TISSUE ANTIGENS
IMMUNE RESPONSE GENETICS

Tissue Antigens ISSN 0001-2815

Assessment of biodiversity in Chilean cattle using the distribution of major histocompatibility complex class II *BoLA-DRB3* allele

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Key words
allele frequency; *BoLA-DRB3* allele; Chilean cattle breeds; polymerase chain reaction; sequence-based typing

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Received 26 May 2014; revised 14 October 2014; accepted 28 October 2014

doi: 10.1111/tan.12481

Abstract

Bovine leukocyte antigens (BoLAs) are used extensively as markers for bovine disease and immunological traits. In this study, we estimated *BoLA-DRB3* allele frequencies using 888 cattle from 10 groups, including seven cattle breeds and three crossbreeds: 99 Red Angus, 100 Black Angus, 81 Chilean Wagyu, 49 Hereford, 95 Hereford × Angus, 71 Hereford × Jersey, 20 Hereford × Overo Colorado, 113 Holstein, 136 Overo Colorado, and 124 Overo Negro cattle. Forty-six *BoLA-DRB3* alleles were identified, and each group had between 12 and 29 different *BoLA-DRB3* alleles. Overo Negro had the highest number of alleles (29); this breed is considered in Chile to be an 'Old type' European Holstein Friesian descendant. By contrast, we detected 21 alleles in Holstein cattle, which are considered to be a 'Present type' Holstein Friesian cattle. Chilean cattle groups and four Japanese breeds were compared by neighbor-joining trees and a principal component analysis (PCA). The phylogenetic tree showed that Red Angus and Black Angus cattle were in the same clade, crossbreeds were closely related to their parent breeds, and Holstein cattle from Chile were closely related to Holstein cattle in Japan. Overall, the tree provided a thorough description of breed history. It also showed that the Overo Negro breed was closely related to the Holstein breed, consistent with historical data indicating that Overo Negro is an 'Old type' Holstein Friesian cattle. This allelic information will be important for investigating the relationship between major histocompatibility complex (MHC) and disease.

Introduction

The major histocompatibility complex (MHC) proteins are cell-surface glycoproteins that bind small peptide fragments derived from host- and pathogen-expressed proteins via proteolysis and are conserved in vertebrates (1). In cattle, the MHC is called the bovine leukocyte antigen (BoLA) complex (2). BoLA molecules are divided into class I (expressed by all nucleated cells) and class II (expressed by antigen-presenting cells and lymphocytes). Compared with other mammals, cattle and sheep have an unusual MHC class II gene structure and organization because they lack the *DP* locus and have novel *DY* genes; moreover, a substantial inversion has split the class II region. In cattle, *DRA, DRB3*, at least two *DQA* genes, and two *DQB* genes are considered classical class II DR and DQ molecules (3). Within BoLA class II genes, *BoLA-DRB3* is the most polymorphic and highly expressed (4). Polymorphism within the MHC genes is mainly located in the peptide-binding sites, and the polymorphisms are maintained by balancing selection (5).

One method that has been used to understand the origins of particular human populations is to determine the frequencies of human leukocyte antigen (*HLA*) gene polymorphisms, which differ considerably between ethnic groups (6). Similarly, many studies have shown that BoLA class II allele frequencies differ between cattle breeds. For example, allele frequencies of the *BoLA-DRB3* gene differ between Jersey, Holstein, Argentine Creole, Japanese Shorthorn, Brazilian dairy Gir, and four Japanese breeds (7–14). Recently, a PCR sequence-based

typing (SBT) method was used to genotype *BoLA-DRB3*, and it showed greater discriminatory power and precision than PCR-restriction fragment length polymorphism (RFLP) for estimating *BoLA-DRB3* diversity in cattle breeds (15). Therefore, as in humans and other animal species, estimating the frequencies of MHC class II alleles can help to differentiate among cattle populations, but the number of populations studied remains limited (8, 14, 16, 17).

The Republic of Chile occupies a long, narrow strip of land between the western side of the Andes Mountains and the Pacific Ocean. Cattle farming is one of the main agricultural activities in the southern part of Chile. The cattle stock largely originates from European breeds brought initially by Spanish conquerors and then by subsequent colonists. Since the end of the 19th century, additional genetic imports have been used to improve the existing cattle (18). There are more than four million bovine heads in Chile, mostly dairy or dual-purpose cattle, with only 25% beef cattle in the national herd (19). Most dairy production is from Holstein cattle or Holstein crossbred with Overo Negro cattle, but some is also from Jersey and pure Overo Negro cattle. Recently, Holstein populations were imported from the United States and Canada to Chile and they are considered the 'Present type' Holstein. Beef production in Chile is from Hereford, Overo Colorado, Angus, Limousine, and Simmental populations, although most of the meat produced is from castrated males on dairy farms (20). Recently, the Japanese Black breed was exported worldwide; Chile first imported its genetics in 1998 and the breed has been used ever since for crossbreeding to produce Wagyu-style beef.

During the 19th century, German immigrants brought two dual-purpose breeds that still influence the Chilean herd, namely, the Overo Colorado breed (or Clavel Alemán), which is related to or descended from German Red Pied cattle, and the Overo Negro breed (also called Black Friesian), which is considered in Chile as an 'Old type' European Holstein Friesian descendant. These breeds were selected for, and adapted to, the Chilean environment and have retained or developed characteristics that differentiate them from the modern imported breeds (21). Little molecular work has been done with Chilean cattle (22). One study investigated the population structure and genetic diversity of bovine beef herds of Southern Chile and found that the Overo Colorado breed presents some level of genetic structure.

