Asian-Aust. J. Anim. Sci.
Vol. 21, No. 4 : 465 - 470
April 2008

www.ajas.info

The Diversity of *BoLA-DRB3* Gene in Iranian Native Cattle

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ABSTRACT: This study describes genetic variability in the *BoLA-DRB3* gene in Iranian native cattle (*Bos Indicus* and *Taurus*) and relationships between these breeds. This is the first study of genetic polymorphism of the *BoLA-DRB3* gene in Iranian native cattle. We examined exon 2 of the major histocompatibility complex (MHC) class II *DRB3* gene from 203 individuals in four populations of Iranian native cattle (52 Sarabi, 52 Najdi, 49 Sistani, 50 Golpayegani cattle) using the hemi-nested PCR-RFLP method. We identified the 36 previously reported alleles and one novel pattern (**eac*). Analysis of the frequencies of the various *BoLA-DRB3.2* alleles in each breed indicated that *DRB3.2*52* in Sarabi cattle (23%), *DRB3.2 *14* and **24* alleles in Najdi cattle (13%), *DRB3.2 *8* allele in Sistani cattle (22%) and *DRB3.2*16* allele in Golpayegani cattle (14%), were the most frequent alleles. Allelic frequencies ranged from 1 to 23% among the 36 alleles and there were some alleles that were found only in Iranian cattle. Effective number of alleles in the four breeds was estimated to be 7.86, 11.68, 7.08 and 3.37 in Sarabi, Najdi, Sistani and Golpayegani, respectively. Observed heterozygosities were the highest in Sarabi (94%) and Najdi (94%). A population tree based on the frequency of *BoLA-DRB3.2* alleles in each breed suggested that Najdi, Sarabi and Golpayegani cattle clustered together and Najdi and Sarabi were the closest breeds. Sistani cattle differed more from these three breeds. These new data suggest that allele frequencies differ between Iranian cattle breeds. (**Key Words :** *BoLA-DRB3*, PCR-RFLP, Iranian Native Cattle, MHC)

INTRODUCTION

Genetic characterization to assess the existing biodiversity and differences among the important cattle breeds is an essential prerequisite to facilitate a conservation program in an effective and meaningful way. More recently, an array of new markers has been developed to carry out genetic variation studies at DNA level (Bradely et al., 1996; Mac-Hugh et al., 1998). Among these, *BoLA-DRB3.2* is considered a suitable marker system for genetic diversity studies owing to abundance in the mammalian genome and high polymorphism for automation (Takeshima et al., 2002). Major histocompatibility complex (MHC) genes, also called bovine lymphocyte antigen (*BoLA*), have received attention because of their high degree of genetic polymorphism and association with immunity. The *BoLA* genes are located on the short arm of bovine chromosome 23. The polymorphic sites of the *BoLA - DRB3* gene are mainly located in exon 2 of class II (Pashmi et al., 2006). The MHC variability in natural populations is of great interest to evolutionary biologists because of the typically high levels of polymorphism. Consequently, representative species of several mammalian orders, including Artiodactyls, Carnivore, Cetacea, Primates and Rodentia, have been characterized with respect to *BoLA-DRB3* allelic diversity (Mikko and Anderson, 1995). Variability within and among populations of a certain species is directly related to the interplay between effective population size, time of divergence and the intensity of selective pressure at a particular locus. Many studies have indicated that differences exist between breeds of cattle and other animals with regard to frequencies of MHC class II alleles. In cattle, interpretations of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing studies indicate there are significant differences in allelic frequencies of *BoLA-DRB3.2* in Jersey (Gilliespie et al., 1999), Holstein (Dietz et al., 1997), Argentine Creole (Gimovambattista et al., 2001), Japanese Shorthorn (Takeshima et al., 2002) and Brazilian dairy Gir cattle (da Mota et al., 2002), Black Pied (Sulimova et al., 1997), Ayrshire (Udina et al., 2003), Hanwoo (Jeong et al., 2007) and Iranian Holstein cattle (Nassiry et al., 2004). Thus, the frequencies of alleles of BoLA-DRB3 genes in different populations allow the differentiation and reconstruction of genetic distance among populations, providing a molecular basis for determination of the possible common origin of

