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# Restriction fragment length polymorphism in the exon 2 of the BoLA-DRB3 gene in Chinese Holstein of the south China

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## ABSTRACT

**The genetic diversity of the exon2 of BoLA-DRB3 (BoLA-DRB3.2) in Chinese Holstein cattle of the south China was investigated by hemi-nested polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Six, four and eleven RFLP patterns were found after digestion with the restriction enzymes Hae III, Bst YI and Rsa I, respectively. The DNA sequence showed and twenty-five DRB3.2 alleles. GLM model analysis indicated that lactation and calving season have positive correlation with SCC (Somatic Cell Count) ($p<0.01$), BoLA-DRB3.2*3, *8, *18 and *26 were associated with lower SCC ($p<0.01$). The present findings concluded that mastitis is a severe hinder of milk production and technology. Therefore, future re-searches should focus on associations of mastitis with BoLA haplotypes rather than single BoLA genes**

**Keywords:** PCR-RFLP; BoLA-DRB3.2; Chinese Holstein; Genetic Polymorphism; SCC

## 1. INTRODUCTION

Major Histocompatibility Complex (MHC), also called Bovine Lymphocyte Antigen (BoLA) has received wide attention because of their association with host immunity. The BoLA gene is located on the short arm of bovine chromosome 23 (BTA23) and consists of three class, I, II and III [1].The class II gene are distributed in two regions, II a and II b, with an approximate recombination frequency of 17% [2]. The DRA, DRB, DQA, and DQB genes are located in the II a region, while the DOB, DYA, DYB and DIB genes in the IIb region. There are at least three DRB-like genes (DRB1, DRB2 and DBR3) in the BoLA region, among which only the DRB3 gene is expressed considerably and is highly polymorphic [1].

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique developed by Van Eijk *et al*. [3] determined alleles in the second exon of BoLA-DRB3 gene (BoL-DRB3.2). This method has advantage of using small amounts of genomic DNA and being adaptable to crude DNA preparations. This advantage will be magnified in case where the studied population is relatively large and the process of extracting DNA with conventional phenol-chloroform methods becomes tedious and labor-intensive. Therefore, PCR-RFLP is a rapid and useful method for DRB3.2 typing in cattle families. But for the outbreed populations sequencing and hybridization techniques are required.

Recently, many researches on BoLA-DRB3 have been reported [4,5,6,7].This study describes genetic variability in the BoLA-DRB3 and its relationship with SCC and production performance in Chinese Holstein cow. This is the first study of the DNA polymorphism of the BoLA-DRB3 gene in Chinese Holstein cows of the south China.

## 2. MATERIALS AND METHODS

### 2.1. Animals and DNA Extraction

Chinese Holstein (n=629) from Shanghai Brightlight Dairy Company were used in the current study. Approximately 5mL of blood was collected from each animal via the caudal rein. The blood was anticoagulated with anticoagulant citrate dextrose (ACD) and stored at -20C.Genomic DNA was extracted from whole blood by the phenol-chloroform extraction method described by Sambrook with some modifications. The quality of the genomic DNA was checked by submarine Agarose gel electrophoresis, purity and concentration were check by spectrophotometer DNA samples with good quality, and purity and concentration were used for further analysis.

### 2.2. PCR Amplification of BoLA-DRB3.2

Exon 2 of BoLA-DRB3 gene (284bp) was amplified by semi-nest PCR, described by Miretti *et al*. [9], to im-

prove the specificity of the PCR product. Primers HL-030 (5'-ATCCTCTCTCTGCAGCACATTTCC-3'), HL-031 (5'-TTTAAATTCGCGCTCACCTCGCCGCT-3') and HL-032 (5'-TCGCCGCTGCACAGTGAAACTCTC -3'), described by Van Eijk [3], were used in the PCR reaction. Briefly, the first stage PCR was performed in a final volume of 20μL containing 50ng of template DNA, 0.5 pm of primer HL-030 and HL-031, 2μL PCR buffer, 1.75mM MgCl2 , 0.25 mM dNTPs, 1.5U Taq DNA Polymerase (Shanghai Sangon). This reaction system was predenatured at 94C for 4min followed by 12 cycles of denaturizing (94C for 1min), annealing (60C for 1min) and elongation (72C for 1min) and a final extension at 72C for5 min. 2μL of the first stage PCR product was used as template DNA.

