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Analysis of Bovine Growth Hormone Gene Polymorphisms in Three Iranian Native Breeds and Holstein Cattle by RFLP-PCR

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Abstract: The aim of this study was to estimate the allelic frequency in polymorphic sites of intron 3 and exon 5 of bovine growth hormone gene in three Iranian native and Holstein cattle. A total of 406 genomic DNA samples were extracted from three Iranian native cattle including, Mazandarani 97, Sarabi 87, Golpaygani 112 and Holstein 110 cattle. The PCR procedure was used to amplify 345 bp of bGH-intron 3 and 404 bp of bGH-exon 5. The frequencies of *Msp*I (+) allele were estimated as 0.55, 0.51, 0.44 and 0.83 in Mazandarani, Sarabi, Golpaygani and Holstein breed, respectively. The allelic frequencies of *Alu*I (+) were calculated as 0.91, 0.84, 0.92, 0.85 and for *Dde*I (+), 0.52, 0.54, 0.47 and 0.86, for these breeds, respectively. Chi-square test showed significant differences ($p<0.01$) in genotypic frequency between native and Holstein breed in *Msp*I and *Dde*I restriction sites. There was significant differences in genotypic frequencies between Mazandarani ($p<0.05$) and Golpaygani ($p<0.01$) with Holstein breed at *Alu*I restriction site. This difference was not significant between Sarabi and Holstein breed. The differences in allelic frequency between native breeds and Holstein cattle at the present study might be due to differences in origin of breeds, selection plans applied to Holstein population for improving milk production.

Key words: Growth hormone, polymorphism, gene frequency, PCR-RFLP

INTRODUCTION

Bovine growth hormone (bGH) is a single peptide of molecular weight equal to 22-kD secreted from pituitary gland, composed of 190 or 191 amino acids residues and containing Ala or Phe at the N-terminus due to alternative processing of bGH precursor (Vukasinovic *et al*., 1999; Dybus, 2002). This gene is a part of multiple gene family that contains prolactin and placental lactogens and is located on BTA19, 66 cM from the centromic marker, BM9202. The gene is approximately 2800 bp with five exons and four introns. Several polymorphic regions have been reported at different regions of bGH gene. Allelic variation in the structural or regulatory sequences would be interesting from several points of view. Firstly, genetic polymorphisms contribute to the genetic characterization of population and could help to identify possible hybridization events in the past. Secondly, they would have possible direct or indirect effects on milk production or growth performance. Also, variations in introns or flanking sequences have potential usefulness as genetic markers and help in the genetic improvement of populations (Mitra *et al*., 1995; Falaki *et al*., 1996). Hetch and Geldermann (1996) reported 6 sites of variable nucleotides in the 5′-flanking region and 1 in intron I. Some of these variable sites are also potential binding sites for trans-acting factors. Sequence analysis revealed differences in mobility of Single Strand Conformation Polymorphism (SSCP) fragments due to deletion or insertion of a TGC repeat at position between 125 and 142 (Yao *et al*., 1996). Ge *et al*. (2003) used denaturating gradient gel electrophoresis method and sequencing to identify three new single nucleotide polymorphisms (SNP) at the promoter region in Angus cattle. Polymorphisms at nucleotide 1547 in intron three creates *Msp*I restriction site. This mutation is due to a T to C transition (Yao *et al*., 1996). Lucy *et al*. (1993) detected a polymorphic site located in exon five, transversion of C to G in position 2141, which substitutes Leu by Val in protein product. This mutation is detectable by *Alu*I endonuclease (Zhang *et al*., 1993). Yao *et al*. (1996) investigated sequence variation in the bGH gene by SSCP method. They reported

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a polymorphism in exon five at position 2291, which was due to a transversion of A to C and detectable by DdeI restriction enzyme. Another type of variation has been reported in Japanese cattle which produce a replacement of Thr by Met amino acids sequence at position 172 (Chikuni *et al*., 1994). There are also other polymorphic regions in nucleotide 1692 of intron three, nucleotide 2017 of intron four (Yao *et al*., 1996) and probably nucleotide 2637 in 3′-flanking region (Unanian *et al*., 1994).

