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Polymorphisms of Intron 1 and the Promoter Region at the *PRNP* Gene in BSE-Free Caracu Cattle

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Abstract The infectious prion protein PrP^{Sc} is encoded by the *PRNP* gene. In cattle, insertion/deletion (indel) polymorphisms are among the changes that occur in this gene, the most studied of which are within intron 1 (12 bp) and the promoter region (23 bp). Sequence variants in this gene may affect the formation of PrP^{Sc}. In the present study, nucleotide variability in specific regions of the *PRNP* gene in Caracu cattle free of bovine spongiform encephalopathy was investigated to determine the genotypic profile of each animal within the group. Caracu cattle exhibited high allele frequency for the two polymorphic regions studied, 12ins (70 %) and 23ins (72.5 %), genotype frequencies of 50 % for 12ins/ins and 50 % for 23ins/del, and a high frequency of the 12ins–23ins haplotype (57.5 %). Of the 40 animals sampled, 15 had the 12ins–23ins/12ins–23ins diplotype.

Keywords Bovine spongiform encephalopathy · *PRNP* · Prion · Indel · Caracu cattle

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Introduction

Bovine spongiform encephalopathy (BSE) belongs to the group of transmissible spongiform encephalopathies (TSEs). Diseases from this group affect various animal species, including humans (Czarnik et al. 2007). For many years, it was believed that these diseases were caused by a small virus known as scrapie (Prusiner and Scott 1997). The agent proposed for TSEs is a prion (proteinaceous infectious particle), or infectious protein, which may be denominated in its form as abnormal scrapie prion protein (PrP^{Sc}) (Prusiner 1982). The normal prion protein, or cellular prion protein (PrP^{C}), is a glycoprotein that is constantly produced and rapidly metabolized by nerve tissue (Moser et al. 1995). It is used as a receptor molecule as well as an adhesion molecule for neurons with neurotransmitter and antioxidant action, which in turn is linked to apoptosis.

PrP^{C} is encoded by the *PRNP* gene located on bovine chromosome BTA13 at position q17 (Schläpfer et al. 2000). In cattle, insertion/deletion (indel) polymorphisms are among the changes that occur in the *PRNP* gene, as a sequence of 12 bp in intron 1 and a sequence of 23 bp in the promoter region. Sander et al. (2004) found that sequence variants in this gene may affect the amino acid sequence or the expression of prion protein and consequent susceptibility to BSE; the haplotype 12del– 23del was more frequent in a group of cattle affected by BSE than in a group of healthy cattle from the same study. Assays with reporter genes demonstrated that indel polymorphisms are associated with expression levels of prion by the bovine *PRNP* gene (Sander et al. 2005; Kashkevich et al. 2007). Epidemiological surveys have shown that the occurrence of the classical form of BSE has no connection with the age or sex of the affected animal but, rather, is connected with the intake of animal protein contaminated with other mutated proteins, and there is no specific denaturing treatment (Wilesmith et al. 1988).

The Caracu cattle breed originates from crosses of the Iberian breeds Alentejana, Minhota, Mirandesa, Arouquesa, and Algaviriano. The first cattle breeds were introduced in Brazil by the Portuguese in 1534 in the state of São Paulo. After nearly five centuries of Caracu breeding in Brazil, the purebred animals have acquired a number of characteristics, such as resistance to certain parasitic diseases and adaptation to differing climates in Brazil, and thus exhibit good productive performance in various regions of the country. These are important factors for the raising and expansion of the Caracu breed in Brazil (Criar e Plantar 2008). The breed exhibits high fertility and longevity. Adult males can weigh as much as 1,100 kg, and adult females 800 kg. The Caracu is used for the production of both milk and meat (Kues et al. 2006). There are records of 80,000 animals of the breed in Brazil for the period between 1973 and 2009 (ABCC 2009).