For the second stage PCR in a final volume of 40μL containing 0.5pM of primer HL-030 and HL-032, 4μL PCR buffer 1.75mM MgCl2, 0.25mM dNTPs, and 2U Taq DNA polymerase. The solution was predenatured at 94C for 4 min followed by 30 cycles of denaturizing (94C for 60s), annealing (63C for 45s), and elongation (72C for 45s) and a final extension (72C for 5min).5μL of the second stage PCR product was electrophoresed on 1.2% agarose gels to check the quality and specificity of DNA fragment amplification.

### 2.3. RFLP

To examine the nucleotide sequence variability at the BoLA-DRB3.2 locus, three end nucleotide restriction enzymes (Hae III, Bst YI and Rsa I) were chosen based on their cut site and ability to cut DNA in this exon. The second stage PCR products were digested with the restriction enzymes according to the manufacture's instructions. A 15μL of the second stage PCR product containing 8μL, 0.5U of restriction enzymes, and 6.8μL of 1×Buffer were digested at 37C for 12h followed by inacting at 80C (Hae III and Bst YI) or 65C (RsaI) for 20min. The resulting DNA fragment were separated on 14% PAGE gels with 1×TBE buffer (0.9M Tris-Base, 0.09M Boric Acid, 2.5mM EDTA) at 150V for 5h, using Msp I digested pBR322/MspI as a molecular marker. After ethidium bromide (EB) staining, the gels were photographed under UV light and the relative migrations of the DNA bonds were estimated. The restriction patterns obtained were compared with previously described restriction maps [10].

### 2.4. DNA Sequencing

BoLA-DRB3.2 DNA sequence was performed base on the restriction enzyme sites. The sequence was compared with the gene Bank sequences.
http://www.projects.roslin.ac.uk/bola/bolanom.html

### 2.5. SCC Analysis

SCC is leukocytes that inter the milk from the alveoli and they are considered an important indicator of mastitis infection. The SCC of the experimental cows was detected every month. In order to express the effect of calving season on SCC, the data are divided into four parts according to calving season.

### 2.6. Statistic Analyses

GLM (General Linear Model) of SCC and production performance was performed by SAS6.12 (SAS Institute, 1996), the models are as follows:

$$Yijkm=\mu+Li+Sj+\sum blBoLAijkl+eijkm$$

Yijkm: SCC, Milk Yield, Fat Percent Protein percent μ: the average value, Li: lactation number, i=1～4, Sj: calv-ing season=1～4, bl: regression coefficient of allele to SCC, l= 1～22, BoLAijkl: the copy number of BoLA al-lele l in individual ijk, the value is 0, 1 and 2, eijkm: random error.

## 3. RESULTS

### 3.1. DRB 3.2 Amplification

DNA bands of the expected size containing 267 bp of exon 2, 3 bp of the 3' intron and 14bp of the 5'intron, were received by semi-nested PCR amplification (**Figure 1**). The specificity of the PCR product was very high, but some nonspecific bands were observed. Although these bands could not be eliminated by modifying PCR conditions, this did not affect the resolution of restriction patterns.

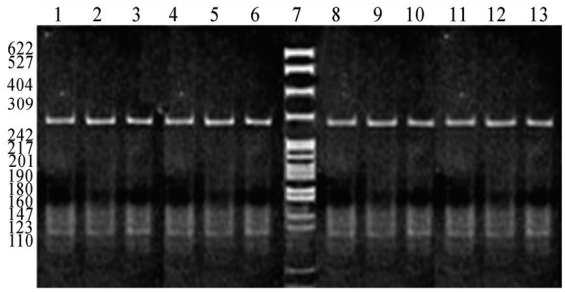


**Figure 1.** Patterns of the semi-nested PCR.

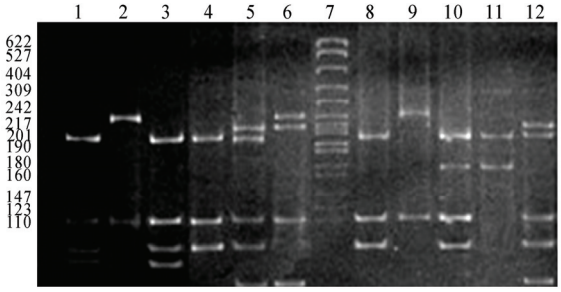


**Figure 2.** Genotypes of BoLA-DRB3.2 locus digested with Hae III.

 