Crossbreeding is an effective method to resolve the economic consequences of suboptimal cow fertility and survival (23). In Chile, crossbred cattle are mainly used for beef and milk production; therefore to study the distribution of Chilean breeds, the diversity of crossbreeds is also important.

In this study, we collected blood from 940 cattle in Chile, including the following breeds: Red Angus, Black Angus, Chilean Wagyu, Hereford, Holstein, Overo Colorado, Overo Negro, Hereford crossbred with Angus (Hereford × Angus), Hereford crossbred with Jersey (Hereford × Jersey), and Hereford crossbred with Overo Colorado (Hereford × Overo Colorado). We performed PCR-SBT for the *BoLA-DRB3* gene, and the frequencies of *BoLA-DRB3* alleles were analyzed. We determined the evolutionary history of Chilean cattle by computing genetic distances among breeds and characterizing the distribution of the *BoLA-DRB3* alleles within each breed by a principal component analysis (PCA). Finally, we compared the 'Old type' Holstein Friesian breed, Overo Negro, with the 'Present type' breed, Holstein. Collectively, we determined that the 'Present type' Holstein cattle, which were recently introduced into Chile mainly from the United States and Canada, had significantly less *BoLA-DRB3* diversity than the 'Old type'.

Materials and methods

Animals and extraction of genomic DNA

All animals were handled by veterinarians from the RIKEN, Universidad Austral de Chile and LAVET, in strict accordance with good animal practice following the Universidad Austral de Chile Institutional guidelines. This experiment was approved by the Committee on the Ethics of Animals for Research at Universidad Austral de Chile (Certificate No. 153-2014).

Blood samples were taken from 940 cattle: 104 Red Angus, 107 Black Angus, 102 Chilean Wagyu, 50 Hereford, 100 Hereford × Angus, 72 Hereford × Jersey, 20 Hereford × Overo Colorado, 117 Holstein, 140 Overo Colorado, and 128 Overo Negro (Table 1). All cattle samples were collected from the Los Ríos Region, which is in the southern part of the Republic of Chile. Genomic DNA was extracted from 40 µl of whole blood that was spotted onto Whatman® FTA® elute cards (GE Healthcare Japan, Tokyo, Japan), according to the manufacturer's instructions.

BoLA-DRB3 genotyping

BoLA-DRB3 alleles were genotyped using PCR-SBT. Briefly, *DRB3* exon 2 was amplified using PCR according to the method of Takeshima et al. (24) using the DRB3FRW and DRB3REV primers designed by Baxter et al. (25). The PCR fragments were purified using an ExoSAP-IT PCR Product Purification Kit (USB Corp., Cleveland, OH) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The raw sequence data were analyzed using ASSIGN 400ATF ver. 1.0.2.41 software (Conexio Genomics, Fremantle, Australia).

Genetic distances, population tree, and principal components analysis

The genetic structure of Chilean cattle breeds was studied. To evaluate the genetic variation at the *BoLA-DRB3* locus, Nei's D_A distances (26) and Jost's differentiation index D (27) were calculated using the allele frequencies, and a cluster analysis was performed using the neighbor-joining (28)

Table 1 Number of heads sampled in each farm

Breed	City	Farm	Number sampled	Number genotyped
Black Angus	Lago Ranco	Soc. Agri. Ganad. Bandurrias	16	16
	Mafil	Humberto Capurro	54	47
	Pozo Brujo	F. Sn. Pedro	36	36
	Rio Bueno	Soc. Agri. Ganad. Bandurrias	1	1
Red Angus	Futrono	Soc. Agric. Los. Corrales	17	16
	Lago Ranco	Soc. Agri. Ganad. Bandurrias	7	7
	Mafil	Humberto Capurro	16	13
	Pozo Brujo	F. Sn. Pedro	62	61
	Valdivia	CIA/UACH	2	2
Chilean Wagyu	Futrono	Soc. Agric. Los. Corrales	100	79
	Valdivia	CIA/UACH	2	2
Hereford	Futrono	Soc. Agric. Los. Corrales	1	1
	Llifen	Soc. Agric. Nilahue/El Mirador	49	48
HF × Angus	Futrono	Soc. Agric. Sta. Izabel	100	95
HF × Jersey	San Jose	Quinthehuegue	72	71
HF × Overo Colorado	Mafil	Fundo Quineo Sur	20	20
Holstein	Mafil	Fundo Molco	30	29
		Fundo Nahuelmo	4	4
	Rio Bueno	ChinChinco	33	32
		Santa Gema	30	28
	San Jose	Agroganad Purento	20	20
Overo Colorado	Lago Ranco	Fundo Santa Marta	21	19
	Mafil	Fundo Nahuelmo	32	32
	Marihue	Baltazar Patino	6	6
	Pangipulli	Casar Barrocal	8	7
		Soc. El Maiten	13	13
	Valdivia	Agroforestal Maco	30	30
		CIA/UACH	5	5
		F. La Dehesa	25	24
Overo Negro	Marihue	Baltazar Patino	54	52
	Pangipulli	Casar Barrocal	19	17
		Soc. El Maiten	33	33
	Rehumen	Cesar Pinner	20	20
	Valdivia	CIA/UACH	2	2
		Total	940	888

HF, Hereford.

algorithm. Confidence in the groupings was estimated by bootstrap re-sampling of the data using 1000 replications. Nei's D_A and phylogenetic trees were estimated using POPTREE2 software (29). Jost's D was calculated using the 'DEMEtics' package (30) implemented in the R statistical environment. The PCA was performed using PAST software (31). In these analyses, previously characterized Japanese cattle were also used, including 102 Holstein, 69 Jersey, 100 Japanese Shorthorn, and 200 Japanese Black samples (16).

