Analysis of *DRB3* gene polymorphisms in Jafarabadi, Mediterranean, and Murrah buffaloes from Brazil

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ABSTRACT. The *DRB3* gene is an MHC class II gene that has a high degree of polymorphism with more than 100 alleles described in cattle. This variation contributes to differences among individuals in immune responsiveness and disease resistance. In this study, we searched for allelic variants in exon 2 of the *DRB3* gene in 80 river buffaloes of three breeds in Brazil using a PCR-RFLP technique. The PCR product showed genetic polymorphism when digested with *Rsa*I, *Pst*I or *Hae*III restriction enzymes. In total, 16 restriction patterns were identified: nine restriction patterns and 16 genotypes were found with *Rsa*I; four restriction patterns and nine genotypes were found with *Hae*III; and, three restriction patterns and four genotypes were found with *Pst*I. Three RFLP patterns were exclusive to Jafarabadi buffaloes (*Rsa*I-b, *Rsa*I-c and *Rsa*I-f) and three

others were only observed in Mediterranean buffaloes (*Rsa*I-g, *Rsa*I-h and *Pst*I-y). Jafarabadi buffaloes had a larger number of RFLP patterns than Mediterranean and Murrah breeds. The analysis showed that the *DRB3* exon 2 was highly polymorphic, with the highest degree of polymorphism in Mediterranean buffaloes. This study provides the first assessment of allelic variation among three different buffalo breeds from Brazil and provides a basis for further investigations into the association between the *DRB3* alleles and disease resistance.

Key words: *Bubalus bubalis*; Major histocompatibility complex; PCR-RFLP; Polymorphism

INTRODUCTION

River buffaloes (*Bubalus bubalis*) were brought to Brazil in the nineteenth century from Asia, Europe (Italy) and the Caribbean for use in meat and milk production (Bernardes, 2007). Currently, Brazil has the largest buffalo herd in South America with a population estimated at 1.33 million (FAOSTAT, 2013). Three river buffalo breeds (Jafarabadi, Murrah, and Mediterranean) and one swamp buffalo breed (Carabao) are officially recognized by the Brazilian Association of Buffalo Breeders.

Infectious diseases in river buffaloes are currently managed much like those in cattle because both are susceptible to similar infectious agents. However, river buffaloes show different susceptibilities and responses to some diseases compared to cattle (Davis et al., 2001). Investigation of the immune responses of river buffalo to infectious agents will be aided by the characterization of the genes of the immune system and identification of the genes responsible for the development of resistance and susceptibility.

The major histocompatibility complex (MHC) is one of the most extensively studied regions in the genome of bovid species, mainly because this region encodes the most important proteins related to both adaptive and innate immune responses. The roles of MHC genes in the innate and adaptive immune responses suggest that they are candidate genes for disease resistance and susceptibility (Kelley et al., 2005).

In buffalo, the MHC genes are located on the short arm of chromosome 2 (BBU2p), which is homologous to bovine chromosome 23 (Rodrigues Filho et al., 2008). The structure and organization of the buffalo and bovine MHC genes are very similar and three distinct classes of gene (I, II, and III) are recognized. Class II genes are separated into two subtypes designated class IIa and class IIb. Class IIa genes are closely associated with class I and class III regions, while class IIb genes are positioned closer to the centromere (Stafuzza et al., 2013).

The extensive allelic diversity at class II MHC loci influences the repertoire of self-, vaccine- or pathogen-derived peptide antigens presented to $CD4^+$ T-lymphocytes as well as maintaining self-tolerance and antibody production (Garcia et al., 1999). This allelic diversity can also be exploited to search for associations between genes and resistance/susceptibility to disease. The *DRB3* gene is an MHC class II gene that has a high degree of polymorphism with more than 100 alleles described in cattle; this variation contributes to differences among individuals in immune responsiveness and disease resistance (Behl et al., 2012), such as to mastitis (Firouzamandi et al., 2010), bovine leukemia virus (Juliarena et al., 2008), dermatophilosis (Maillard et al., 2003), neospirosis (Schwab et al., 2009), bovine neonatal pancytopenia (Ballingall et al., 2011), and tick (*Boophilus microplus*) resistance (Martinez et al., 2006). Additionally, Rupp et al. (2007) also linked

DRB3 alleles to variations in milk, fat, and protein yields in cattle. To date, however, few studies have examined *DRB3* polymorphism in buffalo, although some alleles have been associated with susceptibility to mastitis (Gole et al., 2009; Kumar et al., 2011).