The aims of the present study were to identify indel polymorphisms of the bovine *PRNP* gene at intron 1 (12-bp indel) and the promoter region (23-bp indel) in BSE-free Caracu cattle and to determine the genotypic profile of each animal sampled.

Materials and Methods

Sampling

In this study, we genotyped 40 specimens of Caracu cattle, each from a different breeder in Brazil. The degree of consanguinity (or inbreeding coefficient, F_i) was 0–9 %, and the degree of kinship was 0–7 %. Genetic material was extracted from blood and semen samples. Inbreeding and kinship coefficients were obtained using Matlab routines.

DNA Extraction from Blood and Semen

Genomic DNA was extracted from blood samples using the Easy-DNA kit (Invitrogen). Genomic DNA was extracted from semen samples using a method adapted from Zadworny and Kuhnlein (1990) and previously reported by Regitano and Coutinho (2001).

The purity of total DNA samples from blood and semen was checked using electrophoresis on 0.8 % agarose gel (Promega) stained with SYBR Gold (Invitrogen). The gel was examined under an ultraviolet light transilluminator (Transiluminador, Loccus Biotecnologia) and photographed using a digital camera with the aid of the L-Pix program (Loccus). The concentration of DNA in each sample was measured with a spectrophotometer (NanoDrop).

Design of Primers and Amplification of Target Regions

A pair of specific primers was designed for detecting the 12-bp indel (prnpBovin12F: 5′-GGTCAGCGTAAATAGAGTCCTT-3′; prnpBovin12R: 5′-TCGCCCTTGTTC TGATTCGC-3′), amplifying a sequence of 400 bp, and another pair was designed for detecting the 23-bp indel (prnpBovin23F: 5′-TCTTCCTGGGACAGCTTCT TAGTACAG-3′; prnpBovin23R: 5′-AAGGCATTCATATAGGTGAGGGTTTG AG-3′), amplifying a sequence of 393 bp. These primers were designed by accessing the AJ298878 bovine *PRNP* gene deposited in GenBank and using the Oligo program.

The target regions of the *PRNP* gene were amplified by polymerase chain reaction (PCR) using 40–60 ng DNA per 22-µl reaction volume containing 10× buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 5 pmol of each primer, and 1 U *Taq* DNA polymerase (CenBiot), and the specific primers mentioned above. The amplification consisted of initial denaturation at 94 °C for 5 min, followed by 34 cycles of 94 °C for 45 s, 58 °C (indel 12) and 60 °C (indel 23) for 45 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Genotyping

PCR products underwent electrophoresis on 3 % agarose gel (Promega). The 1 Kb Plus DNA Ladder (Invitrogen) was used to determine the heights of the bands formed, which were viewed with an ultraviolet light transilluminator (Loccus) and photographed using a digital camera and the L-Pix program. Based on the gel

analysis, animals with only the 12-bp indel or the 23-bp indel were designated homozygous, whereas heterozygous animals were those with both the 12-bp indel and the 23-bp indel. The haplotypes and diplotypes of each animal were then determined.

Statistical Analysis

Genotype and allele frequencies were calculated and Hardy–Weinberg equilibrium tested to determine whether the allele frequencies remained constant over generations. Haplotype and diplotype frequencies were determined. The linkage disequilibrium test (Lewontin 1988) was used to analyze the degree of dependence between haplotypes. Of the 40 animals sampled, 15 were double heterozygotes; we estimated their diplotype numbers using conditional probability (P) and haplotype frequencies, as follows:

$$P(12\text{ins}-23\text{del}) = 5/50$$

$$P(12\text{del}-23\text{ins}) = 7/50$$

$$P(12\text{del}-23\text{del}) = 2/50$$

$$P(12\text{ins}-23\text{ins}) = 36/50$$

From the haplotype frequencies, diplotype values were estimated for the 15 animals with the double heterozygous genotype as follows:

$$P(12\text{ins}-23\text{ins}/12\text{del}-23\text{del}) = \frac{[P(12\text{ins}-23\text{ins}) \times P(12\text{del}-23\text{del})] \times 2}{[P(12\text{ins}-23\text{del}) \times P(12\text{del}-23\text{del}) \times 2] + [P(12\text{ins}-23\text{del}) \times P(12\text{del}-23\text{ins}) \times 2]}$$

The correlation test was used to determine the degree of mutual relationship between the two polymorphic regions that formed haplotypes. All calculations were performed in silico with the aid of spreadsheet formulas.

