Diversity of the BoLA-*DRB3* Gene in Cattle Breeds From Tropical and Subtropical Regions of South America

Magali Nicole Valenzano

INTA: Instituto Nacional de Tecnología Agropecuaria <https://orcid.org/0000-0002-8428-3962>

María Eugenia Caffaro

Instituto Nacional de Tecnología Agropecuaria: Instituto Nacional de Tecnología Agropecuaria

Veronica Viviana Lía

Instituto Nacional de Tecnología Agropecuaria: Instituto Nacional de Tecnología Agropecuaria

Mario Poli

Instituto Nacional de Tecnología Agropecuaria: Instituto Nacional de Tecnología Agropecuaria

Silvina Elizabeth Wilkowsky (✉ wilkowsky.silvina@inta.gob.ar)

Instituto Nacional de Tecnología Agropecuaria: Instituto Nacional de Tecnología Agropecuaria

Research

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Abstract

Background

Bovine leukocyte antigens (BoLA) are widely used as markers of disease and immunological traits in cattle because of their primary function in the recognition of pathogens by the immune system. To date, however, the characterization of BoLA gene polymorphisms in Latin American Zebu and mixed zebuine breeds is scarce. By a sequence-based typing method, here we sequenced exon 2 of BoLA class II *DRB3* gene of 264 animals from the five breeds (Creole, Brahman, Braford, Brangus and Nellore) most commonly used in northern Argentina.

Results

The taurine and zebuine breeds contained 61 previously reported alleles. Genetic diversity was high at allelic and molecular levels, particularly in the mixed breeds. Neutrality tests revealed that none of the breeds shows the even gene frequency distribution expected under a balanced selection scenario. The F_{ST} index shows significant differences across all populations ($F_{ST}=0.052$, $P < 0.001$), indicating a substantial differentiation between breeds. UPGMA trees and Metric multidimensional scaling (MDS) analysis show that, as expected, Creole is distantly related to the other zebuine breeds. Among them, Brahman, Braford and Brangus cluster closer together than with Nellore. These data are consistent with the historical and geographical origin of these breeds. A peptide binding region sequence analysis at the amino acid level revealed that, despite the nucleotide diversity among the breeds, the key amino acids involved in peptide recognition are greatly conserved.

Conclusions

This is the first report of BoLA-*DRB3* diversity in pure and mixed zebuine cattle breeds from Argentina. Knowledge of BoLA-*DRB3* variability in breeds adapted to tropical and subtropical environments contributes not only to ongoing efforts to catalog bovine MHC allele frequencies by breed and location but also to the design of peptide-based vaccines.

Background

Increasing livestock productivity requires the use of breeds adapted to specific environments, as this helps the animals to cope with stress and enhances their resistance to circulating pathogens. In this sense, the production, reproduction and survival of cattle breeds in tropical and subtropical climates are seriously compromised by heat stress and enzootic infectious diseases.

According to the United States Department of Agriculture (USDA), Argentina is the third larger producer of bovines worldwide, with a population of 53.8 million heads (1). The breeds Nellore, Brahman, Brangus and Braford are the most widely used by farmers in northern Argentina, which is the second major productivity zone in the country. The selection of these breeds is because of their natural adaptation to the agro-ecological conditions of this subtropical region (2, 3).

The major histocompatibility complex (MHC) has an essential role in the adaptive immune system, and comprises the cell surface glycoproteins necessary for T lymphocytes to recognize antigenic peptides on the surface of

antigen-presenting cells (4). In addition, the MHC genetic variability in native breeds is of great interest in evolutionary biology because of the typically high levels of polymorphism and because of its involvement in host defences against pathogens. Consequently, the MHC allelic diversity of representative species of several mammalian orders, including Artiodactyla, Perissodactyla, Carnivora, Primates, and Rodentia has been thoroughly characterized (5).

The bovine MHC, called Bovine Leucocyte Antigen (BoLA), is encoded in chromosome 23 and consists of three gene classes, I, II and III. MHC class II products are normally expressed by cells of the immune system, namely macrophages, dendritic cells, B and T lymphocytes. In bovines, BoLA class II genes are distributed in two distinct regions, IIa and IIb. The *DRA*, *DRB*, *DQA* and *DQB* genes are located in the IIa region, whereas the *DOB*, *DYA*, *DYB* and *DIB* genes are in the IIb region (6). In particular, the DR and DQ molecules represent the main class II restriction elements for CD4-positive T-helper cells (7). Among them, the BoLA-*DRB3* gene is the only one known to be fully functional (4) and is the most polymorphic class II locus in cattle. This gene product influences the magnitude and epitope specificity of antigen-specific T-cell responses to infectious diseases. Accordingly, differences in susceptibility to different diseases (i.e. dermatophilosis, bovine leucosis virus-induced lymphocytosis and mastitis), production traits (i.e. milk production) and vaccine responses (i.e. foot-and-mouth disease and *Theileria parva* infection) (8–11) have been associated with BoLA-*DRB3* polymorphisms. There is compelling evidence that in mammals the MHC polymorphism is maintained by some form of overdominance or balancing selection (12, 13).

