Genetic Polymorphism at the Candidate Gene in Iranian Sistani Cattle (*Bos indicus*)

Mojtaba Tahmoorespur, Mohammad Reza Nassiry, Mohsen Fathi Najafi and Shahrokh Ghovvati
Department of Animal Science, College of Agriculture, Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, Iran
Razi Vaccine and Serum Research Institute, Mashhad, Iran

**Abstract:** The genotypes for Leptin, Kappa-Casein, Calpastatin and BoLA-DRB3 loci were determined by polymerase chain reaction and restriction enzyme digestion method in native Iranian breed cattle, Sistani. Blood samples were collected from Sistani Breeding Station located in Zahak, Zabol in Iran. The extraction of genomic DNA was based on Guanidin Thiocyanate-Silica gel method. After PCR reaction, amplicons were digested with appropriate restriction enzymes. The Calpastatin locus had 3 genotypes with frequencies of 0.62, 0.29 and 0.09 for MM, MN and NN, respectively; κ-Casein and Leptin had 3 genotypes with frequencies of 0.27, 0.57 and 0.16 for κ-Casein, 0.77, 0.22 and 0.01 for Leptin for AA, AB and BB genotypes, respectively. For BoLA-DRB3 we identified 19 alleles, that DRB3 2*8 had the highest allelic frequency (22.4%) and DRB3 2*3, 2*37 and 2*51 had the lowest allelic frequency (1%). One of the 19 alleles had a new pattern. Average heterozygosity values for all loci were low. χ^2-test did not confirm the Hardy-Weinberg equilibrium for Leptin and Calpastatin in this population. These data provide evidence that Iranian’s Sistani breed have a good genetic variability, which opens interesting prospects for future selection programs, especially marker-assistant selection.

**Key words:** Sistani cattle, candidate genes, RFLP, polymorphism

**INTRODUCTION**

Iranian Sistani cattle is a special breed that shows special features of adaptation to rustic environments, within the last few years it has become a biotype of great interest for the meat production (Tavakkolian, 2000). Marker-Assisted Selection (MAS) is one of the new methods that improve accuracy and progress of selection in genetic resource (Dekkers, 1998). Among specific genes that may affect important economically traits in cattle in MAS method, the Leptin, κ-Casein, Calpastatin and BoLA-DRB3 loci had been extensively studied.

In cattle, the leptin gene is located on chromosome 4 (Pomp et al., 1997; Stone et al., 1996) and consists of three exons. Several polymorphisms in this gene have been found (Madaea et al., 2004). Liefers et al. (2002) reported that heifers with the AB genotype produce 1.32 kg day^-1^ more milk and consume 0.73 kg day^-1^ more food compared with the AA genotype. They suggested that B allele could yield a higher milk production without negatively affecting energy balance and fertility.

Milk protein polymorphisms have effect on the milk yield and processing properties of its products. Caseins are the major constituents of total milk proteins (Kemenes et al., 1999). In bovinae, casein genes are located in chromosome 6 (Ferretti et al., 1990) and 2 polymorphisms of A and B were found for the Kappa-casein (CSN3) gene. Association between CSN3 variants and milk production has been reported by many researchers (Lien and Rogne, 1993). Lien and Rogne (1993) found a significant effect of κ-casein genotype on milk production (p=0.001) and reported that cows of the BB genotype producing 173 kg less milk than AA cows. It has been demonstrated that B variant of CSN3 is associated with a higher protein content, better quality of curd and increased yield of cheese.

Many genes are involved in meat production. Among them, calpastatin is the major constituents of post-mature tenderization. Many studies suggested the calpastatin gene (CAST) as a candidate gene for meat quality (Palmer et al., 1998; Koolmarai et al., 1995). Palmer et al. (1998) have described a two alleles system of polymorphic
variant (M and N) in a region of the ovine CAST (exon and intron regions from a portion of the first repetitive domain) by PCR-RFLP method. Digestion with restriction endonucleases MspI and NcoI differentiated alleles M and N. Polymorphism of CAST has been detected in several cattle breeds (Cockett et al., 1995; Palmer et al., 1998; Chung et al., 2001).