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Received January 23, 2007; Accepted August 26, 2007

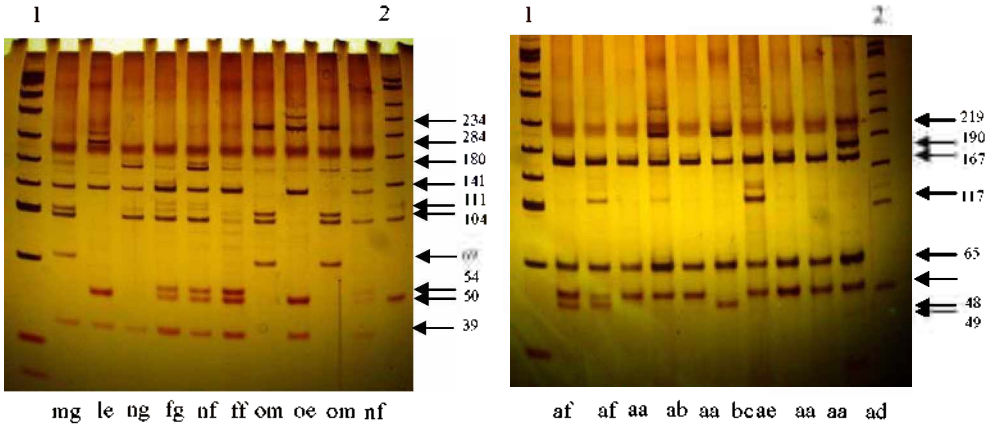

Figure 1. Restriction analysis of amplification products in exon 2 of gene *BoLA-DRB3* in 8% polyacrylamide gel. Right picture: restriction *Hae*III, Left picture: restriction *Rsa*I. As a molecular marker *Msp*I-fragments of plasmid *pUC*19 (lane 1) and M50 bp (lane 2) were used.

populations and can be used to reduce spreading of alleles providing susceptibility to disease in a cattle herd. More than 90 alleles were tested with sequence analysis. By means of PCR (for amplification of exon 2 of the gene) followed by analysis of restriction fragment length polymorphism (RFLP), it is possible to identify the *BoLA-DRB3* alleles (53 alleles) (Van Eijk et al., 1992; Takeshima et al., 2002). Most class II genes show large genetic variation between and within species in both the numbers of loci and alleles.

Further genetic variability and sequencing data from *B. indicus* (Sistani, Golpayegani and Najdi) are needed to characterize the variability of the bovine MHC. The present study, using a PCR-RFLP method, was designed to determine the allelic frequencies of exon 2 of the *BoLA-DRB3.2* gene in a total of 203 individuals belonging to four distinct Iranian cattle breeds, namely, Sarabi (*Bos Taurus*), Najdi, Sistani, Golpayegani.

These four Iranian cattle breeds are located at distinct geographical areas in Iran. Sarabi, Golpayegani and Najdi are located in the Moghan region (eastern Azerbaijan state, Northwest of Iran), Golpayegan region (Esfahan state, center of Iran) and Khouzestan state (Southwest of Iran), respectively. Sistani is a heavy built, dual-purpose (meat and draft) cattle breed of Southeast Iran (Tavakkolian, 2000). Sistani and Najdi are also native to Pakistan and Iraq, respectively. We also estimated the genetic differentiation and the genetic relationship within and between the four native Iranian cattle breeds.

MATERIALS AND METHODS

Animals and DNA extraction

In total, 203 individuals obtained from different regions were examined for the distribution of *BoLA-DRB3.2* alleles. These included 52 Sarabi (Sarab city), 52 Najdi (Azerbaijan state), 49 Siatani (Zabol city) and 50 Golpayegani cattle (Delijan city). Blood samples were collected in 0.5% EDTA and DNA was extracted from 100 µl of blood according to the method of Boom et al. (1989).

