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Characterization of BoLA-DRB3.2 Alleles in Hanwoo (Korean cattle) by Sequence Based Typing (SBT)

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ABSTRACT : A study was conducted with 70 Hanwoo (Korean cattle) for genotyping bovine leukocyte antigen (BoLA)-DRB3.2 gene by using the polymerase chain reaction (PCR) and sequence based typing (SBT). Two-step PCR was carried out for amplifying a 284 bp fragment of the target gene and the PCR products were digested with three restriction enzymes namely *Rsa*I, *Bst*YI and *Hae*III. Seventeen alleles were detected with frequencies ranging from 1.43 to 18.57% and one (x'aa) of these alleles was identified as a new allele that has not been reported before. The frequency of the new x'aa allele identified in this breed was 12.86%. In addition, the seven most frequently observed alleles (DRB3.2 *10, *15, *16, *26, *27, *54 and x'aa) accounted for 74.28% of the alleles in this population. The phylogenetic tree showed that the BoLA-DRB3.2 allele sequences of Hanwoo were shared with other *Bos taurus* breeds and no specific clade for Hanwoo was identified. It indicates high heterogeneity of the BoLA-DRB3 gene in this population and may give some ideas for breeding animals having better disease resistance. (**Key Words** : BoLA-DRB3, PCR-RFLP, Sequence Based Typing (SBT), Hanwoo)

INTRODUCTION

The major histocompatibility complex (MHC) contains a number of genes which have very important roles in immune responses. MHC has been named differently in different animals; human leukocyte antigen (HLA) is for human MHC, H-2 complex for mouse MHC, BoLA for cattle MHC, B and Y complex for chicken MHC and SLA for pig MHC (van Eijk et al., 1992; Bodmer et al., 1996; Miller et al., 2004; Smith et al., 2005). This MHC region encodes cell surface glycoproteins, which bind to foreign antigens of T lymphocytes that are central to the induction and regulation of adaptive immunity (Germain, 1994; Townsend et al., 1989). The MHC class I and class II genes are highly polymorphic and are known to be related to immune response related auto-immune disease, infectious disease, and responses to immunization (Sharif et al., 1998a; Ellis and Ballingall, 1999).

In cattle, BoLA genes are located on bovine chromosome 23 (BTA23) and associations between BoLA alleles and susceptibilities to disease such as mastitis, bovine leukemia virus (BLV) and parasitic infestations were observed (Teale et al., 1991; van Eijk et al., 1992; Park et al., 1993). Also associations between BoLA alleles with fertility, growth and milk production traits have been reported (Beever et al., 1990). Within the Class II region of BoLA, the DR region contained three DRB genes and high expression was observed in peripheral blood lymphocytes (Moon et al., 1997). These results indicated that a high degree of sequence polymorphisms was observed in exon 2 of the DRB3 gene and associations between DRB3 genotypes with production traits, disease and immunological traits were also reported.

In cattle, a number of serological, biochemical and molecular methods have been developed for the efficient typing of DRB3 polymorphisms (Watkins et al., 1989; Davies and Antczak, 1991; Davies et al., 1992; Glass et al., 1992; van Eijk et al., 1992; Paswan et al., 2005). Later on, more efficient methods have been discovered including heteroduplex analysis (Sitte et al., 1995), sequence-specific oligonucleotide typing (Sitte et al., 1996), denaturing gradient gel electrophoresis (Aldridge et al., 1998), and sequencing of the PCR products (Aida et al., 1995). More

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precise sequence-based typing (SBT) of the BoLA-DRB3 gene in cattle has been reported by Takeshima et al. (2001 and 2002) and Miltiadou et al. (2003). At present, 103 BoLA-DRB3.2 alleles have been identified by the sequencing of cloned genomic DNA, cDNA, PCR products and PCR-SBT (Davies et al., 1997; Russell et al., 1997; Takeshima et al., 2002, http://www.projects.roslin.ac.uk/bola/reports.html). The native cattle in Korea known as Hanwoo are mainly used for beef production. The meat of the Hanwoo is considered by Koreans to be a safe source of meat and thus commands a higher price than those from exotic breeds. They are also well adapted to the Korean agro-ecological conditions as compared to other beef breeds. The purpose of this study was to investigate the BoLA-DRB3.2 allele patterns in Hanwoo cattle. This information will be useful for the selection of animals having better disease resistance without compromising their production ability.