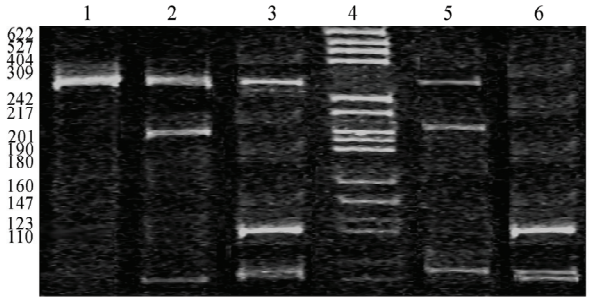


**Figure 3.** Genotypes of BoLA-DRB3.2 locus digested with Bst YI.

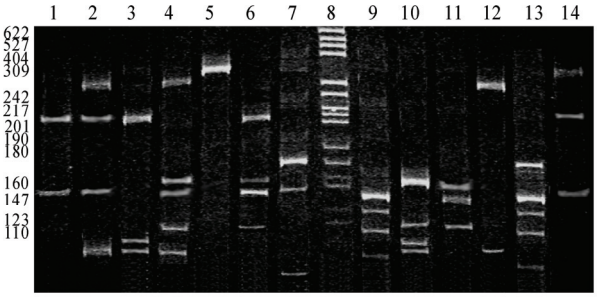


**Figure 4.** Genotypes of BoLA-DRB3.2 locus digested with Rsa I.

The DNA sequence was 284 bp, containing 267 bp of exon 2, 3 bp of the 3' intron and 14bp of the 5'intron.

### 3.2. Identification of Restriction Patterns

The 284 bp fragment of the BoLA-DRB3.2 gene in this study were digested with Rsa I, Hae III and Bst YI respectively. Six restriction patterns were identified with Hae III (See **Table 1** and **Figure 2**). Bst YI resulted in four RFLP patterns a,b,d and e (See **Table 1** and **Figure 3**). RsaI restriction patterns were so complex that eleven patterns were revealed in this study (See **Table 2** and **Figure 2**).

### 3.3. DNA Sequencing and Standard Naming

Combining restriction patterns with Rsa I, Bst YI and Hae III in the whole sample, 22 DRB3.2 alleles found in the study were sequenced (See **Table 2** and **Table 3**). Many mutants were indicated in the cut sites of restriction enzymes by DNA sequencing. The frequency of BoLA-DRB3.2 ranged from 0.003 (2*34) to 0.220 (2*22).

**Table 1.** Fragment Size and Frequencies of Restriction Patterns Digested with Enzymes.

| Enzyme | Restriction Patterns | Fragment Size(bp) | Frequency | Enzyme | Restriction Patterns | Fragment Size(bp) | Frequency |
|---|---|---|---|---|---|---|---|
| Hae III | a | 167/65/52 | 0.598 | Rsa I | b | 111/54/50/39/30 | 0.061 |
| | b | 219/65 | 0.238 | | d | 143/111/30 | 0.024 |
| | d | 190/65/29 | 0.049 | | f | 141/54/50/39 | 0.085 |
| | e | 167/117 | 0.037 | | g | 141/104/39 | 0.082 |
| | f | 167/65/48/4 | 0.061 | | h | 111/69/54/50 | 0.082 |
| | h | 167/65/46/6 | 0.017 | | i | 180/54/50 | 0.006 |
| Bst YI | a | 199/85 | 0.095 | | j | 93/78/63/50 | 0.052 |
| | b | 284 | 0.823 | | l | 234/50 | 0.113 |
| | d | 197/87 | 0.012 | | m | 111/104/69 | 0.241 |
| | e | 112/87/85 | 0.070 | | n | 180/104 | 0.195 |
| | | | | | o | 284 | 0.058 |