Sequencing

The PCR products of some samples were purified using the Qiaex II kit (Qiagen). With purified samples, the sequencing reaction was performed with the BigDye Terminator v3.1 kit (Applied Biosystems), using 1 μl forward primer and 1 μl reverse primer (5 pmol) in separate reactions, 2 μl BigDye, 2 μl 5× buffer, and 5 μl purified PCR. The plate with the reactions was placed in a thermocycler, purified with EDTA and ethanol, and resuspended in formamide. For analysis of the positions of 12-bp and 23-bp indels, the samples were sequenced using the Sanger (2004) procedure in a capillary sequencer (Applied Biosystems model 3130). The sequencing results were analyzed and aligned with accession AJ298878 using the BLASTn program of the National Center for Biotechnology Information. The quality of the waves in the electropherogram was analyzed with the BioEdit program to determine whether the regions were polymorphic in exact locations, as described for other breeds.

Results

From the gel electrophoresis, the amplification pattern of polymorphic regions for heterozygous and homozygous animals was identified based on the specific primers (Fig. 1). The conformation of the bands applies to both indels (12 and 23 bp). Heterozygous animals exhibited the ins/del genotype (one allele with the insertion and the other with the deletion); homozygous animals exhibited either the del/del genotype (deletion in both alleles) or the ins/ins genotype (insertion in both alleles).

In the sequence of each polymorphic region, the online BLASTn and BioEdit programs clearly identified the expected genotype profiles in accordance with the gel analysis: insertion, deletion, and heterozygous polymorphisms of 12 bp in intron 1 (Fig. 2) and insertion, deletion, and heterozygous polymorphisms of 23 bp in the promoter region (Fig. 3). For heterozygous animals, when the BioEdit program was used, nucleotides appeared from or near the region of polymorphism that are not part of the sequence of the bovine *PRNP* gene. When BLASTn was used, the alignment of the sequence from the heterozygous animals stopped at or near the region of polymorphism, and the nucleotides that appeared in the electropherogram after the region of interest were not recognized. This was characteristic in these samples.

For the two polymorphic regions, values were calculated for the degree of inbreeding and kinship of each individual with respect to the remaining animals in the group studied. The animals (numbered 1–40) were divided into groups based on the formation of genotypes, which could also determine their haplotypes and diplotypes (Table 1). Haplotype and diplotype frequencies were estimated for the two indels in the 15 animals that were doubly heterozygous (specimens with the 12ins/del and 23ins/del genotypes), as animals with such profiles generated four haplotypes and two diplotypes. This estimation was necessary before adding their data to those of the other animals and performing further calculations.

Expected genotype and allele frequencies were calculated for the polymorphisms of the two indels among the 40 animals (Table 2), and the frequencies of the haplotype profiles of the two indels in the same chromosome were also calculated (Table 3). An analysis of the polymorphisms in the chromosome pairs of the cattle yielded diplotype frequencies (Table 4). The conditional probability (P) of the 15 doubly heterozygous animals was estimated to be P (12ins–23del) = 0.10, P (12del–23ins) = 0.14, P (12del–23del) = 0.04, and P (12ins–23ins) = 0.72.

