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RESEARCH ARTICLE

Leptin Gene Polymorphism in Lohi, Kajli and Spili Breeds of Sheep

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A B S T R A C T

Pakistan is blessed with rich sheep genetic resources being a source of milk, meat and wool across the country. There has been a very limited work on sheep genetic exploration. The Leptin a 16-kDa protein has been involved milk yield, energy balance, feed intake regulation, fertility and immune functions in animals. This gene is getting importance for animal researches to find useful biomarker for identification of high performing animals with better adaptability and productivity. In the present study, the polymorphism of the leptin gene in Lohi, Kajli and Sipli famous sheep breeds of Pakistan was analyzed by PCR-RFLP technique using six restriction enzymes (*BbvI, SfcI, AccI, Sty1, BsmF1, Ban II*) that showed monomorphic pattern in intron 2 (position A2262T, C2256G, -2730C) and exon 2 (A3201G) while polymorphic trend was observed in intron 2 (at position C1467T and A3050-). *BbvI* was found monomorphic Lohi and Sipli sheep while polymorphic in Kajli sheep at position C1467T. Interestingly the Sty1 enzymes was proved polymorphic (A/-) at position 3050 in Lohi, Kajli and Sipli sheep breeds of Pakistan. The *SfcI, AccI, BsmF1* and *Ban II* enzymes showed monomorphic results in all breeds. The different production traits can be associated with leptin genotypes in future and we recommend that polymorphisms in Leptin gene may be used to develop useful markers for selection of better animals under Maskers Assisted Selection program.

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INTRODUCTION

Genetic characterization to assess the existing biodiversity and differences among the important livestock breeds is an essential pre-requisite to facilitate the conservation program in an effective and meaningful way. Genes affecting polygenic traits and characterizing milk, meat or wool production are difficult to identify. However, a number of potential candidate genes have been recognized which are selected on the basis of a known relationship between physiological or biochemical processes and these traits could be tested as quantitative traits loci (QTLs) (Machugh *et al*., 1998). Among these one of the candidate genes for Marker Assisted Selection is leptin (Nassiry *et al*., 2008).

Leptin is a non-glycosylated polypeptide with a molecular mass of 16 kDa encoded by the obese gene. The leptin gene is highly conserved across species and is located on chromosome 7q31.3 in humans and on chromosome 4q32 in cattle (da Silva *et al*., 2012; Fatima *et al*., 2011). Leptin gene DNA sequence includes 15,000 base pairs and contains 3 exons, which are separated by 2 introns (Green *et al*., 1995). Out of 3 exons and 2 introns, only two exons are translated into protein (Javanmard *et al*., 2008). Exon 1 is a non-coding part (Buchanan *et al*., 2002). Leptin is synthesized as a pro hormone and released in to hormone in 146 amino acid in length. Expression of gene which encodes a Leptin receptor has been confirmed in pituitary (Iqbal *et al*., 2000), adipose tissue, granulosa and theca cells of the ovary (Karlsson *et al*., 1997), interstitial cells in testis (Caprio *et al*., 1999), in heart, liver, lung, kidney, adrenal gland, small intestine and lymph nodes (Hoggard *et al*., 1997). In mammals the 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 and brings the changes in stored energy level (Friedman *et al*., 1998). Leptin also plays a major role in control of body

growth, adoptability, immune function, angiogenesis, renal function, hematopoiesis, reproduction, and not only acts as an endocrine signal in brain and different peripheral tissues in which Leptin receptors are expressed (Zieba *et al*., 2003) fetal tissue, mammary gland, rumen, abomasum, duodenum and pituitary gland (Yuen *et al*., 2002). The Leptin expression is also modulated according to different physiological and growth stages of animal (Yonekura *et al*., 2002, 2003; Wallace *et al*., 2014). Therefore, the Leptin could act as marker for animal growth, feed conversion efficiency and health. The key element of the system regulating food intake has proven to be the Leptin. It act as hunger center in the hypothalamus and affects the regulation of appetite.