MATERIALS AND METHODS

Sampling

Blood samples were collected from 70 Hanwoo bred at the National Institute of Animal Science (NIAS), RDA, Korea. The obtained blood samples were transferred to the laboratory and the isolation of genomic DNA was performed using the MagExtractor kit (TOYOBO Ltd., Japan).

Polymerase chain reaction (PCR) of BOLA DRB3 exon 2

The second exon of the BoLA-DRB3 gene was amplified by hemi-nested PCR using the primers published by van Eijk et al. (1992). The oligonucleotide primers HL-030 (5'-ATCCTCTCTCTGCAGCACATTTCC-3'), HL-031 (5'-TTTAAATTCGCGCTCACCTCGCCGCT-3') and HL-032 (5'-TCGCCGCTGCACAGTGAAACTCTC-3') were used in the PCR reaction. The HL-030 and HL-031 primers were used for the as first round PCR and the HL-030 and HL-032 primers were used for the second round PCR. The first PCR reaction consisted of 50 ng template genomic DNA, 0.2 mM each of dNTP, 5 pM of each primer and 1 U Ampli-Taq Gold DNA polymerase (Perkin Elmer, USA) in a 25 μl reaction volume. The PCR condition included an initial denaturation at 94°C for 4 min followed by 15 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min using a MJ Research thermocycler (MJ Research, USA). The second PCR reaction was carried out in a total volume of 50 μl containing 5 μl of the first PCR product, 0.2 mM of dNTPs, 10 pM of each primer and 2 U of Ampli-Taq Gold DNA polymerase (Perkin Elmer, USA). The second PCR condition included an initial denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 65°C for 30 sec and 72°C for 1 min with a final extension at 72°C for 10 min using a MJ Research thermocycler (MJ Research, USA). The PCR products were run on 2% agarose gels and visualized under UV light.

Restriction endonuclease digestion

All the PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis using *Rsa*I and *Bst*YI (New England Biolabs, UK), and *Hae*III (Promega, USA) restriction enzymes according to the method of van Eijk et al. (1992). The RFLP reaction was performed with 2.5 U each of restriction enzyme, 1×buffer, 15 μl second PCR product and incubated at 37°C for *Rsa*I and *Hae*III and at 60°C for *Bst*YI. The resulting DNA fragments were separated on 12% PAGE gel (Polyacrylamide gel), stained with ethidium bromide (EtBr) and visualized under UV light.

Cloning and sequencing

The BoLA-DRB3 exon 2 PCR products were cloned using the pGEM T-easy vector ligation Kit (Promega, USA) and transformed into DH5α competent cells. Recombinant plasmid DNAs were extracted using the Wizard Plus Minipreps DNA Purification System (Promega, USA) and the DNA sequences of the clone inserts were determined by using a dye terminator kit on an ABI 3100 DNA sequencer (Applied Biosystems, USA).

Bioinformatics

The Nucleotide BLAST program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used for sequence homology searches against public databases. Multiple sequence alignments were performed using the ClustalW program (Thompson et al., 1994) and the aligned sequences were edited by BioEdit package ver. 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/page2.html). Sites representing a gap in any of the aligned sequences were excluded from the analysis. Distances between nucleotide sequences were estimated using the substitution model of Tamura and Nei (1993). Using this distance, a rooted neighbor-joining (NJ) phylogenetic tree was constructed by using the MEGA package ver. 3.1 (Kumar et al., 2004).

