Gene 519 (2013) 150–158

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Characterization of bovine MHC *DRB3* diversity in Latin American Creole cattle breeds

Guillermo Giovambattista [a,*], Shin-nosuke Takeshima [b], Maria Veronica Ripoli [a], Yuki Matsumoto [b], Luz Angela Alvarez Franco [c], Hideki Saito [d], Misao Onuma [b], Yoko Aida [b,**]

[a] *IGEVET, CCT LA PLATA CONICET, FCV, UNLP. La Plata B1900AVW, CC 296, Argentina*
[b] *Viral Infectious Diseases Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*
[c] *Departamento de Ciencia Animal, Facultad de Ciencias Agropecuarias, Universidad Nacional de Colombia, Sede Palmira, Colombia*
[d] *Japan International Cooperation Agency (JICA) Uganda Animal Disease Control Project at Makerere University, College of Veterinary Medicine, Wandegeya, Kampala, Uganda*

ARTICLE INFO

Article history:
Accepted 4 January 2013
Available online 16 January 2013

Keywords:
BoLA-DRB3 polymorphism
Major histocompatibility complex class II
Latin American Creole cattle
Sequence-based typing
Yacumeño
Hartón del Valle

ABSTRACT

In cattle, bovine leukocyte antigens (BoLAs) have been extensively used as markers for diseases and immunological traits. However, none of the highly adapted Latin American Creole breeds have been characterized for *BoLA* gene polymorphism by high resolution typing methods. In this work, we sequenced exon 2 of the *BoLA class II DRB3* gene from 179 cattle (113 Bolivian Yacumeño cattle and 66 Colombian Hartón del Valle cattle breeds) using a polymerase chain reaction sequence-based typing (PCR-SBT) method. We identified 36 previously reported alleles and three novel alleles. Thirty-five (32 reported and three new) and 24 alleles (22 reported and two new) were detected in Yacumeño and Hartón del Valle breeds, respectively. Interestingly, Latin American Creole cattle showed a high degree of gene diversity despite their small population sizes, and 10 alleles including three new alleles were found only in these two Creole breeds. We next compared the degree of genetic variability at the population and sequence levels and the genetic distance in the two breeds with those previously reported in five other breeds: Holstein, Japanese Shorthorn, Japanese Black, Jersey, and Hanwoo. Both Creole breeds presented gene diversity higher than 0.90, a nucleotide diversity higher than 0.07, and mean number of pairwise differences higher than 19, indicating that Creole cattle had similar genetic diversity at *BoLA-DRB3* to the other breeds. A neutrality test showed that the high degree of genetic variability may be maintained by balancing selection. The F_{ST} index and the exact G test showed significant differences across all cattle populations ($F_{ST} = 0.0478$; $p < 0.001$). Results from the principal components analysis and the phylogenetic tree showed that Yacumeño and Hartón del Valle breeds were closely related to each other. Collectively, our results suggest that the high level of genetic diversity could be explained by the multiple origins of the Creole germplasm (European, African and Indicus), and this diversity might be maintained by balancing selection.

1. Introduction

The major histocompatibility complex (*MHC*) is an important component of the immune system. MHC molecules are divided into two groups, class I and class II, and present foreign antigens to T cells, initiating immune responses and the clearance of the foreign materials (Klein et al., 1993). *MHC* genes are extremely polymorphic and their polymorphism occurs predominantly at residues within the peptide-binding groove (PBS) (Brown et al., 1993). There is compelling evidence that the polymorphism is maintained by some form of overdominance or balanced selection (Hughes and Nei, 1989).

The MHC system in cattle is known as the bovine leukocyte antigen (BoLA). A major rearrangement within the class II region has led to the division of the *BoLA* region into two distinct sub-regions, class IIa and class IIb, on chromosome 23. The class IIa sub-region contains the functionally expressed *DR* and *DQ* genes whose gene products, the DR and DQ molecules, represent the main class II restriction elements for CD4-positive T-helper cells (Aida, 1995; Glass et al., 2000). Cattle have one *DRA* gene, three *DRB* genes (only one of which, *DRB3*, is thought to be functionally important), and several *DQA* and *DQB* genes depending on the haplotype (Takeshima and Aida, 2006).

The *BoLA-DRB3* gene is the most polymorphic class II locus in cattle and influences both the magnitude and epitope specificity of antigen-specific T cell responses to infectious diseases. Indeed, 130 *BoLA-DRB3*

Abbreviations: MHC, major histocompatibility complex; BoLA, bovine leukocyte antigen; PCR, polymerase chain reaction; SBT, sequence-based typing; RFLP, fragment length polymorphism; IPD, the Immuno Polymorphism Database; UPGMA, unweighted pair-group method with arithmetic mean; HWE, Hardy–Weinberg equilibrium; PCA, principal components analysis; PCs, principal components; n_a, number of alleles; h_o, observed heterozygosity; h_e, expected heterozygosity; dn, non-synonymous; ds, synonymous; NJ, neighbor-joining.

* Corresponding author. Tel./fax: +54 221 4211799.
** Corresponding author. Tel.: +81 48 462 4408; fax: +81 48 462 4399.
E-mail addresses: ggiovam@fcv.unlp.edu.ar (G. Giovambattista), aida@riken.jp (Y. Aida).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.01.002

alleles have been published in various breeds of cattle by sequencing of cloned genomic DNA, cDNA or cloned polymerase chain reaction (PCR) products (Aida et al., 1995; Ammer et al., 1992; Lee et al., 2012; Maillard et al., 1999, 2001; Mikko and Andersson, 1995; Miyasaka et al., 2011; Russell et al., 1997, 2000; Sigurdardottir et al., 1991; Takeshima et al., 2001, 2002), which are listed in the Immuno Polymorphism Database (IPD)–MHC database (http://www.ebi.ac.uk/ipd/mhc/bola/index.html). *DRB3* polymorphisms have been associated with differences in susceptibility to infectious disease (e.g., bovine leukosis virus lymphocytosis, mastitis, and dermatophilosis), immunological traits, and vaccine responses (foot-and-mouth disease and *Theileria parva*) (Ballingall et al., 2004; Baltian et al., 2012; Baxter et al., 2009; Dietz et al., 1997a, 1997b; Maillard et al., 2002; Sharif et al., 1998; Starkenburg et al., 1997; Sulimova et al., 1995; Takeshima et al., 2008).

The MHC variability in wild and domestic populations is of great interest to evolutionary biologists because of the high levels of polymorphism. In cattle, as well as in other mammals, the allele frequencies of *BoLA class II* genes vary between different breeds. *BoLA-DRB3* polymorphisms have been studied at the population level in less than 30 cattle breeds, including Jersey, Holstein, Black Pied, Ayrshire, Argentinean and Brazilian Creoles, Japanese Shorthorn, Japanese Black, Hanwoo, Nelore, Brazilian dairy Gir, Ongole, Martinique Brahman, and native breeds from Asia (Behl et al., 2007; da Mota et al., 2002, 2004; Dietz et al., 1997a, 1997b; Gilliespie et al., 1999; Giovambattista et al., 1996, 2001; Hernández-Herrera et al., 2009; Lee et al., 2012; Lei et al., 2012; Maillard et al., 1999; Miretti et al., 2001; Miyasaka et al., 2011, 2012; Mohammadi et al., 2009; Nassiry et al., 2005; Ripoli et al., 2004; Rupp et al., 2007; Ruzina et al., 2010; Sharif et al., 1998; Starkenburg et al., 1997; Takeshima et al., 2003, 2008; Udina et al., 1998). Most of these studies used the PCR-fragment length polymorphism (RFLP) method (van Eijk et al., 1992) rather than the PCR-sequence-based typing (SBT) (Lee et al., 2012; Miyasaka et al., 2011, 2012; Takeshima et al., 2001, 2002, 2009, 2011). This technique with RFLP provides a simple method for the rapid analysis of class II polymorphisms, but its utility is limited in the case of alleles that are split into several further alleles at the nucleotide level. By contrast, DNA sequencing allows unequivocal typing of *BoLA-DRB3* alleles, allowing the identification of amino acid motifs that are responsible for the different peptide specificities exhibited by histocompatibility molecules (Brown et al., 1993). Nevertheless, during the last ten years, *BoLA-DRB3* polymorphisms have been determined by PCR-SBT in a small number of breeds, including Japanese Shorthorn, Holstein, Japanese Black, Jersey and Gir, showing significant differences within breeds (da Mota et al., 2002, 2004; Miyasaka et al., 2011; Takeshima et al., 2003, 2008).