Results and discussion

Identification of *BoLA-DRB3* alleles in Chilean cattle breeds

Blood samples were taken from 940 cattle from 21 farms in the southern area of Chile (Table 1); however, for 52 samples, genomic DNA could not be obtained from the FTA elute card. The remaining 888 blood samples came from 10 groups: Red Angus ($n = 99$), Black Angus ($n = 100$), Chilean Wagyu ($n = 81$), Hereford ($n = 49$), Hereford × Angus ($n = 95$), Hereford × Jersey ($n = 71$), Hereford × Overo Colorado ($n = 20$), Holstein ($n = 113$), Overo Colorado ($n = 136$), and Overo Negro ($n = 124$). These 10 groups were genotyped by *BoLA-DRB3* PCR-SBT.

The *BoLA-DRB3* exon 2 genotype was investigated in the 888 cattle (Table 2). Forty-six *BoLA-DRB3* alleles were identified. Red Angus and Overo Negro were the most polymorphic groups, each with 29 different alleles, while the other eight Chilean cattle groups had between 12 and 27 alleles.

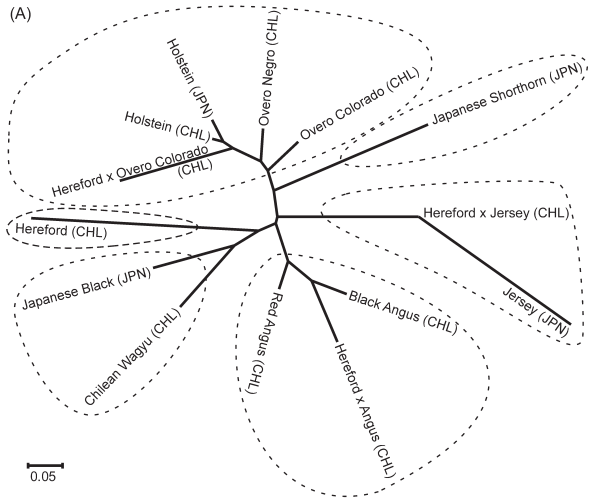
To determine how the 10 groups differed in terms of *BoLA-DRB3* allelic variation, we compared the frequencies of *BoLA-DRB3* alleles among groups (Table 2). High-frequency alleles were found in some strains, as follows: Black Angus cattle and Hereford × Angus cattle each had a high frequency of *BoLA-DRB3*0201* (21.5% and 23.2%, respectively); Chilean Wagyu cattle had a high frequency of *BoLA-DRB3*1001*

Table 2 *BoLA-DRB3* allele frequencies for 10 cattle breeds in Chile

BoLA	Black	Chilean	Hereford	Hereford	Hereford	Hereford	Holstein	Overo	Overo	Red
-*DRB3* allele	Angus	Wagyu		× Angus	× Jersey	× O. Col.		Colorado	Negro	Angus
	n = 100	*n* = 81	*n* = 49	*n* = 95	*n* = 71	*n* = 20	*n* = 113	*n* = 136	*n* = 124	*n* = 99
**0101*	12.5	4.9	0.0	4.7	2.8	17.5	12.4	4.0	6.0	12.6
**0201*	**21.5**	8.0	4.1	**23.2**	12.0	0.0	3.5	5.9	4.4	9.1
**0301*	1.0	0.6	0.0	0.0	3.5	2.5	0.0	2.9	0.4	1.0
**0401*	0.0	0.6	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5
**0501*	0.5	1.9	0.0	4.7	0.7	0.0	0.0	2.6	0.0	3.5
**0503*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0
**0601*	0.0	0.6	0.0	0.5	0.0	0.0	1.3	0.4	2.0	0.0
**0701*	1.0	2.5	0.0	4.2	4.9	2.5	2.2	7.0	6.0	8.6
**0801*	3.0	0.6	0.0	0.5	0.7	0.0	0.4	0.4	1.6	5.1
**0901*	1.5	1.2	2.0	6.8	2.1	2.5	0.9	2.2	0.4	2.5
**0902*	0.0	1.9	1.0	0.0	4.2	**27.5**	8.0	8.8	8.5	0.5
**1001*	1.5	**16.0**	1.0	1.6	0.7	0.0	8.8	1.5	**10.5**	2.0
**1002*	0.5	1.9	8.2	9.5	0.0	0.0	0.0	0.7	0.4	0.0
**1101*	10.0	8.0	0.0	1.1	3.5	7.5	11.9	6.3	8.9	1.5
**1103*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	1.6	0.0
**1201*	0.5	0.0	0.0	0.0	2.8	5.0	8.0	2.9	3.6	1.5
**1301*	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	2.8	1.0
**1302*	0.0	**16.0**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
**14011*	2.5	1.9	12.2	2.6	3.5	15.0	9.3	**9.9**	9.7	3.0
**1501*	5.0	3.1	3.1	4.7	3.5	7.5	**21.7**	9.2	7.3	8.1
**1601*	3.5	10.5	**22.4**	3.7	7.7	0.0	1.3	3.3	4.0	7.6
**1701*	1.0	2.5	1.0	1.1	2.8	7.5	1.8	7.0	4.0	1.5
**1801*	9.5	4.9	8.2	16.3	2.1	0.0	0.9	5.9	4.8	**14.1**
**1901*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0
**20012*	0.0	0.0	12.2	0.0	0.0	0.0	0.4	0.4	0.0	0.0
**2002*	0.0	0.0	0.0	0.0	0.0	0.0	0.9	7.0	0.8	1.5
**2003*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
**2006*	0.0	0.0	0.0	0.0	7.7	0.0	0.0	0.0	0.0	0.0
**2201*	0.0	0.0	0.0	0.0	0.0	0.0	0.4	2.6	2.4	0.0
**2403*	1.5	0.0	0.0	0.5	0.7	0.0	0.0	0.0	0.0	0.5
**2502*	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0
**2601*	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
**2703*	0.5	2.5	1.0	1.6	7.7	2.5	4.9	1.8	4.8	3.0
**2705*	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
**2707*	1.5	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
**2710*	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.5
**2801*	1.0	0.0	0.0	0.0	0.0	2.5	0.0	3.3	0.4	0.0
**2802*	1.5	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.4	1.5
**3101*	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.8	0.0
**3201*	10.0	1.9	0.0	6.8	0.0	0.0	0.4	1.1	0.0	2.5
**3202*	0.5	1.9	21.4	2.1	0.0	0.0	0.0	0.0	0.4	0.0
**3301*	7.0	0.6	0.0	2.6	0.7	0.0	0.0	0.0	0.0	2.0
**3601*	0.0	0.0	1.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0
**3701*	0.0	2.5	0.0	0.0	4.2	0.0	0.0	0.0	0.0	2.0
**4401*	0.0	0.0	1.0	0.0	4.2	0.0	0.0	0.0	0.4	0.0
**4501*	1.0	0.0	0.0	0.0	**14.8**	0.0	0.0	0.0	0.0	0.0
Allele number										
	26	27	15	22	25	12	21	27	29	19

