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Association of growth trait and *Leptin* gene polymorphism in Kermani sheep

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Abstract

Identification of genes affecting energy balance, milk yield and feed intake is an interesting area of researches in animal breeding. *Leptin* gene polymorphism is associated with key economic affair. Considering rich resources for animals, in our country, accomplishing a few assays to identify a gene that controls her traits with molecular genetics, and identifying the candidate genes in sheep breeds using DNA test can greatly help to her breeding progress. For analyzing *Leptin* gene polymorphism and its association with growth traits in Kermani sheep, blood samples of 120 sheep of both genes rearing at breeding centre of Shahre Babak were taken. In addition growth traits were measured. PCR was performed to amplify 275 bp fragments of exon 3 from *Leptin* gene. Then Single Strand Conformation Polymorphism (SSCP) of PCR product was performed and *Leptin* band patterns (genotypes) were obtained using acrylamid gel and silver staining. For *Leptin* gene 10 genotypes including A/A, C/C, A/B, A/C, A/B/C, A/B/E, A/B/F, A/C/F, A/B/D/E and A/B/C/F were obtained. The results of this study showed that the growth traits are significantly affected by the genotypes. Accordingly, A/B/E, A/C, A/B/C/F and A/B/C/F genotypes had higher body weight at 3, 6, 9 and 12 months of ages respectively. The animals with A/B, A/B, A/B/F and A/B/D/E genotypes had the smallest body weight at 3, 6, 9 and 12 months of ages respectively. It is suggested that polymorphism in *Leptin* gene loci can be used as a selective criterion to improve growth traits in Kermani sheep.

Keywords**:** *Growth traits, Kermani sheep, Leptin, PCR-SSCP, Polymorphism.*

Introduction

Genetic diversity in indigenous breeds is a major concern considering the necessity of preserving what may be a precious and irreplaceable richness, regarding new productive demands. Conservation should be based on a deep knowledge of the genetic resources of the specific breed. Therefore, it is important to try to characterize genetically indigenous breeds. Genes affecting polygenic traits and characterizing milk or meat productions are difficult to identify. However, a number of potential candidate genes have been recognized. They may be selected on the basis of a known relationship between physiological or biochemical processes and production traits, and could be tested as quantitative trait *loci* (QTLs). More recently, an array of new markers has been developed to carry out the genetic variation studied at DNA level including, one of the candidate genes for marker assistant selection, *leptin* (Nassiry et al., 2008). However, the major gene model suggests that only a few genes may account for relatively large proportion of the genetic diversity. Such major genes are the genes usually involved in the biology of a trait and are the candidate genes for marker identification. There is also a possibility that the major genes may be linked with some quantitative trait loci (QTL) contributing to a major part of the variation in traits. *Leptin* is a protein involved intricately in growth and metabolism of animals in which plays an important role in the regulation of feed intake, energy metabolism, growth and reproduction of cattle, therefore *leptin* is a potential candidate gene for QTL studies (Choudhary et al., 2005), which is mainly produced by adipose tissue and at lower amounts by tissues such as stomach, skeletal muscle and placenta. *Leptin* is considered as a hormone that regulates the body weight by maintaining the balance between food intake and energy expenditure through signaling to the brain the changes in stored energy levels (Zhou et al., 2009). *Leptin*, the product of the *ob* gene, is secreted from white adipose tissue and regulates food intake and whole-body energy metabolism and consists out of 3 exons and 2 introns of which only 2 exon are translated in to protein (Javanmard et al.,

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2008).