The Leu/Val locus (*Alu*I-/*Alu*I+) was significantly associated with fat and protein Estimated Transmitting Ability (ETA) of the selected Holstein groups and approached significant for milk Predicted Transmitting Ability (PTA), with the Val allele being more frequent in the top than in the bottom group of bulls (Sabour *et al*., 1997). Estimates of transmitting ability for milk production tended to be greater for Holstein cows that were homozygous for Leu (1993). Shariflou *et al.* (2000) investigated the effect of Leu/Val locus on milk production by lactation and test-day data. Results from the two data sets consistently showed that the Leu allele is associated with higher production of milk, fat and protein. The average effects of the gene substitution were 95 L for milk, 7 kg for fat and 3 kg for protein yield per lactation. Dybus (2002) estimated the frequency of Val and Leu alleles of exon five in Danish Black-and-White cattle. The associations between Val/Leu polymorphism and milk production traits were found only in the first lactation. In the first 305-day lactation, the cows of Leu/Leu genotypes produced more milk (+225 kg), fat (+7 kg) and protein (+7 kg) than the Leu/Val individuals. Lee *et al.* (1993) reported PTA-milk and PTA-$ were not associated with genotype for average and high-milk production cows. Presence of Val allele was associated with decrease of PTA-milk in high-milk production cows. Yao *et al.* (1996) found no associations between polymorphism in this locus and milk production traits. They reported that the average effect of the gene substitution for *Msp*I and *Dde*I alleles were similar, with ±300 kg for milk yield, ±8 kg for fat content and ± 7 kg for protein content per lactation.

The aim of this study was to estimate the allelic frequencies in polymorphic sites of intron 3 and exon 5 of bGH gene in three Iranian native and Holstein cattle.

MATERIALS AND METHODS

A total of 406 genomic DNA was extracted from three Iranian native breeds including 97 Mazandarani, 87 Sarabi, 112 Golpaygani and 110 Holstein cattle. Distribution of three native breeds and the region that Holstein cattle were sampled is shown in Fig. 1. DNA was extracted from whole blood by Miller *et al.* (1988) procedure. The selected primers (Yao *et al*., 1996) were used to amplify 345 bp of intron 3 and 404 bp of exon 5 (Table 1). DNA

Table 1: PCR primers and conditions of DNA amplification used for bGH-intron 3 and bGH-exon 5

Gene regions	Sequence of primers	Temperature (°C)/Time (S)
bGH-intron 3	5′-GGACAGAGATACTCCATCCAG-3′ 5′-AGATGCCGAAGCAGCTCCAAGT-3′	92/30 55/80 72/70
bGH-exon 5	5′-TAGGGGAGGGTGGAAAATGGA-3′ 5′-GCAACCTACTCAGACAATGCG-3′	92/30 57/60 72/60

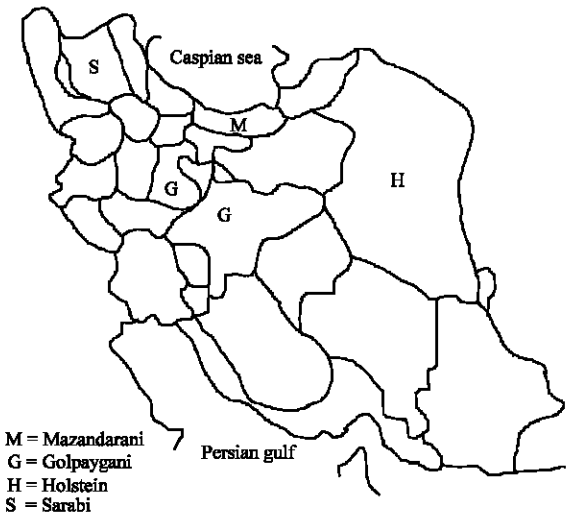

Fig. 1: Geographical distribution of three native breeds in Iran and the region that Holstein cattle were sampled

was amplified in a total volume of 25 μL containing 100 ng genomic DNA, 0.5 μM of each primer, 0.2 mM dNTP, 1x PCR buffer, 2.5 mM $MgCl_2$, 1% DMSO and 0.6 unit Taq DNA polymerase. Amplification was done for 35 cycles followed by 72°C for 5 min. The amplified DNA fragment of the bGH-intron 3 was digested with 10 unit *Msp*I endonuclease. The resulted PCR products of bGH-exon 5 were digested with 5 units of *Alu*I or *Dde*I endonuclease enzymes, separately. Digested products were loaded on 8% acrylamid and the gels were stained with silver staining.

