



## The great diversity of major histocompatibility complex class II genes in Philippine native cattle



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### ABSTRACT

Bovine leukocyte antigens (BoLA) are extensively used as markers for bovine disease and immunological traits. However, none of the BoLA genes in Southeast Asian breeds have been characterized by polymerase chain reaction (PCR)-sequence-based typing (SBT). Therefore, we sequenced exon 2 of the BoLA class II *DRB3* gene from 1120 individual cows belonging to the Holstein, Sahiwal, Simbrah, Jersey, Brahman, and Philippine native breeds using PCR-SBT. Several cross-breeds were also examined. *BoLA-DRB3* PCR-SBT identified 78 previously reported alleles and five novel alleles. The number of *BoLA-DRB3* alleles identified in each breed from the Philippines was higher (71 in Philippine native cattle, 58 in Brahman, 46 in Holstein × Sahiwal, and 57 in Philippine native × Brahman) than that identified in breeds from other countries (e.g., 23 alleles in Japanese Black and 35 in Bolivian Yacumeño cattle). A phylogenetic tree based on the  $D_A$  distance calculated from the *BoLA-DRB3* allele frequency showed that Philippine native cattle from different Philippine islands are closely related, and all of them are closely similar to Philippine Brahman cattle but not to native Japanese and Latin American breeds. Furthermore, the *BoLA-DRB3* allele frequency in Philippine native cattle from Luzon Island, located in the Northern

**Abbreviations:** MHC, major histocompatibility complex; SBT, sequence-based typing; BoLA, bovine MHC; PCR, polymerase chain reaction; HLA, human leukocyte antigen.

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Philippines was different from that in cattle from Iloilo, Bohol, and Leyte Islands, which are located in the Southern Philippines. Therefore, we conclude that Philippine native cattle can be divided into two populations, North and South areas. Moreover, a neutrality test revealed that Philippine native cattle from Leyte showed significantly greater genetic diversity, which may be maintained by balancing selection. This study shows that Asian breeds have high levels of *BoLA-DRB3* polymorphism. This finding, especially the identification of five novel *BoLA-DRB3* alleles, will be helpful for future SBT studies of *BoLA-DRB3* alleles in East Asian cattle.

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## 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. MHC molecules are divided into class I (expressed by all nucleated cells) and class II (expressed by antigen presenting cells and lymphocytes). MHC class I is recognized by CD8 positive cytotoxic T cells and MHC class II by CD4 helper T cells. Binding of peptides with MHC molecules initiates acquired immune responses. Therefore, MHC molecule polymorphism results in diverse immune responses (Germain and Jenkins, 2004).

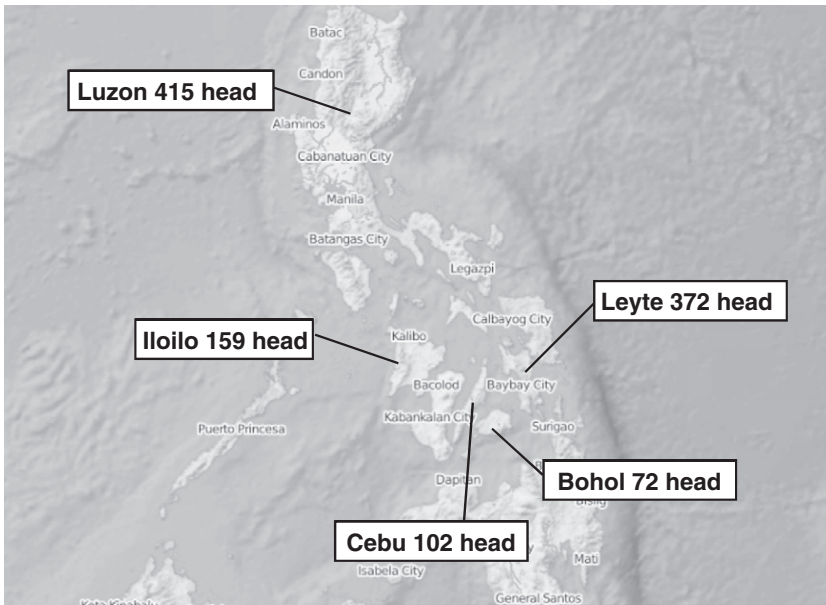
The MHC system in cattle, known as the bovine leukocyte antigen (BoLA) located in chromosome 23, is highly polymorphic and forms an important component of the immune system (Ellis and Ballingall, 1999). The *BoLA-DRB3* gene is the strongest expressed gene with the highest polymorphism level of class II locus in cattle (Aida, 1995), and influences both the magnitude and epitope specificity of antigen-specific T cell responses to infectious diseases. Indeed, 130 *BoLA-DRB3* alleles have been identified in various breeds of cattle by sequencing of cloned genomic DNA, cDNA, or cloned polymerase chain reaction (PCR) products (Aida et al., 1995; Miyasaka et al., 2011; Takeshima et al., 2001, 2002, 2003). These alleles are listed in the Immuno Polymorphism Database (IPD)–MHC database (<http://www.ebi.ac.uk/ipd/mhc/bola/index.html>). *BoLA-DRB3* polymorphisms are associated with differences in susceptibility to infectious diseases (e.g., bovine leukemia virus-induced lymphocytosis, mastitis, and dermatophilosis), immunological conditions (according to 20 indicator traits of innate and adaptive immunity), and vaccine responses (e.g., foot-and-mouth disease and *Theileria parva*) (Ballingall et al., 2004; Baxter et al., 2009; Dietz et al., 1997a,b; Maillard et al., 2002; Miyasaka et al., 2013; Sharif et al., 1998; Takeshima et al., 2008b).

The difference between MHC molecules in wild and domestic cattle 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 East Asia and Latin America, which showed significant differences in the degree of polymorphism (e.g., 18 alleles were determined from 102 Holstein cattle in Japan and 36 alleles were detected from 113 Yacumeño cattle in Bolivia) (da Mota et al., 2002, 2004; Giovambattista et al., 1996, 2001, 2013; Lee et al., 2012; Miyasaka et al., 2011; Takeshima et al., 2003, 2008a).

The number of Philippine cattle was estimated to be 836,300 head in 2011 (FAOSTAT; <http://faostat.fao.org/>), with most being raised in backyard farms. Philippine native cattle are predominantly descended from Chinese and Mexican cattle, which were brought into the country by the Chinese and Spanish (Porter and Mason, 2002). It is thought that they originated from the yellow cattle of Southern China, which evolved from the *Bos indicus* in Java and the Brahmin archipelago (Payne, 1970).