The polymorphism of BoLA-*DRB3* genes occurs predominantly at residues within the peptide-binding groove (14). In cattle, 357 BoLA-*DRB3* alleles have been identified in various breeds up to date; which are listed in the Immune Polymorphism Database (IPD) – MHC database (15). Although seemingly high, this number of alleles encompasses only 12 out of over 800 breeds of cattle recognized worldwide (16). To a large extent, the breeds studied to date include European taurine (i.e., Hereford, Angus, Shorthorn, Holstein, Jersey), Asian taurine (Japanese Black, Philippine Native, and Korean Native), American Creole (i.e., Yacumeño and Hartón del Valle) and different types of cross-breeds (i.e. , Holstein × Sahiwal, Philippine Native × Brahman and Philippine Native × Holstein)(16).

In this context, we aimed at gaining further knowledge about the genetic diversity of the BoLA-*DRB3* gene in Argentine populations of pure *Bos indicus* types Brahman and Nellore as well as in the mixed breeds Braford and Brangus, as genetic data of these breeds are still scarce. We have also investigated the levels and structuring patterns of BoLA allelic variation and the functional diversity of the peptide binding region (PBR) in all these breeds.

Methods

1. Animals and genomic DNA extraction

Blood, hair or semen samples were obtained from 205 *Bos indicus* bovines: Nellore (N = 30) and Brahman (N = 36), as pure breeds, as well as Braford (N = 70) and Brangus (N = 69), as mixed breeds. Individuals (N = 59) of the Creole breed (*Bostaurus*) were added as a different non-related breed, i.e. as an outgroup. The sampled individuals were selected to avoid genetic relatedness and to obtain the maximum representativeness of the breed. The genealogical records and historical data of the formation of the different herds were taken into consideration for the analysis. The procedure for sample collection was approved (protocol number 35/2010) by the Institutional

Committee for Care and Use of Experimental Animals of the National Institute of Agricultural Technology (CICUAE-INTA). All procedures were performed according the guidelines described in the institutional manual. Additional file 1 displays detailed information about all the analyzed breeds.

Whole blood was obtained by venipuncture using EDTA as anticoagulant. Genomic DNA was extracted using a commercial kit (AxyPrep Blood Genomic DNA Miniprep Kit, Axygen Biosciences, Union City, CA) according to the protocol supplied by the manufacturer. Genomic DNA from bull semen was obtained from frozen straws using a standard phenol-chloroform-based extraction method. DNA isolation from hair root samples was done using a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) according to the manufacturer's instructions. In all cases DNA quantity and quality were determined by measuring its UV absorption at 260 and 280 nm and the 260/280 and 260/230 absorbance ratios using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). The purified DNA was stored in aliquots at -20°C until use.

2. BoLA-*DRB3* typing using PCR-sequence based typing (PCR-SBT)

PCR reactions were performed in a Mastercycler (Eppendorf ®) thermocycler in a final volume of 15 µl. The volume mixture contained 6 ng of genomic DNA, 0.03 X PCR buffer (Mg²⁺ + plus), 0.3 mM of each primer (HL030; 5'-ATCCTCTCTGCAGCACATTTCC-3', and HL032; 5'-TCGCCGCTGCYCAGTGAAACTCTC - 3') (17), 0.2 mM of dNTPs, 0.75 U Paq5000® DNA Polymerase (Agilent Technologies, Stratagene, USA) and Milli Q grade water up to the total volume. The conditions for amplification were 94°C for 3 min, followed by 35 cycles of 20 s at 94°C, 20 s at 60°C (annealing temperature) and 1 min at 72°C. The reactions were finished with a 72°C, 5-min extension. Amplified fragments of 247 pb corresponding to exon 2 of the BoLA-*DRB3* gene were visualized in a 1% agarose gel and sequenced using BigDye® chemistry on an ABI3130xl sequencer (Applied Biosystems) following the manufacturer's protocol. The sequencing reactions were performed in both senses using the same primers as for PCR reactions, in a total volume of 10 µl containing a 1:10 dilution of the PCR product as template and 0.1 mM of each of the corresponding primer, in addition to the mix and buffer 5X of the BigDye® Terminator kit. Sequence analysis and genotype calling were performed using Haplofinder (8, 18). Briefly, this program uses all known *DRB3* alleles to create a list of all possible heterozygous combinations using IUPAC ambiguity codes. Haplofinder compares the consensus sequence obtained with the list of all possible combinations list. Alignments were constructed using T-Coffee (19). Additional file 2 displays the list of detected alleles and their IPD-MHC database accession numbers.