n, number of animals; bold face, most frequent allele in each group; underlined, frequent allele (≥10.0%).

and *DRB3*1301* (each 16.0%); Hereford cattle had a high frequency of *BoLA-DRB3*1601* (22.4%); Hereford × Jersey cattle had a high frequency of *BoLA-DRB3*4501* (14.8%); Hereford × Overo Colorado cattle had a high frequency of *BoLA-DRB3*0902* (27.5%); Holstein cattle had a high frequency of *BoLA-DRB3*1501* (21.7%); Overo Colorado cattle had a high frequency of *BoLA-DRB3*14011* (9.9%); Overo Negro cattle had a high frequency of *BoLA-DRB3*1001* (10.5%); and Red Angus cattle had a high frequency of *BoLA-DRB3*1801* (14.1%).



(B)

Breed	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0.28	0.54	0.15	0.33	0.52	0.33	0.37	0.59	0.42	0.31	0.33	0.15	0.44
2		0.44	0.27	0.38	0.54	0.32	0.34	0.66	0.53	0.33	0.26	0.24	0.24
3			0.37	0.54	0.70	0.56	0.55	0.80	0.70	0.47	0.48	0.52	0.54
4				0.40	0.64	0.42	0.47	0.68	0.53	0.31	0.37	0.18	0.50
5					0.43	0.35	0.34	0.22	0.45	0.32	0.31	0.28	0.38
6						0.19	0.23	0.85	0.42	0.23	0.27	0.46	0.42
7							0.05	0.76	0.40	0.17	0.11	0.28	0.20
8								0.72	0.39	0.24	0.15	0.35	0.19
9									0.65	0.68	0.68	0.60	0.66
10										0.33	0.39	0.35	0.46
11											0.13	0.22	0.32
12												0.24	0.22
13													0.37

1,Black Angus (CHL)
2,Wagyu (CHL)
3,Hereford (CHL)
4,Hereford x Angus (CHL)
5,Hereford x Jersey (CHL)
6,Hereford x Ovelo Colorado (CHL)
7,Holstein (CHL)
8,Holstein (JPN)
9,Jersey (JPN)
10,Japanese Shorthorn (JPN)
11,Overo Colorado (CHL)
12,Overo Negro (CHL)
13,Red Angus (CHL)
14,Japanese Black (JPN)

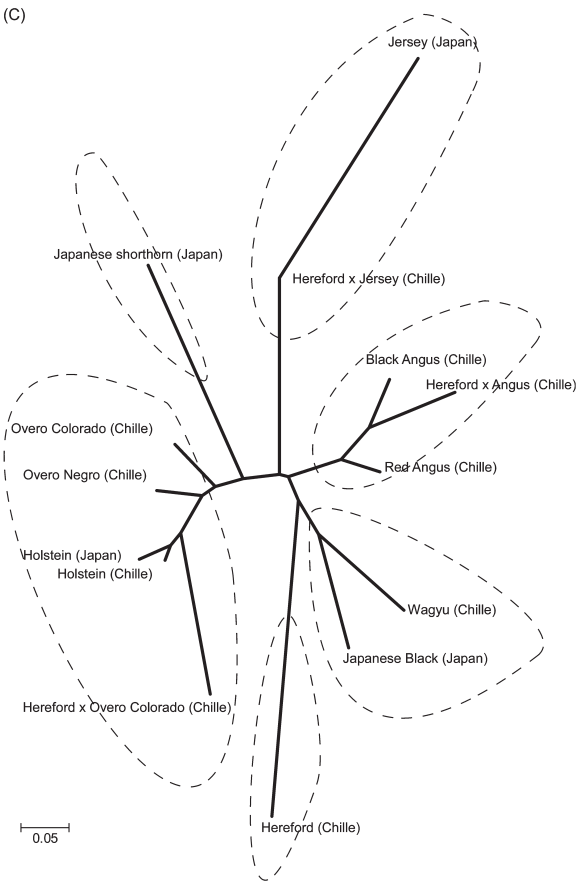


Figure 1 Relationships among populations for 10 cattle breeds in Chile and four in Japan based on *BoLA-DRB3* allele frequencies. A phylogenetic tree (A) showing the relationships between 10 cattle breeds from Chile (designated as CHL) and 4 Japanese breeds (Holstein, Japanese Black, Japanese Shorthorn, and Jersey; designated as JPN) was constructed from a matrix of D_A genetic distances (B). A phylogenetic tree (C) showing the relationships between 10 cattle breeds from Chile and 4 Japanese breeds was constructed from a matrix of Jost's differentiation index D.