It has also been shown that Leptin gene influence milk performance in sheep, cattle and reproduction performance in beef cattle (Almeida *et al*., 2003; Mahmoud *et al*., 2014). Restriction fragment length polymorphism of the bovine leptin gene was reported for the first time by Lien *et al*. (1997). Studies on Leptin gene polymorphism and production traits in dairy cattle (Fiona *et al*., 2002), sheep and poultry (Boucher *et al*., 2006) has been reported with promising results and can be considered as one of the best biological markers in animals and human beings (Nassiry *et al*., 2008).

There are 28.8 million heads of sheep breeds in Pakistan (Anonymous, 2012-13) providing milk, meat and wool to poor and landless farmers across the country. Keeping in view the role of genetic molecular studies especially in combination with reproductive biotechnology practices in improving the productive and reproductive performance of animals, the present study was designed to genotype leptin gene in major Pakistani sheep breeds using PCR-RFLP technique with a view to generate a basic data on biodiversity of leptin gene in Pakistani breeds with an aim to correlate it with animal health, productive and reproductive performance for future breeding reference.

MATERIALS AND METHODS

Animal selection: A total of 82 samples from three famous sheep breeds (Lohi, Kajli, and Sipli) of the Punjab province of Pakistan were selected for this study. Lohi sheep breed was sampled (n= 28) from Livestock Production and Research Institute, Bahadurnagar, Okara. Kajli sheep samples (n=33) were selected from Livestock Experimental Station, Khizerabad District Sargodha, While Sipli sheep breed was sampled (n=21) from Livestock Experiment Station Haroonabad District Bahawalnagar. All the animals were unrelated and selected based upon pure phenotypic characteristics defined for each breed. Lohi and Kajli are the most popular and wide spread native breeds of Punjab. They are large-size sheep, mostly reared in irrigated farmlands. Lohi is mainly reared in the central area of Punjab, while Kajli whose lean meat is favored in the region, is mainly distributed in the northwestern part of the province. Sipli occupy the southern part of Punjab.

Genome extraction and quantification: All the samples were extracted using the method as described by Hussain *et al*. (2013). DNA concentration of all samples was measured using spectrophotometer (Nano Drop-1000, Nano Drop Technologies Inc. Wilmington, DE, USA). The final working solution (50 ng/µl) was prepared by adding filtered distilled water for all samples. The quality of extracted DNA was further analyzed by running on 1% agarose gel electrophoresis along with a size standard.

Alignment and identification of single nucleotide polymorphism in ovine leptin gene: The GenBank NCBI accession no HE605296 and AF310264 was used to assemble different fragments of ovine leptin gene. The NCBI Ovine leptin gene sequences (Accession no HE605296 and AH014693, HE605296 and AY911719, HE605296 and DQ496248, HE605296 and EF534370, HE605296 and EF534372, HE605296 and EF534373) were aligned using Blast Program to identify the SNP's in different fragments.

Restriction enzymes for digestion of leptin gene fragments through RFLP technique: After the alignment of gene sequences in the Blast program the SNP sequence was used in the Web Cutter computer software to identify the suitable restriction enzymes for digestion of the particular sequence. The different SNPs, gene sequence, specific enzymes, cutting position and fragment length have been shown in the Table 1.

Primers optimization and PCR amplification: Two fragments of Ovine Leptin gene Intron 2 (2001 bp) and Exon 2 (1540 bp) were amplified using primers (Table 2) designed by using the Primer3 software v. 0.4.0 (Rozen and Skaletsky 2000). The amplification of 2001 bp (Intron 2) and 1540 bp (Exon 2) of Ovine Leptin gene was carried out in a Bio-Rad thermal cycler using standard PCR procedures.