3. Statistical analysis

3.1 Measures of genetic variability

Allele frequencies and number of alleles (N_a) were obtained by the direct count method. The effective (N_e) and observed (N_o) number of alleles as well as the observed (H_o) and expected unbiased heterozygosity (H_e) for the BoLA-*DRB3* locus were estimated according to Nei (1978) using Genalex (21). Potential deviations from Hardy-Weinberg equilibrium (HWE) were assessed for each breed by FIS statistics (22) using the exact test included in GENEPOP (23), whereas allelic richness was estimated using FSTAT 2.9.3.2 (24).

The number of segregating sites (S), total number of mutations (Θ), number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π), and the average number of nucleotide differences (k) were calculated using DnaSP 5.00.02 (25). Tajima's D test (26) was used for testing the null hypothesis of neutrality (27). The statistical significance of Tajima's D was calculated using a two tailed test and the coalescent simulations algorithm

implemented in DnaSP 5.00.02(25). The mean number of nonsynonymous (dN) and synonymous (dS) nucleotide substitutions per site was estimated for each pair using the Jukes–Cantor’s formula as described by Nei and Gojobori using DnaSP 5.00.02. The Z-test was used for assessing the dN/dS ratio significance using MEGA X (28).

The distribution of alleles across major groups of breeds was represented by a Venn plot using the R package ‘VennDiagram’ (29). For this analysis, we used the data generated here as well as information from previous reports on other south American breeds (16, 30–32)(Additional file 1). In the case of the dataset of Kelly et al.(2002) only the alleles whose PCR-RFLP pattern matched with only one allele in the current nomenclature (33) were used.

3.2 PBR aminoacid analysis

Aminoacid identity and similarity percentages were calculated with Geneious Prime 2020.2.4(33) using a BLOSUM62 substitution matrix. Computations were performed based on the aminoacid sequence of the 31 putative positions constituting the MHC-DRB PBR (35). A logo of PBR positions for each population was created using WebLogo software(36). The height of symbols within the stack indicates the relative frequency of each aminoacid at that position for each population. Only alleles having frequencies greater than 5% were considered in the analysis. The R 4.0.2 package (29) was used to calculate Pearson correlation coefficients (PCC) among breeds and to perform a metric multidimensional scaling (MDS) on the correlation matrix obtained.

3.3 AMOVAanalysis

The partitioning of genetic variation among breeds was performed with an analysis of molecular variance (AMOVA) (37) considering either the sequences or the discrete allelic states of *BoLA-DRB3* genotypes. In the case of sequence analyses, the evaluations were performed based on pairwise differences using Arlequin 3.11 (38). For discrete allelic states, Nei’s genetic distances were used as the starting matrix for AMOVA. Statistical significance of each variance component was assessed based upon 999 permutations of the data.

3.4 Genetic distances, cluster analysis and metric multidimensional scaling

Genetic structure and genetic differentiation among breeds were assessed using Wright’s F statistics using the variance-based method of Weir and Cockerham (22). These parameters and their significance were assessed with FSTAT 2.9.3.2. Nei’s genetic distance was calculated from allele frequencies using GENALEX (21). Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm implemented in Phylip (39). The generated trees were visualized with Figtree (40). Further examination of the relationships among breeds were assessed by using Nei’s genetic distances to perform a MDS analysis using R (29).

4- Phylogenetic analysis

Phylogenetic analysis was performed by constructing Maximum Likelihood (ML) trees from *BoLA-DRB3* nucleotide sequences. Prior to ML analyses, the computer program ModelTest 2.1.9 (41) was used to select the most appropriate model of molecular evolution according to the Akaike Information Criterion. The sequences were analyzed under a Jukes-Cantor model with gamma rate heterogeneity and invariant sites. ML tree searches were performed with PAUP 4.0 (42). Node robustness was assessed with 1000 bootstrap replicates.

Results

1-Distribution of *BoLA-DRB3* alleles among breeds

To determine the diversity and distribution of BoLA-*DRB3* alleles in cattle breeds from North Eastern Argentina, we genotyped 264 individuals using PCR-SBT and therefore identified 61 previously reported alleles, with no new allelic variants (Table 1). Of these 61 variants, five are present only in *B. taurus*, four are present exclusively in *B. indicus* and 22 are present exclusively in mixed breeds. Only nine alleles are shared between the three major bovine types. Meanwhile, the remaining 21 alleles are shared between at least two breeds. All breeds presented exclusive alleles (Additional file 3).

