele was observed in two mythun animals, which were heterozygous for the four- and six-repeat alleles. The four-repeat allele has been reported in an animal of *Bos indicus* × *Bos taurus* composite cattle by Seabury et al. (2004a).

Two SNPs, K3T and S154N, were detected (by PCR–RFLP using *Nmu*CI) in both Asian native cattle and mythun. The K3T SNP site was polymorphic in Vietnam, Laos, Myanmar, mythun, and MCC populations, but not in Mongolia, Bangladesh, and Japanese populations (Table 2). The T allele was a minor allele, with frequencies of 0.00–0.36. The S154N SNP site was polymorphic in all populations, except in the Japanese breeds. The frequency of the N allele encoding Asn at residue 154 ranged from 0.00 to 0.42 (Table 2).

Haplotype Analysis

All six *PRNP* sites involved in this study were assembled into haplotypes. Our present results revealed 33 haplotypes inferred in our populations (Table 3). The greatest number of haplotypes, 17, was found in Myanmar and Laos native cattle. The haplotype 23− 12− K 6 S 14+ was common in all populations, with frequencies from 0.050 in Vietnam native cattle to 0.861 in Japanese Shorthorn (Table 3). The frequencies of the haplotypes with an insertion at both the 23 and 12 bp loci were 13.3–35.5% in Asian native cattle, 0.0–37.5% in Japanese breeds, and 58.4–63.6% in MCC and mythun (Table 3). Haplotypes containing the 23-bp insertion and 12-bp deletion have been reported only by Msalya et al. (2009) and were estimated with low frequencies in 1–2% of Asian native cattle, except Mongolian, 9% of mythun, and 16.6% of MCC (Table 3). These haplotypes were not estimated in Mongolian native cattle and Japanese breeds.

Discussion

In cattle, the deletion allele and deletion/deletion genotype of the 23-bp indel site within the bovine *PRNP* promoter were reported to be tentatively associated with BSE susceptibility (Sander et al. 2004, 2005). In this study, at the 23-bp indel site, the deletion allele was a major allele in all populations excluding mythun, which is similar to observations in other cattle populations worldwide (Brunelle et al. 2007, 2008; Czarnik et al. 2007; Haase et al. 2007; Juling et al. 2006; Muramatsu et al. 2008; Sander et al. 2004). In addition, the genotype distribution for the 23-bp indel site in all populations except mythun and Japanese Shorthorn did not differ statistically from that of cattle with BSE in Germany (Sander et al. 2004). As for the octapeptide repeat units, our results are similar to those reported in earlier studies in both Japan and abroad (Sander et al. 2004; Nakamitsu et al. 2006; Msalya et al. 2009). The PrP with a lower number of octa/nona-peptide repeats appeared in increased numbers in Japanese Black, Mongolia native cattle, MCC, and mythun. Greater numbers of the repeat units were reported to shorten the incubation period in BSE-inoculated transgenic mice (Brun et al. 2007). Mythun with a PrP variant of the four-repeat units may be valuable as a genetic resource in Myanmar. Variability of