Based on the association between *DRB3* alleles and traits related to immunity in bovid species, the aim of this study was to investigate genetic polymorphisms in exon 2 of *DRB3* in Mediterranean, Murrah, and Jafarabadi buffaloes from Brazil. We adopted a PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) approach in order to identify alleles that could provide a basis for further investigation of the association between *DRB3* alleles and resistance or susceptibility to diseases.

MATERIAL AND METHODS

DNA extraction from hair follicle samples

Genomic DNA was extracted from hair follicles collected from the tails of 80 unrelated buffaloes (25 Jafarabadi, 25 Mediterranean, and 30 Murrah animals) from Brazil. The DNA extraction solution contained 80 µL 10X PCR buffer (without $MgCl_2$), 5 µL proteinase K (19.2 mg/mL), 1 µL 1% SDS, and 920 µL ultrapure water. DNA extraction was performed by proteinase K digestion of five hair follicles in 50 µL DNA extraction solution on a Veriti thermal cycler (Applied Biosystems, USA) for 60 min at 56°C. Proteinase K was inactivated by 45 min at 95°C. The quality of the genomic DNA was verified using agarose gel electrophoresis and the concentration of DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific).

Polymerase chain reaction

Exon 2 of *DRB3* (304 bp) was amplified by PCR using a cattle-derived primer pair (Sigurdardóttir et al., 1991); the amplification product comprised 20 bp of the 5'-intron, 267 bp of exon 2, and 17 bp of the 3'-intron. Amplification was carried out on the Veriti thermal cycler. The PCR mixture comprised 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTPs, 0.2 mM forward and reverse primers, 1.5 U GoTaq Hot Start polymerase (Promega, USA), and 50 ng DNA in a 30-µL reaction volume. Amplification was carried out as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s (denaturation), 62°C for 30 s (annealing), extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were separated by 2% agarose gels in 1X TBE buffer containing ethidium bromide to verify the quality and specificity of amplification.

Restriction fragment length polymorphism analysis

For the RFLP analysis, 6 µL of the PCR product was digested with 6 U *Rsa*I (GT↓AC), *Hae*III (GG↓CC), or *Pst*I (CTGCA↓G) restriction enzymes (Promega) in a final volume of 12 µL at 37°C overnight. RFLP detection was carried out by electrophoresis on 4% agarose gels in 1X TBE buffer containing ethidium bromide at 120 V for 90 min. Molecular weight markers of 25 and 50 bp (Promega) were used to identify the size of the restriction fragments on the agarose gel. The agarose gels were photographed under UV light with a Kodak digital camera DC290 and analyzed with the program Kodak Electrophoresis Documentation and Analysis System (EDAS). Allele frequencies and number of alleles were obtained by direct counting and named according to van Eijk et al. (1992) and Aravindakshan et al. (2000).

RESULTS AND DISCUSSION

Here, we present the first data on the distribution of *DRB3* alleles in Jafarabadi, Mediterranean and Murrah buffaloes in Brazil. We found a total of 16 restriction patterns after digestion of PCR products with *Rsa*I, *Hae*III, and *Pst*I. The RFLP patterns in the 80 buffaloes and the allele frequencies among the three breeds are shown in Table 1. Genotype frequencies obtained for each breed are presented in Table 2.

Table 1. Fragment sizes and allele frequencies following digestion of the second exon of *DRB3* with *Rsa*I, *Hae*III, or *Pst*I in Jafarabadi, Mediterranean, and Murrah buffaloes.