**Table 2.** Allele Frequencies of BoLA-DRB3.2 identified by PCR-RFLP analysis.

| DRB3.2 Allele | Patterns | | | Alleles Num | Frequency | DRB3.2 Allele | Patterns | | | Alleles Num | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rsa I | Bst YI | Hae III | | | | Rsa I | Bst YI | Hae III | | |
| *3 | b | b | b | 41 | 0.0652 | *18 | l | b | f | 26 | 0.0413 |
| *6 | d | a | a | 17 | 0.0270 | *21 | l | b | e | 23 | 0.0366 |
| *8 | f | a | a | 5 | 0.0079 | *22 | m | b | a | 148 | 0.2353 |
| *9 | f | d | a | 8 | 0.0127 | *23 | n | b | a | 64 | 0.1017 |
| *10 | f | b | a | 35 | 0.0556 | *24 | n | b | b | 58 | 0.0922 |
| *11 | g | e | a | 55 | 0.0874 | *26 | o | a | b | 5 | 0.0079 |
| *12 | h | a | a | 13 | 0.0207 | *28 | o | b | b | 29 | 0.0461 |
| *13 | h | b | a | 28 | 0.0445 | *32 | m | a | a | 13 | 0.0207 |
| *14 | h | b | b | 14 | 0.0223 | *33 | n | b | f | 19 | 0.0302 |
| *15 | i | b | a | 5 | 0.0079 | *36 | l | b | a | 9 | 0.0143 |
| *16 | j | b | d | 3 | 0.0048 | *42 | f | a | h | 11 | 0.0175 |

 

**Table 3.** The sequences of BoLA-DRB3.2 alleles indicated in the paper.

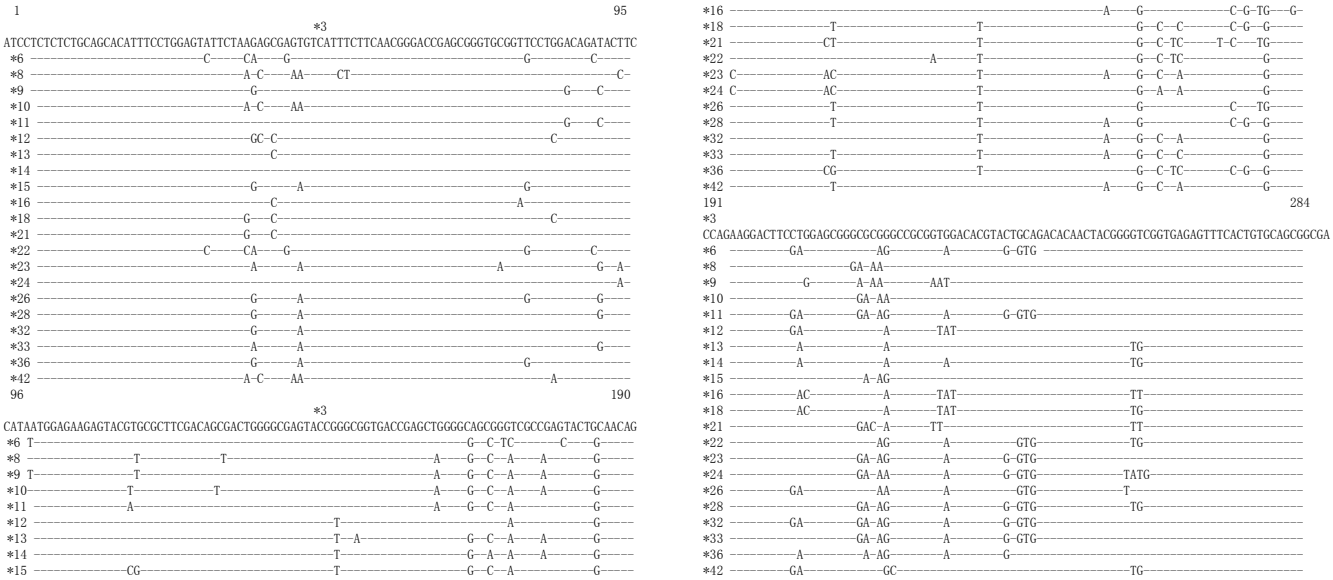

**Table 4.** The correlation between BoLA-DRB3.2 alleles, lactation, seasons of calving and SCC and performance traits in Chinese Holstein.