Major Histocompatibility Complex (MHC) genes, also called bovine lymphocyte antigen (BoLA), have received attention because of high degree of genetic polymorphism and their association with host immunity. The BoLA genes are located on the short arm of bovine chromosome 23. BoLA-DRB3.2 considered suitable marker system for genetic diversity studies owing to their abundance in the mammalian genome. 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) (Takehima et al., 2002; Van Eijk et al., 1992).

Awareness of the values of genetic resource in livestock has estimated the study of the genetic diversity of native breeds. However, most of such studies have been done on European cattle breeds and very little information is available concerning the genetic polymorphism in native cattle breeds of Iran. The objective of this study was to describe the genetic variability in the Leptin, κ-Casein, Calpastatin and BoLA-DRB3 loci in Sistani native cattle (B. indicus) breeds from Iran.

**MATERIALS AND METHODS**

**DNA sampling:** Blood samples (n = 100) were collected from an Iranian Sistani herd in Zheah Research Station located at Zabol (southeast of Iran). Whole blood (100 μL) was used as source of DNA, which was extracted by the modified guanidine isothiocyanate-Silica gel method (Boom et al., 1989).

**Amplification:** Amplification reactions for the Leptin, CSN3, CAST and BoLA DRB3.2 genes were carried out with 100 ng of DNA (5 μL) in a 25 μL total volume containing 1× PCR buffer; 2.5 mM MgCl2; dNTPs, 100 μM of each; 10 pM of each primer (Table 1) and 1 U of Taq DNA polymerase. The thermal cycling profile was an initial denaturation step of 4 min at 94°C followed by 35 cycles 45 s at 94°C, 45 s at specific annealing temperature (Table 1), 45 s at 72°C and final extension step of 10 min at 72°C. Hemi-nested PCR was used for the amplification of the second exon (284 bp) of the BoLA-DRB3 gene. The first PCR stage was performed in a final volume of 20 μL containing 40 ng of template DNA, 10 pM

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>GAGGTGCGTTG</td>
<td>GTGCCGCTCCTT</td>
<td>60</td>
</tr>
<tr>
<td>CSN3</td>
<td>TCATTACATTAG</td>
<td>TTCTTCTAGGTT</td>
<td>56</td>
</tr>
<tr>
<td>CAST</td>
<td>CCTGTGTATGC</td>
<td>TCTCTTTCCTTT</td>
<td>61</td>
</tr>
<tr>
<td>BoLA first</td>
<td>ACTACACATCT</td>
<td>TGAGGAG</td>
<td>65°C</td>
</tr>
<tr>
<td>stage</td>
<td>ATCCCTCTGCTC</td>
<td>TCTGCTGCGCC</td>
<td></td>
</tr>
<tr>
<td>BoLA second</td>
<td>ATTCCCTCTGCT</td>
<td>TCGGCGCTGAC</td>
<td>65°C</td>
</tr>
<tr>
<td>stage</td>
<td>CAGGCATTCTTC</td>
<td>AGTGAACCTCTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Restriction enzymes and reaction condition for RFLP analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Enzyme</th>
<th>Time (h)</th>
<th>Digestion length (bp)</th>
<th>Optimum temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>HaeIII</td>
<td>6</td>
<td>390, 303, 88 and 32</td>
<td>65</td>
</tr>
<tr>
<td>CSN3</td>
<td>HaeIII</td>
<td>3</td>
<td>228, 135 and 95</td>
<td>37</td>
</tr>
<tr>
<td>CAST</td>
<td>MspI</td>
<td>4</td>
<td>622, 336 and 286</td>
<td>37</td>
</tr>
</tbody>
</table>

of each primers. Subsequently, 2 μL of the first-stage PCR product was used as template DNA for the second-stage PCR in a final volume of 20 μL containing 10 pM of each primers, 1 U Taq DNA polymerase and the remaining components in the concentrations stated above. The initial denaturation (94°C for 2 min), was followed by 25 cycles of denaturation (94°C for 40 sec) and annealing-extension (65°C for 30 sec) and a final extension (72°C for 5 min).