Leptin can be considered as one of the best biological markers reflecting body fatness both in animals and human being (Nassiry et al., 2007). Plasma *leptin* level in cattle and sheep increase linearly with body fat mass and with energy balance (Nassiry et al., 2007). It has been shown that *leptin* gene influences milk performance in cattle and it's reproduction in beef cattle (Nassiry et al., 2007). In cattle, *leptin* gene has been mapped to chromosome 4 and its full sequence is available in GenBank database at accession number U50365. Variation of LEP gene in the human is reported to be associated with low *leptin* levels, overweight or obesity and non-insulin-dependent diabetes mellitus (van der Lende et al., 2005). Polymorphisms in the bovine *LEP* gene have been described and an association with food intake, milk production and carcass and meat quality traits (Schenkel et al., 2005). In order to investigate the role of *LEP* in sheep, its gene needs to be identified and the occurrence of sequence polymorphism needs to be confirmed (Zhou et al., 2009). Although many polymorphic studies on the bovine *leptin* gene have been reported, the sequence of this gene has not yet been published for sheep. Nassiry et al., (2008) using PCR-RFLP method, showed three genotypes (CC, CT and TT) for *leptin* exon II gene in six Iranian native cattle breeds. Choudhary et al. (2005) for the 522 bp PCR *leptin* fragment found three *Bsa*AI digestion patterns (genotypes) in the *Leptin* gene of *Bos indicus*, *Bos taurus* and their crossbred cattle. Buchanan et al. (2002) described a cytosine (C) to thymine (T) substitution (C→T substitution) in intron 2 of *leptin* gene *in B. taurus* breeds suggesting the existence of C and T alleles and therefore CC, TT and CT genotypes. Javanmard et al., (2008) found three genotypes, AB, AA, and BB with 81.30, 18.50 and 0.20 frequencies respectively in the intron region of the bovine *Leptin* gene in Iranian Sarabi cattle. The single-strand conformation polymorphism (SSCP) is based on changes in secondary structure(s) in single-stranded DNA fragments caused by a change in sequence which are detected as alterations in the fragment mobility by gel electrophoresis. This method is one of the easiest screening procedures to perform and very cost effective, and has a reportedly high mutation detection rate (70–95% for some genes). However, SSCP is labour intensive, and analytical steps are performed before and during electrophoresis (concentrations of DNA template and primers, running temperature and time) may affect the detection of mutations and reproducibility. Only a few studies have been conducted on polymorphism in the Ovin *Leptin* gene using SSCP-PCR (Zhou et al., 2009;Tahmurespoor et al., 2009) and only one study of *Leptin* (Exon 3) genotyping in the Iranian important sheep breeds (Tahmures poor et al., 2009). The objectives of this study were to characterize potential variation in the ovine *LEP* gene using PCR–single-strand conformational polymorphism (PCR–SSCP) analysis and to investigate their associations with growth traits in Kermani sheep.

Materials and Methods

Animals and DNA extraction

For this study blood samples were randomly collected from 120 Kermani sheep (96 females and 24 males, ranging from 3 months to 2 years of age) reared at the breed conservation station in Shahre Babak, Kerman, Iran. The body weight at birth, 3, 6, 9 and 12 months of age were measured on these animals. Blood samples were stored at -20°C for DNA extraction. Genomic DNA was extracted from blood leukocytes using a Diatom kit (Boom et al., 1990). Purity of DNA was assessed by 2% Agarose gel stained with etithium bromide.

Amplification of Leptin exon 3

A 275 bp region of exon3 of *Leptin* gene was amplified using primers described by Tahmures Poor et al. (2009) with the following sequence; LeptF: 5'-GCT CCA CCC TCT CCT GAG TTT GTC C-3', LeptR: 5'-TGT CCT GTA GAG ACC CCT GTA GCC G-3'. The amplification of this region was performed by thermal cycling and using PCR master kit (sinnagen Co., Tehran). PCR reactions were performed in a 25 µl mixture containing 2 µl DNA (50 ng), 12.5 µl Master Mix, 1 µl of each primer (LepF and LepR) and 8.5 µl ddH2O. The thermal cycling profile consisted of an initial denaturation step of 95^{o}C for 4 min, 38 cycles of denaturation at 95^{o}C for 45 seconds; annealing at 66.5^{o}C for 55 seconds and extension at 72^{o}C for 75 seconds followed by a final extension at 72^{o}C for 10 min. Products of amplification were recognized by electrophoresis on 2% agarose gel stained with etithium bromide.

Single-Strand Conformational Polymorphism Analysis (SSCPA)

PCR products were resolved by SSCPA. For SSCPA a 7 µl aliquot of each amplicon was mixed with 7 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene-cyanol). After denaturation at 95°C for 5 min, samples were rapidly cooled on wet ice for 10

min to prevent reannealing of the single-stranded product (Zhou et al., 2009) and then loaded on 8% acrylamide: bisacrylamide (38:2) gels. Electrophoresis was performed using Vertical Slab Unit, VSS-100 (Akhtarian), at 4-5°C with 200 V for 4-5 h in 1% TBE buffer. A constant temperature was essential for band sharpness and reproducibility of strand separation. Then the electrophoresis unit was coupled to a thermostatic bath (Mousavizadeh et al., 2009). DNA fragments were visualized by the silver staining (Basam et al., 1991).