The gene frequencies were calculated by counting method as:

$$P = \frac{2(AA) + (Aa)}{2N}, \quad q = \frac{2(aa) + (Aa)}{2N}$$

Where, p = the gene frequency of allele (+), q = the gene frequency of allele (-) and N = the total number of cattle tested.

RESULTS

The amplified bGH-intron 3 resulted in a DNA fragment with 345 bp length. Homozygous genotype (+/+) digested with *Msp*I endonuclease enzyme showed 3 fragments with 177, 109, 59 bp in length, heterozygous 4

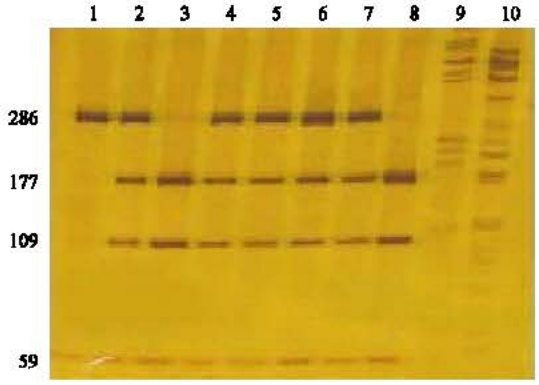

Fig. 2: Different genotypes resulted from *Msp*I restriction enzyme. Columns 9 and 10 DNA marker, columns 3 and 8 are homozygous +/+, columns 2, 4, 5, 6 and 7 are heterozygous +/- and column 1 is homozygous -/- RFLP-genotyping of the bGH-intron 3 gene

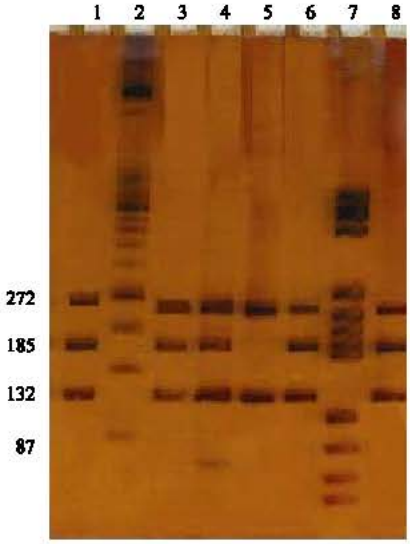

Fig. 3: Different genotypes resulted from *Alu*I endonuclease. Columns 2 and 7 are DNA markers. Columns 1, 3, 6 and 8 are homozygous *Alu*I (+/+), columns 4 is heterozygous *Alu*I (+/-) and column 5 is homozygous *Alu*I (-/-) RFLP-genotyping of the bGH-exon 5 gene

fragments with 286, 177, 109, 59 bp and not digested homozygous (-/-) genotype, 2 fragment with 286 and 59 bp length (Fig. 2). From the 404 bp PCR product of the bGH-exon 5, the homozygous (+/+) genotype digested with *Alu*I endonuclease enzyme showed 3 fragments with the length of 132, 87, 185 bp, heterozygous *Alu*I (+/-) with

Table 2: Distribution of genotypes (number of animals) and gene frequencies (%) of RFLP polymorphic at the *Msp*I, *ALu*I and *Dde*I loci in the bGH gene

		Genotypes			Gene frequency	
Breeds	Locus Intron 3, Exon 5	+/+	+/-	-/-	f (+)	f (-)
Mazandarani	*Msp*I	30	46	21	0.55	0.45
	*Alu*I	80	17	0	0.91	0.09
	*Dde*I	27	45	24	0.52	0.48
Sarabi	*Msp*I	23	43	21	0.51	0.49
	*Alu*I	61	25	1	0.84	0.16
	*Dde*I	25	43	18	0.54	0.46
Golpaygani	*Msp*I	14	70	28	0.44	0.56
	*Alu*I	95	17	0	0.92	0.08
	*Dde*I	20	66	26	0.47	0.53
Holstein	*Msp*I	68	29	2	0.83	0.17
	*Alu*I	76	34	0	0.85	0.15
	*Dde*I	79	31	0	0.86	0.14