Amplification of *BoLA-DRB3* exon 2

Exon 2 of the *DRB3* gene was amplified by the hemi-nested polymerase chain reaction. Primers HL030 (5'-ATCCTCT CTCTGCAGCACATTTCC-3') and HL031 (5'-TTTAATT CGCGCTCACCTCGCCGCT-3'), previously published by Van Eijk et al. (1992), were used in the first amplified round for all individuals of different breeds. Amplification reactions were carried out with 50 ng of DNA (5 µl) in a 25 µl total volume containing 1×PCR buffer; 2.5 mM $MgCl_2$; dNTPs, 100 µM of each; 0.5 µM of each primer and 1 unit of *Taq* DNA polymerase. The thermal cycling profile for the first round of amplification was an initial denaturation step of 3 min at 94°C followed by 10 cycles of 25 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final extension step of 5 min at 72°C. The second round of PCR was carried out with 3 µl of first-round product into one new tube, with the same volume and concentration of contents as described above, and using primers HL030 and HL032 (5'-TCGCCGCTGCACAGTGAAACTCTC-3'). Primer HL032 is internal to the sequence of the amplified product of the first-round PCR and has eight bases that overlap with primer HL031 (underlined in the text above). The thermal cycling profile for the second round was 25 cycles of 40 s at 94°C for the denaturation step and 30 s at 65°C for the annealing step, followed by a final extension step of 5 min at 72°C. PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

BoLA-DRB3 typing

PCR products were digested with *Rsa*I, *Hae*III and *BstY21* enzymes (Sibenzyme, Moscow). Restriction

Table 1. Frequencies and heterozygosities of *BoLA-DRB3.2* alleles for studied cattle breeds

DRB3 alleles	Sarabi (*N* = 52*2)	Najdi (*N* = 52*2)	Sistani (*N* = 49*2)	Golpayegani (*N* = 50*2)
2	10	2	-	4
3	2	2	1	2
4	-	-	2	3
7	-	-	4	11
8	2	8	22	-
10	-	-	4	6
11	18	11	5	7
12	10	5	-	6
13	-	-	3	-
14	2	13	-	-
15	-	5	8	2
16	-	-	-	14
17	2	2	-	-
19	2	-	-	10
20	2	-	-	2
21	-	-	2	-
22	2	2	-	2
23	15	11	-	-
24	-	13	2	2
25	3	-	-	2
28	-	-	-	8
29	-	-	1	-
31	-	-	-	4
34	-	-	21	-
35	-	-	-	3
36	-	11	2	-
37	-	-	1	-
43	6	3	-	-
44	-	-	6	-
45	-	-	2	6
47	-	-	3	-
48	5	-	-	-
51	-	-	1	-
52	23	6	-	6
53	-	5	-	-
54	-	5	-	-
Eac	-	-	8	-
No. of alleles	15	16	19	19
Ne	7.86	11.68	7.08	13.37
$H^{observed}$	0.94	0.94	0.20	0.52
$H^{expected}$	0.87	0.91	0.91	0.93

[a] *N* = Number of individuals. [b] Ne = Effective number of alleles.
[c] H = Heterozygosity rate. [d] X = A new allele.
[e] The most frequent alleles in each breed are showed in boldface.

fragments were revealed by gel electrophoresis on 8% acrylamide gel and visualized with silver staining. *pUC19/Msp*I and *M50* size markers were used as molecular weight markers.

BoLA-DRB3.2 typing was performed using a PCR-RFLP method developed by Van Eijk et al. (1992). To date, more than 53 alleles have been identified by restriction enzyme digestion of a 284 bp PCR product of *DRB3* exon 2 and 103 alleles have been identified by PCR-sequence-based typing (SBT) (Takeshima et al., 2001). The nomenclature for alleles of *BoLA-DRB3* defined by the PCR-RFLP method is indicated by the format locus.exon.allele, e.g., *DRB3.2*16*.

Statistical analysis

Allele frequencies (*f*) were obtained by direct counting. The observed frequencies of heterozygotes ($H^{observed}$), were obtained directly by dividing the number of heterozygous individuals by the total number of individuals. The expected frequencies of heterozygote ($H^{expected}$) and the effective number of alleles were calculated using Arlequine ver. 2.000 (Schneider et al., 2000). Nei's genetic identity, genetic distance and dendrogram based Nei's genetic distance were assessed using the Popgene ver. 1.32 software. The observed heterozygosity (H^{obs}), expected heterozygosity (H^{exp}) and effective number of alleles (Ne) were calculated with the same program.