The first cattle were brought to America by Iberian conquerors in the 16th century (Primo, 1992). The founding population of Creole cattle, introduced into America by the Spanish and Portuguese during the first 50 years of colonization, consisted of between 300 and 1000 animals of Iberian origin (Primo, 1992; Wilkins, 1984). Over several years, these animals were taken to Central and South America, and to the southern United States. They spread over the South American continent. The Creole cattle were the only bovines bred in Latin America for more than 300 years until selected European and Zebu breeds were introduced. American Creole cattle evolved under low levels of breeding management and, as a result of natural selection, became adapted to different environments, such as tropical rainforest, subtropical dry forest, highland steppe, and Patagonian steppe, and exhibit a high degree of resistance to tropical disease and a high level of fertility. Today, almost all North, Central and South American countries have Creole cattle breeds (http://www.ansi.okstate.edu/breeds/cattle/). The Bolivian Yacumeño Creole breed has adapted to the tropical seasonal flood plain of northern Bolivia (Beni Department). The breed population is approximately 1200 animals, raised primarily for beef. The Colombian Hartón del Valle cattle are a dual purpose (beef and dairy) breed adapted to the dry tropical forest of the Cauca Valley Department (Colombia). Currently, the population size of this breed is about 5000 head.

Latin American Creole cattle have been extensively characterized using molecular markers (STRs, mitochondrial D-Loop, Y-chromosome, and SNPs) (Groeneveld et al., 2010). However, none of the Latin American Creole breed has been characterized by PCR-SBT. Therefore, in the present work, we determined the *BoLA-DRB3* gene frequencies and distribution within two Latin American Creole cattle breeds (Yacumeño and Hartón del Valle) highly adapted to a dry tropical climate using PCR-SBT. Furthermore, we compared the degree of genetic variability and the genetic distance with SBT data previously reported for five other cattle breeds (Holstein, Japanese Shorthorn, Japanese Black, Jersey and Hanwoo) (Miyasaka et al., 2011; Takeshima et al., 2002, 2003). This is the first study to genetically characterize the *BoLA-DRB3* gene in Latin American Creole cattle breeds by SBT.

2. Material and methods

2.1. Animals and extraction of genomic DNA

Blood samples from 179 cattle, comprising 113 Bolivian Yacumeño cattle and 66 Colombian Hartón del Valle cattle, were obtained in the Santa Cruz Department (Bolivia) and Valle del Cauca (Colombia), respectively (Fig. S1). Genomic DNA was extracted from (a) blood samples using Wizard® Genomic DNA Purification kits (Promega, Madison, WI, USA) or (b) 40 μl whole blood spotted on FTA elute cards (Whatman, Tokyo, Japan), following the manufacturer's instructions.

2.2. BoLA-DRB3 typing using PCR-SBT

BoLA-DRB3 alleles were genotyped using PCR-SBT. Briefly, DRB3 exon 2 was amplified using two different methods (single PCR (Takeshima et al., 2011) and allele group-specific PCR (Takeshima et al., 2001)) to avoid mistyping. Single PCR was performed using primers DRB3FRW and DRB3REV (Baxter et al., 2009). Allele group-specific PCR was performed using primers ERB3N and HL031 for the first round of amplification, and group-specific primers (DRB3sp1 to DRB3sp8) and DRB3B for the second round (Takeshima et al., 2001). The PCR fragments were purified using an ExoSAP-IT PCR product purification kit (USB Corp., Cleveland, OH) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Alternatively, amplification products were purified with polyethylene glycol 8000 and sequenced in an automatic DNA sequencer MegaBACE 1000 (GE Healthcare) using a DYEnamic ET Terminator Kit (GE Healthcare). The raw sequence data were analyzed using Assign 400ATF ver. 1.0.2.41 software (Conexio Genomics, Fremantle, Australia).

2.3. Statistical analysis

2.3.1. Measures of genetic variability

Allele frequencies and number of alleles (n_a) were obtained by direct counting. 95% confidence intervals for allele frequencies were computed using the binomial Distribution implemented into R with the binom.confint function (http://cran.r-project.org/web/packages/binom/). The observed (h_o) and unbiased expected heterozygosity (h_e) for the *BoLA-DRB3* locus were estimated according to Nei (1978) using the ARLEQUIN 3.5 software for population genetic analyses (Schneider et al., 2000). Potential deviations from Hardy–Weinberg equilibrium (HWE) were estimated by F_{IS} statistics (Weir and Cockerham, 1984) for each breed using the exact test included in GENEPOP 4.0 (Rousset, 2008). The Ewens–Watterson–Slatkin exact neutrality test was estimated using the method described by Slatkin (1996), and implemented in the Arlequin 3.5 program.

2.3.2. Genetic structure and population differentiation

Genetic structure and genetic differentiation among breeds were assessed through standard Wright's F_{ST} statistics using the variance-

based method of Weir and Cockerham (1984) and the exact G test (Goudet et al., 1996) for population differentiation. These parameters were estimated using ARLEQUIN 3.5 and GENEPOP 4.0. Levels of genetic differentiation between populations were described through population pairwise F_{ST} indices and represented graphically using an R-function (pairFstMatrix.r).

2.3.3. Genetic distances, population tree and principal components analysis

To condense the genetic variation revealed by the *BoLA-DRB3* polymorphisms, allele frequencies were used to perform a principal components analysis (PCA) according to Cavalli-Sforza et al. (1994), implemented using PAST software (Hammer et al., 2001). Nei's Standard genetic distances (Ds) (Nei, 1972) and D_A distances (Nei et al., 1983) were calculated from allele frequencies to perform a cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA (Sneath and Sokal, 1973) and the neighbor-joining (NJ) (Saitou and Nei, 1987)) algorithms. Confidence for the groupings was estimated through bootstrap re-sampling of the data using 1000 replications. Genetic distances and trees were computed using POPULATIONS 1.2.28 software (Langella, 1999). The trees were then visualized using TREEVIEW (Page, 1996).

2.3.4. Genetic diversity at sequence level

Nucleotide diversity (π) was calculated using Arlequin 3.5 (Schneider et al., 2000). Pairwise comparisons of nucleotide substitutions between alleles were conducted according to the average number of differences between pairs of DNA sequences. The mean number of non-synonymous (dn) and synonymous (ds) nucleotide substitutions per site was estimated for each pair as described by Nei and Gojobori (1986) using the Jukes–Cantor's formula. These parameters were estimated using ARLEQUIN 3.5 and MEGA 5 (Kumar et al., 2001).

Pairwise genetic distances between DNA sequences were estimated on the basis of Kimura's two-parameter model. The gene tree was constructed from a distance matrix that was based on the NJ method of Saitou and Nei (1987). To test the significance of the branches, 1000 bootstrap replicate calculations were performed.

3. Results

3.1. Distribution of BoLA-DRB3 alleles within the Yacumeño and Hartón del Valle cattle breeds

To determine the distribution of allele frequencies for *BoLA-DRB3* in Yacumeño and Hartón del Valle cattle breeds, we determined the genotype of the *BoLA-DRB3* allele exon 2 in 179 individuals using PCR-SBT. Thirty-nine *BoLA-DRB3* alleles were identified, of which 36 were previously reported alleles and three were new variants (Table 1). Thirty-five alleles (32 previously reported and three new) were detected in Yacumeño cattle and 24 (22 reported and two new) were detected in Hartón del Valle.

Nucleotide and deduced amino acid sequences of the three new alleles are shown in Fig. 1. These new alleles were assigned allele names by the BoLA nomenclature committee (http://www.projects.roslin.ac.uk/bola/bolahome.html). One new allele was designated *BoLA-DRB3*0704* and differed from DRB3*0701 at position 85. The second new allele was designated *BoLA-DRB3*1104* and differed from *BoLA-DRB3*1103* at position 57. The third new allele, *BoLA-DRB3*2902*, differed from *BoLA-DRB3*1801* at five positions (positions 55, 56, 57, 85, and 86). The new *DRB3* alleles were about 91% identical at the nucleotide level and 84% identical at the amino acid level to the *BoLA-DRB3* cDNA clone NR1 (Aida et al., 1995).