Philippine cattle have been characterized using molecular markers for mitochondrial DNA (Watanabe et al., 1989) and by blood group DNA typing (Namikawa et al., 1984); however, the BoLA genes have not been characterized. Therefore, the present study used PCR-sequence-based typing (SBT) to examine the frequency and distribution of *BoLA-DRB3* genes within Philippine native cattle breeds that become highly adapted to living on certain islands. PCR-SBT can identify specific *BoLA-DRB3* alleles at the nucleotide sequence level, allowing the accurate detection of *DRB3* alleles (Lee et al., 2012; Miyasaka et al., 2011,

(A)



(B)

Island	Breed	Number of investigated cow
Luzon	Brahman	184
	Holstein	3
	Holstein x Sahiwal	151
	Native	29
	Native x Brahman	48
Leyte	Brahman	52
	Brahman x Sahiwal	3
	Brahman x Simbrah	1
	Native	267
	Native x Brahman	47
	Native x Holstein	2
Bohol	Native	66
	Native x Brahman	5
	Native x Holstein	1
Iloilo	Native	120
	Native x Brahman	32
	Native x Holstein	4
	Native x Holstein x Brahman	1
	Native x Holstein x Sahiwal	2
Cebu	Holstein x Jersey	1
	Holstein x Sahiwal	98
	Holstein x Brahman x Sahiwal	3

**Fig. 1.** (A) Map of the Philippine islands showing the number of cattle from each that were included in the study. (B) The number of cattle on each island belonging to each breed.

2012; Takeshima et al., 2001, 2002, 2009a,b, 2011). Furthermore, we compared the *DRB3* allele frequency data with those derived from other cattle breeds to examine the degree of genetic diversity and genetic distance between breeds (Bolivian Yacumeño, Holstein, Japanese Shorthorn and Japanese Black) (Giovambattista et al., 2013; Miyasaka et al., 2011; Takeshima et al., 2002, 2003). This is the first study to use SBT to characterize the *BoLA-DRB3* gene in Philippine cattle.

## Materials and methods

### *Animals and extraction of genomic DNA*

Blood samples were taken from 1120 cattle: 236 Brahman cattle, 249 Holstein × Sahiwal cattle, 482 Philippine native cattle, 132 Philippine native × Brahman, seven Philippine native × Holstein, one Philippine native × Holstein × Brahman, two Philippine native × Holstein × Sahiwal, two Sahiwal × Brahman, one Simbrah × Brahman, one Holstein × Jersey, one Brahman × Sahiwal, three Holstein, and three Holstein × Brahman × Sahiwal. The samples were collected from Luzon Island, Cebu Island, Bohol Island, and Leyte Island (Fig. 1). Genomic DNA was extracted from 40 µl of whole blood spotted onto FTA elute cards (Whatman, Tokyo, Japan) according to manufacturer's instructions.

### *BoLA-DRB3 typing*

*BoLA-DRB3* alleles were genotyped using PCR-SBT. Briefly, *DRB3* exon 2 was amplified using two different methods to avoid mistyping: 1) single PCR (Takeshima et al., 2011) and 2) allele group-specific PCR (Takeshima et al., 2001). Single PCR was performed according to the method of Takeshima et al. (2011) using primers *DRB3FRW* and *DRB3REV*, which were designed by 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). The raw sequence data were analyzed using Assign 400ATF ver. 1.0.2.41 software (Conexio Genomics, Fremantle, Australia). When the sequences could not be splitted into two known alleles, we predicted the existence of new allele in the cattle, and determined the sequences as following procedure.

### *Identification of new alleles by PCR cloning*

When new alleles were identified, the PCR products were cloned into pBluescriptII SK(+), and the PCR products of at least three positive clones were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits. The sequence data were then analyzed using Assign 400ATF ver. 1.0.2.41 software. Finally, the predicted alleles which are derived from >1 individual were determined as new allele.

### *Measures of genetic variability*

The allele frequencies and the number of alleles ( $n_a$ ) were obtained by direct counting. The observed ( $h_o$ ) and unbiased expected heterozygosity ( $h_e$ ) for the *BoLA-DRB3* locus were estimated according to the method of Nei (1978) using the ARLEQUIN 3.5 software for population genetic analyses (Schneider et al., 2000).

### *Genetic distances, population tree, and principal components analysis*

To evaluate the genetic variation revealed by the *BoLA-DRB3* polymorphisms, Nei's  $D_A$  distances (Nei et al., 1983) were calculated from the allele frequencies and cluster analysis performed using neighbor-joining (NJ) (Saitou and Nei, 1987) algorithms. Confidence in the groupings was estimated by bootstrap re-sampling of

**(A)**

	1								80
DRB3*1601	TCTCTCTGCA	GCACATTTC	TGGAGTATAC	CAAGAAAGAG	TGTCATTTC	TCAACGGGAC	CGAGCGGGTG	CGGTTCTCTGG	
DRB3*7201	.....	.....	.....T.	T....GC...	.....	.....	.....	.....	
DRB3*7301	.....	.....	.....G.	T.C..GC...	.....	.....	.....	.....C	
DRB3*2008	.....G	.....	.....	..CA T...GGC...	.....	.....	.....	.....G...	
DRB3*3403	.....	.....	.....TG	T...GC...	.....	.....	.....	.....	
DRB3*50011	.....	.....	.....T.	T.C.GGC...	.....	.....	.....	.....A...	
	81								160
DRB3*1601	ACAGATACTT	CCATAATGGA	GAAGAGTTCG	TGCGCTTCGA	TAGCGACTGG	GGCGAGTACC	GGGCGGTGAC	CGAGCTAGGG	
DRB3*7201	.....A	..AC.....	.....AC..	.....	C.....	.....T.	.....T...	.....G...	
DRB3*7301	..G.....	..T.....	.....A..	.....	C.....	.....T..	.....	.....G...	
DRB3*2008	.....	.....T.....	.....CG..	.....	C.....	.....T..	.....	.....G...	
DRB3*3403	..G...C...	..T.....	.....	.....	C.....	.....	.....	.....	
DRB3*50011	.....	.....	.....C..	.....	C.....	.....T..	.....	.....G...	
	161								240
DRB3*1601	CGGCCGGACG	CCAAGTACTG	GAACAGCCAG	AAGGACTTCC	TGGAGGAGAA	GCGGGCCGCG	GTGGACACGT	ACTGCAGACA	
DRB3*7201	.....	..G.....	.....	.....G.	.....CG...	.....AGT	.....	.....	
DRB3*7301	.....	..G..C.G.	.....	.....AC..	.....CG.GC	.....TAT	.....	.....	
DRB3*2008	.....	..G.....	.....	.....	.....C..G	.....	.....	.....	
DRB3*3403	.....G..T..	..G..C.G.T	.....	.....A..	.....CG..	.....AAT	.....	.....	
DRB3*50011	.....	..G..C...T	..G.....	.....AC..	.....CG.G.	.....TAT	.....	.....	
	241				281				
DRB3*1601	CAACTACGGG	GTCGGTGAGA	GTTTCACTGT	GCAGCGGCCA	G	Id. (%)			
DRB3*7201	.....	..G..TG...	.....	.....		.91.8			
DRB3*7301	.....	.....	.....	.....		.91.5			
DRB3*2008	.....	..G..TG...	.....	.....T.		.92.5			
DRB3*3403	.....	.....	.....	.....		.92.5			
DRB3*50011	.....	.....T.....	.....	.....		.91.5			