RESULTS

Allelic frequencies

Distribution of alleles of *BoLA-DRB3* in the studied groups of native animals of Sarabi, Najdi, Sistani and Golpayegani are presented in Table 1. The spectrum of the most frequent alleles was different in the four breeds. The genotypes of 203 individuals from four different Iranian populations of cattle were determined for exon 2 of the *BoLA-DRB3* allele by PCR-RFLP typing. The 36 previously reported alleles and one novel allele were identified: 15 previously reported alleles were obtained from 52 Sarabi cattle, 16 from Najdi cattle, 19, including one new allele (**eac*), from Sistani cows and 19 from Golpayegani cows (Table 1). Allelic frequency ranged from 1 to 23% and almost 39% of the alleles were accounted by fourteen alleles (*DRB3.2*2, *7, *8, *11, *12, *14, *15, *16, *19, *23, *24, *34, *36, *54* alleles) in these four Iranian native cattle. The frequency of new pattern (**eac*) was 8.1%.

Effective number

Effective numbers of alleles in the four breeds were estimated 7.86, 11.68, 7.08 and 3.37 in Sarabi, Najdi, Sistani and Golpayegani, respectively.

Heterozygosity

Observed frequency of heterozygotes and expected frequency of hemozygotes were computed to assess genetic variability in the four Iranian native cattle breeds. The values are shown in Table 1. Observed heterozygotes were the highest in Sarabi (94%) and Najdi (94%).

Genetic distance and population tree

The Nei's genetic distance values and genetic identity

Table 2. Nei's genetic identity and genetic distance among four Iranian native cattle breeds

Breeds	Sarabi	Najdi	Sistani	Golpayegani
Sarabi	****	0.5798	0.1102	0.4161
Najdi	0.5451	****	0.3209	0.2543
Sistani	2.2055	1.1365	****	0.1596
Golpayegani	0.8768	1.3691	1.8353	****

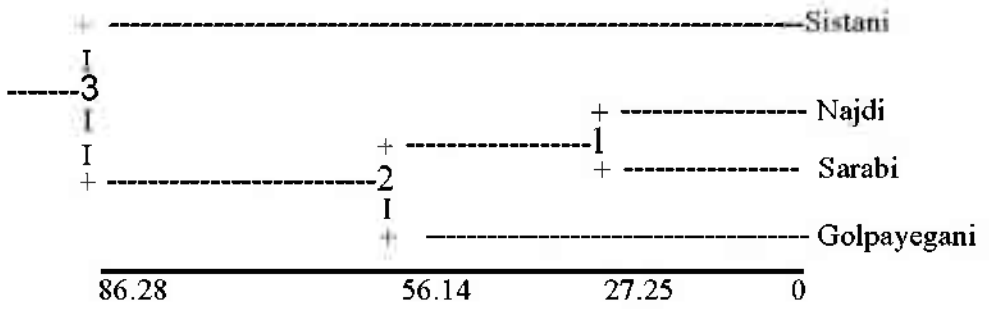

Figure 2. Dendrogram Based on Nei's (1978) genetic distance clustered by unweighted pair group method with arithmetic mean for BoLA-DRB3.2 alleles.

for the four Iranian native cattle are shown in Table 2. A dendrogram, based on UPGMA cluster analysis using Nei's genetic distance, revealed the genetic relationship among the breeds. Nei's genetic distance within the four Iranian cattle breeds ranged from 0.5451 to 2.2055. The highest value of genetic identity was observed between Sarabi and Najdi. The lowest was found between Sarabi and Sistani. A population tree based on the frequency of *BoLA-DRB3.2* alleles in each breed suggested that Najdi, Sarabi and Golpayegani cattle clustered together and Najdi and Sarabi were the closest. Sistani cattle differed most from these three breeds.