For the diplotype 12ins–23ins/12del–23del, P was 0.67, equivalent to 10 of the 15 doubly heterozygous animals. This allowed us to calculate P (12ins–23del/

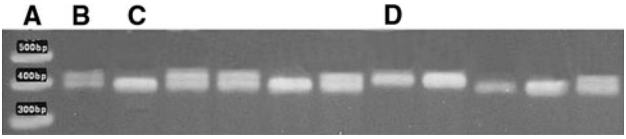

Fig. 1 Electrophoretic profile of the *PRNP* gene in Caracu cattle. *Lanes A* base pair pattern; *B* band characteristic of heterozygous animals; *C* band characteristic of homozygous animals with deletion; *D* band characteristic of homozygous animals with insertion

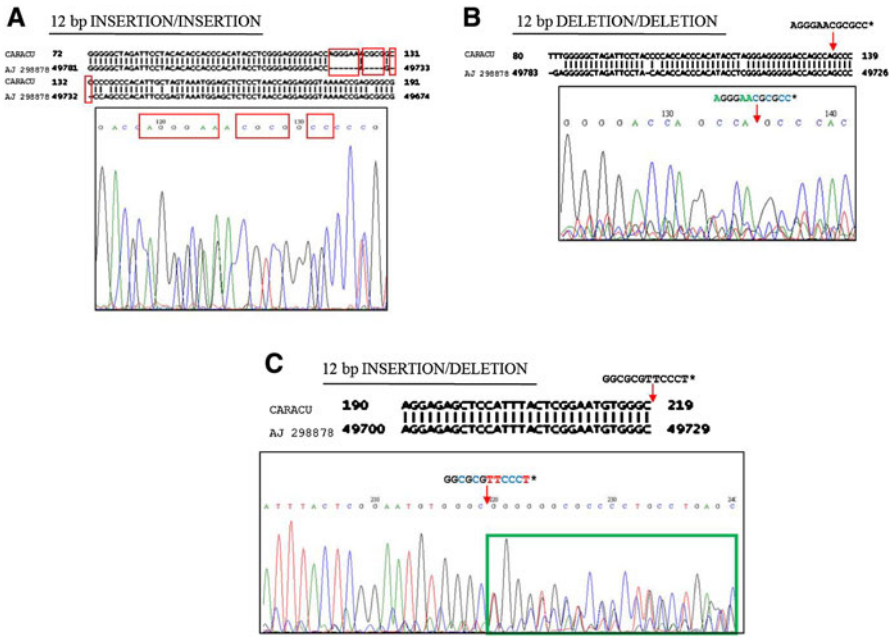

Fig. 2 Comparison of 12-bp polymorphisms in a specific region of the *PRNP* gene in Caracu cattle. BLASTn alignments of sequences from Caracu animals and the bovine *PRNP* gene AJ298878, deposited in GenBank, appear above an electropherogram of the same region of the Caracu gene. **A** Homozygous Caracu, with 12-bp insertion (in both alleles) *boxed*; sequence obtained from reverse direction (3′–5′). **B** Homozygous Caracu, with *arrow* indicating position (in both alleles) where 12-bp sequence (*asterisk*) is deleted; *asterisk* sequence obtained from reverse direction (3′–5′). **C** Heterozygous Caracu, with *arrow* indicating position of 12-bp insertion (*asterisk*) in one allele and deletion in the other; overlap and differences in wave characteristics are *boxed*; *asterisk* sequence obtained from forward direction (5′–3′) (color figure online)

12del–23ins) as 1.00 – 0.67, or 0.33, which equates to 5 of 15 heterozygous animals. Following the principle of chromosome distribution ($n + n = 2n$), we estimated the number of haplotypes that originated from the diplotypes of these 15 animals (Table 3).

Discussion

The insertion and/or deletion of 12 bp in intron 1 and 23 bp in the promoter region of the bovine *PRNP* gene is one of the most studied polymorphisms used to determine a possible genetic predisposition toward developing BSE. Previous studies have compared these polymorphisms in healthy cattle with those in BSE-infected cattle, and it is now possible to distinguish polymorphisms that may be associated with the traits of resistance and susceptibility to BSE (Sander et al. 2004).

