The allele and genotype frequencies of bovine pituitary-specific transcription factor and leptin genes in Iranian cattle and buffalo populations using PCR-RFLP

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Abstract
The use of polymorphic markers in breeding programmes could make selection more accurate and efficient. A total of 324 individuals from six Iranian cattle populations (Sarabi, Golpayegani, Sistani, Taleshi, Mazandarani, Dashtiyari), F_1 Golpayegani × Brown Swiss and Iranian buffalo populations were genotyped for the Pit-1 HinfI and leptin Sau3AI polymorphisms by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The genotype and gene frequencies for each breed were determined and shown to be quite variable among the breeds. The highest frequencies of allele B for the leptin gene and allele A for the Pit-1 gene were found in Dashtiyari and Sistani cattle, respectively. According to our results, the highest AB genotype frequencies were found in the Taleshi and F_1 Golpayegani x Brown Swiss cross for the leptin and Pit-1 genes, respectively. These allele frequencies were comparable to previously published data on exotic breeds. The highest and lowest heterozygosities were found in Taleshi and Dashtiyari cattle for the leptin gene and in F_1 Golpayegani x Brown Swiss cross and Sistani cattle for the Pit-1 gene, respectively. These values indicated the presence of low variation for these genes in the studied populations. The possible association between molecular polymorphisms within these candidate genes and economic traits for the studied populations should be further investigated.
Keywords: *Pit-1; Leptin; Iranian cattle; buffalo; PCR-RFLP; Polymorphism.*

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INTRODUCTION

Pit-1 gene has been identified as the pituitary specific transcription factor that regulates the expression of the growth hormone (*GH*) and prolactin (*PRL*) genes in the anterior pituitary (Tuggle *et al.*, 1993). Renaville *et al*. (1997) showed that the A allele (especially the AB genotype) may have an effect on milk yield and its components. Leptin is a 16 KDa proteins synthesized by adipose tissue (Itosser, 1998) and it is involved in the regulation of feed intake (Lagonigro *et al.,* 2003), energy balance, fertility and immune function (Lieferes *et al*., 2002). Leptin gene consists of three exons and two introns. Liefers *et al.* (2002) reported that heifers with the *Sau3A*I-AB genotype produce 1.32 kg/d more milk and consume 0.73 kg/d more food compared to those with the AA genotype. They also suggested that the B allele may have a role in improving milk production without negative energy balance and low fertility. Buchanan *et al.* (2003) reported that the T allele of the bovine leptin gene resulted in the production of more milk and higher somatic cell count but did not have a significant effect on milk fat or protein during lactation. Lagonigro *et al*. (2003) reported an association between five single nucleotide polymorphisms within the leptin gene and feed intake as well as fat traits. Almedia *et al.* (2003) found two alleles responsible for increasing the calving interval, thus, they suggested selection against these carriers could improve calving interval at least 2 months.

So far there are no reports on the study of these genes in Iranian cattle and buffalo populations (*Bos*

indicus). This study intends to identify the *Pit-1-Hinf*I and leptin-*Sau3A*I polymorphisms and estimate allelic and genotypic frequencies and heterozygosities (as inter-population variation measurement) in six Iranian cattle populations, F_1 Golpayegani × Brown Swiss crosses and Iranian buffalo population. The data obtained from this research will also be compared with the results of similar studies carried out on exotic cattle and buffalo breeds.

MATERIALS AND METHODS

Samples and Bleeding Locations: A total of 324 individuals were used in this study. Whole blood samples were collected from the following populations: Sarabi (n=82), Golpayegani (n=57), Sistani (n=38), Taleshi (n=70), Mazandarani (n=26), Dashtiyari (n=8), F_1 offspring of Golpayegani x Brown Swiss crosses (n=13) and Iranian buffalo (n=30). The samples were randomly obtained from Shabestar, Sarab, Uremia (Jabal), Delijan, Golpayegan, Zehak, Talesh and Mazandaran Jahad- e- Keshavarzi animal breeding stations.