Table 1
Frequencies of BoLA-DRB3 alleles detected by PCR-SBT.

DRB3.2 Allele	Creole (N = 59)	Nellore (N = 30)	Brahman(N = 36)	Braford(N = 69)	Brangus (N = 70)	Reference
BoLA-DRB3*001:01	0.203			0.022	0.171	Mikko& Anderson,(43)
BoLA-DRB3*002:01	0.017			0.014	0.164	Takeshima et al., (44)
BoLA-DRB3*003:01	0.008			0.007		Mikko& Anderson, (43)
BoLA-DRB3*005:01	0.068		0.014	0.022	0.007	Mikko& Anderson, (43)
BoLA-DRB3*005:02	0.008					Mikko& Anderson, (43)
BoLA-DRB3*005:04				0.007		Takeshima et al., (45)
BoLA-DRB3*007:01	0.008		0.042	0.014	0.007	Takeshima et al., (44)
BoLA-DRB3*008:01				0.036	0.029	Mikko& Anderson, (43)
BoLA-DRB3*009:01				0.021	0.021	Mikko& Anderson, (43)
BoLA-DRB3*009:02	0.008		0.014	0.014	0.007	Mikko& Anderson, (43)
BoLA-DRB3*010:01					0.007	Mikko& Anderson, (43)
BoLA-DRB3*010:02				0.094	0.007	Mikko& Anderson, (43)
BoLA-DRB3*011:01	0.051			0.007	0.021	Takeshima et al., (44)
BoLA-DRB3*011:03		0.017		0.007		Maillard et al., (46)
BoLA-DRB3*012:01	0.034	0.017	0.194	0.065		Mikko& Anderson, (43)
BoLA-DRB3*013:01	0.025			0.014		Mikko& Anderson, (43)
BoLA-DRB3*014:01:01				0.014	0.021	Mikko& Anderson, (43)
BoLA-DRB3*014:02					0.007	De S &Sing, (47)
BoLA-DRB3*015:01	0.017			0.051	0.043	Mikko& Anderson, (43)
Total number of alleles: 61. Most frequent alleles in each breed are highlighted in bold.						

DRB3.2 Allele	Creole (N = 59)	Nellore (N = 30)	Brahman(N = 36)	Braford(N = 69)	Brangus (N = 70)	Reference
BoLA-DRB3*015:02				0.007		Xu A et al.,(48)
BoLA-DRB3*016:01		0.033		0.051		Mikko& Anderson, (43)
BoLA-DRB3*016:02					0.007	Mikko et al., (49)
BoLA-DRB3*017:01					0.014	Mikko& Anderson,(43)
BoLA-DRB3*017:03					0.021	Maillard et al., (46)
BoLA-DRB3*018:01	0.110	0.017		0.043	0.029	Takeshima et al., (44)
BoLA-DRB3*019:01			0.014			Mikko& Anderson, (43)
BoLA-DRB3*019:02					0.007	Takeshima et al., (45)
BoLA-DRB3*020:01:02	0.042		0.042	0.007		Gelhaus et al., (50)
BoLA-DRB3*020:03	0.059					Maillard et al., (46)
BoLA-DRB3*021:01	0.042					Mikko& Anderson, (43)
BoLA-DRB3*022:01		0.083	0.069	0.022		Mikko& Anderson, (43)
BoLA-DRB3*023:01	0.085					Mikko& Anderson, (43)
BoLA-DRB3*024:03				0.007		
BoLA-DRB3*026:01	0.008					Mikko& Anderson, (43)
BoLA-DRB3*027:03				0.043	0.007	Takeshima et al., (44)
BoLA-DRB3*027:04					0.014	Gelhaus et al., (50)
BoLA-DRB3*027:05			0.028			Maillard et al., (46)
BoLA-DRB3*027:07				0.022	0.021	Maillard et al., (46)
BoLA-DRB3*027:10					0.021	Takeshima et al., (51)

Total number of alleles: 61. Most frequent alleles in each breed are highlighted in bold.