RESULTS AND DISCUSSION

The genotypes of 70 Hanwoo individuals were determined for exon 2 of the BoLA DRB3 allele by PCR SBT. The PCR products gave only single bands with the expected size of 284 bp in each reaction, indicating the high efficiency of the PCR reactions. The second PCR products of the BoLA-DRB3.2 gene were digested with *Rsa*I, *Hae*III and *Bst*YI restriction enzymes. The restriction patterns of *Hae*III and *Bst*YI were found to be identical type with the

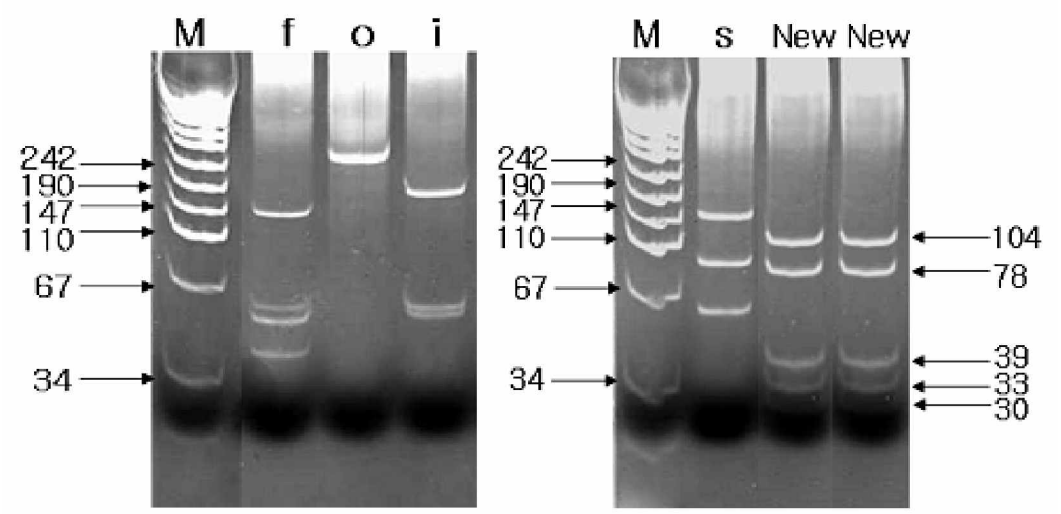

Figure 1. PCR-RFLP patterns of BoLA-DRB3 exon 2 region using *Rsa*I restriction enzyme in Hanwoo cattle. Lane M: Molecular weight marker (Sun Genetics, Korea). Lane f, i, o and s represent respective patterns obtained by *Rsa*I restriction enzyme. Lane new denotes the allele not reported previously in the scientific literature. Numbers indicate the sizes of DNA fragments in base pairs.

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Bovine ---------------------------GAGTATTCTACGGGCGAGTGTCATTTCTTCAACG 34
Hanwoo ATCCTCTCTCTGCAGCACATTTCCTGGAGTATCATAAGAGTGAGTGTCATTTCTTCAACG 60
                                  ********************************

Bovine GGACCGAGCGGGTGCGGTACCTGGACAGATACTTCTATAATGGAGAAGAGTACGCGCGCT 94
Hanwoo GGACCGAGCGTTTGCGGTACCTGGACAGATACTTCTATAATGGAGAAGAGTACGTGCGCT 120
       ********** ********************************************* *******

                                Sequence change T→A
Bovine TCGACAGCGACTGGGGCGAGTTCCGGGCGGTGACCGAGCTGGGGCGGCCGGACGCCAAGT 154
Hanwoo TCGACAGCGACTGGGGCGAGTACCGGGCGGTGACCGAGCTGGGGCGGCCGGACGCCAAGT 180
       ********** ********* ********************************** *

Bovine ACTGGAACAGCCAGAAGGACTTCCTGGAGCAGAAGCGGGCCGAGGTGGACAGGGTGTGCA 214
Hanwoo ACTGGAACAGCCAGAAGGAGATCCTGGAGCGGAGGCGGGCCGAGGTGGACACGGTGTGCA 240
       ***********************************************************