The alleles with frequencies >5% were *BoLA-DRB3*0201*, **0701*, **0902*, **14011* and **1801* in Yacumeño; and *BoLA-DRB3*1002*, **1101*, **1501*, **1601*, **2006*, **2201*, **2703*, **2801*, **3001* and **3601* in Hartón del Valle (Table 1). These common alleles account for 41.15% and 73.48% of the cumulative gene frequencies in Yacumeño and Hartón del Valle cattle, respectively (Fig. S2). In Yacumeño, an additional 19 alleles were detected with frequencies between 1% and 5% (*BoLA-DRB3*0101*, **0501*, **0601*, **0901*, **1001*, **1101*, **1201*, **1301*, **1501*, **1601*, **1701*, **2201*, **2710*, **2703*, **2801*, **3301*, **4802*, **1104* and **0704*), while the remaining 11 variants exhibited gene frequencies lower than 1% (*BoLA-DBR3*20012*, **2101*, **2502*, **2802*, **3001*, **3202*, **3501*, **3601*, **3701*, **3901* and **2902*). In Hartón del Valle, eight alleles were detected with frequencies between 1% and 5% (*BoLA-DRB3*1001*, **1801*, **20012*, **2201*, **25011*, **2802*, **4401* and **2902*), while the remaining seven variants exhibited gene frequencies lower than 1% (*BoLA-DBR3*0101*, *0501, **14011*, *3501, **3901*, **4802* and **1104*). Twenty out of 39 alleles were shared between both Creole breeds, including two of the three new alleles (**2902* and **1104*; Table 1). However, the most common alleles detected in each breed were absent or present at frequencies lower than 5% in the other.

Table 1
Gene frequencies (in percentage) of BoLA-DRB3 alleles detected by PCR-SBT in Yacumeño and Hartón del Valle Creole cattle breeds. In bracket, confidence intervals for allele frequencies were detailed.

DRB3 Allele	Yacumeño[a] (N = 113) (allele no. 226)	Hartó del Valle[a] (N = 66) (allele no.132)
DRB3*0101	2.21 (0.72–5.09)	0.76 (0.02–4.15)
DRB3*0201	7.08 (4.10–11.24)	0 (0.00–2.76)
DRB3*0501	3.98 (1.84–7.42)	0.76 (0.02–4.15)
DRB3*0601	3.10 (1.25 –6.28)	0 (0.00–2.76)
DRB3*0701	**10.62[b] (6.92–15.39)**	0 (0.00–2.76)
DRB3*0704	1.33 (0.27–3.83)	0 (0.00–2.76)
DRB3*0901	4.42 (2.14–7.99)	0 (0.00–2.76)
DRB3*0902	8.41 (5.14–12.82)	0 (0.00–2.76)
DRB3*1001	3.10 (1.25–6.28)	3.79 (1.24–8.62)
DRB3*1002	0 (0.00–1.62)	6.82 (3.16–12.55)
DRB3*1101	3.54 (1.54–6.85)	**12.12[b] (7.09–18.94)**
DRB3*1104	4.87 (2.45–8.54)	0.76 (0.02–4.15)
DRB3*1201	1.77 (0.48–4.50)	0 (0.00–2.76)
DRB3*1301	1.33 (0.27–3.83)	0 (0.00–2.76)
DRB3*14011	6.64 (3.76–10.71)	0.76 (0.02–4.15)
DRB3*1501	4.42 (2.14–7.99)	7.58 (3.69–13.49)
DRB3*1601	4.42 (2.14–7.99)	6.06 (2.65–11.59)
DRB3*1701	1.77 (0.48–4.47)	0 (0.00–2.76)
DRB3*1801	8.41 (5.14–0.13)	3.79 (1.24–8.62)
DRB3*20012	0.44 (0.01–2.44)	1.52 (0.18–5.3)
DRB3*2006	0 (0.00–1.62)	9.85 (5.35–16.25)
DRB3*2101	0.44 (0.01–2.44)	0 (0.00–2.76)
DRB3*2201	1.77 (0.48–4.47)	5.3 (2.16–10.62)
DRB3*25011	0 (0.00–1.62)	2.27 (0.47–6.50)
DRB3*2502	0.88 (0.12–3.16)	0 (0.00–2.76)
DRB3*2703	2.21 (0.72–5.09)	9.85 (5.35–16.25)
DRB3*2710	1.77 (0.48–4.47)	0 (0.00–2.76)
DRB3*2801	1.33 (0.27–3.83)	7.58 (3.69–13.49)
DRB3*2802	0.88 (0.12–3.16)	1.52 (0.18–5.3)
DRB3*2902	0.88 (0.12–3.16)	1.52 (0.18–5.3)
DRB3*3001	0.88 (0.12–3.16)	6.06 (2.65–11.59)
DRB3*3202	0.44 (0.01–2.44)	0 (0.00–2.76)
DRB3*3301	2.21 (0.72–5.09)	0 (0.00–2.76)
DRB3*3501	0.88 (0.12–3.16)	0.76 (0.02–4.15)
DRB3*3601	0.88 (0.12–3.16)	7.58 (3.69–13.49)
DRB3*3701	0.88 (0.12–3.16)	0 (0.00–2.76)
DRB3*3901	0.44 (0.01–2.44)	0.76 (0.02–4.15)
DRB3*4401	0 (0.00–1.62)	1.52 (0.18–5.3)
DRB3*4802	1.33 (0.27–3.83)	0.76 (0.02–4.15)

[a] N, number of typed unrelated individuals.
[b] The most frequent alleles in each breed are given in boldface. New alleles are underlined.

3.2. Identification of Creole cattle-specific alleles

With the aim of identifying Creole cattle-specific alleles, we generated an NJ tree using the 235-base pair (bp) nucleotide sequences of