DRB3.2 Allele	Creole (N = 59)	Nellore (N = 30)	Brahman(N = 36)	Braford(N = 69)	Brangus (N = 70)	Reference
BoLA-DRB3*028:01	0.076	0.100		0.065		Gelhaus et al., (50)
BoLA-DRB3*028:02				0.007		Takeshima et al., (45)
BoLA-DRB3*029:01	0.025	0.017				Gelhaus et al., (50)
BoLA-DRB3*030:01		0.100	0.375	0.087	0.121	Gelhaus et al., (50)
BoLA-DRB3*030:21		0.017			0.021	Takeshima et al., (44)
BoLA-DRB3*030:22			0.014			De S &Sing, (47)
BoLA-DRB3*031:01			0.042	0.014	0.014	Takeshima et al., (44)
BoLA-DRB3*032:01		0.017		0.036	0.050	Takeshima et al., (52)
BoLA-DRB3*032:02		0.116	0.028		0.007	Takeshima et al., (45)
BoLA-DRB3*032:03					0.007	Maillard et al., (46)
BoLA-DRB3*033:01				0.022	0.043	Sitte et al., (53)
BoLA-DRB3*034:01			0.014	0.014		Takeshima et al., (44)
BoLA-DRB3*035:01	0.017	0.183	0.014	0.007	0.007	Maillard et al., (46)
BoLA-DRB3*036:01	0.034	0.233	0.042	0.065	0.043	Maillard et al., (46)
BoLA-DRB3*037:01		0.017				Takeshima et al., (52)
BoLA-DRB3*039:01			0.014			
BoLA-DRB3*042:01			0.028	0.029	0.007	Maillard et al., (46)
BoLA-DRB3*043:01				0.007		Maillard et al., (46)
BoLA-DRB3*044:01	0.034			0.014		Maillard et al., 1999
BoLA-DRB3*048:01	0.017				0.007	De S &Sing, (47)

Total number of alleles: 61. Most frequent alleles in each breed are highlighted in bold.

DRB3.2 Allele	Creole (N = 59)	Nellore (N = 30)	Brahman(N = 36)	Braford(N = 69)	Brangus (N = 70)	Reference
BoLA-DRB3*048:02				0.014		Takeshima et al., (45)
BoLA-DRB3*057:02		0.033	0.014		0.007	Das et al., (54)
Number of alleles	23	15	18	37	34	
Number of most frequent alleles	7	6	3	7	4	
Total number of alleles: 61. Most frequent alleles in each breed are highlighted in bold.						

To better analyze allele distribution, we subsequently constructed a Venn diagram using a larger dataset of 70 alleles from ten breeds grouped in three major bovine types (*Bos taurus*, *Bos indicus* and mixed breeds) (see materials and methods and Additional file 1). The plot shows that 26 alleles (37.1%) are shared by three major groups, whereas seven alleles (10%) are present exclusively in *Bos taurus*, three (4.3%) in *Bos indicus* and seven (10%) in the mixed breeds (Fig. 1).

The identity of high frequency alleles (> 5%) also varies depending on the breed. Creole and Braford have the highest number of alleles above the 5% threshold (n = 7 each), followed by Nellore (n = 6), Brangus (n = 4) and Brahman (n = 3). The allele*030:01 is the only high-frequency allele that is shared by almost all breeds, except for Creole (Table 1). Only the alleles *035:01 and *036:01 are shared by all the breeds.

2-BoLA-DRB3 diversity and Tajima'sD.

The genetic diversity was assessed taking into account several parameters (Table 2). The Na ranges from 15 in Creole to 37 in Braford. It is noteworthy that in all the breeds the Ne is lower than the allelic richness. The He values ranges from 0.806 in Brahman to 0.953 in Braford, whilst the Ho values ranges from 0.643 in Brangus to 0.855 in Braford. Overall, Braford is the most diverse breed, as evidenced by the number of alleles, the heterozygosity and the allelic richness.

Table 2: Measures of genetic variability.

	N	Na	Ne	Ho	He	uHe	F _{IS}	Allelic richness
Creole	59	23	11.488	0.814	0.913	0.921	0.117*	19.113
Nellore	30	15	7.531	0.733	0.867	0.882	0.170*	15.000
Brahman	36	18	5.153	0.694	0.806	0.817	0.152*	16.574
Braford	69	37	21.350	0.855	0.953	0.960	0.110	27.108
Brangus	70	34	11.681	0.643	0.914	0.921	0.303*	22.990
References: Number of individuals (N), Number of alleles (Na), effective number of alleles (Ne), observed (Ho), expected (He) and unbiased expected (uHe) heterozygosity, Hardy-Weinberg equilibrium (HWE) measured using F _{IS} , *P < 0.05.								

The BoLA-DRB3 diversity was also examined at the DNA level using seven indexes (Table 3). The π value varies from 0.074 in Nellore and Creole to 0.082 in Braford. The ranking of Hd is in line with that of the He. The S number

varies from 56 in Nellore and Brahman to 65 in Braford, whilst the number of mutations varies from 67 in Nellore to 82 in Braford. All the breeds present more mutations than segregating sites; which shows that there are sites that do not conform to the infinite site model. The average number of nucleotide differences (k) is lower in pure breeds than in mixed breeds, as expected because of their admixed nature. Tajima's D test shows no departures from neutral expectations.