Statistical analysis

Of the 120 animals analyzed by PCR-SSCP, only 118 of them were statistically analyzed. The observed band patterns were genotyped by comparing them with the patterns used by Mousavizadeh et al. (2008) (figure 1) and Genotypic frequencies, Allele frequencies and diversity measurements were obtained. The observed frequency of heterozygotes was obtained directly by dividing the number of individual heterozygous by the total number of individuals. The following equation was used to calculate the genotype frequency of A/B genotype:

$$F(A/B) - \frac{n(A/B)}{N}$$

The diversity measures were obtained using POP GENE32 (Yeh et al., 1999). Association of *Leptin* gene genotypes with growth traits including body weight measured at birth, 3, 6, 9 and 12 months of age were investigated using a univariate animal model. Data were analyzed using ASReml (Gilmour et al., 2002). The mixed model used was as follow:

$$Y = Xb+Z_1a+ Z_2m+ Z_3mp_e+ e$$

Where y is individuals records on body weight; b is the fixed effects; a, direct additive genetic effects; m, maternal genetic effects; mp_e, maternal permanent environmental effects; e, residual effect and X, Z_1, Z_2 and Z_3 are the incidence matrixes for the fixed and random effects. The observed associations of candidate gene genotypes with growth traits were evaluated for individual genotypes using LSM ± SE (least squares means ± standard error).

Results

The fragment of *Leptin* gene (275 bp) was characterized successfully and amplified from each sample DNA (120 samples) used in the present study. The agarose gel electrophoresis of PCR that amplified 275 bp fragment of sheep *Leptin* gene for Kermani sheep is presented in figure 2. After optimization of the parameters affecting the detection of SSCPs, the PCR products were analyzed for 118 animals. Figure 3 shows the SSCP analysis for the 275 bp fragment of exon 3 of the *Leptin* gene. This fragment showed a high level of polymorphism allowing the detection of different conformational patterns (figure 3). In this study 10 conformational patterns, A/A, C/C, A/B, A/C, A/B/C, A/B/E, A/B/D/E, A/B/C/F, A/C/F and A/B/F (Table 1), were observed and only two of them were homozygous. Distributions of the alleles of *Leptin* gene in different animal groups and other diversity measurements are presented in table 2. The following equation was used to calculate genotype frequency of the A/B genotype:

$$F(A/B) = \frac{n(A/B)}{N}$$

Where *F* (*A/B*) is the genotype frequency for the A/B genotype, n is the number of genotypes for the A/B genotype and N is the total number of genotypes in the population. The frequency of the alleles was calculated as follow:

F(A)=0.02542+0+0.050845/2+0.09745/2+0.07626/3+0.11016/3+0.09322/3+0.0762/3+0.0847/4+0.06779/4=0.38

F(B)=0+0+0.050845+0+0.07626+0.036705+0+0.0254+0.021175+0.01694=0.23

F(C)=0+0.01694+0+0.09745+0.076266+0+0.031073+0+0+0.01694=0.24

F(D)=0+0+0+0+0+0+0+0+0.021175+0=0.021

F(E)=0+0+0+0+0+0.036705+0+0+0.021175+0=0.058

F(F)=0+0+0+0+0+0+0.0310733+0.0254+0+0.01694=0.074

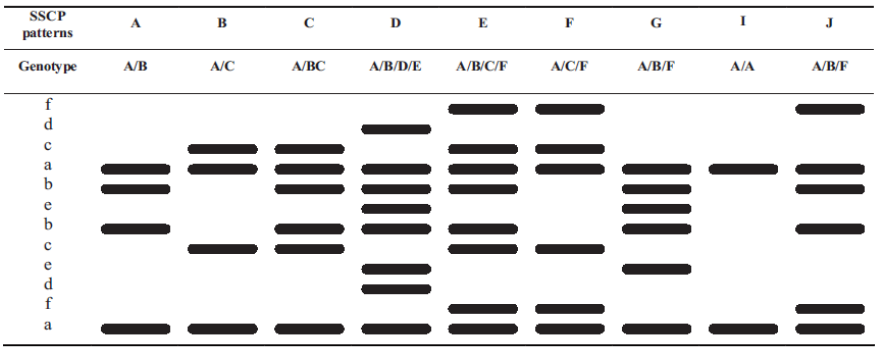

Figure 1. Proposed patterns for genotyping of the observed bands.