Electrophoresis of the amplification of target regions in 3 % agarose gel is an important tool for detecting resistant genotypes. In the present study, homozygous genotypes resistant to BSE (ins/ins), homozygous genotypes susceptible to BSE

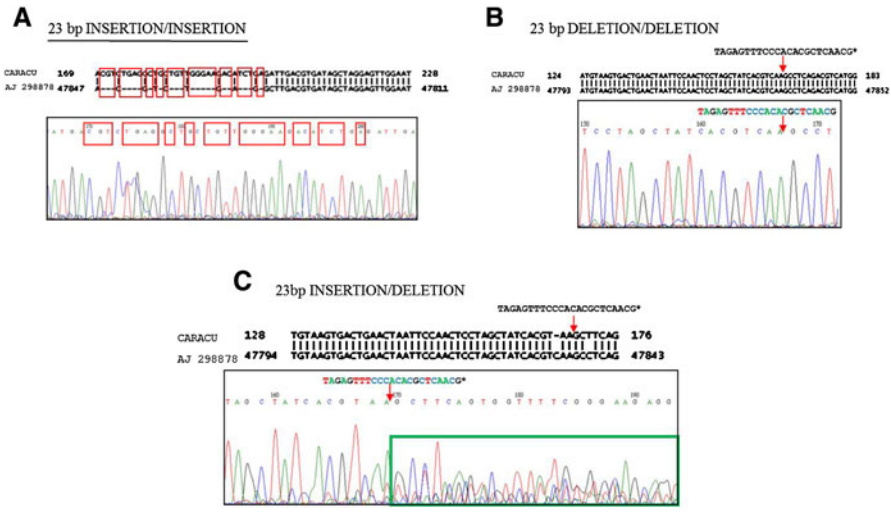

Fig. 3 Comparison of 23-bp polymorphisms in a specific region of the *PRNP* gene in Caracu cattle. BLASTn alignments of sequences from Caracu animals and the bovine *PRNP* gene AJ298878, deposited in GenBank, appear above an electropherogram of the same region of the Caracu gene. **A** Homozygous Caracu, with 23-bp insertion (in both alleles) *boxed*; sequence obtained from reverse direction (3′–5′). **B** Homozygous Caracu, with *arrow* indicating position (in both alleles) where 23-bp sequence (*asterisk*) is deleted; *asterisk* sequence obtained from forward direction (5′–3′). **C** Heterozygous Caracu, with *arrow* indicating position of 23–bp insertion (*asterisk*) in one allele and deletion in the other; overlap and differences in wave characteristics are *boxed*; *asterisk* sequence obtained from forward direction (5′–3′) (color figure online)

(del/del), and heterozygous genotypes (ins/del) were identified from the differing heights of the bands formed by 12- and 23-bp insertions and deletions (Fig. 1). Recent studies have reported the results of electrophoresis used to identify these genotypes in German and Japanese cattle (Sander et al. 2004; Nakamitsu et al. 2006), which served as a basis for our analysis of polymorphisms in Caracu cattle.

The sequencing was used as an auxiliary tool for viewing the polymorphisms and confirming both the electrophoretic results and the alignment of the polymorphisms with those of accession AJ298878 (Sander et al. 2004). The BLASTn results allowed visualization of the polymorphisms, especially the insertion of base pairs in the two alleles (Figs. 2, 3). The heterozygous profile for both the 12- and 23-bp sequences merits attention. One allele of the heterozygote has the insertion, and the other has the deletion. Thus, two bands are formed in the agarose gel, one corresponding to the amplified fragment with the insertion and the other corresponding to the amplified fragment with the deletion (Fig. 1). An analysis of the specific polymorphic region in the electropherogram reveals overlapping waves formed by nucleotides and a subsequent disorder of nucleotides and their respective waves due to the alleles having different numbers of nucleotides in the region amplified by the PCR (Figs. 2, 3).