In addition, we calculated the number of dN and dS for each breed (Table 3). The dN values ranges from 0.096 in Nellore and Brahman to 0.104 in Braford, whilst the dS values ranges from 0.024 in Nellore to 0.037 in Brahman, with a significant dN/dS ratio ($\neq 1$) in all populations. The ratio between dN and dS is more pronounced when only considering PBR codons.

Table 3
Genetic diversity at the sequence level.

	N	S	Eta	h	Hd	π	k	Tajima's D	Total		PBR	
									dN	dS	dN	dS
Creole	59	58	74	10	0.786	0.074	17.419	1.114	0.100	0.036*	0.217	0.082*
Nellore	30	56	67	15	0.882	0.074	17.356	0.701	0.096	0.024*	0.197	0.051*
Brahman	36	56	68	10	0.817	0.076	17.924	0.930	0.096	0.037*	0.198	0.072*
Braford	69	65	82	37	0.960	0.082	19.184	0.908	0.104	0.035*	0.228	0.071*
Brangus	70	62	76	33	0.918	0.078	18.108	0.989	0.097	0.035*	0.196	0.071*
References: number of individuals (N), number of segregating sites (S), number of mutations (Eta), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), mean number of pairwise differences (k), Tajima's D Neutrality test, mean number of nonsynonymous (dN) and synonymous (dS) nucleotide substitutions per site in the complete BoLA sequence (Total) and in the peptide binding site (PBR). The asterisks indicate a statistically significantly different from 1 dN/dS ratio.												

3- PBR similarity analysis

The BoLA-*DRB3* PBR aminoacidic sequence was analyzed for each breed as an indicator of functional diversity. To this purpose, we assessed PBR position identity and similarity for all alleles (Additional file 4). According to the identity analysis, most (59/61) of the alleles possessa different PBR. Regarding similarity, Brahman and Brangus have the highest percentages (80.8%), thus indicating low diversity in these mixed breeds. Conversely, Braford had the lowest similarity percentage (74.7%), and it is the most diverse breed at the PBR level. This finding is consistent with the nucleotide diversity results. According to the PCC analysis and MDS, Braford is the most different breed at the PBR level in comparison to all the analyzed breeds (Fig. 2 B and C).

The logo representation of PBR shows very similar substitution patterns between breeds (Fig. 2A). The most variable positions were 11, 13, 37, 70, 71 and 74, as reported previously (Bohorquez et al., 2020). Aminoacids present at position 86 (binding pocket 1) are reported to frequently be either a valine (V) or a glycine (G) (55). We also found *phenylalanine* (F) in this position, but only in the Nellore breed. Other study has previously reported the same result for this breed (4). Aminoacids frequently found in position 70 of the binding pocket 4 are frequently either glutamic acid (E), arginine (R) or glutamine (Q). Accordingly, in our samples we have found all these

aminoacids. However, a high proportion of Brahman samples also presented aspartic acid (D). In the other binding pockets (6, 7 and 9), the aminoacids found in our samples are quite conserved as the ones already reported (4,55).

4-Genotypic frequencies of BoLA-DRB3 alleles and population structuring

According to an analysis of potential deviations from HWE performed by computing FIS indexes for each breed, Creole, Nellore, Brahman and Brangus exhibit a significant excess of homozygotes, while Braford does not, although the FIS is also higher than zero (Table 2).

The F_{ST} index was used to analyze the degree of genetic differentiation among the 5 breeds studied. This index shows significant differences for all cattle breeds ($F_{ST}=0.052$, $P<0.001$) (Fig. 3A). Moreover, significant values are also observed for all pairwise comparisons ($P<0.001$) with values ranging from 0.029 between Braford and Brangus to 0.117 between Brahman and Creole. These results demonstrate that, although rather low in most instances, there is significant genetic differentiation between all the breeds.

The analysis of the levels of genetic differentiation between breeds estimated using AMOVA reveals that the percentage of variation between breeds was 5.60% when alleles were coded as discrete states and 4.50% when the sequence divergence was included in the analysis ($P<0.001$).

5-Population relationships based on frequencies of BoLA-DRB3 alleles

To determine the genetic relationship between the breeds under study, we used genetic distances and a metric MDS (Fig. 3). In agreement with the historical and geographical origin of the breeds examined here, the genetic distance between the group of pure and mixed *Bos indicus* breeds and Creole is quite large (Fig. 3A and B). Fig. 3C displays the results from the MDS and illustrates the first and second principal coordinates. The first two coordinates account cumulatively for 98.07 % of the variability in the data. In accordance with the UPGMA tree (Fig. 3B), the first coordinate accounts for 77.30% of the total variance and shows a differentiation pattern between Creole (*Bos taurus*) and the other breeds. Brahman is located at the opposite end of this coordinate. The second coordinate explains 20.77% of the total variation and clearly distinguishes Nellore (*Bos indicus*) from the remaining breeds. The third coordinate (not shown in the figure) accounts for 1.93% of the variance and it fails to provide any information regarding the potential origin of the breeds.