**(B)**

	6								94
DRB3*1601	HFLEYTKKECHFNGTERVRF	LDRYFHNGEEFVRFDS	DWGEYRAVTE	LGPRDAKYWNSQKDF	FLEEKRAAVDTY	CRHNYGVGESFTVQRR	Id. (%)		
DRB3*7201	.....S.S.....	.....YT.....	T.....	F.L.....	E.....	L.R...S.....	GV.....	84.6	
DRB3*7301	.....ATS.....	.....Q...Y...Y.....	.....F.....	.....EQ.....	T...RA..Y.....	.....	.....	84.6	
DRB3*2008	.....H.G.....	.....L...Y...R.....	.....F.....	.....E.....	.....QR.....	.....GV.....	L	85.9	
DRB3*3403	.....C.S.....	.....E.S.Y.....	.....RV.EQL.....	I.R..N.....	.....	.....	.....	84.6	
DRB3*50011	.....STG.....	.....Y.....	.....L.....	.....F.....	.....EHL.G...T...RE..Y.....	.....V.....	.....	82.1	

**Fig. 2.** Alignment of the nucleotide (A) and predicted amino acid (B) sequences of the beta 1 domains encoded by the five novel *BoLA-DRB3* alleles (accession numbers as follows: AB820282 for *BoLA-DRB3\*7201*, AB820283 for *BoLA-DRB3\*7301*, AB820284 for *BoLA-DRB3\*2008*, AB820285 for *BoLA-DRB3\*3403*, and AB820286 for *BoLA-DRB3\*50011*) identified in 980 cattle: Brahman, Holstein × Sahiwal, Philippine native cattle, Philippine native × Brahman, Philippine native × Holstein, Philippine native × Holstein × Brahman, Philippine native × Holstein × Sahiwal, Sahiwal × Brahman, Simbrah × Brahman, Holstein × Jersey, Brahman × Sahiwal, Holstein, and Holstein × Brahman × Sahiwal. The numbering indicates the positions of the amino acids in the mature protein. The amino acid residues identical to those encoded by the *BoLA-DRB3\*1601* cDNA clone NR-1 are indicated by dots (Aida et al., 1995). The 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 AB820282 through AB820286.

**Table 1**  
Allele frequencies of Philippine breed.

DRB3	Holstein × Sahiwal (N = 249 <sup>a</sup> )	Brahman (N = 236 <sup>a</sup> )	Native (N = 482 <sup>a</sup> )	Native × Brahman (N = 132 <sup>a</sup> )	Yacumeno (N = 113 <sup>a</sup> )
*0101	<b>13.05<sup>b</sup></b>	–	0.10	0.38	2.21
*0201	<b>5.82<sup>b</sup></b>	<b>5.72<sup>b</sup></b>	<b>8.30<sup>b</sup></b>	<b>7.95<sup>b</sup></b>	<b>7.08<sup>b</sup></b>
*0301	0.20	0.85	<b>11.72<sup>b</sup></b>	4.92	–
*03021	0.20	1.48	0.93	0.76	–
*0501	1.20	0.42	0.73	–	3.98
*0502	–	–	0.10	–	–
*0503	0.40	–	1.04	–	–
*0601	0.40	–	–	–	3.10
*0701	1.41	1.91	2.18	1.14	<b>10.62<sup>b</sup></b>
*0704	–	–	–	–	1.33
*0801	–	0.21	0.21	0.76	–
*0901	2.61	–	0.31	0.38	4.42
*0902	<b>5.22<sup>b</sup></b>	3.39	0.62	0.76	<b>8.41<sup>b</sup></b>
*1001	<b>5.02<sup>b</sup></b>	2.33	0.31	–	3.10
*1101	<b>5.22<sup>b</sup></b>	0.21	0.93	1.52	3.54
*1103	0.20	–	2.39	1.89	–
*1104	2.61	0.64	–	0.38	4.87
*1201	<b>13.65<sup>b</sup></b>	<b>7.20<sup>b</sup></b>	1.24	4.92	1.77
*1301	4.02	–	0.10	–	1.33
*1302	–	0.21	0.10	0.76	–
*14011	3.21	1.91	0.62	0.76	<b>6.64<sup>b</sup></b>
*1501	<b>6.02<sup>b</sup></b>	2.12	<b>5.29<sup>b</sup></b>	4.55	4.42
*1502	–	–	0.10	0.38	–
*1601	0.40	0.85	2.49	1.14	4.42
*1602	0.20	0.42	0.21	1.14	–
*1701	0.20	–	0.10	–	1.77
*1703	2.81	4.66	1.45	2.65	–
*1801	4.82	2.54	0.21	0.76	<b>8.41<sup>b</sup></b>
*1901	0.80	1.27	0.10	1.14	–
*1902	0.20	–	–	0.38	–
*20012	2.81	0.21	0.21	0.76	0.44
*2002	–	0.42	0.10	0.76	–
*2003	–	1.06	0.41	0.38	–
*2005	0.20	0.42	0.41	–	–
*2006	1.20	–	–	–	–
*2008 <sup>c</sup>	–	0.64	0.62	0.38	–
*2101	–	0.42	0.10	–	0.44
*2201	0.40	<b>6.36<sup>b</sup></b>	2.07	3.79	1.77
*2301	–	0.42	0.31	0.76	–
*2403	0.20	–	–	–	–
*25011	–	1.48	4.36	3.79	–
*25012	0.60	–	0.31	0.76	–
*2502	0.40	–	0.10	–	0.88
*2601	–	0.85	–	–	–
*2701	–	0.21	0.10	–	–
*2703	4.02	–	0.31	0.38	2.21
*2704	–	0.42	0.31	0.38	–
*2705	3.01	1.69	0.10	–	–
*2707	0.20	0.64	0.31	–	–
*2710	–	0.42	1.35	–	1.77
*2801	–	4.45	1.35	2.27	1.33
*2802	–	1.06	0.21	1.14	0.88
*2902	–	–	–	–	0.88
*3001	0.60	<b>13.77<sup>b</sup></b>	3.94	<b>8.71<sup>b</sup></b>	0.88
*3002	–	0.21	–	–	–
*3101	0.20	3.39	1.35	3.79	–
*3102	–	0.21	–	–	–
*3201	0.40	–	0.10	0.38	–

(continued on next page)