Enzyme	Restriction patterns	Fragment size (bp)	Allele frequency			Allele frequency (N = 80)
			Jafarabadi (N = 25)	Mediterranean (N = 25)	Murrah (N = 30)	
*Rsa*I	*Rsa*I-b	114/67/54/39/30	0	0.040	0	0.012
	*Rsa*I-c	114/93/67/30	0	0.060	0	0.019
	*Rsa*I-f	144/67/54/39	0	0.040	0	0.012
	*Rsa*I-h	114/69/67/54	0.080	0	0	0.025
	*Rsa*I-l	237/67	0.300	0.180	0.200	0.225
	*Rsa*I-m	121/114/69	0.100	0	0	0.032
	*Rsa*I-n	183/121	0	0.160	0.150	0.106
	*Rsa*I-o	304	**0.500***	**0.460***	**0.533***	0.500
	*Rsa*I-s	144/93/67	0.020	0.060	0.117	0.069
*Hae*III	*Hae*III-a	170/82/52	0	0.240	0.083	0.106
	*Hae*III-b	222/82	0.440	**0.360***	**0.534***	0.450
	*Hae*III-d	193/82/29	0	0.080	0.083	0.056
	*Hae*III-e	170/134	**0.560***	0.320	0.300	0.388
*Pst*I	*Pst*I-z	230/68/6	0.160	0.080	0.050	0.094
	*Pst*I-y	216/88	0.060	0	0	0.019
	*Pst*I-o	304	**0.780***	**0.920***	**0.950***	0.887

*The most frequent alleles in each breed are shown in bold.

*Rsa*I polymorphisms

Nine restriction patterns were identified using *Rsa*I: *Rsa*I-b (114/67/54/39/30 bp), *Rsa*I-c (114/93/67/30 bp), *Rsa*I-f (144/67/54/39), *Rsa*I-h (114/69/67/54 bp), *Rsa*I-l (237/67 bp), *Rsa*I-m (121/114/69 bp), *Rsa*I-n (183/121 bp), *Rsa*I-s (144/93/67 bp), and *Rsa*I-o (304 bp). These nine patterns resulted in 16 different genotypes: *Rsa*I-b/c, *Rsa*I-c/n, *Rsa*I-f/*s*, *Rsa*I-h/l, *Rsa*I-h/m, *Rsa*I-h/o, *Rsa*I-l/l, *Rsa*I-l/m, *Rsa*I-l/n, *Rsa*I-l/o, *Rsa*I-l/s, *Rsa*I-m/m, *Rsa*I-n/o, *Rsa*I-o/o, *Rsa*I-o/s, and *Rsa*I-s/s.

The *Rsa*I-o pattern is characterized by the absence of an *Rsa*I restriction site and was the most frequent in all analyzed breeds (Table 1); the *Rsa*I-o/o genotype was likewise the most frequent (Table 2).

Mediterranean buffaloes had seven RFLP patterns (*Rsa*I-b, *Rsa*I-c, *Rsa*I-e, *Rsa*I-l, *Rsa*I-n, *Rsa*I-o, and *Rsa*I-s) combined into eight genotypes. The *Rsa*I-b, *Rsa*I-c, and *Rsa*I-f patterns were only found in Mediterranean buffaloes and therefore could be used as a genetic marker for this breed.

Jafarabadi buffaloes had five RFLP patterns (*Rsa*I-g, *Rsa*I-h, *Rsa*I-l, *Rsa*I-s, and *Rsa*I-o) and 11 genotypes. The *Rsa*I-m and *Rsa*I-h patterns were only found in Jafarabadi buffaloes and could be used as genetic markers for this breed. Murrah buffaloes had four RFLP patterns (*Rsa*I-l, *Rsa*I-n, *Rsa*I-o, and *Rsa*I-s) and six different genotypes.

Aravindakshan et al. (2000) found that *Rsa*I-o and *Rsa*I-s patterns were the most frequent in Murrah buffaloes with a frequency of 0.25 for each patterns, while *Rsa*I-l was the most frequent in Surti buffaloes (0.24). Kumar et al. (2011) reported *Rsa*I-i (183/67/54 bp) and *Rsa*I-f digestion

patterns as the most common in Murrah with a frequency of 0.22. The most frequent pattern in Nili-Ravi buffaloes was *Rsa*I-f (0.26) (Kumar et al., 2008), while *Rsa*I-g and *Rsa*I-w (81/69/67/54/33 bp) digestion patterns were most frequent in Mehsani (0.28) and Jafarabadi (0.23) buffaloes, respectively (Acharya et al., 2002). The *RsaI-w* and *RsaI-i* digestion patterns were not found in our study.