DISCUSSION

Iranian cattle (*Bos indicus* and *Bos taurus*) have unique features like adaptability to extreme climatic conditions, subsistence on poor feed and fodder and better resistance capabilities to withstand environmental stress and tropical disease. There are several diverse cattle breeds in Iran that are primarily being used for draught, milk and meat. Although, cattle in Iran is the most important livestock species and plays a major role in agricultural economy, the population of some of the important cattle breeds is either declining or breed characters are being diluted under the present production system. To avoid further loss of important gene/gene-pool and preserve maximum amount of genetic diversity, an objective breed classification based on genetic uniqueness is of priority (Hall and Bradley 1995). In cattle, analysis of allelic variation at *BoLA-DRB3.2* loci could potentially be used to evaluate temporal changes in genetic diversity. There are no studies of *BoLA-DRB3* genotyping in the important Iranian cattle breeds. This study determined the *BoLA-DRB3.2* allelic frequencies, heterozygosity and genetic distance. In addition, we identified the 36 previously reported alleles (http://www.projects.roslin.ac.uk/bola/drb3pcr.html) and one novel pattern (**eac*). Results of the present study indicate that the *BoLA-DRB3* locus is highly polymorphic in Iranian native *Bos indicus* (Sarabi) and *Bos taurus* (Sistani, Najdi and Golpayegani). There are significant differences in frequencies of *BoLA-DRB3* allelic frequencies between Iranian cattle breeds. The frequencies of the *DRB3.2 *2, *7, *8, *11, *12, *14, *16, *23, *24, *3* and **52* alleles were higher than 10% in Iranian cattle. Similarly, the five most frequent detected alleles in Holstein were *DRB3.2 *8, *11, *16, *2* and **24*). On the other hand, the other six most frequent isolated alleles in Jersey cows were *DRB3.2 *8, *10, *21, *36*, and **ibe*.

Also, differences between Iranian cattle breeds existed. For example: *DRB3.2*52* was the most frequent allele in Sarabi cattle (23%), *DRB3.2 *14* and **24* were the most frequent allele in Najdi cattle (13%). *DRB3.2 *8* was the most frequent allele in Sistani cattle (22%) and *DRB3.2*16* was the most frequent allele in Golpayegani cattle (14%), indicating that the frequencies of alleles differed in each breed. Among 36 alleles, *DRB3.2 *52* was the most frequent allele in Sarabi (52%) but was found in Najdi and Golpayegani with equal frequency of 6%. The *DRB3.2 *14* and **24* were the most frequent alleles in Najdi (13%), but had the least frequency in other breeds (allele **14* was not present in both Golpayegani and Sistani and allele **24* was not present in Sarabi). The *DRB3.2*8* was the most frequent in Sistani, was not detected in Golpayegani cattle and had the least frequency in other breeds. In addition, *DRB3.2*16* was the most frequent in Golpayegani cattle, but was not found in other breeds. This investigation showed that the most frequent allele in each population is specific for breed.

Additionally, some of these alleles were only in a special breed of Iranian cattle. For example, the allele *DRB3.2*48* was found only in Sarabi and alleles *DRB3.2*53* and **54* were found only in Najdi. The eight alleles (*DRB3.2 *34, *44, *47, *13, *21, *51, *37, *eac*) were observed only in Sistani. Also, in the 203 Iranian native cattle examined, *DRB3.2 *3, *11* alleles (with higher frequency) and **22* allele (with lower frequency) were common alleles. A high degree of *BoLA-DRB3.2* polymorphism has also been reported in studies of Holstein, Jersey, Japanese Shorthorn and Argentine Creole cattle (Giovambattista et al., 1996; Dietzet al., 1997a; Dieta et al., 1997b; Sulimova et al., 1997; Gilliespie et al., 1999; Takeshima et al., 2002; Udina et al., 2003; Nassiry et al., 2005).There were significant differences in frequencies of *BoLA-DRB3* alleles between Iranian and other world cattle breeds. Only seven (*DRB3.2 *34, *44, *47, *13, *21, *51, *37*) of 11 alleles occurred at high frequency in Jersey, Japanese and Argentine breeds.

The most frequently detected *BoLA* alleles of Iranian Holstein Cattle were *DRB3.2 *8, *11, *12* and **23* (Nassiry et al., 2005). In the present study, similar results were observed in Sarabi cows and only allele **8* was detected at a high frequency in Sistani cows. Thus, it would appear that differences in allelic frequencies exist between the *Indicus* and *Taurus*.