**Table 1** (continued)

DRB3	Holstein × Sahiwal (N = 249 <sup>a</sup> )	Brahman (N = 236 <sup>a</sup> )	Native (N = 482 <sup>a</sup> )	Native × Brahman (N = 132 <sup>a</sup> )	Yacumeno (N = 113 <sup>a</sup> )
*3202	0.20	1.27	0.41	0.76	0.44
*3301	2.01	2.12	1.14	0.76	2.21
*3401	–	–	0.52	0.76	–
*3403 <sup>c</sup>	0.40	0.42	3.32	2.27	–
*3501	–	1.91	1.97	1.14	0.88
*3601	1.81	<b>8.69<sup>b</sup></b>	2.49	<b>6.44<sup>b</sup></b>	0.88
*3701	0.80	–	–	–	0.88
*3901	–	0.64	0.31	–	0.44
*4002	–	0.21	2.28	2.27	–
*4101	–	0.85	<b>5.19<sup>b</sup></b>	3.79	–
*4201	–	0.64	0.41	1.52	–
*4301	–	1.27	3.11	1.14	–
*4302	–	0.21	1.97	3.03	–
*4303	–	0.42	0.62	–	–
*4401	–	0.42	0.73	0.76	–
*4501	0.20	–	–	–	–
*4801	0.20	–	0.21	–	–
*4802	–	1.06	0.52	0.38	1.33
*5001	0.20	–	2.59	0.38	–
*50011 <sup>c</sup>	–	–	3.84	2.27	–
*5702	–	1.69	4.56	2.27	–
*6101	–	0.21	–	–	–
*6201	–	–	0.21	0.38	–
*6301	–	–	0.31	–	–
*6401	–	0.42	–	0.38	–
*7201 <sup>c</sup>	–	0.42	1.97	0.76	–
*7301 <sup>c</sup>	–	–	0.93	0.76	–
Total no. of allele	46	58	71	57	35

<sup>a</sup> N, number of cattle investigated.

<sup>b</sup> Frequent alleles in each population are given in bold and underlined (>5%).

<sup>c</sup> Novel alleles identified in this study.

the data using 1000 replications. Genetic distances and phylogenetic trees were derived by POPTREE2 software (Takezaki et al., 2010).

## Results and discussion

### Distribution of *BoLA-DRB3* alleles in Philippine cattle breeds

The genotypes of *BoLA-DRB3* exon 2 from a total of 1120 individuals belonging to various cattle breeds living in Luzon, Leyte, Bohol, Iloilo, and Cebu Islands (Fig. 1) were investigated. Eighty-three *BoLA-DRB3* alleles were identified, of which 78 were previously reported alleles and five were novel (Fig. 2).

The five new alleles were named by the ISAG *BoLA* nomenclature committee ([http://www.ebi.ac.uk/ipd/mhc/bola/nomen\\_committee.html](http://www.ebi.ac.uk/ipd/mhc/bola/nomen_committee.html)). The first was designated as *BoLA-DRB3\*7201*, which differed from *DRB3\*6201* (AY847715) at the positions 30 (T to C), 87 (C to A), 92 and 93 (TA to AC), 108 (A to C), 219 (A to G), and 252 (T to G). The second was designated as *BoLA-DRB3\*7301*, differentially from the *BoLA-DRB3\*1701* at the positions 82 (C to G), 111 (C to T), 173 (A to G), 176 (T to C), 178 (C to G), 196 (G to C), 198 (T to C), and 210 (A to C). The third, named *BoLA-DRB3\*2008*, differed from the *BoLA-DRB3\*2005* at the positions 30 and 31 (TC to CA), 33 (C–A), 35 (A to G), and 252 (T to G). The fourth, *BoLA-DRB3\*50011*, differed from the *BoLA-DRB3\*5001* at the position 37 (G to C) and the fifth, *DRB3\*3403*, differed from the *BoLA-DRB3\*3402* at the positions 172 and 172 (GA to CG), 197 (T to A), 199 (G to C), 207 (A to G), and 255–256 (TG to GT). The five new *DRB3* alleles were 91.5–92.5% identical to the *BoLA-DRB3* cDNA clone NR1 allele (designated *DRB3\*1601*) (Aida et al., 1995) at the nucleotide level and 82.1–85.9% identical at the amino acid level. These high similarities suggested that all new detected alleles were *BoLA-DRB3* alleles.

Next, we compared the distribution of *BoLA-DRB3* allele frequencies in 236 Brahman cattle, 249 Holstein × Sahiwal cattle, 482 Philippine native cattle, and 132 Philippine native × Brahman cattle (Table 1). Breeds which collected less than 10 cows per breed were excluded from calculation of allele frequency. Forty-six alleles (45 previously reported and one novel) were identified in Holstein × Sahiwal cattle, 58 alleles (55 previously reported alleles and three novel) were identified in Brahman cattle, 71 alleles (66 previously reported and five novel) were detected in Philippine native cattle, and 57 (52 previously reported and five novel) were detected in Philippine native × Brahman cattle. The alleles showing frequencies >5% were as follows: *BoLA-DRB3\*0101*, *\*0201*, *\*0902*, *\*1001*, *\*1101*, *\*1201* and *\*1501* in Holstein × Sahiwal cattle; *BoLA-DRB3\*0201*, *\*1201*, *\*2201*, *\*3001* and *\*3601* in Brahman cattle; *BoLA-DRB3\*0201*, *\*0301*, *\*1501*, and *\*4101* in Philippine native cattle; and *BoLA-DRB3\*0201*, *\*3001*, and *\*3601* in Philippine native × Brahman cattle. These common alleles accounted for 54.0% and 41.7% of the cumulative gene frequencies in the Holstein × Sahiwal and Brahman cattle breeds, respectively, and for 30.5% and 23.1% in Philippine native and Philippine native × Brahman cattle, respectively.

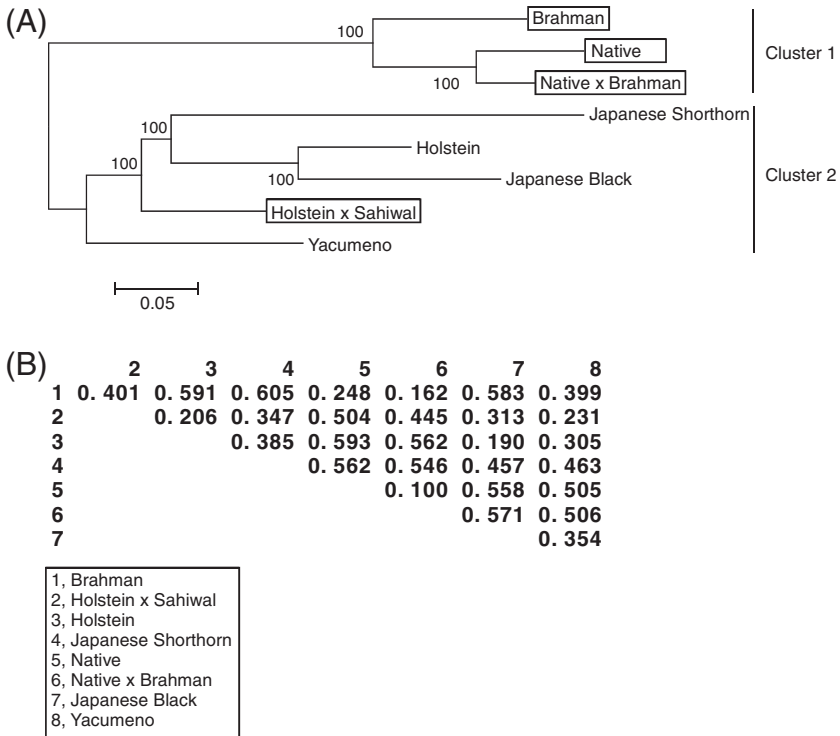
A high number of *BoLA-DRB3* alleles (71 alleles) were detected in Philippine native cattle compared with Brahman (58 alleles) and Holstein × Sahiwal (46 alleles) cattle. In addition, though a few number of samples from Philippine native × Brahman cattle (132 heads) compared with Brahman (N = 236) or native (N = 482), 57 alleles were identified in Philippine native × Brahman cattle. Increasing of polymorphism may be due to the effect of crossbreed between Brahman and Philippine native cattle. To date, 130 of the *BoLA-DRB3* alleles have been registered in IPD-MHC *BoLA* allele database. The identification of 71 *BoLA-DRB3* alleles means that half of those detected worldwide appear in the Philippine native breed. In addition, we identified 20 previously reported alleles in 100 Japanese Shorthorn cattle, 14 (13 previously reported and one novel) in 69 Jersey cattle, 18 previously reported alleles in 101 Holstein cattle, 24 alleles (21 previously reported and three novel) in 201 Japanese Black cattle, 35 (32 previously reported and three novel) in 113 Bolivian Yacumeño cattle, and 24 (22 previously reported and two novel) from 66 Colombian Hartón del Valle cattle (Giovambattista et al., 2013; Takeshima et al., 2003). Therefore, we may conclude that Philippine native cattle show a high degree of genetic diversity.