DNA Extraction: DNA extraction was carried out by the method of Boom *et al*. (1989) as follows: Briefly, to an aliquot of 100 µl blood (after thawing), 400 µl of lysis buffer (Guanidin Thiocyanate, 20 mM; EDTA, 20 mM; Tris-HCl, 10 mM; Triton X_{100}, 40 g/l; DTT, 10 g/l) was added, the mixture was vortexed and incubated at 65°C for 5 min. The cells were resuspended in 20 µl of nuclease solution (Silica gel: 4g, Guanidine solution: 100 ml) and spun for 10 sec at 12,000 ×g. The pellet was resuspended in 200 µl of lysis buffer again. The suspended white blood cell suspension was then added to 400 µl of saline buffer (NaCl, 1M; Tris-HCL, 10 mM; KCl, 1M and EDTA, 20 mM), the mixture was vortexed and then spun for 10 sec at 5,000 ×g. The DNA was precipitated with 45-55 µl of extra gene solution (Ion Exchange Resin): 10%, Orange G color: 0.02%, Triton X_{100}: 0.01%) and was incubated in 65°C for 3-5 min. Then protein was precipitated by centrifugation (3 min at 1000 ×g) and the upper layer containing the DNA was transferred to another tube. The relative purity of DNA was determined using a spectrophotometer based on absorbances at 260 and 280 nm, respectively.

PCR–RFLP Analysis: The sequences of the forward and reverse primers for the amplification of the *Pit-1* gene were:
*Pit1*F 5´-GAGCCTACATGAGACAAGCATC-3´
*Pit1*R 5´-AAATGTACAATGTGCCTTCTGA-3´

The polymerase chain reaction for the *Pit-1* gene was performed in a 25 µl reaction mixture, containing 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.3 µM of each primers, 1X PCR buffer, 1U *Taq* polymerase (Cinagen, Iran) and 100 ng of genomic DNA template. The reaction mixture was placed in a DNA thermal cycler (Perkin Elmer 9700). Thermal cycling conditions included: an initial denaturation step at 95°C for 2 min followed by 30 cycles of 95°C for 45 sec, 60°C for 1 min, 72°C for 1 min and a final extension at 72°C for 3 min. The PCR products were digested with 10 U of *Hinf*I (Gibco BRL, life Technologies, USA) at 37°C for at least 14h.

One part of second intron of leptin gene was amplified using the following primers:
LepF: 5´-TGGAGTGGCTTGTTATTTCTTCT-3´
LepR: 5´-GTCCCCGCTTCTGGCTACCTAACT-3´

PCR reaction was performed using 0.5 U *Taq* polymerase and 50 ng of DNA. Thermal cycling conditions were as follows; initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C, 55°C and 72°C (each for 1 min) and a final extension at 72°C for 15 min. PCR products were digested overnight at 37°C with 10 U of *Sau3A*I (Roche, Germany). Restriction fragments from the above PCR reactions were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Statistical analysis: The allele and genotype frequencies were estimated by direct counting. The heterozygosities (as gene variation indicates) were calculated using the POPGENE software version 1.31 (Yeh *et al*., 1999), according to Nei procedure (1978).

RESULTS

One RFLP in the bovine leptin and *Pit-1* genes was detected. There was one polymorphic *Hinf*I site in the 600 bp PCR product within exon 6 of *Pit-1* gene. The digested AA PCR product exhibited one fragment of 600 bp and for the BB PCR product, the 600 bp fragment was cleaved into two fragments of 357 and 243 bp. Figure 1 shows the restriction pattern of three genotypes AA, AB and BB upon digestion of the *PIT-1 Hinf*I. Sequence analysis of the polymorphic *Hinf*I site revealed a mutation at position 1256. The mutation was a G to A transition. The 442bp fragment of the leptin gene also contained two restriction sites of *Sau3AI*. AB PCR product was cleaved into three fragments of 303, 88 and 32 bp (bands not detected on the gel). Sequence analysis of *Sau3A*I site in the leptin gene revealed a mutation at position 1180 that was a C to T