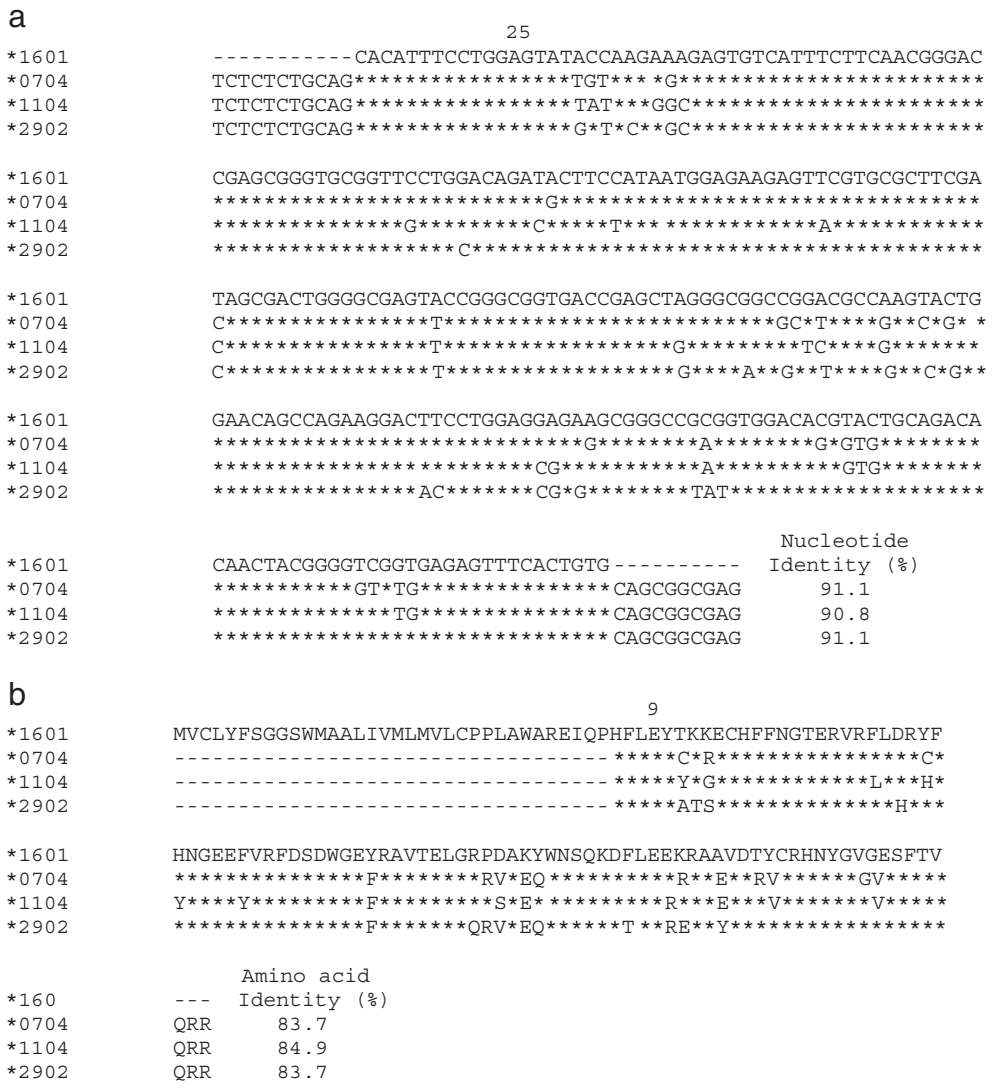

Fig. 1. Alignment of the nucleotide (a) and predicted amino acid (b) sequences of the β1 domains encoded by three new *BoLA-DRB3* alleles (accession numbers are AB554653 for *BoLA-DRB3*1104*, AB554654 for *BoLA-DRB3*2902*, and AB554655 for *BoLA-DRB3*0704*) derived from 179 cattle, comprising 113 Bolivian Yacumeño Creole cattle and 66 Colombian Hartón del Valle Creole cattle. The numbering refers to amino acid positions in the mature protein. Amino acid residues identical to those encoded by the *BoLA-DRB3* cDNA clone NR-1 are indicated by dots (Aida et al., 1995). Homology scores also refer to this cDNA clone. The nucleotide sequences reported in this paper have been submitted to the International Nucleotide Sequence Database and have been assigned accession numbers AB554653 through AB554655.

the h1 domains of all reported alleles and the three new variants (Fig. 2). In addition, alleles detected in seven breeds (Yacumeño, Hartón del Valle Creole, Holstein, Japanese Black, Japanese Shorthorn, Jersey and Hanwoo) were underlined in the tree. According to this tree, 43 *BoLA-DRB3* sequences were shared by two to seven breeds, and alleles detected within each of the studied breeds were interspersed along the clusters. Only 10 variants, including the three new alleles, were private in the studied Creole cattle populations (*BoLA-DRB3*2101*, **2802*, **3301*, **3501*, **3601*, *3901, *4802, **2902*, **1104* and **0704*), and this result may provide evidence of the influence of African and Zebuine genes in the Creole cattle germplasm.

3.3. Nucleotide and amino acid diversity of BoLA-DRB3 alleles

We also examined the genetic diversity at the DNA and amino acid level by comparing the average amino acid and nucleotide substitutions for all pairs of alleles in Yacumeño and Hartón del Valle Creole cattle. The results obtained were compared with those previously reported in five other breeds (Table 2). The nucleotide diversity (π) within Yacumeño and Hartón del Valle breeds was 0.079 and 0.076, respectively, while the mean number of pairwise differences for Yacumeño and Hartón del Valle was 19.78 and 19.00, respectively. These values were similar to those estimated for other cattle breeds using SBT methods, with Shorthorn cattle being an exception. We also calculated the average numbers of dn and ds in both Creole breeds. The dn/ds differences were more prominent when only the antigen-binding site was analyzed. As shown in Table 2, values obtained for Yacumeño and Hartón del Valle were similar to those estimated for other cattle breeds.

3.4. Population study: gene diversity, Hardy–Weinberg equilibrium and neutrality testing of BoLA-DRB3 in the Yacumeño and Hartón del Valle Breeds

To estimate the degree of gene diversity and evaluate the presence of evolutive forces (selection, inbreeding, population structure, etc.), we calculated gene diversity (h_e and h_o) and HWE (F_{IS} index) and performed a neutrality test for *BoLA-DRB3*. As expected, the high number of alleles in Yacumeño and Hartón del Valle Creole cattle breeds resulted in an extremely high diversity value (Table 3). In both breeds, h_e and h_o were higher than 0.90, were similar to the values previously reported for other bovine breeds studied by

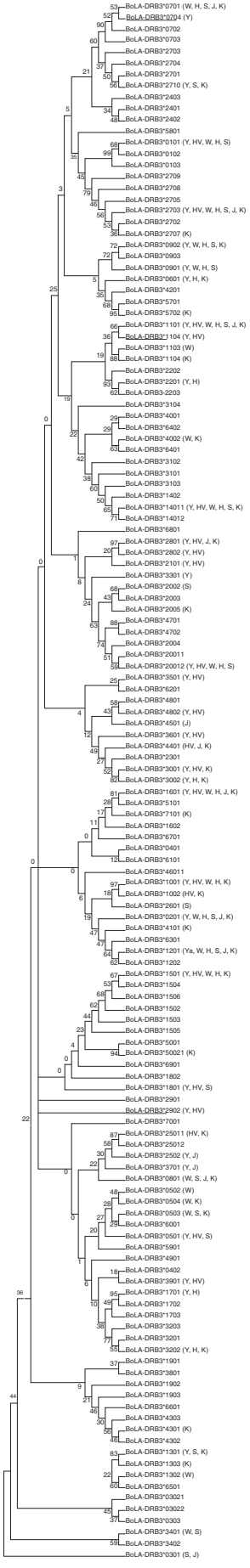

Table 2
Values of nucleotide diversity (Π), mean number of pairwise differences (NPD), the mean numbers of non-synonymous (dn) and synonymous (ds) nucleotide substitutions per site. Ds and dn were estimated for the entire sequence and for the antigen biding sites (ABS).

Breed	Π	NPD	Total		ABS	
			ds	Dn	ds	dn
Yacumeño[a]	0.079	19.78	0.039	0.105	0.151	0.484
Hartón del Valle[a]	0.076	19.00	0.034	0.109	0.140	0.484
Japanese Shorthorn[b]	0.146	36.56	0.047	0.106	0.188	0.504
Holstein[b]	0.082	20.50	0.092	0.151	0.198	0.520
Japanese Black[b]	0.071	18.59	0.093	0.149	0.174	0.519
Jersey[b]	0.086	19.20	0.109	0.164	0.175	0.547
Hanwoo[c]	0.072	18.06	0.047	0.111	0.153	0.505

[a] Present work,
[b] Takeshima et al. (2003) and Miyasaka et al. (2011).
[c] Lee et al. (2012).

PCR-SBT (Lee et al., 2012; Miyasaka et al., 2011, 2012; Takeshima et al., 2002, 2003) (Table 3), and were characteristic of *MHC class II DR* genes. An HWE test showed that Yacumeño did not deviate significantly from the theoretical proportions, even when heterozygote deficit or excess was tested ($F_{IS\ deficit} = 0.034$, $p = 0.159$; and $F_{IS\ excess} = 0.034$, $p = 0.937$). By contrast, Hartón del Valle exhibited significant deviation from HWE (Table 3). As shown in Table 2, four out of five previously studied breeds were also in HWE, with Jersey being an exception.

It is widely accepted that *MHC class II* gene diversity can be maintained by balancing selection. Therefore, to test this hypothesis, Slatkin's exact neutrality test was performed for *BoLA-DRB3* gene frequency distributions. The result showed an even *BoLA-DRB3* gene frequency distribution in Yacumeño breed, in spite of the HWE, under balancing selection rather than the allelic profiles expected under positive selection against one or few alleles or under neutral selection (Table 3). P values lower than 0.025 are expected if the sampled population is under relatively recent balancing selection rather than positive or neutral selection. A similar even gene frequency distribution was observed in Japanese Black cattle. However, in Hartón del Valle cattle, only a non-significant trend toward gene frequency distribution under balancing selection was observed, similar to the results obtained for Japanese Shorthorn, Holstein, and Hanwoo cattle.