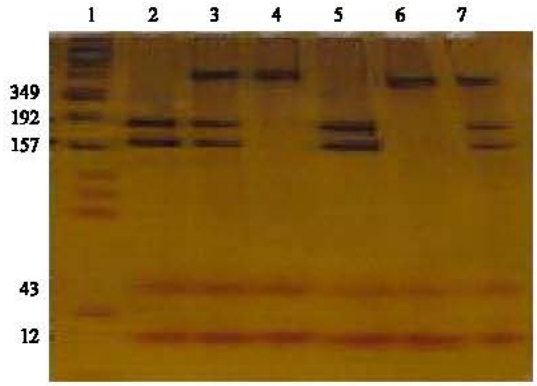

Fig. 4: Different genotypes resulted from *Dde*I endonuclease. Column 1 is DNA. Marker. Column 2 and 5 are homozygous *Dde*I (+/+), columns 3 and 7 are heterozygous *Dde*I (+/-) and column 4 and 6 are homozygous *Dde*I (-/-) RFLP-genotyping of the bGH-exon 5 gene

4 fragments of 132, 272, 87, 185 bp and homozygous *Alu*I (-/-) genotype 2 fragments with 132 and 272 bp in length (Fig. 3). *Dde*I endonuclease enzyme showed three restriction sites in the amplified PCR product of bGH-exon 5. Homozygous *Dde*I (+/+) genotype with 4 fragments of 192, 157, 43, 12 bp long, heterozygous *Dde*I (+/-) with 5 fragments of 349, 192, 157, 43, 12 bp and homozygous *Dde*I (-/-) with 3 fragments of 349, 43 and 12 bp in length (Fig. 4).

The gene frequencies of three loci for each breed and the summary of chi-square test for genotypic frequencies are presented in Table 2 and 3. The frequency of *Msp*I (-) allele ranged from 0.17-0.56 in Holstein and Golpaygani breed, respectively. The frequency of *Alu*I (-) allele ranged from 0.08 in Golpaygani to 0.16 in Sarabi breed. The highest and lowest value for *Dde*I (-) frequency were

Table 3: Chi-square test of genotypic frequencies of *Msp*I, *Alu*I and *Dde*I loci between breeds

	Holstein			Golpaygani			Sarabi		
Breed	*Msp*I	*Alu*I	*Dde*I	*Msp*I	*Alu*I	*Dde*I	*Msp*I	*Alu*I	*Dde*I
M	34.3a	4.97	51.4a	10.8a	0.21NS	3.9NS	0.48NS	63.5a	0.43NS
S	40.1a	1.3NS	45.7NS	6.6b	6.9b	3.5NS	-	-	-
G	74.6a	7.8a	73.8a	-	-	-	-	-	-

M = Mazandarani, S = Sarabi, G = Golpaygani, NS = Non Significant, a = ($p<0.01$), b = ($p<0.05$)

observed in Golpaygani and Holstein cattle respectively. There was a significant difference in genotypic frequencies between the cattle groups studied.

DISCUSSION

bGH/*MspI* polymorphism: Relationships between polymorphic sites with milk production traits have been much studied. Falaki *et al.* (1996) reported that *Msp*I and *Taq*I restriction sites had no significant effect on milk production. They found that the *Msp*I (-) allele was associated with higher fat and protein milk yield and percentage. They concluded lack of variation in PTA for the animals might be because of using positively tested bulls. The effect of bGH gene polymorphism using *Taq*I endonuclease on milk production trait of Simmental cattle was not significant, but bulls with BB genotype had a higher breeding value for milk yield than AA bulls (Falaki *et al.*, 1996). It was reported that *Msp*I(-) allele was favorable for milk fat percentage in Gyr breed (Mattos *et al*., 2004) and milk fat production in high-fat selected groups of Red dairy breed (Høj *et al*., 1993). This polymorphic locus was also significantly associated with differences among the top, middle and bottom group of Holstein bulls for milk ETA, but not significantly associated with fat and protein ETA (Sabour *et al*., 1997). Lagziel *et al.* (1999) reported five intragenic haplotypes, A to E, in Israeli Holstein dairy cattle. Haplotype E which carried *Msp*I (-) allele, showed a significant increasing effect on protein percentage and kg protein per year and a decreasing effect on milk somatic cell counts. In contrast, Yao *et al.* (1996) reported that *Msp*I (-) allele decreased milk, fat and protein yields in Holstein bulls. Polish Black-and-White homozygous *Msp*I (+/+) cows yielded higher milk than heterozygous genotype, whereas *Msp*I (+/-) cows showed higher milk fat content than homozygous individuals (Yao *et al*., 1996).