#### Population relationships based on the frequencies of *BoLA-DRB3* alleles

Variations in mitochondrial DNA are usually used to deduce evolutionary trees. Although such analyses show that *Bos taurus* can clearly be differentiated from *B. indicus*, differences in the evolution of different breeds are unclear (Achilli et al., 2009). In a previous study, we calculated the putative evolutionary relationships between different cattle breeds using genetic distances estimated from the allele frequencies and succeeded to construct a phylogenetic tree (Giovambattista et al., 2013; Miyasaka et al., 2011; Takeshima et al., 2003, 2008a). Therefore, to assess the genetic relationship between Philippine cattle breeds and previously studied Japanese and Bolivian breeds using SBT, we performed a dendrogram analysis (Fig. 3). First, dendrograms were constructed from the distance matrix using NJ algorithms (Fig. 3A). The  $D_A$  genetic distances for each pair were generated using the *BoLA-DRB3* allele frequencies obtained from 249 Philippine Holstein × Sahiwal cattle, 236 Brahman cattle, 132 Philippine native × Brahman cattle, and 482 Philippine native cattle (Table 1), and four previously reported breeds (Japanese Shorthorn, Japanese Black, Holstein and Yacumeño cattle) (Giovambattista et al., 2013; Takeshima et al., 2003) (Fig. 3B). NJ cluster analysis using  $D_A$  genetic distances identified congruent topologies, which were consistent with the historical and geographical origins of the breeds.

The phylogenetic tree clearly divided into two clusters: Cluster 1 comprised Brahman cattle, Philippine native cattle and Brahman × Philippine native cattle, whereas Cluster 2 comprised Holstein, Japanese Shorthorn, Japanese Black, Holstein × Sahiwal, and Yacumeño cattle (Fig. 3A). As shown in Fig. 3B, the genetic distances between Philippine native cattle and Japanese Shorthorn, Japanese Black, Yacumeño, and Holstein cattle were 0.562, 0.558, 0.505, and 0.593, respectively, indicating that Philippine native breeds are genetically different from other breeds. In addition, the genetic distance between Philippine native cattle and Brahman cattle was 0.248, indicating that Philippine native cattle resemble the Brahman breed, which is a member of *B. indicus*. By contrast, the genetic distances between Holstein × Sahiwal cattle and breeds including Yacumeño, Japanese Black, Japanese Shorthorn, and Holstein range from 0.206 to 0.347,





**Fig. 3.** A phylogenetic tree (A) showing the relationship between four Philippine breeds (□), the Bolivian native Yacumeño breed, and three Japanese breeds (Holstein, Japanese Black, and Japanese Shorthorn) was constructed from a matrix of  $D_A$  genetic distances (shown in B). Abbreviations: 1, Brahman; 2, cross between Holstein cattle and Sahiwal cattle; 3, Holstein; 4, Japanese Shorthorn; 5, Philippine native; 6, cross between Philippine native cattle and Brahman cattle; 7, Japanese black; and 8, Bolivian native Yacumeño breed.

indicating that Holstein × Sahiwal cattle clustered within the branches comprising Bolivian and Japanese breeds, which are members of *B. taurus*.

These results show that both Philippine native and Philippine native × Brahman cattle can be classified within the species *B. indicus*, but Holstein × Sahiwal are classified within the species *B. taurus*, despite being crossed with Sahiwal, which is classified within the species *B. indicus*. Namikawa et al. (1984) also compared three proteins (hemoglobin, albumin, and transferrin) in South Asian breeds and reported that, whereas Philippine cattle resemble Brahman breeds, they differ from the Japanese Black and Holstein breeds. Our results clearly support this observation, and show that this tendency has been conserved for at least 25 years (from 1982 to 2007).

#### Distribution of Philippine native cattle on four islands

To determine the allele distribution in Philippine native cattle, we estimated the allele frequency in native and its cross-breed cattle from four islands (Table 2). As shown in Table 1, *BoLA-DRB3\*0301* was the most frequent allele, identified in 482 Philippine native cattle from the four islands. *BoLA-DRB3\*0301* showed the highest frequency in Philippine native cattle from Bohol Island and Leyte Island, but was the sixth of the most frequent allele in Philippine native cattle from Iloilo Island; while the allele was not determined in cattle from Luzon (Table 2). These results suggest that the population of Philippine native cattle on each island shows a distinct allele frequency. Moreover, *DRB3\*1201*, *\*2201*, and *\*3001* were common allele (>5%) in Philippine native cattle from Luzon Island same as in Brahman cattle. Taken