Bovine GACACAACTACGGGGGTGTGGAGAGTTTCACTGTG--------- 249
Hanwoo GACACAACTACGGGGTCGGTGAGAGTTTCACTGTGCAGCGGCGA 284
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Figure 2. Sequence alignment of BoLA-DRB3 between Hanwoo (x'aa) and the published *Rsa*I x type sequence (GenBank accession number Z82025). Squares indicate the *Rsa*I restriction enzyme recognition sequence and the arrow indicates the new site of *Rsa*I restriction enzyme giving different RFLP patterns between the x and the x' types.

previous results of van Eijk et al. (1992), Gelhaus et al. (1995) and Maillard *et al.* (1999). Based on the SBT of BoLA-DRB3 exon 2 in each individual, a new pattern for *Rsa*I restriction enzyme was identified (Figure 1). The nucleotide sequence of the new allele is shown in Figure 2. The allelic nomenclature of *x'aa is based on the restriction endonuclease enzyme patterns described by van Eijk et al. (1992). A transversion mutation (T→A) had occurred at 142 nucleotide base position in the BoLA-DRB3 gene of Hanwoo and thus resulted in one more cutting site for the *Rsa*I enzyme than the x type allele. Therefore, we called this type as x' type. The *Rsa*I restriction enzyme cuts a 69 bp fragment into two fragments having 30 bp and 39 bp in Hanwoo (Figure 1).

In total, 17 BoLA-DRB3.2 alleles were identified in 70 Hanwoo with frequencies ranging from 1.43 to 18.57% (Table 1). Sixteen alleles, representing an allele frequency of 87.14% were the same as previously described by van Eijk et al. (1992), Gelhaus et al. (1995) and Maillard et al. (1999). The new x'aa allele comprising 12.86% of the total alleles studied was different from the previously characterized BoLA-DRB3.2 alleles. The seven most frequently observed BoLA alleles in Hanwoo population (BoLA-DRB3.2*10, *15, *16, *26, *27, *54 and x'aa)

Table 1. Allele types and frequencies for BoLA-DRB3.2 in 70 Hanwoo as identified by PCR-RFLP analysis

DRB3.2 allele	RFLP type[a]			No. of animals	Frequency (%)
	*Rsa*I	*Bst*YI	*Hae*III		
02[b]	b	b	a	1	1.43
03[b]	b	b	b	4	5.71
08[b]	f	a	a	2	2.86
10[b]	f	b	a	6	8.57
12[b]	h	a	a	2	2.86
15[b]	i	b	a	5	7.14
16[b]	j	b	d	9	12.86
19[b]	s	b	b	1	1.43
20[b]	l	b	b	2	2.86
21[b]	l	b	e	1	1.43
26[b]	o	a	b	5	7.14
27[b]	o	b	f	5	7.14
28[b]	o	b	b	2	2.86
37[b]	o	b	a	1	1.43
45[c]	s	d	b	2	2.86
54[c]	j	d	b	13	18.57
x`aa[d]	x'	a	a	9	12.86

[a] RFLP types as described by van Eijk et al. (1992).
[b] Allele type designation based on nomenclature described by van Eijk et al. (1992) and Gelhaus et al. (1995).
[c] Allele type described by Maillard et al. (1999).
[d] New allele type observed in Hanwoo which was not reported previously.