Table 3 Haplotypes and their frequencies in cattle populations

Haplotype	Population									
	Asian native					Mythun	MCC[a]	Japanese		
	Vietnam	Laos	Myanmar	Mongolia	Bangladesh			Black	Polled	Shorthorn
23+ 12+ K 6 N 14+	0.065	0.089	0.046	0.012	0.067	–	0.167	–	–	–
23+ 12+ K 6 N 14−	0.089	0.158	0.108	0.022	0.017	–	–	–	–	–
23+ 12+ K 6 S 14+	0.115	0.040	0.018	0.183	–	0.409	–	0.221	0.375	–
23+ 12+ K 6 S 14−	0.021	0.006	0.005	0.037	–	–	–	–	–	–
23+ 12+ T 6 N 14+	0.030	0.030	–	–	–	0.182	0.167	–	–	–
23+ 12+ T 6 S 14+	0.005	0.012	0.009	–	–	–	–	–	–	–
23+ 12+ T 6 S 14−	–	0.013	–	–	–	–	–	–	–	–
23+ 12+ K 5 N 14+	–	0.007	–	–	–	–	–	–	–	–
23+ 12+ K 5 N 14−	0.005	–	–	–	0.049	0.045	–	–	–	–
23+ 12+ K 5 S 14+	–	–	–	0.042	–	–	0.250	–	–	–
23+ 12+ T 5 S 14+	–	–	–	–	–	0.045	–	–	–	–
23+ 12+ T 4 S 14+	–	–	–	–	–	0.045	–	–	–	–
23+ 12− K 6 N 14+	–	0.016	–	–	–	–	0.083	–	–	–
23+ 12− T 6 N 14+	–	–	0.004	–	–	–	–	–	–	–
23+ 12− T 6 S 14+	–	–	0.005	–	–	–	–	–	–	–
23+ 12− K 6 S 14+	–	0.002	–	–	–	0.045	0.083	–	–	–
23+ 12− K 6 S 14−	0.010	–	–	–	0.017	–	–	–	–	–
23+ 12− T 4 N 14+	–	–	–	–	–	0.045	–	–	–	–
23− 12+ K 6 N 14+	0.006	0.048	0.007	0.03	0.067	–	–	–	–	–
23− 12+ K 6 N 14−	–	0.005	0.021	0.007	–	–	–	–	–	–
23− 12+ K 6 S 14+	0.539	0.482	0.542	0.030	0.516	–	0.167	0.013	–	0.081
23− 12+ K 6 S 14−	0.055	0.025	0.035	0.053	–	–	–	–	–	–

Table 3 continued

Haplotype	Population									
	Asian native					Mythun	MCC[a]	Japanese		
	Vietnam	Laos	Myanmar	Mongolia	Bangladesh			Black	Polled	Shorthorn
23− 12+ K 5 S 14+	–	–	0.013	0.065	0.017	0.045	–	0.214	0.036	0.152
23− 12+ K 5 S 14−	–	–	–	–	–	–	–	–	0.026	–
23− 12+ T 6 N 14+	–	–	0.005	–	–	–	–	–	–	–
23− 12+ T 6 S 14+	–	0.008	–	–	–	–	–	–	–	–
23− 12+ K 5 N 14−	–	–	–	0.008	–	–	–	–	–	–
23− 12+ K 5 N 14+	–	–	–	–	0.017	–	–	–	–	–
23− 12− K 5 S 14+	0.005	–	–	0.023	–	–	–	0.013	–	0.042
23− 12− K 6 S 14+	0.050	0.054	0.173	0.387	0.233	0.091	0.083	0.539	0.495	0.861
23− 12− K 6 S 14−	–	0.005	0.004	0.069	–	–	–	–	0.067	–
23− 12− T 6 S 14+	–	–	0.005	–	–	0.045	–	–	–	–
23− 12− T 6 N 14+	0.005	–	–	–	–	–	–	–	–	–
23− 12− K 5 S 14−	–	–	–	0.032	–	–	–	–	–	–
Total haplotypes	14	17	17	15	9	10	7	5	5	4

[a] Mythun × cattle composite population

the octapeptide repeat has not been linked to BSE incidence (Hunter et al. 1994; Neibergs et al. 1994; Sander et al. 2004). In this study, several results regarding *PRNP* variability indicated these differences between Asian native populations and Japanese breeds: (1) The two SNP sites (K3T and S154N) and the 14-bp indel site in 3′ UTR were polymorphic in Asian native populations but not in Japanese breeds. (2) Haplotype analysis revealed that Asian native populations had a higher number of haplotypes than the Japanese breeds. (3) The haplotype 23− 12− K 6 S 14+ was common in all groups, as a major haplotype in Japanese breeds and the Mongolia native population, and as a minor haplotype in other Asian native populations. These results are thought to be associated with differences in the genetic background of the populations. Therefore, the Asian native cattle are valuable as genetic resources because of the possibility of obtaining new *PRNP* variants.

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