In contrast to Holstein cattle, which are referred to as the 'Present type' and generally have a low number of *BoLA-DRB3* alleles (21 alleles in this study and 18 in the Japanese study (16)), Overo Negro, the 'Old type' Holstein Friesian breed, had the highest number of alleles (29). As shown in Table 2, 10 alleles, namely, *BoLA-DRR3*0301*, **0503*, **1002*, **1103*, **1301*, **1901*, **2801*, **2802*, **3202*, and **4401*, were detected in Overo Negro but not in Holstein cattle. Only one allele, *BoLA-DRB3*20012*, was detected in Holstein but not in Overo Negro cattle.

We also collected samples from Hereford cattle, which are used for crossbreeding with other Chilean breeds such as Angus, Jersey, and Overo Colorado (Table 1); however, the four alleles with the highest frequency (>10%) in Hereford cattle (*DRB3*14011*, *DRB3*1601*, *DRB3*20012*, and *DRB3*3202*) were not detected at a high frequency in the three Hereford crossbreeds, except *DRB3*14011* (15.0%) in Hereford × Overo Colorado (Table 2). In this study, we collected 48 of 49 Hereford samples from a single farm, and this collection strategy likely resulted in biased Hereford *BoLA-DRB3* allele frequencies. Further sampling is needed to obtain unbiased allele frequency estimates for the Chilean Hereford breed.

Relationships among populations based on the frequencies of *BoLA-DRB3* alleles

Genetic distance can be used as a tool for comparing evolutionary divergence between two populations, and in cattle it is related to factors such as breeding history, geology, and breeding objectives (32). To assess the genetic relationships between the cattle groups, we estimated Nei's D_A (26) using the *BoLA-DRB3* allele frequencies of each group, and constructed a neighbor-joining tree (Figure 1A, B). The population tree included the 10 Chilean cattle groups and 4 Japanese breeds for which we have previously determined the *BoLA-DRB3* allele frequencies (32). These 14 cattle groups were divided into six major clusters as follows: (1) Jersey-influenced clade (Jersey and Jersey × Hereford), (2) Angus-influenced clade (Red, Black, and Hereford × Angus), (3) Japanese Black-influenced clade (Chilean Wagyu and Japanese Black), (4) clade of

Holstein and related breeds (Holstein, Overo Negro, Overo Colorado, and Overo Colorado × Hereford), (5) Japanese Shorthorn cluster, and (6) Hereford cluster. To confirm this result, we also estimated the population tree using Jost's D (27), and confirmed that the 14 cattle groups were divided into six major clusters, consistent with those estimated using Nei's D_A. The population tree showed that populations within a breed clustered, and that breeds clustered with their crossbreeds, for example, Holstein populations from Chile and Japan, Chilean Wagyu and Japanese Black, Jersey and Hereford × Jersey, Black Angus, and Red Angus with Hereford × Angus. This suggests that the tree provides a good description of the genetic history of the breeds. The tree also indicated that the Angus and Jersey breeds are genetically similar and that Holstein populations are more distantly related to Angus than to Hereford. These relationships were consistent with those reported by MacHugh et al. (33), who analyzed samples from breeds at their places of origin. Therefore, we consider that the current allele frequency distribution of the Chilean breeds reflects that of the original breeds. In addition, both the Overo Colorado and Overo Negro breeds were placed within the same cluster as Holstein populations. Historical data suggest that Overo Negro is the 'Old type' Holstein Friesian cattle imported to Chile at the end of the 19th century by German settlers, while Overo Colorado, a dual-purpose breed, is related to the German Red Pied (Deutsche Rotbunte) (21).

Previously, we reported that Holstein and Japanese Black cattle are the most closely related in a comparison of Jersey and Japanese Shorthorns (16). However, in this study, Japanese Black cattle did not cluster with Holstein cattle because the 14 breeds used to construct the population tree enabled a more precise analysis.

Principal components analysis based on the frequencies of *BoLA-DRB3* alleles

Unlike the analysis based on genetic distances, PCA cannot compute evolutionary history, but it is a good method for characterizing the current distribution of alleles. Because the diversity of MHC alleles is important for estimating the immunological background of each population, PCA is also used for characterizing the distribution of MHC alleles (9, 34). We used PCA to analyze the *BoLA-DRB3* allelic diversity for each cattle group, and the results are shown in Figure 2, which illustrates the first and second principal components (PCs) (Figure 2A) and the third and fourth PCs (Figure 2C) for *BoLA-DRB3* allele frequencies. The first two PCs accounted cumulatively for only 48.01% of the variability in the data (the variance for PC1 was 29.48% and for PC2 was 18.53%). Therefore, we also illustrated the third and fourth PCs, and these four components accounted cumulatively for 74.69% of the variance (the variance for PC3 was 14.14% and for PC4 was 12.54%).