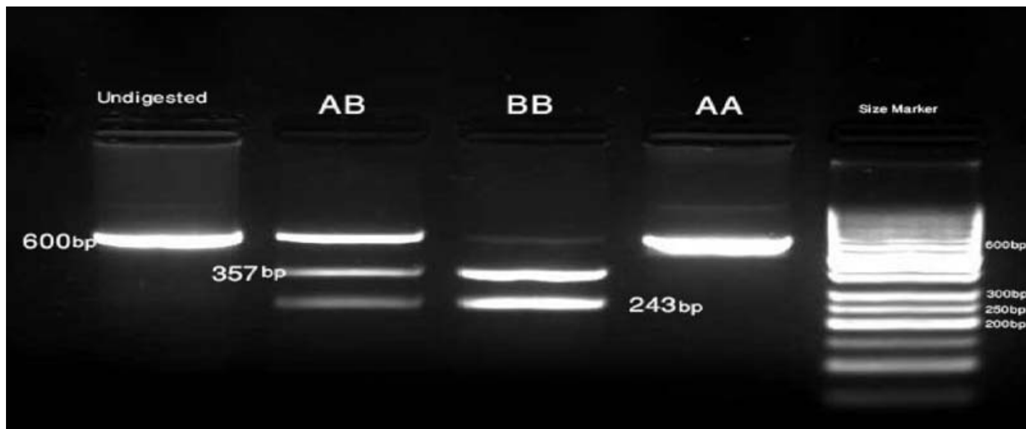

Figure 1. Analysis of *Hinf*I polymorphism at the bovine *Pit-1*gene*:* Electrophoretic patterns of three genotypes separated on 2% agarose gel. Lane M is molecular weight marker (SM037 from MB1 fragments).

Figure 2. Analysis of *Sau3A*I polymorphism at the bovine leptin gene: Electrophoretic patterns of three genotypes separated on 2% agarose gel. Lane M is molecular weight marker (SM037 from MB1 fragments).

transition. Figure 2 shows the restriction pattern of three genotypes of the leptin gene. Allele and genotype frequencies and the average heterozygosity in six Iranian cattle, F_1 Golpayegani × Brown Swiss crosses and buffalo populations for *Pit-1* and leptin loci were analyzed (Table 1 and 2). Although the B allele is favorable for the leptin gene (Pomp *et al.,* 1997 and Almedia *et al*., 2003) and A allele for the *Pit-1* gene (Sabour *et al*., 1996), AB genotype is preferred by both the candidate genes (Woolard *et al*., 1994; Renaville *et al.,* 1997; Pomp *et al.,* 1997 and Almedia *et al*., 2003). The highest B allele frequency was estimated in Dashtiyari cattle for the leptin gene (0.875) and A allele in Sistani cattle (0.9211) for the *Pit-1* gene. However, the highest AB genotype frequency was found in Taleshi and F_1 Golpayegani x Brown Swiss for the leptin and *Pit-1* genes, respectively.

The heterozygosity was varied from 0.2188 to 0.4991 for the leptin gene in Dashtiyari and Taleshi cattle and from 0.1454 to 0.4734 for the *Pit-1* gene in Sistani cattle and F_1 Golpayegani × Brown Swiss cross, respectively.

DISCUSSION

A main goal of the animal breeder is to select superior animals for breeding. Screening favorable alleles for selection at the DNA level provides an ideal tool for marker-assisted selection. RFLP polymorphism within the bovine *Pit-1* gene was first detected with *Hinf*I nuclease by Woolard *et al.* (1994). Sabour *et al.* (1996) showed that allele A in *Pit-1* locus positively affected milk production traits in Friesian cattle. This allele (frequency of 0.18) showed a significant superiority over allele B for milk and milk protein yields and body conformation traits within Italian Holstein Friesian cattle.