| Factor | SCC | Factor | SCC |
|---|---|---|---|
| μ | +580.61 | μ | +580.61 |
| BoLA-DRB3.2*3 | -336.95** | BoLA-DRB3.2*21 | -503.95* |
| BoLA-DRB3.2*6 | NS | BoLA-DRB3.2*22 | -413.38* |
| BoLA-DRB3.2*8 | -623.01** | BoLA-DRB3.2*23 | -345.89 |
| BoLA-DRB3.2*9 | NS | BoLA-DRB3.2*24 | NS |
| BoLA-DRB3.2*10 | NS | BoLA-DRB3.2*26 | -878.14** |
| BoLA-DRB3.2*11 | -341.62 | BoLA-DRB3.2*28 | -386.66 |
| BoLA-DRB3.2*12 | NS | BoLA-DRB3.2*32 | NS |
| BoLA-DRB3.2*13 | NS | BoLA-DRB3.2*33 | NS |
| BoLA-DRB3.2*14 | NS | BoLA-DRB3.2*36 | -395.54 |
| BoLA-DRB3.2*15 | NS | BoLA-DRB3.2*42 | NS |
| BoLA-DRB3.2*16 | -353.07 | Lactation | +195.19** |
| BoLA-DRB3.2*18 | -511.33** | Calving Season | +49.91** |

Note:*means significant difference, P<0.05; **means significant difference, P<0.01; NS means no significant difference. The values means regression coefficient, +means positive correlation, -means negative correlation.

### 3.4. Effect of BoLA-DRB3.2, Lactation and Caving Season on SCC

The effect of BoLA-DRB3.2, lactation and caving season on SCC and production performance are displayed in **Table 4** Lactation and calving season have positive correlation with SCC (p<0.01), most alleles of BoLA-DRB3.2 have negative correlation with SCC, among them, BoLA-DRB3.2*3, *8, *18 and *26 are the most associated with SCC (p<0.01).

## 4. DISCUSSION

Taking an important role in the immune system of animals and having a close relationship with resistance to diseases, MHC has become a candidate gene and a hot point of recent researches. It is widely said that the high polymorphism of MHC-DRB3 genes was decided by its important function made by class II antigen of MHC in the immune system. In order to adapt to various geographic and climatic conditions, the immune system is highly polymorphic. So the polymorphism of BoLA-DRB3 gene, encoding the main functional area of MHC antigen, is very high.

Miretti *et al.* [9] identified the polymorphism of BoLA-DRB3 gene in Argentinean Holstain using PCR-RFLP. Four patterns a, b, d and e were found with Hae III, three (a, b and d) and eleven patterns (b, c, d, f, g, h, I, j, l, m, n and o) were found with Bst YI and RsaI, respectively. In this research, Hae III f, h and BstYI d were detected.

 

The most frequent (frequency＞0.05) BoLA-DRB3.2 alleles of 835 Holstein dairy cattle re-ported by Sharif *et al*. [11] were BoLA-DRB3.2*3(0.0652), *8(0.0079), *11(0.0874), *16(0.0048), *22(0.2353), *23(0.1017), *24(0.0922). In the present study, the most frequent alleles were BoLA-DRB3.2*3(0.0652), **11(0.0874), *16(0.0048), *22(0.2353), *23(0.1017), *24(0.0922). Sharif *et al* reported that BoLA-DRB3.2 *8 was the most common allele type in Holstein, However, in this study, the BoLA-DRB3.2 *16 was at low frequency (0.0048), while the BoLA-DRB3 *22 was the most common allele (0.2353). Therefore, BoLA polymorphism information from the research herd seemed representative of the regional Holstein population. These differences may be largely due to the long-term adaptation to different geographical and climatic conditions. Therefore, BoLA polymorphism information from the research herd seemed representative of the regional Holstein population. These differences may be largely due to the long-term conditions.

Many mutants, changing the restriction site and the restriction maps, were identified in the cut sites by DNA sequencing. RFLP, as a method to identify the polymorphism of BoLA-DRB3, can only define the position of mutants. Other methods must be used to find the detail mutants. Result of our study demonstrated that the BoLA-DRB3.2 locus is highly polymorphic in Chinese Holstein. PCR-RFLP may therefore be a rapid and useful method for DRB3 typing and studying the evolutionary changes in dairy cattle.

Sharif reported calving season had no significant effect on SCC, but this research indicated that calving season significantly affect SCC; there was a trend that cows calving in spring and summer have high SCC. Related to BoLA-DRB3.2 and SCC, the result in this research is different from Sharif *et al.* [11], among the most frequently detected alleles, BoLA-DRB3.2*3 has significant correlation to low SCC, BoLA-DRB3.2*8, 18 and 26 maybe have same function, but their frequencies are very low.

Contradictory results from different studies investtigating associations between BoLA-DRB3.2 alleles and mastitis indicate that future studies should focus on associations of mastitis with BoLA heliotypes rather than with single BoLA genes.

## 5. ACKNOWLEDGEMENTS

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