**Table 2**  
Allele frequencies of Philippine native breed.

Breed	Holstein × Sahiwal		Brahman		Native				
	Island	Cebu	Luzon	Luzon	Leyte	Bohol	Iloilo	Luzon	Leyte
DRB3	(N = 98 <sup>a</sup> )	(N = 151 <sup>a</sup> )	(N = 184 <sup>a</sup> )	(N = 52 <sup>a</sup> )	(N = 66 <sup>a</sup> )	(N = 120 <sup>a</sup> )	(N = 29 <sup>a</sup> )	(N = 267 <sup>a</sup> )	
*0101	<b>14.29<sup>b</sup></b>	<b>12.25<sup>b</sup></b>	–	–	–	0.42	–	–	
*0201	4.08	<b>6.95<sup>b</sup></b>	<b>6.52<sup>b</sup></b>	2.88	<b>7.58<sup>b</sup></b>	<b>8.75<sup>b</sup></b>	<b>8.62<sup>b</sup></b>	<b>8.24<sup>b</sup></b>	
*0301	–	0.33	0.82	0.96	<b>18.18<sup>b</sup></b>	<b>5.42<sup>b</sup></b>	–	<b>14.23<sup>b</sup></b>	
*03021	0.51	–	1.90	–	0.76	1.25	–	0.94	
*0501	2.04	0.66	–	1.92	0.76	0.42	–	0.94	
*0502	–	–	–	–	–	–	–	0.19	
*0503	1.02	–	–	–	–	4.17	–	–	
*0601	0.51	0.33	–	–	–	–	–	–	
*0701	1.53	1.32	2.45	–	0.76	<b>7.92<sup>b</sup></b>	1.72	–	
*0801	–	–	0.27	–	–	–	–	0.37	
*0901	<b>6.12<sup>b</sup></b>	0.33	–	–	–	–	1.72	0.37	
*0902	<b>5.61<sup>b</sup></b>	4.97	0.54	<b>13.46<sup>b</sup></b>	–	0.42	1.72	0.75	
*1001	2.55	<b>6.62<sup>b</sup></b>	1.09	<b>6.73<sup>b</sup></b>	–	–	1.72	0.37	
*1101	3.06	<b>6.62<sup>b</sup></b>	0.27	–	–	3.75	–	–	
*1103	0.51	–	–	–	0.76	0.83	–	3.75	
*1104	–	4.30	0.82	–	–	–	–	–	
*1201	<b>14.80<sup>b</sup></b>	<b>12.91<sup>b</sup></b>	<b>5.43<sup>b</sup></b>	<b>13.46<sup>b</sup></b>	2.27	–	<b>8.62<sup>b</sup></b>	0.75	
*1301	3.57	4.30	–	–	–	–	–	0.19	
*1302	–	–	0.27	–	–	–	1.72	–	
*14011	3.57	2.98	1.90	1.92	–	–	–	1.12	
*1501	<b>5.10<sup>b</sup></b>	<b>6.62<sup>b</sup></b>	2.45	0.96	<b>10.61<sup>b</sup></b>	<b>8.33<sup>b</sup></b>	1.72	3.00	
*1502	–	–	–	–	–	–	–	0.19	
*1601	0.51	0.33	1.09	–	–	0.42	–	4.31	
*1602	–	0.33	0.54	–	–	–	–	0.37	
*1701	–	0.33	–	–	–	0.42	–	–	
*1703	3.57	2.32	<b>5.98<sup>b</sup></b>	–	<b>8.33<sup>b</sup></b>	–	3.45	0.19	
*1801	2.04	<b>6.62<sup>b</sup></b>	1.90	4.81	–	0.42	–	0.19	
*1901	–	1.32	1.63	–	–	0.42	–	–	
*1902	0.51	–	–	–	–	–	–	–	
*20012	3.57	2.32	0.27	–	–	0.83	–	–	
*2002	–	–	0.54	–	–	0.42	–	–	
*2003	–	–	1.09	0.96	–	1.25	1.72	–	
*2005	0.51	–	0.54	–	–	1.67	–	–	
*2006	1.53	0.99	–	–	–	–	–	–	
*2008 <sup>c</sup>	–	–	0.82	–	–	2.50	–	–	
*2101	–	–	0.54	–	–	–	1.72	–	
*2201	–	0.66	<b>7.34<sup>b</sup></b>	2.88	–	–	<b>6.90<sup>b</sup></b>	3.00	
*2301	–	–	0.27	0.96	–	0.83	1.72	–	
*2403	0.51	–	–	–	–	–	–	–	
*25011	–	–	1.90	–	4.55	<b>5.83<sup>b</sup></b>	1.72	3.93	
*25012	1.53	–	–	–	–	0.83	–	0.19	
*2502	–	0.66	–	–	–	0.42	–	–	
*2601	–	–	1.09	–	–	–	–	–	
*2701	–	–	0.27	–	–	–	1.72	–	
*2703	<b>6.63<sup>b</sup></b>	2.32	–	–	–	–	–	0.56	
*2704	–	–	0.54	–	–	–	1.72	0.37	
*2705	2.55	3.31	1.90	0.96	–	–	1.72	–	
*2707	0.51	–	0.82	–	–	–	1.72	0.37	
*2710	–	–	0.54	–	1.52	1.25	3.45	1.12	
*2801	–	–	<b>5.71<sup>b</sup></b>	–	0.76	0.42	3.45	1.69	
*2802	–	–	1.36	–	–	–	1.72	0.19	
*3001	–	0.99	<b>9.51<sup>b</sup></b>	<b>28.85<sup>b</sup></b>	3.79	2.50	<b>10.34<sup>b</sup></b>	3.93	
*3002	–	–	0.27	–	–	–	–	–	
*3101	0.51	–	4.08	0.96	–	–	–	2.43	
*3102	–	–	0.27	–	–	–	–	–	
*3201	1.02	–	–	–	–	0.42	–	–	

(continued on next page)

**Table 2** (continued)

Breed	Holstein × Sahiwal		Brahman		Native				
	Island	Cebu	Luzon	Luzon	Leyte	Bohol	Iloilo	Luzon	Leyte
DRB3	(N = 98 <sup>a</sup> )	(N = 151 <sup>a</sup> )	(N = 184 <sup>a</sup> )	(N = 52 <sup>a</sup> )	(N = 66 <sup>a</sup> )	(N = 120 <sup>a</sup> )	(N = 29 <sup>a</sup> )	(N = 267 <sup>a</sup> )	
*3202	0.51	–	1.36	0.96	–	–	–	0.75	
*3301	2.04	1.99	2.17	1.92	–	0.42	3.45	1.50	
*3401	–	–	–	–	1.52	1.25	–	–	
*3403 <sup>c</sup>	1.02	–	–	1.92	<b>5.30<sup>b</sup></b>	<b>5.83<sup>b</sup></b>	–	2.06	
*3501	–	–	2.45	–	1.52	–	<b>5.17<sup>b</sup></b>	2.62	
*3601	1.02	2.32	<b>8.42<sup>b</sup></b>	<b>9.62<sup>b</sup></b>	<b>6.82<sup>b</sup></b>	0.83	<b>6.90<sup>b</sup></b>	1.69	
*3701	–	1.32	–	–	–	–	–	–	
*3901	–	–	0.82	–	–	0.83	–	0.19	
*4002	–	–	0.27	–	–	2.92	1.72	2.62	
*4101	–	–	0.54	1.92	2.27	4.58	–	<b>6.74<sup>b</sup></b>	
*4201	–	–	0.82	–	–	–	1.72	0.56	
*4301	–	–	1.36	0.96	<b>8.33<sup>b</sup></b>	2.50	1.72	2.25	
*4302	–	–	0.27	–	2.27	3.75	1.72	1.12	
*4303	–	–	0.54	–	–	2.08	1.72	–	
*4401	–	–	0.54	–	–	–	–	1.31	
*4501	–	0.33	–	–	–	–	–	–	
*4801	0.51	–	–	–	–	0.83	–	–	
*4802	–	–	1.36	–	–	–	1.72	0.75	
*5001	0.51	–	–	–	4.55	0.83	–	3.18	
*50011 <sup>c</sup>	–	–	–	–	3.03	2.92	–	4.87	
*5702	–	–	2.17	–	3.03	3.33	1.72	<b>5.81<sup>b</sup></b>	
*6101	–	–	0.27	–	–	–	–	–	
*6201	–	–	–	–	–	0.42	–	0.19	
*6301	–	–	–	–	–	0.42	–	0.37	
*6401	–	–	0.54	–	–	–	–	–	
*7201 <sup>c</sup>	–	–	0.54	–	0.76	0.83	1.72	2.81	
*7301 <sup>c</sup>	–	–	–	–	–	2.92	–	0.37	
Total no. of allele	36	32	56	21	24	46	33	50	

<sup>a</sup> N, number of cattle investigated.