The concentration and purity of the obtained DNA were assessed by spectrophotometer and electrophoresis in 1% agarose gels, respectively.

**RFLP analysis:** For RFLP analysis, 5 μL of PCR products were incubated with 5 units of respective restriction enzymes for definite time (Table 2). To examine the nucleotide sequence variability at the DRB3.2 locus, three restriction enzymes, Rsal (GT1AC), HaeIII (GG1CC) and BstYI (TaiGATC1Py) were chosen based on their ability to cut DNA in this exon. Digestion products of leptin, CSN3 and DRB3.2 were separated by electrophoresis on 8% non-denaturant polyacrylamid gel and visualized by silver staining. For the calpastatin, Restriction fragments were revealed electrophoresis on 2% agarose gel stained with ethidium bromide.

**Statistical analysis:** Genotypic and allelic frequencies, Nier's heterozygosity value and χ² test for all loci were analyzed using the PopGen32 software (V. 1.31). A χ² test was performed to verify if genotype frequencies agreed with Hardy-Weinberg expectations. Average heterozygosity (h) was employed to estimate genetic diversity within the population (Nei and Li, 1979).

**RESULTS**

**Leptin:** The results showed that there were two BsmMBI cutting sites in 422 bp fragment of leptin gene. The AA
Table 3: Allelic and genotypic frequencies for Leptin, κ-casein and Calpstatin loci in Iranian Sistani cattle

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypic frequency</th>
<th>Allelic frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.77</td>
<td>0.22</td>
</tr>
<tr>
<td>CSN3</td>
<td>0.27</td>
<td>0.57</td>
</tr>
<tr>
<td>Calpstatin</td>
<td>0.62</td>
<td>0.29</td>
</tr>
</tbody>
</table>

*: The 1, 2 and 3 are the respective AA, AB and BB genotypes for Leptin and CSN3 and MM, MN and NN genotypes for calpstatin. **: The 4 and 5 are the respective A and B alleles for Leptin and CSN3 and M and N alleles for calpstatin, ns: non significant

Fig. 1: Allelic frequency of BoLA-DRB3.2 in Sistani cattle breed. *: Names of DRB3.2 alleles

- **genotype pattern exhibited two fragments of 390 and 32 bp (not detected on the gel), BB genotype pattern exhibited 303, 88 and 32 bp and AB genotype exhibited 390, 303, 88 and 32 (Fig. 1). Present results confirmed transversion mutation (GAC→GAT) in the leptin gene. The allelic frequencies for A and B alleles were 0.88 and 0.12, respectively. The genotypes AA, AB and BB were observed with respective frequencies of 0.77, 0.22 and 0.01 (Table 3).**

- **CSN3:** The TaqI and Hind3 digestions of the 228 bp fragment of exon 4 from CSN3 gene revealed two alleles with respective allelic frequencies of A = 0.25 and B = 0.75. The A allele had no restriction site for TaqI and Hind3, whereas B allele characterized by the presence of two fragments of 135 and 95 bp. Genotypic frequencies were 0.27, 0.57 and 0.16 for AA, AB and BB, respectively. Frequencies of alleles A and B were 0.55 and 0.45, respectively (Table 3).**

- **Calpstatin:** Digestion of the 622 bp fragment from CAST locus with restriction endonuclease MspI differentiates two M and N alleles. The MspI enzyme did not digest the allele N amplicon and allele M digestion exhibited two fragments of 336 and 286 bp. The allelic frequencies were 0.764 and 0.236 for M and N, respectively. The genotypic frequencies for MM, MN and NN were 0.62, 0.29 and 0.09, respectively (Table 1).**