3.5. Genetic structure and levels of population differentiation of BoLA-DRB3 in the Yacumeño and Hartón del Valle Breeds

The F_{ST} index and the exact G test for population differentiation were used to analyze the degree of genetic differentiation among the cattle breeds studied. The F_{ST} parameter showed significant differences across all cattle populations ($F_{ST} = 0.0478$; $p < 0.001$), ranging from 0.024 between Holstein and Japanese Black to 0.091 between Holstein and Jersey (Fig. 3). The exact G test for population differentiation indicated that, on average, differences in allele frequency distributions among populations were highly significant (exact p value ≤ 0.0001). When all pairs of breeds were tested, significant results ($p < 0.001$)

Fig. 2. Neighbor-joining (NJ) tree constructed from the 235 bp nucleotide sequences of the β1 domain encoded by all reported *BoLA-DRB3* alleles. Numbers are bootstrap percentages that support each node. Bootstrapping was performed with 1000 replicates to assess the reliability of individual branches. Alleles that were only found in Creole cattle in this study are shown in boldface. New alleles are underlined. Letters in parentheses indicate the breeds in which each allele was found: Yacumeño (Y), Hartón del Valle (HV), Holstein (H), Japanese Shorthorn (S), Japanese Black (W), Jersey (J), and Hanwoo (K).

Table 3
Number of alleles (n_a), observed (h_o) and expected heterozygosity (h_e), and Hardy–Weinberg equilibrium (HWE), measure through F_{IS}, and Slatkin's exact neutrality test calculated for the cattle breeds studied. N = sample size, ND = not determined.

Breed	N	n_a	h_o	h_e	HWE	Slatkin's exact p value
					F_{IS}–p value	
Yacumeño[a]	113	36	0.92	0.95	0.034–0.78	**0.006**
Hartón del Valle[a]	66	24	0.97	0.94	**−0.036–0.0004**	0.136
Japanese Shorthorn[b]	100	20	0.92	0.91	−0.009–0.095	0.061
Holstein[b]	102	18	0.92	0.90	−0.021–0.358	0.083
Japanese Black[b]	200	23	0.90	0.91	0.009–0.362	**0.003**
Jersey[b]	69	14	0.91	0.89	**−0.030–0.0005**	0.055
Hanwoo[c]	359	39	ND	0.90	ND	0.186

Significant differences with p<0.01 are indicated by boldface.
[a] Present work.
[b] Takeshima et al. (2003) and Miyasaka et al. (2012).
[c] Lee et al. (2012).

were obtained in all comparisons, indicating a significant level of genetic structure for *BoLA-DRB3* among breeds.

3.6. Population relationship based on frequencies of BoLA-DRB3 alleles

To assess the genetic relationship between Creole cattle and five breeds previously studied by SBT, two type of analysis were performed: dendrograms and PCA. First, *BoLA-DRB3* allele frequencies were used to generate the D_A and D_S genetic distances for each pair, including Yacumeño and Hartón del Valle and five previously reported breeds (Jersey, Japanese Shorthorn, Holstein, Japanese Black, and Hanwoo; (Lee et al., 2012; Miyasaka et al., 2011, 2012; Takeshima et al., 2002, 2003)). Dendrograms were constructed from the distance matrix using UPGMA and NJ algorithms. The NJ cluster analysis using D_A genetic distance revealed congruent topologies, which were consistent with the historical and geographical origin of the breeds. This tree revealed three clusters: Yacumeño/Hartón del Valle/Hanwoo, Japanese Black/Holstein, and Japanese Shorthorn/Jersey (Fig. 4). The smallest genetic distances for *BoLA-DRB3* alleles were found between Holstein and Japanese Black ($D_A = 0.190$; Table 4). This cluster was observed in the four performed trees and was in agreement with data reported by Takeshima et al. (2002). Jersey cattle was the most divergent breed ($D_A = 0.645$) and was clustered with Japanese Shorthorn or located on a different branch from the other breeds. The remaining breeds (Yacumeño, Hartón del Valle, Japanese Shorthorn, and Hanwoo) changed their relative position depending upon the genetic distance and the method used to build the tree.

Second, to condense the genetic variation revealed for *BoLA-DRB3* and assess the relationship between breeds, PCA was performed from allele frequencies. Results from the PCA are reported in Fig. 3, which illustrates the first and the second principal components (PCs) for the *BoLA-DRB3* gene frequency. The first two components accounted cumulatively for 56.63% of the variability in the data. The first PC accounted for 31.35% of the total variance and showed a differentiation pattern between Jersey and the other breeds. Japanese Black and Holstein were located at opposite ends of this PC, while the Creole cattle, Japanese Shorthorn, and Hanwoo were located in the middle. The first PC was determined mainly by 16 alleles, eight with a positive value (*BoLA-DRB3*0101*, **0902*, **1001*, **1101*, **1201*, **1501*, **1601* and **2703*) and eight with a negative value (*BoLA-DRB3*0201*, **0301*, **0801*, **2006*, **2502*, **3701*, **4401* and **4501*). The second PC explained 27.28% of the total variation and clearly distinguished Shorthorn and Hanwoo breeds from the remaining breeds. The second PC was mainly determined by differences in the frequencies of 11 alleles, seven with a positive value (*BoLA-DRB3*0101*, **0201*, **0301*, **0501*, **0801*, **1101* and **1201*) and four with a negative value (*BoLA-DRB3*0701*, **1002*, **1601* and **4301*). Although the third PC accounted for 16.54% of the variance, it did not provide any information regarding the potential origin of the breeds. Results from the PCA were consistent with the overall results of the cluster analyses generated using the UPGMA and NJ algorithms.

4. Discussion

This is the first report of the allelic distribution of the *BoLA-DRB3* gene, using PCR-SBT, in two Latin American Creole cattle: Yacumeño and Hartón del Valle. The present analysis allowed us to detect 39 *BoLA-DRB3* alleles, including 36 previously published and three new variants. Thirty-five (32 previously reported and three new) and 24 alleles (22 reported and two new) were detected in Yacumeño and Hartón del Valle breeds, respectively. Ten alleles, including the three new ones, were detected only in the Latin American native population and not in the other breeds (Holstein, Japanese Shorthorn, Japanese Black, Jersey and Hanwoo). Comparison of allele frequency of the *BoLA-DRB3* locus between all pairs of breeds revealed significant differences in all analyzed cases due to differences in allele combination and gene frequency profile. These significant differences could be due to several factors, such as breed origin, natural or artificial selection, inbreeding, and/or founder effect. It is noteworthy that some of the alleles detected only in Creole cattle had previously been sequenced in African (Boran, N'Dama, Ethiopian Arsi and gudali) and *Bos indicus* (Brahman) breeds. These results are in agreement with previous mitochondrial and microsatellite data that suggest the influence of African and Zebuine genes in the Creole cattle germplam due to breed origin and modern gene introgression (Liron et al., 2006a, 2006b). Unfortunately, studies performed on African and Zebuine animals have been limited to small numbers of animals, making it impossible to conduct a more extensive comparative study.

The population tree and PCA based on the *BoLA-DRB3* allele frequencies in Yacumeño and Hartón del Valle and the previously published allele frequencies of *BoLA-DRB3* indicated that Holstein and

a

Breed	Ya	HV	Wa	Ho	Sh	Je	Han
Ya	0	0.449	0.354	0.305	0.463	0.645	0.508
HV	0.034	0	0.538	0.492	0.745	0.694	0.563
Wa	0.031	0.041	0	0.190	0.457	0.660	0.460
Ho	0.036	0.038	0.024	0	0.385	0.718	0.524
Sh	0.042	0.063	0.054	0.039	0	0.652	0.721
Je	0.058	0.069	0.082	0.091	0.077	0	0.755
Han	0.048	0.056	0.061	0.074	0.084	0.094	0

b

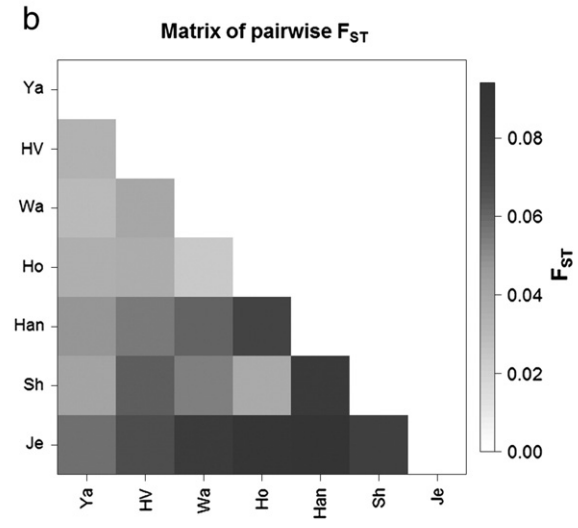

Fig. 3. (a) Genetic distance between pairs of populations estimated through F_{ST} (below) and Nei D_A distance (above); (b) graphic representation of calculated F_{ST} between population pairs using an R-function: pairFstMatrix.r. Ya = Yacumeño, HV = Hartón del Valle, Wa = Japanese Black, Ho = Holstein, Sh = Japanese Shorthorn, Je = Jersey, and Han = Hanwoo.