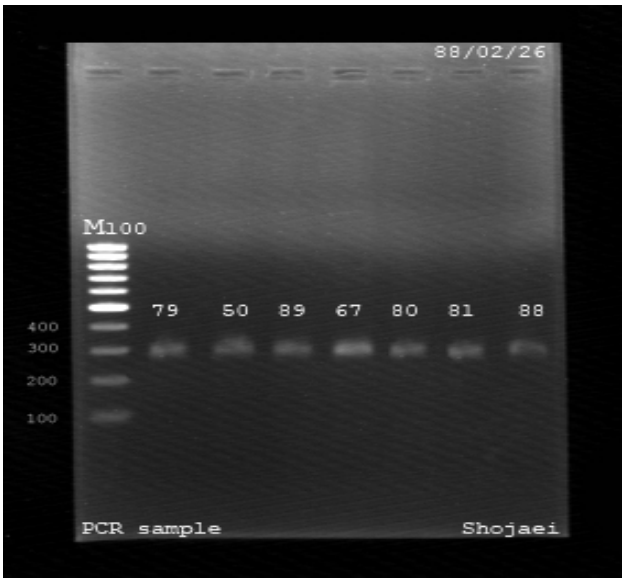

Figure 2. Agarose gel electrophoresis of PCR products of 275 bp fragment of exon 3 of *Leptin* gene. Lane 1: M100 bp ladder, lane 2-8: PCR products of 79, 50, 89, 67, 80, 81 and 88 samples.

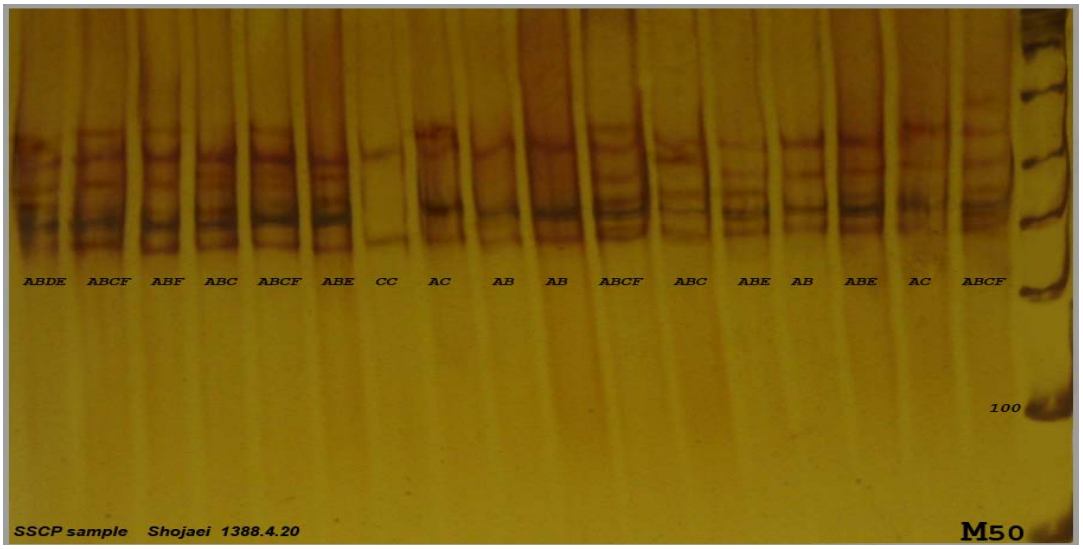

Figure 3. SSCP analysis of the 275 bp fragment of *Leptin* gene (exon 3). Lanes from left to right (1-18): ABDE, ABCF, ABF, ABC, ABCF, ABE, CC, AC, AB, AB, ABCF, ABC, ABE, AB, ABE, AC, ABCF, Ladder (M50).

Discussion

The most common allele present in Kermani sheep was allele A with a frequency of 0.38, and the second one was allele C with a frequency of 0.24. PCR-SSCP analysis shows that there is a high level of polymorphism in Kermani sheep for *Leptin* gene. There is only one study about *Leptin* (Exon 3) genotyping of the Iranian important sheep breeds (Tahmures poor et al., 2009). Comparing with the previous studies (Zhou et al., 2009 and Tahmures poor et al., 2009) we detected more polymorphisms in the fragments of *Leptin* genes. Variations detected here might have an impact on *leptin* activity and function. Tahmures poor et al. (2009) analyzed the same exon with the same methodology and primers and found 3 patterns in the Baluchi sheep (L_1, L_2, and L_3), while Zhou *et al.* (2009) showed five electrophoretic variants (01, 02, 03, 04 and 05) for exon 3 of *Leptin* gene in New Zealand breeds. Further, in our study, the association of this candidate gene with the growth traits was tested. The results of this study indicate that LEP gene contributes the body weight measured at birth to 12 months of ages. The LEP genotypes had significantly different effects on body weight measured at 3 months of age ($P \leq 0.01$). But the association was not significant for birth weight. Body weight measured at the different ages for different genotypes of LEP gene are shown in Table 3.