The genotype and allele frequencies were based on the electrophoretic results (Table 2) and were compared with those of previous studies of other cattle breeds (Table 5). Despite differences in the number of samples between studies, the

Table 1 Kinship of 40 Caracu cattle genotyped for two *PRNP* polymorphisms

Animal ID no.	F_i (%)[a]	Kinship (%)[b]	Genotype		Haplotype 12–23	Diplotype 12–23/12–23
			12/12	23/23		
8	0	1.4	Del/del	Del/del	12del–23del	12del–23del/12del–23del
					12del–23del	
1	0.29	7.03	Ins/ins	Ins/del	12ins–23ins	12ins–23ins/12ins–23del
6	2.84	7.03			12ins–23del	
12	1.21	2.15				
15	0	5.29				
29	0	1.35				
3	2.39	5.29	Ins/ins	Ins/ins	12ins–23ins	12ins–23ins/12ins–23ins
7	0.78	3.22			12ins–23ins	
11	2.99	4.86				
20	4.33	5.09				
21	4.75	3.19				
22	0	2.51				
23	0	0				
24	0.90	2.87				
25	0.04	3.83				
26	0	3.28				
27	0	0				
32	0.99	5.85				
37	0	1.74				
39	3.98	4.20				
40	2.83	3.98				
28	1.66	3.54	Del/del	Ins/ins	12del–23ins	12del–23ins/12del–23ins
30	0.53	3.93			12del–23ins	
31	0.94	2.90				
35	4	4.39	Ins/del	Ins/ins	12ins–23ins	12ins–23ins/12del–23ins
					12del–23ins	
2	**0**	**0**	**Ins/del**	**Ins/del**	**12ins–23ins**	**12ins–23ins/12del–23del**
4	**0**	**0**			**12del–23del**	**12ins–23del/12del–23ins**
5	**0**	**3.49**			**12ins–23del**	
9	**0**	**1.28**			**12del–23ins**	
10	**9.02**	**5.68**				
13	**0**	**1.28**				
14	**2.15**	**4.45**				
16	**0**	**0.66**				
17	**0.96**	**5.10**				
18	**1.90**	**2.44**				
19	**0**	**1.06**				
33	**0**	**1.51**				

Table 1 continued

Animal ID no.	F_i (%)[a]	Kinship (%)[b]	Genotype		Haplotype 12–23	Diplotype 12–23/12–23
			12/12	23/23		
34	**1.61**	**5.71**				
36	**2.25**	**3.40**				
38	**0.01**	**5.96**				

[a] Degree of consanguinity (inbreeding); mean $F_i = 1.33$ %

[b] Mean kinship = 3.27 %

Bold type indicates data for the 15 animals that were doubly heterozygous (number of haplotypes and estimated diplotypes were used in calculations of frequencies, Hardy–Weinberg, linkage disequilibrium, and correlation)

majority of breeds exhibit a high frequency of 12del and 23del alleles (Kerber et al. 2008). The breed with the highest frequency is the Japanese Holstein, at 74 % for 12del and 79 % for 23del (Nakamitsu et al. 2006). The Polish Holstein–Friesian exhibits frequencies of 54 % for 12del and 63 % for 23del (Czarnik et al. 2007).

In the present study, the Caracu breed exhibited a high frequency of 12ins (70 %) and 23ins (72.5 %) alleles. Compared with zebu breeds (*Bos indicus*), taurines (*B. taurus*) have a greater percentage of alleles for susceptibility, especially in the promoter region (23del) (Brunelle et al. 2008). The Hardy–Weinberg test revealed that the animals of the present study were in equilibrium at both regions ($P > 0.05$), indicating that there was no selection, mutation, and/or migration that could affect their frequencies over generations.