<sup>b</sup> Frequent alleles in each population are in bold and underlined (>5%).

<sup>c</sup> Novel alleles identified in this study.

together with the fact that most native cattle on Luzon Island are crossed with Brahman cattle (Fig. 1B), and *DRB3\*1201*, *\*2201* and *\*3001* are common in the Brahman breed (Table 1), we can conclude that *DRB3\*1201*, *\*2201* and *\*3001* alleles were passed to Philippine native cattle from Brahman cattle. Despite the high frequency of *DRB3\*0301* on Bohol, Iloilo, and Leyte Islands which are located in the Southern Philippines, the allele was not detected in cattle from Luzon Island, which is located in the Northern Philippines. Contrary to non-frequently *DRB3\*0301* allele in Luzon native cattle, *DRB3\*1201*, *\*2201*, *\*3001* and *\*3501* were specifically high frequency in Luzon native cattle, but not in other native breed from the other three islands. The differences reflect the genetic makeup of the founder population that was introduced to the island and also this difference may have arisen because cattle have adapted to live in either the Southern or the Northern Philippines.

Table 3 summarizes the number and heterozygosity of the alleles identified in native cattle from four islands. Twenty-four *DRB3* alleles were detected in 66 native cattle from Bohol Island, 46 in 120 native cattle from Iloilo Island, 50 in 267 native cattle from Leyte Island, and 33 in 29 native cattle from Luzon Island. As mentioned above, we detected five novel alleles, designated *DRB3\*7201*, *\*7301*, *\*2008*, *\*50011* and *\*3403*, in 482 Philippine native cattle (Fig. 1B). The frequency of new alleles was different on each island: 3/24 (12.5%) on Bohol, 5/46 (10.9%) on Iloilo, 4/50 (8.0%) on Leyte, and 1/33 (3.0%) on Luzon. The observed heterozygosity ranged from 0.86667 to 0.95455. Native cattle from Leyte and Iloilo showed a level of heterozygosity significantly below the expected heterozygosity. Thus, these results suggested that some alleles identified in Philippine native cattle from Leyte and Iloilo Island were subjected to negative selection. From the same analysis, Brahman cattle from Luzon and Holstein × Sahiwal from Cebu were under negative selection. Additionally, comparing of the *DRB3\*2201* allele frequencies between the Luzon's breed and other island's breeds, Luzon's breed had higher frequency of *DRB3\*2201* than the other

**Table 3**  
Hardy–Weinberg equilibrium calculated for Philippine native cattle from four islands.

Island	N	Detected allele (novel allele)	Observed heterozygosity	Expected heterozygosity	P-value <sup>a</sup>
Holstein					
× Sahiwal	249	46(1)	0.87149	0.93919	<b>0.00680</b>
Cebu	98	36(1)	0.80612	0.93689	<b>0.00000</b>
Luzon	151	32(0)	0.91391	0.93741	0.05964
Brahman	236	58(3)	0.88559	0.95077	<b>0.00000</b>
Luzon	184	56(2)	0.89674	0.95852	<b>0.00054</b>
Leyte	52	21(1)	0.84615	0.86856	0.47739
Native	482	71(5)	0.91286	0.95880	<b>0.00257</b>
Bohol	66	24(3)	0.95455	0.92563	0.44032
Iloilo	120	46(5)	0.86667	0.95900	<b>0.01915</b>
Leyte	267	50(4)	0.92135	0.94965	<b>0.00237</b>
Luzon	29	33(1)	0.93103	0.96733	0.54427

<sup>a</sup> P value > 0.05 were assigned as significant difference and revealed as bold face.

island's breeds (Holstein × Sahiwal; Luzon = 2.88% vs. Cebu = 0.66%, Brahman; Luzon = 7.34% vs. Leyte = 2.88%, Philippine native cattle; Luzon = 6.9% vs. breeds from three southern islands = 0 to 3%) (Table 2). This may indicate that *DRB3\*2201* allele has the advantage to adopt in the northern part of the Philippines.

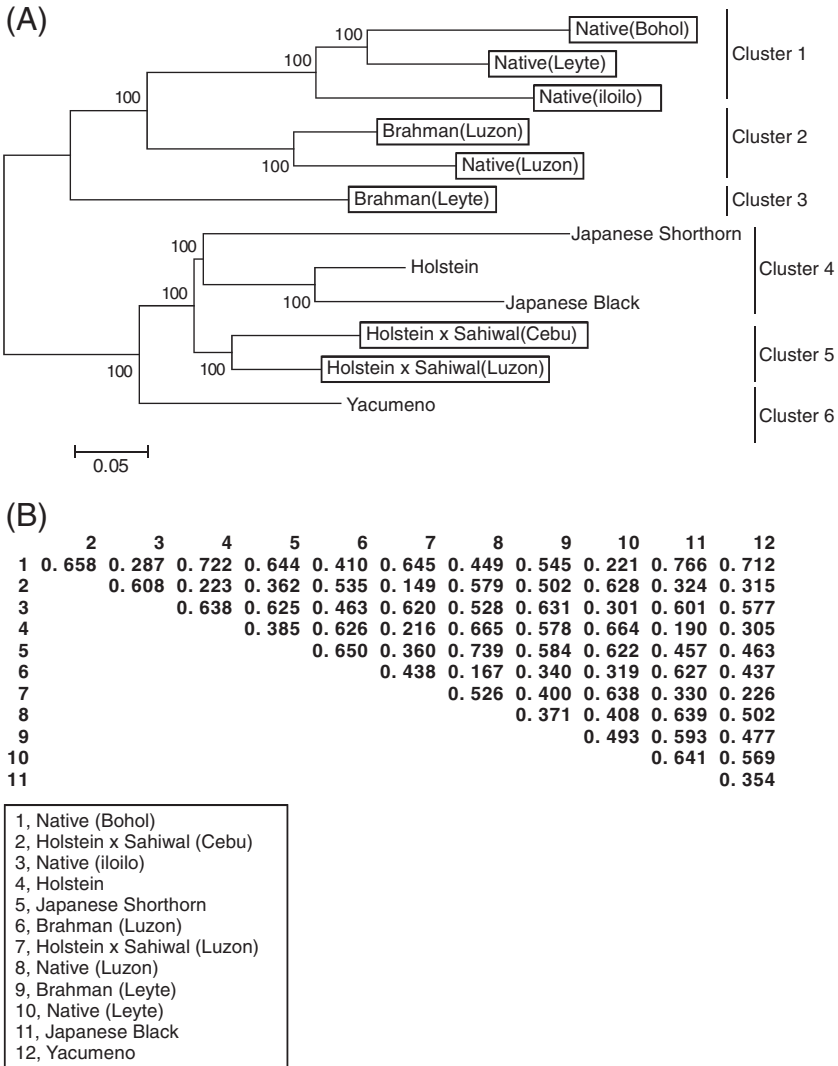
#### *Population relationships according to the frequencies of BoLA-DRB3 alleles on different islands*