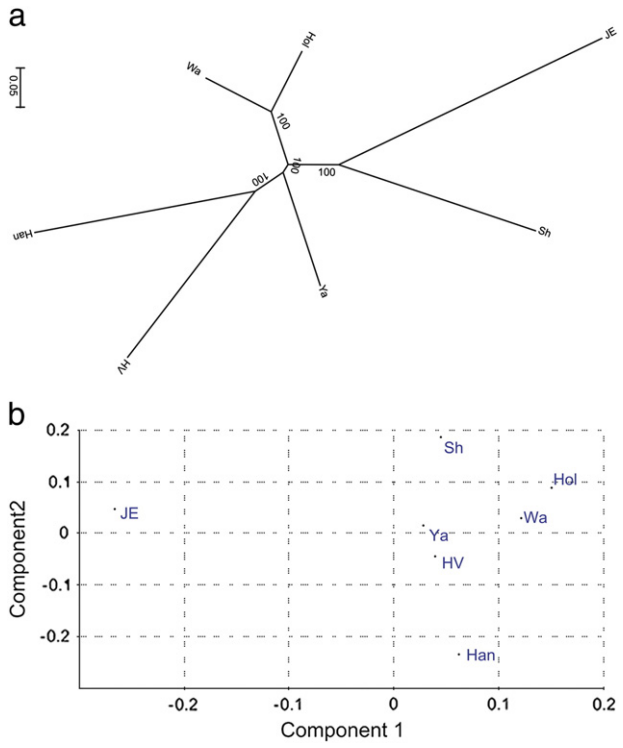

Fig. 4. (a) NJ ss constructed from a matrix of D_A genetic distances. (b) Principal components analysis of allele frequencies from six genotyped SNPs in eleven analyzed populations. Ya = Yacumeño, HV = Hartón del Valle, Wa = Japanese Black, Ho = Holstein, Sh = Japanese Shorthorn, Je = Jersey, and Han = Hanwoo.

Japanese Black clearly clustered into a different branch to the other breeds. The Latin American Creole cattle tended to group together, and Japanese Shorthorn and Jersey exhibited higher genetic distance, measure through F_{ST}, Ds and Da, than other breeds included in the present analysis. Our results are in concordance with data reported by Takeshima et al. (2008) and support the hypothesis that the genetic diversity of the studied native breeds was explained at least partially by their historical origin.

In spite of the small population size of Yacumeño and Hartón del Valle (several thousand adult head), our results revealed that these breeds exhibited a high degree of allelic and gene diversity at the *BoLA-DRB3* locus. The values obtained were similar to those reported for other cattle breeds and are not unexpected for the *MHC class II* genes. It is noteworthy that Yacumeño exhibited a large number of alleles comparable only with the values observed in the Hanwoo breed. A high degree of diversity was also observed in the DNA sequences when nucleotide diversity and a mean number of pairwise differences were estimated, and the observed values were similar to those reported for other breeds. As expected, more dn than ds changes were observed, with the ds/dn being greater within sites corresponding to antigen peptide binding. A possible explanation for the observed high degree of genetic diversity is the multiple origins of the founder population of Creole cattle (Liron et al., 2006a, 2006b) and the further maintenance of genetic diversity through some selection mechanism. At least three mechanisms have been previously proposed: a reproductive mechanism, overdominance, and frequency-dependent selection. To determine the mechanism, we evaluated different statistical methods.

Table 4
Genetic distance between pair of populations estimated through F_{ST} (below) and Nei Da distance (above). Ya = Yacumeño, HV = Hartón del Valle, Wa = Japanese Black, Ho = Holstein, Sh = Japanee Shorthorn, Je = Jersey, Han = Hanwoo.

Breed	Ya	HV	Wa	Ho	Sh	Je	Han
Ya	0	0.449	0.354	0.305	0.463	0.645	0.508
HV	0.034	0	0.538	0.492	0.745	0.694	0.563
Wa	0.031	0.041	0	0.190	0.457	0.660	0.460
Ho	0.036	0.038	0.024	0	0.385	0.718	0.524
Sh	0.042	0.063	0.054	0.039	0	0.652	0.721
Je	0.058	0.069	0.082	0.091	0.077	0	0.755
Han	0.048	0.056	0.061	0.074	0.084	0.094	0

First, we considered reproductive mechanisms including MHC-based mating preferences and selective abortion, both of which have been documented in mouse and human populations and may be sufficiently strong to maintain MHC diversity (Potts et al., 1991; Wedekind and Furi, 1997). However, the extensive genetic diversity at the *BoLA-DRB3* locus in the Yacumeño and Hartón del Valle cattle, as well as in other farm animals, cannot be explained by mating-type preferences since the reproduction of these breeds has been controlled by breeders since the herds were established.

Second, in large populations such as humans, a significant homozygote deficit has been reported for *MHC* genes (Black and Salzano, 1981; Boyce et al., 1997; Markow et al., 1993). The excess of heterozygosity has been interpreted to be a consequence of overdominance because heterozygous individuals are able to recognize a broader spectrum of foreign antigens, thereby increasing their relative fitness compared with homozygotes (Hedrick et al., 1991; Hughes and Nei, 1989). Several studies have shown that heterozygosity confers a selective advantage against infectious diseases (heterozygote advantage or homozygote disadvantage). For example, human leukocyte antigen class II heterozygosity was associated with resistance to infections with hepatitis B and hepatitis C viruses (Hraber et al., 2007; Thursz et al., 1997). Takeshima et al. (2008) showed a significant deviation from HWE in the *bovine class II BoLA-DQA1* gene in cows with mastitis caused by *Escherichia* or *Streptococcus* bacteria. Based on the above findings and hypotheses, we analyzed HWE deviations in the Creole cattle population. While Yacumeño was in HWE for the *BoLA-DRB3* gene, Hartón del Valle differed significantly from the theoretical proportions. However, when heterozygote deficit or excess was tested in this breed, no significant difference from zero was observed. These results are in agreement with data observed in other cattle populations. The most likely explanation for the absence of heterozygote excess in the studied breeds is that the overdominance selection coefficient at *MHC* loci is quite low (probably lower than 0.02; (Mikko et al., 1999)). Such weak selection would only be enough to increase the number of heterozygotes in large populations and in the absence of high rates of stochastic forces (population bottlenecks, genetic drift, and inbreeding). For this reason, and because the HWE method may suffer from low resolving power, such effects could not be observed.

A third hypothesis that could explain the high level of *MHC* polymorphisms proposes that the even allele frequency distribution observed at *MHC* loci could be explained by balancing selection assuming that the presence of a particular allele, rather than heterozygosity, is the critical factor determining survivorship and fitness differences. If so, each *MHC* allele could be related to protection against a different infectious disease or associated with distinct fitness traits such as survival or fecundity. An appropriate test of this hypothesis is the neutrality test. Our results in the Yacumeño breed showed an even gene frequency distribution, in spite of the HWE, expected under balancing selection rather than allelic profiles expected under positive selection against one or few alleles or under neutral selection. The most common alleles account for 41.15% of the cumulative gene frequencies. This result would be expected if the sampled population is under relatively recent balancing selection rather than positive or neutral selection. However, in the Hartón del Valle cattle breed, only a non-significant trend toward balancing selection was observed. This type of finding has also been reported in other cattle breeds and ruminants. For example, Paterson et al. (1998) observed a very even allele frequency distribution at MHC linked microsatellite markers in the Soay sheep population on St. Kilda (Scotland). Furthermore, Kantanen et al. (2000) reported that the microsatellite

BoLA-DRBP1, located within the *BoLA* locus, is not neutral in several Scandinavian bovine breeds (Eastern Finncattle, Northern Finncattle, Doela cattle, Blackside Troender and Norland cattle, and Danish Shorthorn). In summary, our results are consistent with the third hypothesis which proposes that *MHC* polymorphisms could be explained by balancing selection.