6-Phylogenetic analysis

The phylogenetic analysis was performed by constructing [Maximum Likelihood](#) (ML) trees from *BoLA-DRB3* [nucleotide sequences](#) (Additional file 5). The resulting tree shows no relationships between allele lineages and breeds. As expected, most clusters are formed according to their respective allele nomenclature, which is based on the nucleotide sequence (10,33) and thus confirms the correct assignation of the alleles.

Discussion

Knowledge of MHC diversity has important implications for the improvement of animal health and breeding since histocompatibility molecules play a key role in pathogen recognition. Therefore, information about breeds frequently used in specific geographic regions and whose *BoLA-DRB3* genetic background is currently unknown is crucial for the update of allele catalogues and the design of better breeding strategies.

In this study, we report the BoLA-*DRB3* diversity of five cattle breeds currently raised in northeast Argentina but that are also used in many temperate regions of south America. These breeds are selected by Argentine farmers because of their adaptation to the warm climate of the region as well as their natural resistance against certain diseases and arthropods, including the bovine tick *Rhipicephalus microplus*, which is widely distributed in the area (2,3). This tick is the vector of pathogens such as the bacteria *Anaplasma marginale* and the protozoa *Babesia bovis* and *Babesia bigemina* (3). Some reports have associated certain BoLA-*DRB3* alleles of pure and mixed breeds from tropical environments with susceptibility or resistance to these tick-borne diseases (12,56). For instance, the alleles *042:01, *011:01 and *020:01:02 were associated with tolerance to *B. bigemina* infections, whilst the alleles *027:03 and *013:01 were linked to *B. bigemina* and *A. marginale* susceptibility, respectively. All these alleles occurred in low frequencies in the samples assessed in our study. This may be related to a bias in the types of breeds sampled for the research. It is noteworthy that the allele *011:01 was highly frequent in Creole, whereas the other alleles only occurred in low frequencies in Brangus and Braford. If *B. bigemina* tolerance alleles are further confirmed in other bovine populations, our data would reinforce the utility of characterizing BoLA-*DRB3* diversity as a routine procedure of animal breeding programs.

The distribution of BoLA-*DRB3* alleles reveals that frequencies differed significantly between breeds. All breeds have exclusive alleles, suggesting that they are well differentiated populations with very low levels of gene flow.

An analysis of the expanded data matrix including data from *Bos taurus*, *Bos indicus* and mixed breeds from other Latin American countries shows that the exclusive alleles from mixed breeds decrease in comparison to our own data (Fig. 1) (Additional file 1). This may indicate that the *Bos indicus* individuals involved in the origin of the mixed breeds would be different from the ones that are present in the region today.

The genetic diversity within breeds was measured by the number of alleles, allelic richness and heterozygosity at the BoLA-*DRB3* gene. In all the breeds, N_e is lower than the allelic richness, probably due to an uneven distribution of the allele frequencies. Overall, Braford is the most genetically diverse breed. Although the detected number of alleles was higher than those estimated for other south American cattle breeds (4,31,57), the heterozygosity values obtained here are lower, indicating the contribution of low frequency alleles. An analysis of the genetic diversity at the nucleotide level retrieved similar results. In our data, the H_d , π and k values result higher in mixed breeds than in pure ones, thus showing that mixed breeds capture more diversity, as expected.

The diversity at PBR positions determines which peptides a given BoLA-*DRB3* allele can bind and this information is crucial to increase peptide-histocompatibility complex affinity for peptide-based vaccine design. Despite the fact that *Bos taurus* and *Bos indicus* are well differentiated bovine types, our correlation analysis shows that they cluster together based on PBR aminoacid sequences. Moreover, the sequence analysis of binding pockets, the PCC and MDS show that with exception of Braford, the remaining breeds tend to have a similar peptide repertoire in the PBR, in spite of the observed genetic differentiation. This conservation extends to the breeds studied here, and is particularly evident in aminoacids at positions 70, 71, and 74 of pocket 4, which occupies a central position within the peptide binding groove. These aminoacids have been implicated as playing a significant role in human immune responsiveness (58), but also correlated with vaccine responses and infectious disease susceptibility or tolerance in bovines (55,59–61). In this sense, it could be hypothesized that even though bovine breeding programs make use of extensive crossing, there may be a functional constraint in the PBR to BoLA-*DRB3* aminoacid variability.