accounted for 74.28%. These findings partially agree with the previous results (Sharif et al., 1998a; Gilliespie et al., 1999; Takeshima et al., 2002; Mosafer and Nassiry, 2005; Pasmi et al., 2006). They found that the most frequently detected BoLA alleles in Canadian Jersey cows BoLA-DRB3.2*8, *10, *15, *21, *36 and *ibe accounted for 73.9% of the alleles (Gilliespie et al., 1999); *8, *11, *16, *22, *23 and *24 in Holstein cattle population (Sharif et al., 1998a); *7, *8, *9, *21, *24 and *27 representing 70% of the alleles in Japanese Shorthorn cattle (Takeshima et al., 2002) and *6, *16, *23, *46, *kba and *vaa accounted for 64.7% of the alleles in Iranian native cows (Pasmi et al., 2006). We observed that only 4 (DRB3.2*10, *15, *16 and *27) out of the seven alleles of the present study occurred at high frequency in Jersey, Holstein, Japanese Shorthorn and Iranian native cows as mentioned by previous researchers. The most frequently detected allele BoLA-DRB3.2*54 (18.57%) and the other two frequently occurring alleles DRB3.2 *3 and *26 were not found in other *Bos taurus* breeds. On the other hand, the frequency of the remaining nine alleles (DRB3.2*02, *08, *12, *19, *20, *21, *28, *37 and *45) varied from 1.43 to 2.86%. Our present study demonstrated that a substantial variation exists in allele frequencies between Hanwoo and other cattle breeds. Takeshima et al. (2003) reported that the remarkably dissimilar patterns of distribution of BoLA-DRB3.2 alleles in different breeds might be the result of differential selection after the separation of the major cattle population. The allelic frequencies of BoLA-DRB3.2 may also depend on breed, population and selection pressure (Mosafer and Nassiry, 2005).

Significant associations have been found between some infectious diseases and production traits and the BoLA genes. BoLA-DRB3.2*3 and *16 and *22 alleles were associated with lower risks of retained placenta and cystic ovarian disease in Holstein cows, respectively (Sharif et al., 1998a), innate and adaptive immunity in Holstein cows were related with DRB3.2*2 allele (Dietz et al. 1997), and *22, *23, *24 and *16 alleles have associations with mastitis in cattle (Starkenburg et al., 1997; Ledwidge et al., 2001). Moreover, DRB3.2*10 and *22 alleles have significant associations with reduced fat yield, and decreased milk and protein yield, respectively, in dairy cattle (Sharif et al., 1998b; Ledwidge et al., 2001). The frequencies of BoLA-DRB3.2*2, *3, *10 and *16 were 1.43, 5.71, 8.57 and 12.87%, respectively, in Hanwoo. However, the BoLA-DRB3.2*22, *23 and *24 alleles were not detected in our study.

In order to identify the relationships of the BoLA-DRB3 gene in Hanwoo with other taurine breeds, a phylogenetic tree was constructed using the NJ method with known BoLA-DRB3 sequences from different *Bos taurus* breeds. This phylogeny displayed 4 distinct lineages (Figure 3). Moreover, there is no specific clade for Hanwoo cattle rather the sequences of this breed were intermingled with the sequences of other taurine cattle. Similar phylogenetic patterns were found by Takeshima et al. (2003) with 4 *Bos taurus* breeds including Holstein, Jersey, Japanese Shorthorn and Japanese Black cattle. The low bootstrap values also indicated that there was no significant branch on this tree. It may be concluded that Hanwoo has diverse genetic background for this gene even though it has been