The present work was the first report of the allelic distribution of the *BoLA-DRB3* gene, using PCR-SBT, in Latin American Creole cattle. We reported three new *BoLA-DRB3* alleles and demonstrated that both Creole breeds exhibit a high degree of genetic diversity in this gene, despite these breeds suffering a drastic reduction in their population size during the last century. Furthermore, we provided evidence suggesting that this genetic diversity could be maintained by balancing selection. Finally, dendrograms and PCA showed that Latin American Creole cattle were closely related to each other. Therefore, the present characterization of the distribution of the *BoLA-DRB3* gene in two Latin American Creole cattle breeds expands our knowledge about the variability of bovine MHC, which are important loci for the immune response and protection against pathogens. This will allow design of breeding strategies that produce more resistant livestock for the future.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.01.002.

Acknowledgments

We thank Dr. Mariluz Arainga for excellent technical assistance. We are grateful to the Support Unit for Bio-material Analysis and RIKEN BSI Research Resources Center, for help with sequence analysis. This work was supported by Grants-in-Aid for Young Scientists (B) and for Scientific Research (A and B) from the Japan Society for the Promotion of Science (JSPS), by the RIKEN ASI Invitation Program, by a grant from the Program for the Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry, and by theNational Council of Sciences and Technology of Argentina.