- **BoLA-DRB3:** Figure 1 shows the patterns of digesting DRB3 284bp fragment with Rsal, Hae3 and BsrYI. Of all the restriction patterns described in the literature (24 patterns for Rsal, 10 for Hae3 and 5 for BsrYI) only 14, 5 and 5 patterns were identified for Rsal, Hae3 and BsrYI, respectively (Table 1). In the present study, 19 alleles of the 53 previously reported BoLA-DRB3.2 alleles were detected (Fig. 1). As shown in Fig. 1, 70% of the cumulative gene frequencies corresponded to the six most common alleles, which are defined as variants with gene frequencies higher than 5% (DRB3.2 number 8, 11, 15, 34, 44 and new pattern of each). Four alleles exhibited gene frequencies of 1% (DRB3.2*3, *29, *37 and *51). Digestion of PCR products from four animals showed a new combination of endonucleases patterns. By using Rsal and Hae3, these samples produced patterns of le and bc, respectively. According to PCR-RFLP patterns published by BoLA Nomenclature Committee (http://www.projects.roslin.ac.uk/bo1a/bolaacom.html) expected alleles must be #7 (ecc) for 1 allele and #20 (lbb) or #34 (lab) for another one that the later must be finalized with BsrYI digestion. Surprisingly when we digested these samples with BsrYI, it revealed that they have a new digestion pattern (aa) rather than what expected (ac or bc). Therefore, the first allele for these animals is a new pattern (eac) that has not been published yet and the second allele was #20.**

**DISCUSSION**

It was the first study using polymorphism of Leptin, κ-Casein, Calpstatin and BoLA-DRB3 loci to understanding genetic polymorphism of Sistani cattle in Iran. Therefore the present study may be regarded as the beginning of attempts to understand the genetic variability of native cattle breeds in Iran.
Leptin: The genotypes AA, AB and BB were observed with respective frequencies of 0.77, 0.22 and 0.01, respectively (Table 3). Liebers et al. (2002) in Holstein cattle breed observed similar results. They observed the AA, AB and BB genotypes with respective frequencies of 0.813, 0.185 and 0.002. Similar with our results only one animal with BB genotype was observed in this study. Moussavi et al. (2006) investigated similar region of leptin gene in Iranian Holstein cattle. They observed the AA and AB genotypes with respective frequencies of 0.86 and 0.14. The BB genotypes not observed in this study that confirmed our results with low BB genotypic frequencies. In contrast Javannard et al. (2005) reported genotypic frequencies of 0.32, 0.43 and 0.25 for AA, AB and BB in Sarabi cattle breed, respectively. Madeja et al. (2004) investigated three restriction fragment length polymorphisms (RFLP): HphI, KpnI and Sau3AI in the leptin gene in Polish Black and White cattle. They reported the allelic frequencies of 0.54 (C) and 0.46 (T) for KpnI, 0.66 (C) and 0.34 (T) for HphI and 0.86 (A), 0.11 (B) and 0.03 (C) for Sau3AI. The polymorphisms of leptin gene were reported by several studies. Buchanan et al. (2002) reported a missense mutation in the bovine leptin gene and described cytosine (C) to thymine (T) substitution in exon 2 of the leptin gene of the B. taurus breeds. Pomp et al. (1997) using PCR-based polymorphism showed a rare polymorphism in the leptin gene. They reported that the Sau3AI restriction enzyme digested the PCR product four times instead of three times.

CSN3: α-Casein A allele frequencies of Sistani cattle in this study are lower than the frequency reported in Iranian Holstein by Nassiri et al. (2003), but similar to frequency in German Black and White reported by Freyer et al. (1999). A rare polymorphism at an additional Sau3AI restriction site (the 690-bp fragment was digested into two fragments of approximately 470 and 220 bp) was observed in cattle from Simmental, Gelbvieh and Angus breeds. Present result did not show this polymorphism in Iranian Holstein dairy cows which is in agreement with other studies (Pomp et al., 1997). Prinzenberg et al. (1997) was developed PCR-RFLP method to detecting CSN3 alleles (CSN3A, CSN3B, CSN3C, CSN3E, CSN3F and CSN3G). In addition to RFLP, polymorphisms of the CSN studied using other methods. Barroso et al. (1999) investigated CSN polymorphisms in various breeds of cattle using SSCP method. They detected four variants (A, B, C and D) in this gene and each variant yielded patterns clearly distinguishable from the others. The results showed that the frequency of B allele in Sistani cows is high. Therefore, there is a probability of selection the better quality for higher protein content and cheese yield.