Sumathi et al. (2010) identified five digestion patterns in Indian Murrah buffaloes (*Rsa*I-g, *Rsa*I-l, *Rsa*I-m, *Rsa*I-o, and *Rsa*I-s) with the *Rsa*I-s digestion pattern as the most frequent (0.44) and three digestion patterns in Indian Surti buffaloes (*Rsa*I-g, *Rsa*I-l, and *Rsa*I-s) with the *Rsa*I-l digestion pattern as the most frequent (0.5).

Table 2. Genotype frequencies identified after digestion of the second exon of *DRB3* with *Rsa*I, *Hae*III, or *Pst*I in Jafarabadi, Mediterranean, and Murrah buffaloes.

Genotype	Fragment size (bp)	Genotype frequency		
		Jafarabadi	Mediterranean	Murrah
*Rsa*I-b/c	114, 93, 67, 54, 39, 30	0	0.080	0
*Rsa*I-c/n	183, 121, 114, 93, 67, 30	0	0.040	0
*Rsa*I-f/s	144, 93, 67, 54, 39	0	0.080	0
*Rsa*I-h/l	237, 114, 69, 67, 54	0.040	0	0
*Rsa*I-h/m	121,114/69/67/54	0.040	0	0
*Rsa*I-h/o	304, 114, 69, 67, 54	0.080	0	0
*Rsa*I-l/l	237, 67	0.160	0	0
*Rsa*I-l/m	237, 121, 114, 69, 67	0.080	0	0
*Rsa*I-l/n	237, 183, 121, 67	0	0.240	0.267
*Rsa*I-l/o	304, 237, 67	0.120	0.120	0.100
*Rsa*I-l/s	237, 144, 93, 67	0.040	0	0.033
*Rsa*I-m/m	121, 114, 69	0.040	0	0
*Rsa*I-n/o	304, 183, 121	0	0.040	0.033
*Rsa*I-o/o	304	**0.400***	**0.360***	**0.467***
*Rsa*I-o/s	304, 144, 93, 67	0	0.040	0
*Rsa*I-s/s	144, 93, 67	0	0	0.100
*Hae*III-a/a	170, 82, 52	0	0.080	0.033
*Hae*III-a/b	222, 170, 82, 52	0	0.120	0.067
*Hae*III-a/e	170, 134, 82, 52	0	**0.200***	0.033
*Hae*III-b/b	222, 82	0.240	0.160	**0.467***
*Hae*III-b/d	222, 193, 82, 29	0	0.080	0
*Hae*III-b/e	222, 170, 134, 82	**0.400***	**0.200***	0.067
*Hae*III-d/d	193, 82, 29	0	0	0.067
*Hae*III-d/e	193, 170, 134, 82, 29	0	0.080	0.033
*Hae*III-e/e	170, 134	0.360	0.080	0.233
*Pst*I-z/z	230, 68, 6	0.080	0	0
*Pst*I-z/y	230, 216, 88, 68, 6	0.120	0	0
*Pst*I-z/o	304, 230, 68, 6	0.040	0.160	0.100
*Pst*I-o/o	304	**0.760***	**0.840***	**0.900***

*The most frequent genotypes in each breed are shown in bold.

*Hae*III polymorphisms

Four *Hae*III restriction sites were identified, namely *Hae*III-a (170/82/52 bp), *Hae*III-b (222/82 bp), *Hae*III-d (193/82/29 bp), and *Hae*III-e (170/134 bp). These patterns resulted in nine different genotypes: *Hae*III-a/a, *Hae*III-a/b, *Hae*III-a/e, *Hae*III-b/b, *Hae*III-b/d, *Hae*III-b/e, *Hae*III-d/d, *Hae*III-d/e, and *Hae*III-e/e.