References

Aida, Y., 1995. Characterization and expression of bovine MHC class II genes. Bull. Soc. Fr. Jpn. Sci. Vet. 6, 17–24.
Aida, Y., Niimi, M., Asahina, M., Okada, K., Nakai, Y., Ogimoto, K., 1995. Identification of a new bovine MHC class II DRB allele by nucleotide sequencing and an analysis of phylogenetic relationships. Biochem. Biophys. Res. Commun. 209, 981–988.
Ammer, H., et al., 1992. Exonic polymorphism vs intronic simple repeat hypervariability in MHC-DRB genes. Immunogenetics 35, 332–340.
Ballingall, K.T., et al., 2004. Bovine leukocyte antigen major histocompatibility complex class II DRB3*2703 and DRB3*1501 alleles are associated with variation in levels of protection against Theileria parva challenge following immunization with the sporozoite p67 antigen. Infect. Immun. 72, 2738–2741.
Baltian, L.R., Ripoli, M.V., Sanfilippo, S., Takeshima, S.N., Aida, Y., Giovambattista, G., 2012. Association between BoLA-DRB3 and somatic cell count in Holstein cattle from Argentina. Mol. Biol. Rep. 39, 7215–7220.
Baxter, R., Craigmile, S.C., Haley, C., Douglas, A.J., Williams, J.L., Glass, E.J., 2009. BoLA-DR peptide binding pockets are fundamental for foot-and-mouth disease virus vaccine design in cattle. Vaccine 28, 28–37.
Behl, J.D., Verma, N.K., Behl, R., Mukesh, M., Ahlawat, S.P., 2007. Characterization of genetic polymorphism of the bovine lymphocyte antigen DRB3.2 locus in Kankrej cattle (Bos indicus). J. Dairy Sci. 90, 2997–3001.
Black, F.L., Salzano, F.M., 1981. Evidence for heterosis in the HLA system. Am. J. Hum. Genet. 33, 894–899.
Boyce, W.M., Hedrick, P.W., Muggli-Cockett, N.E., Kalinowski, S., Penedo, M.C., Ramey 2nd, R.R., 1997. Genetic variation of major histocompatibility complex and microsatellite loci: a comparison in bighorn sheep. Genetics 145, 421–433.
Brown, J.H., et al., 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364, 33–39.
Cavalli-Sforza, L.L., Menozzi, P., Piazza, A., 1994. The History and Geography of Human Genes. Princeton University Press, United States of America.
da Mota, A.F., Gabriel, J.E., Martinez, M.L., Coutinho, L.L., 2002. Distribution of bovine lymphocyte antigen (BoLA-DRB3) alleles in Brazilian dairy Gir cattle (Bos indicus). Eur. J. Immunogenet. 29, 223–227.
Da Mota, A.F., Martinez, M.L., Coutinho, L.L., 2004. Genotyping BoLA-DRB3 alleles in Brazilian Dairy Gir cattle (Bos indicus) by temperature-gradient gel electrophoresis (TGGE) and direct sequencing. Eur. J. Immunogenet. 31, 31–35.
Dietz, A.B., Cohen, N.D., Timms, L., Kehrli Jr., M.E., 1997a. Bovine lymphocyte antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. J. Dairy Sci. 80, 406–412.
Dietz, A.B., Detilleux, J.C., Freeman, A.E., Kelley, D.H., Stabel, J.R., Kehrli Jr., M.E., 1997b. Genetic association of bovine lymphocyte antigen DRB3 alleles with immunological traits of Holstein cattle. J. Dairy Sci. 80, 400–405.
Gilliespie, B.E., Jayarao, B.M., Dowlen, H.H., Oliver, S.P., 1999. Analysis and frequency of bovine lymphocyte antigen DRB3.2 alleles in Jersey cows. J. Dairy Sci. 82, 2049–2053.
Giovambattista, G., Golijow, C.D., Dulout, F.N., Lojo, M.M., 1996. Gene frequencies of DRB3.2 locus of Argentine Creole cattle. Anim. Genet. 27, 55–56.
Giovambattista, G., Ripoli, M.V., Peral-Garcia, P., Bouzat, J.L., 2001. Indigenous domestic breeds as reservoirs of genetic diversity: the Argentinean Creole cattle. Anim. Genet. 32, 240–247.
Glass, E.J., Oliver, R.A., Russell, G.C., 2000. Duplicated DQ haplotypes increase the complexity of restriction element usage in cattle. J. Immunol. 165, 134–138.
Goudet, J., Raymond, M., de Meeus, T., Rousset, F., 1996. Testing differentiation in diploid populations. Genetics 144, 1933–1940.
Groeneveld, L.F., et al., 2010. Genetic diversity in farm animals—a review. Anim. Genet. 41 (Suppl. 1), 6–31.
Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 9.
Hedrick, P.W., Whittam, T.S., Parham, P., 1991. Heterozygosity at individual amino acid sites: extremely high levels for HLA-A and -B genes. Proc. Natl. Acad. Sci. U. S. A. 88, 5897–5901.
Hernández-Herrera, D.Y., Posso Terranova, A.M., Benavides, J.A., Muñoz-Flórez, J.E., Giovambattista, G., Álvarez-Franco, L.A., 2009. Polimorfismos del gen BoLA-DRB3.2 en razas criollas y Colombianas. Rev. Colomb. Cienc. Pecuarias 22, 469.
Hraber, P., Kuiken, C., Yusim, K., 2007. Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. Hepatology 46, 1713–1721.
Hughes, A.L., Nei, M., 1989. Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc. Natl. Acad. Sci. U. S. A. 86, 958–962.
Kantanen, J., et al., 2000. Genetic diversity and population structure of 20 North European cattle breeds. J. Hered. 91, 446–457.
Klein, J., Satta, Y., O'HUigin, C., Takahata, N., 1993. The molecular descent of the major histocompatibility complex. Annu. Rev. Immunol. 11, 269–295.
Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.
Langella, O., 1999. Populations: a population genetic software.
Lee, B.Y., Hur, T.Y., Jung, Y.H., Kim, H., 2012. Identification of BoLA-DRB3.2 alleles in Korean native cattle (Hanwoo) and Holstein populations using a next generation sequencer. Anim. Genet. 43, 438–441.
Lei, W., Liang, Q., Jing, L., Wang, C., Wu, X., He, H., 2012. BoLA-DRB3 gene polymorphism and FMD resistance or susceptibility in Wanbei cattle. Mol. Biol. Rep. 39, 9203–9209.
Liron, J.P., Bravi, C.M., Mirol, P.M., Peral-Garcia, P., Giovambattista, G., 2006a. African matrilineages in American Creole cattle: evidence of two independent continental sources. Anim. Genet. 37, 379–382.
Liron, J.P., Peral-Garcia, P., Giovambattista, G., 2006b. Genetic characterization of Argentine and Bolivian Creole cattle breeds assessed through microsatellites. J. Hered. 97, 331–339.
Maillard, J.C., Renard, C., Chardon, P., Chantal, I., Bensaid, A., 1999. Characterization of 18 new BoLA-DRB3 alleles. Anim. Genet. 30, 200–203.
Maillard, J.C., Chantal, I., Berthier, D., 2001. Sequencing of four new BoLA-DRB3 and six new BoLA-DQB alleles. Anim. Genet. 32, 44–46.
Maillard, J.C., Chantal, I., Berthier, D., Thevenon, S., Sidibe, I., Razafindraibe, H., 2002. Molecular immunogenetics in susceptibility to bovine dermatophilosis: a candidate gene approach and a concrete field application. Ann. N. Y. Acad. Sci. 969, 92–96.
Markow, T., et al., 1993. HLA polymorphism in the Havasupai: evidence for balancing selection. Am. J. Hum. Genet. 53, 943–952.
Mikko, S., Andersson, L., 1995. Extensive MHC class II DRB3 diversity in African and European cattle. Immunogenetics 42, 408–413.
Mikko, S., Roed, K., Schmutz, S., Andersson, L., 1999. Monomorphism and polymorphism at Mhc DRB loci in domestic and wild ruminants. Immunol. Rev. 167, 169–178.
Miretti, M.M., Ferro, J.A., Lara, M.A., Contel, E.P., 2001. Restriction fragment length polymorphism (RFLP) in exon 2 of the BoLA-DRB3 gene in South American cattle. Biochem. Genet. 39, 311–324.
Miyasaka, T., et al., 2011. The diversity of bovine MHC class II DRB3 and DQA1 alleles in different herds of Japanese Black and Holstein cattle in Japan. Gene 472, 42–49.
Miyasaka, T., Takeshima, S.N., Sentsui, H., Aida, Y., 2012. Identification and diversity of bovine major histocompatibility complex class II haplotypes in Japanese Black and Holstein cattle in Japan. J. Dairy Sci. 95, 420–431.
Mohammadi, A., Nassiry, M.R., Mosafer, J., Mohammadabadi, M.R., Sulimova, G.E., 2009. Distribution of BoLA-DRB3 allelic frequencies and identification of a new allele in the iranian cattle breed sistani (Bos indicus). Genetika 45, 224–229.
Nassiry, M.R., et al., 2005. Analysis and frequency of bovine lymphocyte antigen (BoLA-DRB3) alleles in Iranian Holstein cattle. Genetika 41, 817–822.
Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283.
Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.
Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3, 418–426.
Nei, M., Tajima, F., Tateno, Y., 1983. Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. J. Mol. Evol. 19, 153–170.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
Paterson, S., Wilson, K., Pemberton, J.M., 1998. Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population. Proc. Natl. Acad. Sci. U. S. A. 95, 3714–3719.
Potts, W.K., Manning, C.J., Wakeland, E.K., 1991. Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature 352, 619–621.
Primo, A.T., 1992. El ganado bovino ibérico en las Américas 500 años después. (The IBERIC cattle in the Americas: 500 years later). Archivos de Zootecnia 41, 421–432.
Ripoli, M.V., Liron, J.P., De Luca, J.C., Rojas, F., Dulout, F.N., Giovambattista, G., 2004. Gene frequency distribution of the BoLA-DRB3 locus in Saavedreno Creole dairy cattle. Biochem. Genet. 42, 231–240.
Rousset, F., 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resour. 8, 103–106.
Rupp, R., Hernandez, A., Mallard, B.A., 2007. Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis, and production and type traits in Canadian Holsteins. J. Dairy Sci. 90, 1029–1038.
Russell, G.C., et al., 1997. BoLA class II nucleotide sequences, 1996: report of the ISAG BoLA Nomenclature Committee. Anim. Genet. 28, 169–180.
Russell, G.C., Fraser, D.C., Craigmile, S., Oliver, R.A., Dutia, B.M., Glass, E.J., 2000. Sequence and transfection of BoLA-DRB3 cDNAs. Anim. Genet. 31, 219–222.
Ruzina, M.N., Shtyfurko, T.A., Mohammadabadi, M.R., Gendzhieva, O.V., Tsedev, T., Sulimova, G.E., 2010. Polymorphism of the BoLA-DRB3 gene in the Mongolian, Kalmyk, and Yakut cattle breeds. Genetika 46, 517–525.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Schneider, S., Roessli, D., Excoffier, L., 2000. Arlequin version 2.000: a software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.
Sharif, S., et al., 1998. Associations of the bovine major histocompatibility complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Anim. Genet. 29, 185–193.
Sigurdardottir, S., Borsch, C., Gustafsson, K., Andersson, L., 1991. Cloning and sequence analysis of 14 DRB alleles of the bovine major histocompatibility complex by using the polymerase chain reaction. Anim. Genet. 22, 199–209.
Slatkin, M., 1996. A correction to the exact test based on the Ewens sampling distribution. Genet. Res. 68, 259–260.
Sneath, P.H.A., Sokal, R.R., 1973. In: Hardcover (Ed.), Numerical Taxonomy: The Principles and Practice of Numerical Classification. Freeman & Company Limited, W H.
Starkenburg, R.J., Hansen, L.B., Kehrli Jr., M.E., Chester-Jones, H., 1997. Frequencies and effects of alternative DRB3.2 alleles of bovine lymphocyte antigen for Holsteins in milk selection and control lines. J. Dairy Sci. 80, 3411–3419.
Sulimova, G.E., Udina, I.G., Shaikhaev, G.O., Zakharov, I.A., 1995. DNA polymorphism of the BoLA-DRB3 gene in cattle in connection with resistance and susceptibility to leukemia. Genetika 31, 1294–1299.
Takeshima, S.N., Aida, Y., 2006. Structure, function and disease susceptibility of the bovine major histocompatibility complex. Anim. Sci. J. 77, 138–150.
Takeshima, S.N., Ikegami, M., Morita, M., Nakai, Y., Aida, Y., 2001. Identification of new cattle BoLA-DRB3 alleles by sequence-based typing. Immunogenetics 53, 74–81.
Takeshima, S.N., Nakai, Y., Ohta, M., Aida, Y., 2002. Short communication: characterization of DRB3 alleles in the MHC of Japanese shorthorn cattle by polymerase chain reaction-sequence-based typing. J. Dairy Sci. 85, 1630–1632.
Takeshima, S.N., Saitou, N., Morita, M., Inoko, H., Aida, Y., 2003. The diversity of bovine MHC class II DRB3 genes in Japanese Black, Japanese Shorthorn, Jersey and Holstein cattle in Japan. Gene 316, 111–118.
Takeshima, S.N., Matsumoto, Y., Chen, J., Yoshida, T., Mukoyama, H., Aida, Y., 2008. Evidence for cattle major histocompatibility complex (BoLA) class II DQA1 gene heterozygote advantage against clinical mastitis caused by Streptococci and Escherichia species. Tissue Antigens 72, 525–531.
Takeshima, S.N., Matsumoto, Y., Aida, Y., 2009. Short communication: establishment of a new polymerase chain reaction-sequence-based typing method for genotyping cattle major histocompatibility complex class II DRB3. J. Dairy Sci. 92, 2965–2970.
Takeshima, S.N., et al., 2011. A new method for typing bovine major histocompatibility complex class II DRB3 alleles by combining two established PCR sequence-based techniques. Tissue Antigens 78, 208–213.
Thursz, M.R., Thomas, H.C., Greenwood, B.M., Hill, A.V., 1997. Heterozygote advantage for HLA class-II type in hepatitis B virus infection. Nat. Genet. 17, 11–12.
Udina, I.G., et al., 1998. Comparative analysis of Ayrshire and Black Pied cattle breeds by histocompatibility markers. Genetika 34, 1668–1674.
van Eijk, M.J., Stewart-Haynes, J.A., Lewin, H.A., 1992. Extensive polymorphism of the BoLA-DRB3 gene distinguished by PCR-RFLP. Anim. Genet. 23, 483–496.
Wedekind, C., Furi, S., 1997. Body odour preferences in men and women: do they aim for specific MHC combinations or simply heterozygosity? Proc. Biol. Sci. 264, 1471–1479.
Weir, B., Cockerham, C., 1984. Estimating F-statistics for the analysis of population structure. Evolution 38, 1358–1370.
Wilkins, J.V., 1984. Criollo cattle of the Americas. Anim. Genet. Resour. Inform. 2, 1–19.