Calpastatin: A TaqI Restriction Fragment Length Polymorphism was identified in the Bovine Calpastatin Gene (Cockett et al., 1995) and three alleles (1, 2 and 3) were resulted. Chung et al. (2001) detected a genetic polymorphism in intron 6 of bovine Calpastatin gene by PCR-RFLP in purebred Angus cattle. Two alleles of A and B were observed with 0.75 and 0.25 respective frequencies. Recently, Schenkel et al. (2006) were identified a single nucleotide polymorphisms (SNP) in the CAST gene (G to C substitution) and genotyped on crossbred commercially fed heifers, steers and bulls from beef feedlots and steers. The allele frequencies of CAST C and G were 0.63 and 0.27, respectively. Chung et al. (1999) were identified two alleles (A and B), resulting in three genotypes (AA, AB and BB) using SSCP method in purebred Angus cattle. Allelic frequencies for A and B were 0.29 and 0.71, respectively.

BoLA-DRB3: Comparison between the present results with the allele spectrum and the gene frequencies profile previously reported in Bos taurus showed that 4 alleles (DRB3.2*47, *34, *45 and *51) were found in Sistani and Zebu Brahman Cattle. The most frequently detected BoLA alleles of Kenya Boran cattle, Caracu and Saavedres Creole was DRB3.2*8. In present study, the same results were observed with Sistani cows. In present study with Iranian Sistani meat breed, alleles DRB3.2*27, *33, *42, *27, *18, *31, *5, *35, *6, *20, *16, *2, *6 were not observed. In contrast, allele *8, which had a frequency of 0.70, 2.94 and 3.45 on Gir breed, Curraleiro and Argentinean Creole (Machado et al., 2005), respectively, was the most frequent allele (0.22) in our study. Similarly, Miretti et al. (2001) showed that BoLA-DRB3.2*8 accounted for 23.07% of the alleles in a population of Caracu cows in Brazil. The four most frequently alleles in Gir cows were BoLA-DRB3.2*16, *20, *2 and *29 and these accounted for about 51.7% of the alleles in this population (Machado et al., 2005). BoLA-DRB3.2 alleles number 6,*2 and *27 were found in Kenya Boran cattle, not observed in Sistani cattle. The three least frequently isolated alleles in Golpayegani (Mosafer and Nassiri, 2005) and Sistani were BoLA-DRB3.2*4,*3 and *24. DRB3.2*7 Allele, which was a low frequency allele in Sistani cattle is the most common in Ayrshire cattle. More significant distinctions have been found between Iranian Sistani cattle and Bos taurus breeds studied. Polymorphism of the BoLA-DRB3 gene has been reported in the studies of Holstein-Friesian (Nassiri et al., 2005); Jersey (Gilliespie et al., 1999); Japanese Black, Japanese Shorthorn and Japanese Jersey cattle (Takeshima et al., 2002) and Russian Ayrshire and Russian Black Pied cattle breeds (Udina et al., 1998).
The Sistani cattle breed represented excellent source of biological information for studies on genetic characterization. This breed has been kept in closed station with no selection for these loci; therefore, we observed a low degree of genetic variability for the Leptin, κ-Casein and Calpastatin loci. This may be explained by conservation and breeding methods that have been carried out. Here, a few sires were selected and used for breeding. With respect to low effective number of population the inbreeding rate is high and so heterozygosity and genetic variability is low. For this problem it seems that in this station must be used the sires from other population. Although low variables were found for the those loci, in the other hand, these data provided an evidence some that Iranian’s Sistani cattle had a good polymorphism for Leptin, κ-Casein and Calpastatin loci, which opens interesting prospects for future selection programs, especially marker-assistant selection between different genotypes of different locus and reproduction, milk traits, gain and meat traits. The present study may be regarded as the beginning of attempts to understand the genetic variability of Iranian native cattle breeds.

REFERENCES