The first PC (Figure 2B) showed a clear pattern of differentiation between British breeds (Angus, Hereford, and Jersey) and continental breeds (Holstein and related breeds). The Angus breed originated in the northeast of Scotland, the Hereford breed were bred in the English county of Herefordshire, and the Jersey breed originated in Jersey, a small British island in the English Channel, off the coast of France. The original stock of Holstein were the black and white animals of the Batavians and Friesians, migrant European tribes who settled in the Rhine delta region approximately 2000 years ago, and finally in the Netherlands (Breed of Livestock website: http://www.ansi.okstate.edu/breeds/cattle/). The PC1 in this study, similar to the results of MacHugh et al. (33), shows the differences between Chilean breeds originating from the British islands and European continental breeds. Japanese Black and similar breeds (Chilean Wagyu) were intermediately related to the British and European breeds (Figure 2A). Sixteen alleles strongly contributed to PC1 (loading value >|0.1|), nine with a positive value (*BoLA-DRB3*0201*, **1002*, **1601*, **1801*, **2006*, **2502*, **3202*, **3701*, and **4501*) and seven with a negative value (*BoLA-DRB3*0101*, **0902*, **1001*, **1101*, **1201*, **14011*, and **1501*) (Figure 2B). The Hereford × Overo Colorado group showed an extremely low PC1 value because it had a high frequency of *DRB3*0902*; however, sampling of this crossbreed included only 20 animals from a single farm, so the results are likely affected by sampling error. Therefore, the allele frequencies of Hereford × Overo Colorado cattle should be measured in future studies using a greater sample size.

The second PC showed a high degree of differentiation between the Jersey and Hereford groups (Figure 2A). Seventeen alleles strongly contributed to PC2 (loading value >|0.1|), nine with a positive value (*BoLA-DRB3*0101*, **0201*, **0301*, **0801*, **1201*, **2006*, **2502*, **3701*, and **4501*) and eight with a negative value (*BoLA-DRB3*1001*, **1002*, **1302*, **14011*, **1601*, **1801*, **20012*, and **3202*) (Figure 2B).

The third PC showed separated Jersey and Hereford from Red Angus and Black Angus cattle, with the remaining groups in intermediate positions (Figure 2C). PC3 (loading value >|0.1|) was determined by 15 alleles, seven with a positive value (*BoLA-DRB3*0101*, **0201*, **0501*, **1501*, **1801*, **3201*, and **3301*) and eight with a negative value (*BoLA-DRB3*0902*, **1601*, **20012*, **2006*, **2502*, **3202*, **3701*, **4401*, and **4501*) (Figure 2D).

The fourth PC showed patterns that clearly differentiated Japanese Black/Chilean Wagyu (originated from Japanese Black) from the other groups (Figure 2C). Eleven alleles strongly contributed to PC4 (loading value >|0.1|), seven with a positive value (*BoLA-DRB3*0301*, **0902*, **14011*, **1701*, **1801*, **20012*, and **3201*) and four with a negative value (*BoLA-DRB3*1001*, **1101*, **1302*, and **1501*) (Figure 2D).

Figure 2A shows that Jersey cattle presented high values for PC1 and PC2. Alleles *DRB3*0201* and *DRB3*4501*, which strongly influenced the PC1 value, were detected in Jersey cattle from Japan with high frequencies (16.7% and 18.1%, respectively) (16). Those same alleles were also detected in the Hereford × Jersey crossbreed at high frequencies (12.0% and