Mediterranean and Murrah buffaloes had all four polymorphisms and eight genotypes. Genotypes *Hae*III-b/d and *Hae*III-d/d were not observed in Mediterranean and Murrah buffaloes, respectively. The *Hae*III-b pattern was the most frequent in Mediterranean and Murrah buffaloes with frequencies of 0.36 and 0.53, respectively. Jafarabadi buffaloes showed only *Hae*III-b and *Hae*III-e patterns and three different genotypes (*Hae*III-b/b, *Hae*III-b/e, and *Hae*III-e/e). The *Hae*III-e polymorphism was the most frequent in Jafarabadi buffaloes with a frequency of 0.56.

Aravindakshan et al. (2000) and Kumar et al. (2011) found the same four patterns in Murrah buffaloes, but additionally identified a pattern not found here; this pattern was characterized by the absence of an *Hae*III restriction site (304 bp). Kumar et al. (2011) reported that *Hae*III-a was most common in Murrah buffaloes (0.46), while Aravindakshan et al. (2000) found the *Hae*III-b pattern to be most frequent in this breed (0.38). In Surti buffaloes, the *Hae*III-a pattern was the most frequent (0.43) (Aravindakshan et al., 2000). Kumar et al. (2008) found that the *Hae*III-b pattern was the most frequent in Nili-Ravi buffaloes (0.60) while Acharya et al. (2002) reported *Hae*III-a as the most frequent in Jafarabadi (0.42) and *Hae*III-b as the most frequent in Mehsani buffaloes (0.64).

Sumathi et al. (2010) identified three alleles in Indian Murrah buffaloes (*Hae*III-a, *Hae*III-b, and *Hae*III-e) and four in Indian Surthi buffaloes, with the pattern *Hae*III-b most frequent in both buffalo breeds (~0.63).

*Pst*I polymorphisms

Digestion of the PCR amplicon with *Pst*I yielded three restriction sites named *Pst*I-z (230/68/6 bp), *Pst*I-y (216/88 bp), and *Pst*I-o (304 bp). These patterns resulted in four different genotypes: *Pst*I-z/z, *Pst*I-z/y, *Pst*I-z/o, and *Pst*I-o/o. Thus, the level of *Pst*I polymorphism was lower than found for *Rsa*I and *Hae*III restriction enzymes. A similar outcome was reported by Kumar et al. (2011) in a study in Murrah buffaloes. The cause of this relatively low level of polymorphism is that *Pst*I recognizes and cuts a 6-bp site while *Rsa*I and *Hae*III recognize 4-bp sites.

The *Pst*I-o restriction pattern was the most common with frequencies of 0.78, 0.92, and 0.95 in Jafarabadi, Mediterranean, and Murrah buffaloes, respectively. Mediterranean and Murrah buffaloes had only two patterns (*Pst*I-z and *Pst*I-o) and two genotypes (*Pst*I-z/o and *Pst*I-o/o), whereas Jafarabadi buffaloes had all three restriction patterns and all four different genotypes. The high frequency of the *Pst*I-o pattern might be due to a high degree of inbreeding.

Gole et al. (2009) identified three digestion patterns, *Pst*I-s (170/68/66 bp), *Pst*I-y and *Pst*I-z, and six genotypes in Indian Murrah buffaloes. The *Pst*I-z restriction pattern was the most common (0.58). We did not identify the *Pst*I-s pattern in the present study.

We present the first analysis of DNA polymorphisms in the *DRB3* gene of Brazilian buffaloes. Our results on the allelic and genotype variability of the buffalo population in Brazil will be of value to conservation programs, for example, by enabling the evaluation of genetic distances and the choice of the best animals for mating to avoid an increase in inbreeding. The high number of alleles and genotypes found in this study indicates that *DRB3* is highly polymorphic in buffaloes in Brazil. Additionally, the allele frequencies showed significant differences among breeds enabling some digestion patterns to be used as genetic markers for a breed.

All 16 digestion patterns found in this study correspond to patterns previously described in cattle (van Eijk et al., 1992; Gelhaus et al., 1995). The high degree of similarity between buffalo and cattle provides evidence for genetic conservation among members of the Bovidae family.

This preliminary study on polymorphism of *DRB3* in Jafarabadi, Mediterranean, and Murrah buffaloes provides a basis for further research into the association between *DRB3* alleles and economically important traits such as disease resistance.

Conflicts of interest

The authors declare no conflict of interest.

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