The allele and genotype frequencies are variable among different studied populations and also the favorable allele and genotype frequencies in Iranian populations (*Bos indicus)* were comparable to published results, especially on *Bos taurus*. The *Pit-1* A allele frequencies have been estimated to be 0.45 in Angus; 0.26 in Holstein; 0.21 in Herford; 0.28 in Gelbvieh; 0.1 in Brahman; 0.25 in Polish and 0.95 in Gry cattle (Zwierzchowski *et al.,* 2001). Pomp *et al.*

Table 1. The allele and genotype frequencies and the average heterozygosities in six Iranian cattle, F_1 Golpayegani x Brown Swiss and buffalo populations for the leptin gene.

		Genotype Frequency			Allele Frequency		
Population	No. of samples	AA	AB	BB	A	B	Heterozygosity
Sarabi	82	0.561	0.378	0.061	0.75	0.25	0.3750
Golpayegani	57	0.544	0.351	0.105	0.7193	0.2807	0.4038
Sistani	38	0.211	0.263	0.526	0.3421	0.6579	0.4501
Taleshi	70	0.100	0.757	0.143	0.4786	0.5214	0.4991
Manzadrani	26	0.231	0.308	0.462	0.3846	0.6154	0.4734
Dashtiyari	8	0.000	0.250	0.750	0.125	0.875	0.2188
F1 Golpayegani x Brown Swiss	13	0.000	0.308	0.692	0.1538	0.846	0.2604
Iranian Buffalo	30	0.100	0.100	0.800	0.15	0.85	0.2550

Table 2. The allele and genotype frequencies and the average heterozygosities in six Iranian cattle, F_1 Golpayegani x Brown Swiss and buffalo populations for the *Pit-1* gene.

		Genotype Frequency			Allele Frequency		
Population	No. of samples	AA	AB	BB	A	B	Heterozygosity
Sarabi	82	0.451	0.341	0.207	0.622	0.378	0.4703
Golpayegani	57	0.614	0.263	0.123	0.7556	0.2544	0.3793
Sistani	38	0.842	0.158	0.000	0.9211	0.789	0.1454
Taleshi	70	0.614	0.314	0.071	0.7714	0.2286	0.3527
Manzadrani	26	0.692	0.269	0.038	0.826	0.173	0.2862
Dashtiyari	8	0.625	0.000	0.375	0.625	0.375	0.4688
F_1 Golpayegani x Brown Swiss	13	0.000	0.769	0.231	0.384	0.615	0.4734
Iranian Buffalo	30	0.567	0.400	0.033	0.766	0.233	0.3578

(1997) have reported leptin B allele frequencies of 0.3 in Limousine; 0.21 in Simmental; 0.28 in Gelbvieh; 0.29 in Holstein; 0.5 in Hereford, 0.27 in Angus, 1.0 in Brahman and 0.4 in Branguse. Therefore, breeding strategies could be designed for introregression of the A allele for *Pit-1* and B allele for leptin from Iranian populations to exotic breeds such as Holestein.

A heterozygosity of less than 0.5 indicated low variation for these genes in studied populations. It is suggested that the strategies such as migration, introduction of new diversity and crossbreeding for increasing gene diversity and its conservation besides exploration of this potential genetic diversity should be adapted. Although the allele frequency of B is high for some Iranian populations, the AB genotype (favorable genotype) frequency is not too high. Therefore, it is suggested that crossbreeding should be done between these populations and/or with exotic breeds to increase the frequency of the favorable genotype. For example, crossing of the Golpayegani with the Brown Swiss stock has increased both B allele and AB genotype frequencies in F_1 the generation.

In conclusion, the possible association between molecular polymorphisms within these candidate genes and economic traits for the studied populations should be further investigated.

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