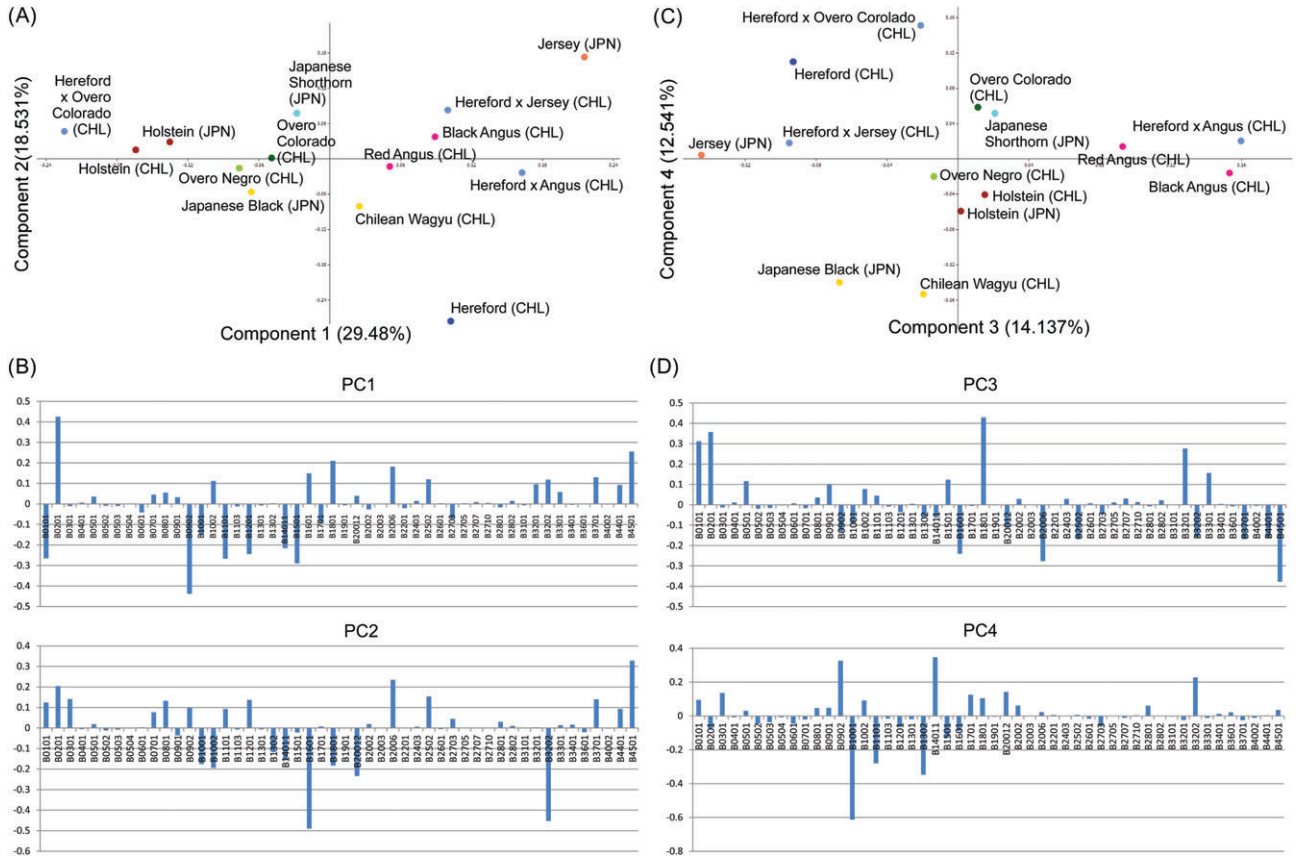


Figure 2 Principal component analysis of 10 cattle breeds in Chile and four in Japan based on *BoLA-DRB3* allele frequencies. (A) A principal component analysis (PCA) plot, in which 10 cattle breeds in Chile and 4 in Japan are plotted according to the eigenvectors corresponding to the first (PC1) and second (PC2) principal components. The dot colors represent different breeds. (B) The loading values of PC1 and PC2 calculated by PCA. (C) A PCA plot, in which 10 breeds in Chile and 4 in Japan are plotted according to the eigenvectors corresponding to the third (PC3) and fourth (PC4) principal components. The dot colors represent different breeds. (D) The loading values of PC3 and PC4 as calculated by PCA analysis.

14.8%, respectively) (Table 2). *DRB3*4501* was rarely detected in other groups in this and other studies (8, 12, 14, 16, 17). Figure 2A also shows that Hereford was characterized by a low PC2 value, which was strongly influenced by the frequencies of *BoLA-DRB3*1601* and *DRB3*3202*. *DRB3*1601* was also detected at a high frequency in Japanese Black (16) and in Chilean Wagyu cattle (Table 2).

Figure 2A (PC1 and PC2) clearly shows the genetic structure of the crossbreed populations. Hereford × Angus was positioned between Black Angus and Hereford populations, Hereford × Jersey was positioned between Jersey and Hereford, and Chilean Wagyu was positioned between Angus and Japanese Black. In Chile, imported Japanese Black semen was used with Angus females to make crossbreeds and thus the Chilean Wagyu breed was greatly influenced by Angus; however, Chilean Wagyu still retains many genes of the Japanese Black breed, as shown in Figure 2C where Japanese Black and Chilean Wagyu clustered in low PC4 values. PC4 was mainly constructed with the frequencies of *BoLA-DRB3*1001*, *DRB3*1101*, and *DRB3*1302* (Figure 2D). A high frequency of *DRB3*1302* was only detected in Japanese Black cattle (16), which suggests that this allele arose after native Japanese cattle became isolated from other breeds.

Overo Negro, which is an 'Old type' Holstein Friesian breed, had much higher diversity at the *DRB3* allele than the 'Present type' Holstein breed, which was recently introduced into Chile (29 alleles for Overo Negro and 21 alleles for Holstein) (Table 1). However, Overo Negro cattle were closely related to the Holstein group for all clustering methods used in this study, including methods based on genetic diversity and a four-component PCA analysis (Figures 1 and 2). To determine whether the difference between Overo Negro and Holstein populations were due to sampling effect, we divided the samples by farm and performed a PCA analysis (Figure 3A). Almost all farms clustered in the low Component 1 area expect Overo Negro number 4 farm, which had a sample size of only two cattle, suggesting that the allele frequencies represented by Holstein and Overo Negro farms were similar. We compared the number of alleles detected among farms (Figure 3B). Because the number of alleles was influenced by population size, we