There are hypotheses that these and other polymorphisms in the bovine *PRNP* gene have an Indian origin. Zebu breeds, which contributed to the improvement of European breeds (*Bos taurus*), could have passed down deletion alleles (Richt and Hall 2008). One study found 10 polymorphisms in a single nucleotide in the exon 3 region of the *PRNP* gene in Caracu cattle; one of them encodes a change in the expression of an amino acid, and nine were determined to be silent mutations that could be related to BSE resistance and susceptibility (Kues et al. 2006).

The haplotypes described in the present study indicate that the 12–bp and 23–bp indels occur together in a single allele (Table 1). It is therefore possible to obtain an overview of how many alleles, or chromosomes, are associated with the two polymorphisms. The specimens of Caracu cattle exhibited 46 chromosomes (57.5 %) with 12ins–23ins, which is a high frequency in the group of 40 animals examined (Table 3). This chromosome with two insertions is important in that it can segregate in future generations.

The 12ins–23ins haplotype has been reported with a frequency of 46 % in healthy Swiss cattle and 37 % in those infected with BSE (Haase et al. 2007); lower frequencies have been reported for healthy specimens of Aberdeen Angus (25 %), Charolais (31 %), and Franqueiro (36 %) (Kerber et al. 2008). The frequency of the resistant haplotype is reported to be 24 % in the UK Holstein, 30 % in the German Holstein, 45 % in the German Brown, and 27 % in the Fleckvieh (Juling et al.

Table 2 Genotype and allele frequencies for *PRNP* indels in 40 Caracu cattle

PRNP indel	Genotype (%)			Allele (%)	
	Ins/ins	Del/del	Ins/del	Ins	Del
12 bp (400–412 bp)	50	10	40	70	30
23 bp (393–416 bp)	47.5	2.5	50	72.5	27.5

Hardy–Weinberg test, 12-bp indel ($\chi^2 = 0.09$), $P > 0.05$; 23-bp indel ($\chi^2 = 2.58$), $P > 0.05$

Table 3 Haplotype frequency in Caracu cattle

Haplotype	Number of chromosomes (*n*)			Frequency (%)
	n	Doubly heterozygous *n*[a]	Total[b]	
12ins–23del	5	5	10	12.5
12del–23ins	7	5	12	15
12del–23del	2	10	12	15
12ins–23ins	36	10	46	57.5

[a] Estimated from 15 doubly heterozygous animals (bold type in Table 4)

[b] Used for calculating frequencies; linkage disequilibrium test, $D = 0.0675$ and $D' = 0.3506$; correlation test, $r = 0.33$

Table 4 Diplotype frequency in Caracu cattle

Diplotype	Number of animals	Frequency (%)
12del–23del/12del–23del	1	2.5
12ins–23ins/12del–23ins	1	2.5
12ins–23del/12del–23ins	**1**	**2.5**
12del–23ins/12del–23ins	3	7.5
12ins–23ins/12ins–23del	5	12.5
12ins–23ins/12del–23del	**14**	**35**
12ins–23ins/12ins–23ins	15	37.5

Bold type indicates data for the 15 doubly heterozygous animals (bold type in Table 1)

2006). In the Polish Holstein–Friesian, the haplotype was present at a frequency of 36.3 % (Czarnik et al. 2011).

The haplotypes calculated from 40 animals in the present study showed a linkage disequilibrium of 35.06 % ($D' = 0.3506$). This disequilibrium is fairly low in comparison with the values for the Aberdeen Angus (87 %), Charolais (95 %), and Franqueira (99 %) breeds (Kerber et al. 2008). In the Caracu breed, these haplotypes have a certain dependence, and the loci that form the different haplotypes tend toward a medium degree of correlation ($r = 0.33$).