In order to assess the structuring of *BoLA-DRB3* variation, we computed Wright's fixation indices (22). Previous studies reported both positive and negative deviations from HWE in different breeds; which suggests an interplay between selection and inbreeding, or either that some breeds were more admixed than the others (57). Although the individuals selected for this research were not genetically related, the excess of homozygotes observed in most of the breeds may be also indicative of the inbreeding derived from the small effective population sizes generally associated with cattle breeding.

We also performed a neutrality test for *BoLA-DRB3* within each population. In contrast to what was described for other organisms, no excess of alleles is detected in intermediate frequencies, which would be expected if there were balancing selection. Previous results from other cattle breeds have shown variable results (16,31,57). This variability may indicate that selective pressures acting since before domestication may not leave in detectable signatures in the current populations.

The F_{ST} index shows significant differences across all cattle breeds. This is similar to previous results for Latin American breeds (16,31). The differentiation levels are in accordance with what was expected considering the diverse origin of the breeds. Although Nellore and Brahman are both *Bos indicus* breeds, they have a very different historical and geographical origin. Whereas Nellore breed has its origins in India, Brahman was created in the United States from selected and directed crosses between zebu breeds (Guzerat, Nelore, Gyr, Krishna Valley and Sindhi Red). On the other hand, Brangus and Braford are the result of the crosses between Brahman with Angus or Hereford, respectively. The Creole bovine is the descendent of one brought by the conquerors to America, installed in the Caribbean and then scattered throughout the continent. This breed was mainly under natural selection for at least four centuries and it is a *Bos taurus* type.

It is known that individuals or populations having highly divergent *BoLA-DRB3* alleles will have a broader peptide-binding repertoire than those having very similar alleles (62,63). In this sense, AMOVA analysis reveals that the differentiation between breeds is lower when analyzed at the sequence level. This indicates that the populations tend to have alleles more functionally similar, which can eventually decrease the response capacity.

The Nei's genetic distances are visualized by performing UPGMA trees and MDS analysis. The results of the analysis of both methods are concordant and show a strong distinction between *Bos taurus* and *Bos indicus* breeds. In the UPGMA tree, the closer distance between Braford and Brahman, with respect to Brangus, could indicate that the zebuine ancestor used for the creation of Braford was genetically different than the one used for Brangus since intervals of evolutionary times, i. e. the timeframe is too short for the appearance of new alleles. The finding of alleles absent from the Brahman individuals in the mixed breeds may support this hypothesis but further studies are required.

Finally, the results obtained in the phylogenetic analysis performed by constructing Maximum Likelihood trees from *BoLA-DRB3* nucleotide sequences must be taken cautiously because bootstrap values are low, as expected when studying intraspecific variation. We did not find clusters that grouped alleles by breeds and alleles grouped according to their nomenclature as expected (33).

Conclusions

In conclusion, we have genetically characterized five bovine breeds of extensive use in northern Argentina and other subtropical regions of South America. The results show differences across all cattle populations and Braford is the

breed with the highest genetic diversity. However, PBR sequence analysis at the aminoacid level shows no differences in functionality between populations, except for Braford. Altogether, these results could contribute to an ongoing effort to catalogue bovine MHC allele frequency according to breed and location. Moreover, the identification of the alleles present in these breeds could be of great help on the bioinformatic prediction of T cell epitopes for the rational design of vaccines for bovine tropical diseases.

List Of Abbreviations

MHC: Major Histocompatibility Complex

BoLA: Bovine Leukocyte Antigens

dN: number of nonsynonymous nucleotide substitutions per site

dS: number of synonymous nucleotide substitutions per site

PCC: Pearson Correlation Coefficients

MDS: Multidimensional Scaling

AMOVA: Analysis of Molecular Variance

HWE: Hardy-Weinberg equilibrium

ML: Maximum Likelihood

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by the Institutional Committee for Care and Use of Experimental Animals of the National Institute of Agricultural Technology (CICUAE-INTA). Protocol number 35/2010.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MP and SW designed the study. MC and MV carried out experimental procedures. VL, MP, SW and MV analyzed the data. MV, VL and SW wrote the manuscript. All authors approved the final version of the manuscript.

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Figures

Figure 1

Venn diagram showing BoLA-DRB3 allele distribution among cattle breeds according to their major type: Bos Taurus, Bos indicus and mixed breeds.

Figure 2

PBR analysis. (A) Peptide-binding region logos, (B) Pearson correlation coefficients among breeds and (C) metric MDS based on BoLA-DRB3 pocket position variability among populations. (B) shows identical values above and below the diagonal.

Figure 3

Population structuring and relationship. (A) Genetic distance between pairs of breeds estimated by Wright's F statistics (F_{ST}) (above) and Nei's DA distance (below) (B) Nei's distance tree (C) Metric multidimensional scaling analysis (MDS) of BoLA-DRB3 genotypes in five cattle breeds.

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