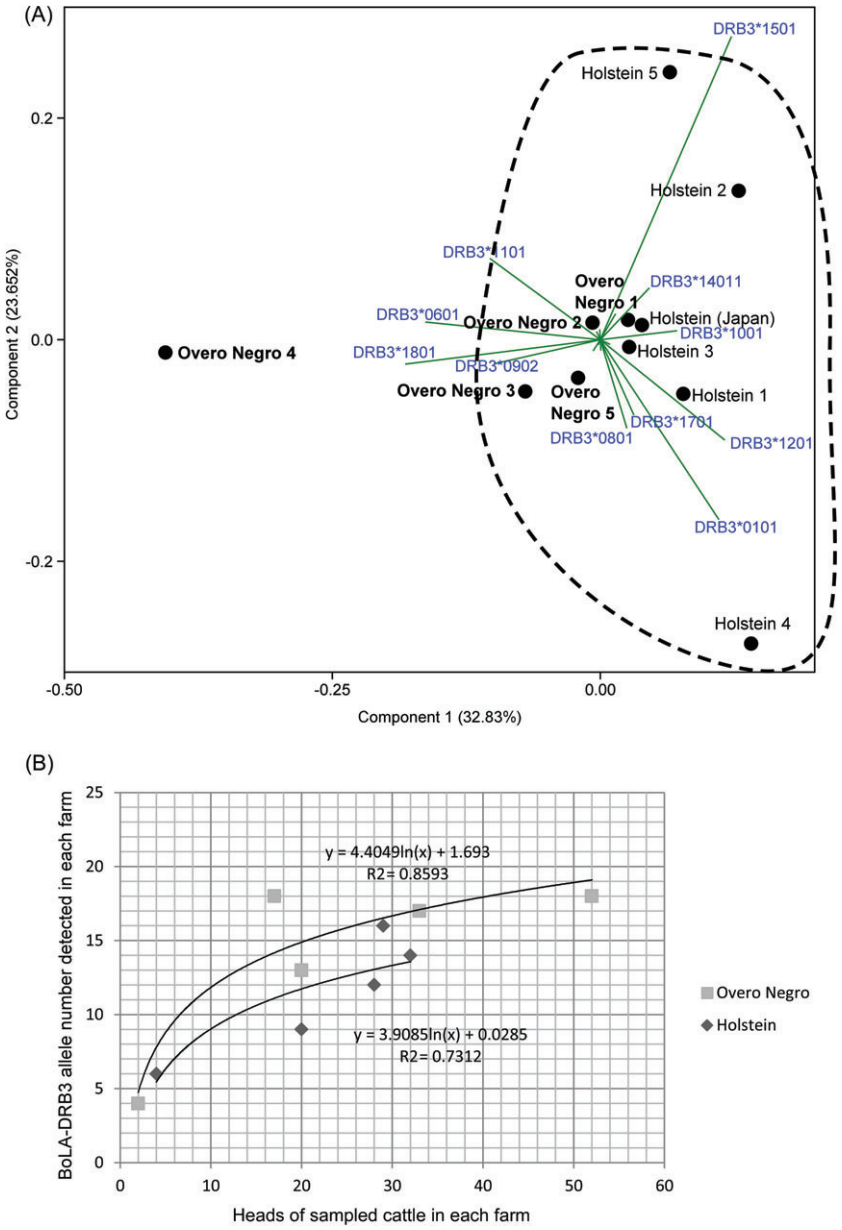


Figure 3 Comparison of the allele frequencies between Holstein and Overo Negro farms. (A) A principal component analysis (PCA) of five Overo Negro farms, five Holstein farms and Japanese Holstein cattle based on *BoLA-DRB3* allele frequencies. The farm names (and numbers of heads) used in this study were as follows: Overo Negro 1, Baltazar Patino (52 heads); Overo Negro 2, Caser Barrocal (17 heads); Overo Negro 3, Cesar Pinner (20 heads); Overo Negro 4, CIA/UACH (2 heads); Overo Negro 5, Soc. El Maiten (33 heads); Holstein 1, Agroganad Purento (20 heads); Holstein 2, ChinChinco (32 heads); Holstein 3, Fundo Molco (29 heads); Holstein 4, Fundo Nahuelmo (4 heads); Holstein 5, Santa Gemma (28 heads). (B) A scatter plot shows the number of *BoLA-DRB3* alleles detected in each farm (*y*-axis) and the number of heads sampled for each farm (*x*-axis).

used a regression analysis to estimate the relationship between the number of alleles and the sample size. Figure 3B clearly shows that fewer alleles were detected in Holstein farms than in Overo Negro farms. This result indicates that the Holstein breed may have lost a number of *DRB3* alleles that have been retained over time in the Chilean breed. This pattern can occur for a few reasons, for example, if (1) high selection pressure in present-day Holstein cattle reduced *BoLA-DRB3* diversity, (2) imported Holstein genes represented only a portion of the total Holstein diversity, or (3) Overo Negro acquired the alleles through interbreeding.

MHC diversity is important for pathogen and parasite resistance (35); in particular, the expressions of genes in the MHC class IIa region containing all functional class II genes, which are associated with antigen-specific acquired immunity, are strongly correlated with each other (36). Consistent with this, several infectious diseases are associated with polymorphism in the class IIa region in cattle (3); however, polymorphism is low in domesticated and artificially selected populations, especially in livestock. Therefore, assessing biodiversity using MHC class IIa genes as markers is important. In cattle, the current level of diversity within breeds is as great as that observed within humans (37), and diversity is maintained when breeds are imported. We consider it important to use cattle gene pools worldwide for artificial selection to conserve biodiversity.

In conclusion, we genotyped the *BoLA-DRB3* locus in Chilean cattle breeds and characterized the allele frequency distribution for each breed by evolutionary methods and a

multivariate analysis. The structure of the population tree provided a thorough description of breed history, and we successfully estimated the genetic distances among breeds based on *BoLA-DRB3* frequencies. We also investigated the *BoLA-DRB3* alleles by a PCA. *BoLA-DRB3* alleles have been associated with susceptibility to several diseases, such as mastitis, bovine leukemia, and dermatophilosis (3). Therefore, the allelic information for *BoLA-DRB3* in each breed will be important for investigating the relationship between host factors and disease.

Acknowledgments

We thank the veterinarians in Valdivia and members of CIA, Universidad Austral de Chile, for kindly assisting with sampling from many farms in Chile. We are grateful to Drs. Jiyun Kim, Tomoyuki Murakami, Kazunori Yamada, Mariluz Arainga, and Mayuko Jimba for helpful experimental assistance. We are grateful to the Support Unit at the Bio-material Analysis, RIKEN BSI Research Resources Center, for help with the sequence analysis. This work was supported by Grants-in-Aid for Scientific Research (A, B, and C) from the Japan Society for the Promotion of Science (JSPS), and by a grant from the Program for the Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry of Japan.

Conflict of interest

Authors have no conflict of interest to declare.

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