Polymorphisms in the chromosome pair were analyzed in the diplotypes (Table 1). Of the 40 animals, 15 (37.5 %) were found to exhibit the 12ins–23ins/12ins–23ins diplotype (Table 4). Lower frequencies of this diplotype have been

Table 5 Allele frequencies for the 12- and 23-bp *PRNP* indels reported in various cattle breeds

Breed (reference)	Sample size	Allele			
		12 bp		23 bp	
		Ins	Del	Ins	Del
German (Sander et al. 2004)	48	49	51	43	57
US beef (Clawson et al. 2006)	96	37	63	26	74
US dairy (Clawson et al. 2006)	96	53	47	38	62
German Holstein (Juling et al. 2006)	127	47	53	38	62
German Brown (Juling et al. 2006)	43	86	14	65	35
Fleckvieh (Juling et al. 2006)	106	38	62	32	68
Japanese Holstein (Nakamitsu et al. 2006)	278	26	74	21	79
Japanese Brown (Nakamitsu et al. 2006)	186	43	57	41	59
Polish Holstein–Friesian (Czarnik et al. 2007)	234	46	54	37	63
Swiss (Haase et al. 2007)	294	54	46	46	54
German Holstein (Kashkevich et al. 2007)	17	47	53	42	58
Braunvieh (Kashkevich et al. 2007)	17	84	16	60	40
Fleckvieh (Kashkevich et al. 2007)	44	36	64	29	71
Zebu (*Bos indicus*) (Brunelle et al. 2008)	116	87	13	12	88
Bos indicus × *B. taurus* (Brunelle et al. 2008)	76	47	53	20	80
Taurine (*Bos taurus*) (Brunelle et al. 2008)	4,552	45	55	38	62
Aberdeen Angus (Kerber et al. 2008)	99	44	56	27	73
Charolais (Kerber et al. 2008)	82	42	58	22	68
Franqueiro (Kerber et al. 2008)	73	67	33	36	64
Vietnamese dairy (Muramatsu et al. 2008)	206	52	48	15	85
Korean (Kim et al. 2009)	437	44	56	44	56
Polish Holstein–Friesian (Czarnik et al. 2011)	837	47.3	52.7	37.8	62.2
Caracu (this study)	40	70	30	72.5	27.5

reported for Aberdeen Angus (3 %), Charolais (10 %), and Franqueiro (16 %) breeds (Kerber et al. 2008). Juling et al. (2006) reported frequencies in healthy specimens of other breeds, including UK Holstein (3 %), German Holstein (15 %), German Brown (46 %), and Fleckvieh (11 %).

There have been no cases of BSE in Brazil, and therefore we made no comparisons with affected animals. Our aim was to gather information on the degree of inbreeding and kinship among Caracu cattle from 40 breeders, because data on polymorphisms of the bovine *PRNP* gene are useful to genetic improvement programs. Breeders are an integral part of an important Brazilian genetic improvement program aimed at the productive development of the Caracu breed. Caracu cattle are of European origin and, after more than 400 years of establishment in Brazil, are fully adapted to the country's various environments. Over all that time, 12ins and 23ins alleles have been passed from generation to generation, down to the cattle analyzed in the present study. As demonstrated by the Hardy–Weinberg test,

the loci are in equilibrium; that is, these regions have not undergone significant changes that would affect their allele frequencies over generations.

The BSE cases recorded so far merit attention. Most cases of the disease in the countries where the precursor breeds of the Caracu originated (Portugal and Spain) have been traced to the importation of animals infected with the prion (OIE 2007). There are no reports of this disease in Caracu cattle.

Thus, based on our analysis of the *PRNP* gene in the present study, the cattle raised by these Caracu breeders have a genotypic profile similar to that of healthy cattle from other studies and significantly different from that of BSE-affected cattle. The search for indel and other polymorphisms of the bovine *PRNP* gene is important for eventually forming a screening program of cattle resistant to BSE, in order to control and reduce cases of this disease worldwide. From there, breeding would be a new tool to combat BSE, contributing to public health and lessening the economic impact of the disease.

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