The *Msp*I (-) allele frequency within breed in relation to their geographic origin have been reported earlier. It has been shown a low frequency of the *Msp*I (-) allele for breeds originated in Northern Europe, moderate frequencies for breeds originating in Eastern Europe or countries surrounding the Mediterranean basin and very high allele frequency for breeds originating in the Indian subcontinent (Lagziel *et al*., 2000). The allele frequencies varied widely among breeds, from 0.0 in the Herford to 0.81-0.89 in the Gyr breed (Mattos *et al*., 2004; Lagziel *et al*., 2000; Zhang *et al*., 1993). It was also reported 0.86 for Sahiwal Zebu (1995), 0.13 for Polish Black-and-White cattle (Dybus, 2002), 0.16 for Piemontese (2003), 0.05-0.28 in lines of Red Danish (1993), 0.32 for Simmental (1994), 0.88 for Iranian Sistani and 0.83 for Iranian Dashtyari breed (Masoudi *et al*., 2002). Frequency of *Msp*I (-) allele in Holstein breed is in agreement with earlier reports, 0.15 (Yao *et al*., 1996), 0.13 (Sabour *et al*., 1997) and 0.26 (Zhang *et al*., 1993). It was proposed that *Msp*I (-) allele may be diagnostic for divergence of *Bos taurin* and *Bos indicus* lineage about 400000 years ago (Lagziel and Soller, 1999). At the present study significant ($P<0.01$) differences were found in genotypic frequency between all native breeds and Holstein cattle at *Msp*I locus (Table 3). The frequency of *Msp*I (-/-) genotype in all native breeds was higher than that for Holstein cattle. The low level of homozygous (-/-) genotype in Holstein cattle may be due to the selection pressure applied for higher milk production in this breed.

bGH/*AluI* polymorphism: The *Alu*I (-) allele frequency in Mazandarani and Golpaygani breeds were less than 0.1. There was a significant ($P<0.01$) difference in genotypic frequencies between these breeds and Holstein at this locus, but this differences was not significant between Sarabi and Holstein cattle (Table 3). Only one homozygous *Alu*I (-/-) genotype was observed in Sarabi breed. The *Alu*I(-) allele frequency has been reported 0.04 in Sahiwal Zebu (Mitra *et al*., 1995), 0.19 in Polish Black-and-White (Dybus *et al*., 2002), 0.32 in Simmental (1994), 0.24 and 0.29 in Jersy and Ayrshire (Sabour *et al*., 1997), 0.0 in Iranian Sistani and Dashtyari breeds (Masoudi *et al*., 2002), 0.33 in Limousine (Abbasi *et al*., 1999) and 0.18 in Holstein (Shariflou *et al*., 2000). The Low frequency of *Alu*I (-) allele at the studied cattle populations can be due to low number of samples, low actual allele frequency or the effect of reverse natural selection at this locus.

bGH/*DdeI* polymorphism: The *Dde*I(-) allele frequency varied between 0.14 in Holstein cattle and 0.48 in Mazandarani breed. Significant ($p<0.01$) differences in genotypic frequency were observed among all three native breeds and Holstein cattle (Table 3). The frequency

of *Dde*I (-) allele in Holstein breed is in agreement with the finding of Yao *et al.* (1996). The observed *Dde*I (-) allele frequency at the present study is the same as nearly with the results of Abbasi *et al.* (1999), who has found 0.12 for Iranian Holstein bulls.

The differences in allelic frequency between native breeds and Holstein cattle at the present study might be due to differences in origin of breeds, selection plans applied to Holstein population for improving milk production traits, low number of samples and/or as the effect of natural selection in native breeds. A non-significant difference in genotype frequency between Sarabi and Holstein in *Msp*I and *Dde*I restriction sites might be due to the same genetic lineage, both as *Bos taurus*.

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