Table 1. Genotypic frequencies

Genotypes	A/A	C/C	A/B	A/C	A/B/C	A/B/E	A/C/F	A/B/F	A/B/D/E	A/B/C/F	Total
Number	3	2	12	23	27	13	11	9	10	8	118
Frequency (%)	0.54	1.69	10.17	19.49	22.88	11.02	9.32	7.63	8.48	6.78	100

Table2. Allele frequencies and diversity measurement

Allele	A	B	C	D	E	F	total
Frequency (%)	0.381	0.227	0.239	0.021	0.058	0.073	1
							Average
na	2	2	2	2	2	2	2
ne	1.293	1.498	1.884	1.100	1.215	1.297	1.456
h	0.227	0.478	0.470	0.091	0.177	0.229	0.280
I	0.387	0.680	0.662	0.192	0.321	0.390	0.439

na = Observed number of alleles, ne = Effective number of alleles, h = Nei's, gene diversity, I = Shannon's Information index

Table 3. least square mean and standard errors of the body weight measured at birth3, 6, 9 and 12 months of ages for the LEP genotypes[1] and the number of animals (in bracket)

			Traits = weights (Number) [2]		
Genotypes	**Birthday**	**3 months**	**6 months**	**9 months**	**12 months**
	[3] NS	**	*	*	*
A/B	3.43±0.57 (6)	15.80±4.40 (12)	16.16±3.65 (12)	21.17±7.44 (8)	15.07±5.63(7)
A/C	3.54±0.50 (21)	17.40±4.14 (24)	18.61±3.35 (24)	25.01±6.88 (21)	16.83±5.23 (2)
A/B/C	3.43±0.49 (27)	17.05±4.06 (32)	18.18±3.28 (30)	24.25±6.79 (26)	17.14±5.17(26)
A/B/E	3.42±0.54 (10)	18.19±4.38 (12)	18.45±3.63 (12)	24.06±7.29 (10)	16.84±5.47(10)
A/B/F	3.32±0.55 (8)	16.29±4.63 (7)	17.58±3.91 (7)	21.12±7.49 (7)	15.94±5.62 (7)
A/C/F	3.23±0.57 (6)	17.38±4.46 (10)	17.59±3.72 (10)	24.52±7.64 (6)	17.23±5.72 (6)
A/B/D/E	3.46±0.59 (5)	16.58±4.46 (10)	17.48±3.73 (10)	21.52±7.77 (5)	14.97±5.80 (5)
A/B/C/F	3.46±0.58 (6)	17.09±4.56 (8)	18.50±3.84 (8)	26.57±8.35 (3)	21.44±6.18 (3)

[1]Least square means ± Standard error
[2]Number of individual records with specific genotype
[3]NS: Not significant P>0.05, *P<0.05, ** P<0.01, ***P<0.001

Only a few studies have been conducted to investigate the association between *Leptin* gene polymorphism and performance traits in sheep. Tahmures Poor et al. (2009) analyzed the association of *Leptin* polymorphism with average daily gain (ADG) of Baluchi sheep. They reported a significant association between *Leptin* polymorphism and ADG at birth to 3 months of age ($P<0.10$). Leifers et al. (2002) have reported on the association between the *Leptin* genotype and milk production traits in Holstein-Friesian cows in which AB genotype was associated with higher milk yield. Yang et al. (2007) analyzed genetic polymorphisms in *Leptin* gene and their association with fatness in pig breeds. Duncan's multiple range test was used to evaluate the effect of different genotypes on the body weight measured at different ages ($P<0.05$). The results of this study show that growth traits are significantly affected by the genotypes. Accordingly, A/B/E, A/C, A/B/C/F and A/B/C/F genotypes had higher body weight at 3, 6, 9 and 12 months of ages respectively. The animals with A/B, A/B, A/B/F and A/B/D/E genotypes had the smallest body weight at 3, 6, 9 and 12 months of ages respectively. In comparison with other genotypes, A/B/C/F and A/C genotypes had significantly higher body weight at different ages ($P<0.05$).

Because of its simplicity, the PCR-SSCP procedure has been applied successfully in many laboratories to the routine clinical diagnostic testing for hereditary diseases or cancer. However, factors such as temperature, running time, buffer concentration, gel composition, position of the base change in the analyzed fragment, length fragment, etc. can influence the sensitivity of conformation-based methods. When screening for unknown genetic mutations in large genes, the number of different conditions must be controlled for reproducibility. The association of *Leptin* polymorphism with the growth traits in kermani sheep indicates that the gene can be used as a selective criterion to improve body weight genetically.

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