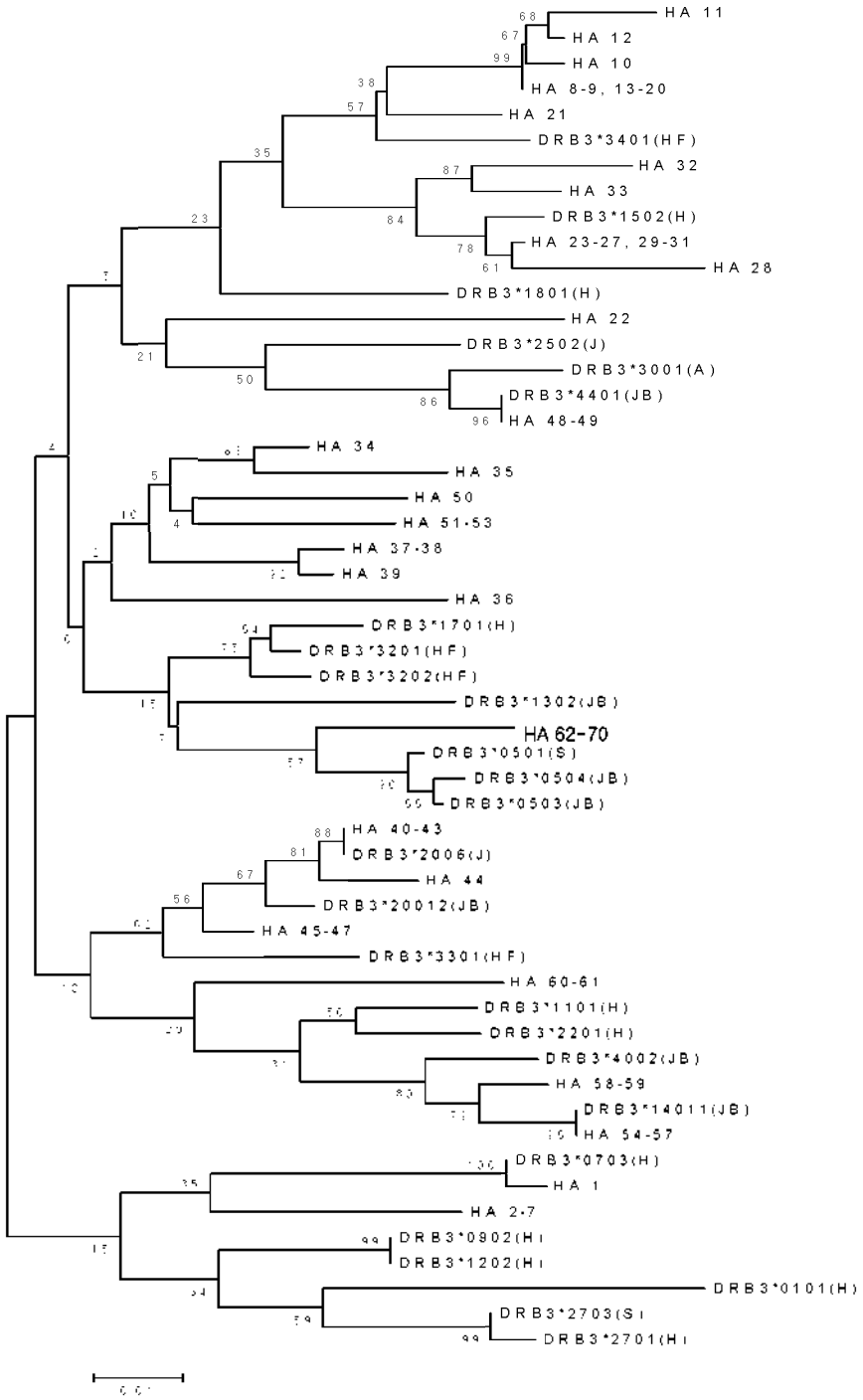

Figure 3. Neighbor-Joining phylogenetic tree constructed from BoLA-DRB3.2 nucleotide sequences of Hanwoo with different taurine cattle breeds using Tamura-Nei distance (Tamura and Nei, 1993). Breed abbreviations are as follows: Holstein (H), Simmental (S), Japanese Black (JB), Hereford (HF), Jersey (J), Black Angus (A) and Hanwoo (HA). The percent numbers at nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets. The scale bar indicates (0.01) nucleotide substitutions per each nucleotide position. Bold letters indicate new type (x`aa) of BoLA-BRB3.2 allele identified in Hanwoo.

bred in the Korean peninsula for a long time. The new alleles found in Hanwoo cattle were shared with Japanese Black and Simmental cattle suggesting that this lineage might be derived from common ancestral alleles that existed prior to the divergence of the breed.

The results of the present study indicated that the BoLA-DRB3 locus is highly polymorphic in Hanwoo and a large variation exists with other breeds of cattle regarding allele frequencies. However, further studies need to be conducted to more preciously define the extent of

polymorphism in Hanwoo. The relationship between the BoLA-DRB3 alleles and disease resistance traits will also be investigated in near future.

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