The sequence of 1 of 36 alleles was different from previously characterized BoLA-DRB3 alleles (van Eijk et al., 1992; and Gilliespie et al., 1999). A new pattern of **eac* was observed for the first time in our study with high frequency (8.1%) in Sistani cattle. The obtained sequence of the new pattern was submitted to the NCBI with accession number DQ486519. The new pattern differs from reference sequence in 24 nucleotides resulting in 16 amino acid substitutions. These replacements include 3 A-G; 8 G-C; 4 C-T; 4 A-T; 3 G-T; and 2 C-A across the sequence.

Comparison of the frequencies of *BoLA-DRB3* alleles in *B. taurus* and *B. indicus* shows that five alleles (DRB3.2. **35, *34, *47, *44* and **53*) were found were found only in *B. indicus* breeds (Sistani, Golpayegani, Najdi, Gir, Gudali Zebu, Zebu Brahman, Caracu, Pantaneiro and Curraleiro (Nassiry et al., 2005). These results provide evidence that breeds of *B. taurus* and *B. indicus* can be clearly differentiated and indicate that many more alleles remain to be discovered. Therefore, further sequencing data from *B. indicus* and *B. taurus* is needed to characterize the variability of bovine MHC.

The number of alleles per breed ranged from 7.08 to 13.37 with an average of 10 alleles per Iranian breed. The highest number of effective alleles was observed in Golpayegani (Ne = 13.37) and Najdi (Ne = 11.68). In Sarabi and Sistani the frequency of the effective number of alleles reached almost 7.86 and 7.08, respectively. These numbers are also reflected in the mean heterozygosity.

Variation across the four populations was not homogeneously distributed. The Sarabi and Najdi showed the maximum mean observed heterozygosity (H^{obs} = 0.94) while the Sistani population showed the minimum H^{obs} at 0.20. The heterozygote deficiency observed in the Sistani and Golpayegani might be explained by inbreeding due to small number of reproducers and genetic drift.

For finding the evolutionary relationships among closely related populations, *BoLA-DRB3.2* is rated the more suitable and informative marker system. The diversity data generated for Iranian native cattle (*B. indicus*, except Sarabi breed) may be utilized for characterizing the genetic relationships with *B. indicus* and *B. taurus* from other countries as well. The smallest genetic distance for *DRB3* alleles were those between Sarabi and Najdi (0.5451). The genetic distance between Sistani cattle and the other three breeds was large and Sistani were clustered on a different branch from the other three breeds. Collectively, the results suggested that the Sarabi and Najdi breeds were closer to each other; with Sistani being further from these two breeds than Golpayegani. A similar relationship between Sarabi, Najdi and Golpayegani cattle may be the result of the massive introgression between these populations due to being geographically adjacent to each other. On the other hand, Sistani cattle are from a geographical area relatively far from the location of the other three breeds. Thus, the genetic relationship of these four native Iranian cattle breeds corresponds to their breeding history and geographic origins. The usefulness of *BoLA-DRB3.2* for the estimation of genetic distances among populations has been documented by numerous studies. Takeshima et al. (2003) constructed a population tree on the frequencies of *BoLA-DRB3.2* alleles. On this tree, the smallest genetic distances were between Holstein and Japanese Blacks (0.2803). In this work, Jerseys were clustered on a different branch from the other three Japanese cattle (Japanese Black, Japanese and Shorthorn).

Thus, the frequencies of alleles of MHC genes allowed the differentiation and reconstruction of genetic distance among different populations, providing a molecular basis for determination of the possible common origin of populations. This study is the first using *BoLA-DRB3.2* polymorphism to understand genetic diversity of native cattle breeds in Iran. Very little information is currently available to compare different cattle populations from Iran. Although we have used only four representative breeds, the present study may be regarded as the beginning of attempts to understand the genetic diversity of local cattle breeds in Iran. Further investigations including more native Iranian cattle breeds would be useful to clarify their recent origin and relationships between them. Also, our analysis showed that breeds of *B. taurus* and *B. indicus* can be differentiated in *BoLA-DRB3* variability. Altogether, this analysis defines

one novel PCR-RFLP type that may be added to the *BoLA-DRB3.2* allele list. Therefore, it may be concluded that the *BoLA-DRB3* locus is effective in detecting polymorphism between cattle breeds, and provide a potential tool for studying inter- and intra-breed genetic variability and for establishing genetic relationships.

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