We performed dendrogram analysis to assess the genetic relationship between Philippine native cattle and native breeds from Japan and Latin America (Fig. 4). The Philippine native cattle living in four Philippine islands were clearly genetically distinct from native Japanese (Japanese Shorthorn and Japanese Black) and Latin American (Yacumeño) breeds. A comparison of Philippine native cattle showed that the cattle in Luzon mostly resembled the Brahman breeds, whereas those living in Bohol, Iloilo and Leyte showed different allele frequencies from those of foreign cattle. These results showed that differences in the allele frequencies within Philippine native breed from Luzon were strongly affected by the Brahman breeds. However, a principal component analysis performed by Namikawa et al. showed that the Philippine native cattle from Luzon was a little bit different from those of Cebu Island located at the Southern Philippines (Namikawa et al., 1984). These results suggest that Philippine native cattle are divided into two populations: Northern and Southern.

#### *Neutrality tests for Philippine native cattle*

In this study, we identified 71 *DRB3* alleles in Philippine native cattle (including five novel alleles), suggesting that Philippine native cattle show great genetic diversity. The Leyte and Iloilo population in particular showed an observed heterozygosity that was significantly higher than the expected heterozygosity ( $P < 0.05$ ) (Table 3). To examine whether native Philippine cattle were subjected to selection pressure, we performed selective neutrality tests on four populations of Philippine cattle and compared the results with those obtained from Bolivian native cattle (Table 4). Based on Chakraborty's population amalgamation test (Chakraborty, 1990), we confirmed that there were no significant population amalgamation in these cattle ( $P = 0.93495, 0.87813, 0.96884, \text{ and } 0.23445$  for Bohol, Iloilo, Leyte and Luzon, respectively). Slatkin's exact test (Slatkin, 1994; Slatkin and Excoffier, 1996), which was used to detect neutrality, indicated that only Philippine native cattle from Leyte Island formed a non-neutral population ( $P = 0.04500$ ). Taken together with the results presented in Table 3, these suggest that Philippine native cattle from Leyte show a significantly greater level of genetic diversity because of natural selection.

The present study is the first to report the allelic distribution of the *BoLA-DRB3* gene in Philippine native cattle. First, we detected 80 alleles, including five novel *BoLA-DRB3* alleles from 1120 cattle (Holstein, Simbrah, Jersey, Sahiwal, Brahman, Philippine native, and several cross-breeds) living on five Philippine islands. Our results clearly identified 71 *BoLA-DRB3* alleles and five novel *DRB3* alleles in 482 Philippine native cattle. Thus, we conclude that Philippine native cattle show a high degree of genetic



**Fig. 4.** A phylogenetic tree (A) showing the relationship between Philippine native cattle from four islands (□), Bolivian native Yacumeño cattle, and two Japanese breeds (Japanese Black and Japanese Shorthorn) was constructed from a matrix of  $D_A$  genetic distances (shown in B). Abbreviations: 1, Philippine native cattle in Bohol island; 2, cross between Holstein and Sahiwal in Cebu island; 3, Philippine native cattle in Iloilo island; 4, Holstein; 5, Japanese Shorthorn; 6, Brahman in Luzon island; 7, cross between Holstein and Sahiwal in Luzon island; 8, Philippine native cattle in Luzon island; 9, Brahman native cattle in Leyte island; 10, Philippine native cattle in Leyte island; 11, Japanese Black; and 12 Bolivian native Yacumeño cattle.

diversity for this gene. Second, we calculated the *BoLA-DRB3* allele frequency in Philippine native cattle from four islands: Luzon, Leyte, Bohol, and Iloilo. We then constructed a phylogenetic tree based on these allele frequencies. The results showed that Philippine native cattle from all four islands are closely related, and closely resemble Brahman cattle, rather than native Japanese or Latin American breeds. Third, Philippine native cattle from Luzon showed different allele frequencies from those on Iloilo, Bohol, and Leyte. Thus, we concluded that Philippine native cattle split into two populations: North and South. Fourth, a neutrality test revealed that Philippine native cattle from Leyte showed significantly higher levels of genetic diversity, which may be maintained by balancing selection. Taken together, the results of

**Table 4**

Neutrality tests performed using the Ewens–Watterson–Slatkin exact test and Chakraborty's test to examine population amalgamation in Philippine native cattle.

Breed	No. of gene copy	No. of allele	No. of expected allele	Slatkin's exact P-value <sup>a</sup>	Chakraborty's P-value <sup>b</sup>
Island					
Holstein					
× Sahiwal	498	46	50.62959	0.56400	0.80054
Cebu	196	36	37.16559	0.22300	0.94092
Luzon	302	32	42.91205	0.07200	0.98508
Brahman					
Luzon	472	58	59.23213	0.14500	0.60017
Luzon	368	56	62.61915	0.10100	0.86524
Leyte	104	21	16.91612	0.81500	0.14210
Native					
Bohol	964	71	82.99150	0.08900	0.94726
Bohol	132	24	28.67687	0.11000	0.89096
Iloilo	240	46	54.50035	0.22300	0.94092
Leyte	534	50	60.24175	0.04500	0.95233
Luzon	58	33	31.70025	0.86600	0.41057
Native					
× Brahman	264	57	64.03908	0.11000	0.88641
Yacumeno	226	35	47.96352	0.00500	0.99421

<sup>a</sup> Slatkin's exact test (Slatkin, 1994; Slatkin and Excoffier, 1996) was performed for Philippine native cattle from Bohol, Iloilo, Leyte, and Luzon, and on Yacumeño cattle from Bolivia.  $P > 0.05$ ; the population showed significant natural selection pressure.

<sup>b</sup> Chakraborty's test for population amalgamation (Chakraborty, 1990) was performed for Philippine native cattle from Bohol, Iloilo, Leyte, and Luzon, and for Yacumeño cattle from Bolivia.  $P > 0.05$ ; the population showed significant amalgamation.

the present study will increase our knowledge about the diversity of the bovine MHC complex, which comprises important loci involved in immune responses and protection against invading pathogens, and may allow us to design effective breeding strategies to produce disease-resistant livestock in the future.

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