RFLP analysis and gel electrophoresis: Polymerase chain reaction fragment length polymorphism (PCR-RFLP) technique was applied to explore the polymorphism in Ovine Leptin gene. The restriction digestion of PCR product was carried out with enzymes (*BbvI, SfcI, AccI, Sty1, BsmF1, Ban II*) provided by (New England Bio Lab). The incubation time and temperature were followed as instructions provided by the company. Restricted fragments were resolved on 4% agarose gel electrophoresis and were visualized by Ethidium Bromide staining. The restricted digested gene fragments were visualized on UV transilluminator and photographed with gel documentation system (Gel Doc TM x R system BioRad).

RESULTS AND DISCUSSION

In the present study PCR-RFLP analysis using the selected restriction enzymes revealed monomorphic pattern at position A2262T, C2256G, -2730C in intron 2 sequence of Leptin gene and A3201G at exon 2 sequence. However, a polymorphic trend was recorded at position C1467T and A3050- in intron 2.

More specifically the *BbvI* was found monomorphic at position C1467T in Lohi and Sipli sheep while Kajli sheep was polymorphic at this position. Interestingly the *Sty1* enzymes was proved polymorphic (A/-) at position

Table 1: The size of various electrophoretic bands observed by PCR-RFLP analysis of Ovine Leptin gene with selected restriction enzymes in Lohi, Kajli and Sipli sheep breeds

Mutation	Enzyme	Incubation Temp. (°C)	Incubation time (Mins)	Restriction length	Genotype Pattern		
					Lohi	Kajli	Sipli
1467 C/T	*Bbvl*	37	15	C: 06, 128, 159, 173, 186, 507,843 T : 06, 159, 173, 314, 507, 843	A	B	A
2262 A/T	*SfcI*	37	60	A: 392, 718, 893 T: 201, 392, 692, 718	A	A	A
2256 C/G	*AccI*	37	15	C: 724, 1279 G: 191, 724, 1088	A	A	A
3050 A/-	*Sty I*	37	15	A : 286, 298, 1419 _ : 286, 1716	B	B	B
2730 -/C	*BsmF I*	65	60	_ : 98, 131, 180, 332, 351, 911 C : 98, 131, 152, 180, 200, 332, 911	A	A	A
3201 A/G	*Ban II*	37	60	A : 63, 109, 126, 141, 247, 307, 1010 G : 30, 63, 79, 126, 141, 247,307,1010	A	A	A

Table 2: Primers used for amplification of selected region of Leptin gene

Primer name	5'-3' Sequence	Product size (bp)
21F/21R Intron 2	Fw-CCCCTCATCAAGACGATTGTCAC Rev-GCGAGGATCTGTTGGTAGA	2001 bp
31F/31R Exon 2	Fw-AGAGGGTCACTGGTTTGGACTT Rev-CCCACGCTGGAATACTT	1540 bp

3050 in all the three selected Lohi, Sipli and Kajli sheep breeds of Pakistan. In contrast the *SfcI, AccI, BsmF1* and *Ban II* enzymes were found monomorphic for Lohi, Kajli and Sipli sheep breeds (Table 1).

Position A2262T, C2256G, -2730C in intron 2 and A3201G in exon 2 of Ovine Leptin gene in Lohi, Kajli and Sipli breeds proved monomorphic during PCR-RFLP analysis of the enzymes *SfcI, AccI, BsmF1* and *Ban II. The* position C1467T and A3050- were found polymorphic using *BbvI* and *Sty1* enzymes.

Economical traits are among quantitative traits that are controlled by many genes each having a small effect. The major gene model suggests that only a few genes may account for relatively large proportion of the genetic variation. Such major genes being the genes usually involved in the biology of the trait and are the candidate gene for marker identification. There is also the possibility that major genes may be linked with the some quantitative trait loci (QTL) contributing to a major part of the trait (Fruhbeck *et al*., 1998). One of the candidate genes for marker assisted selection is Leptin gene. Plasma Leptin levels in cattle and sheep increases the linearly with increased body fat mass and energy balance. Leptin gene has also been shown to influence production and reproduction in beef cattle (Ngu *et al*., 2015).

In livestock variation in Leptin gene has been characterized in cattle and pig but scanty information is available in Ovine. Positive correlation between Leptin serum levels and carcass traits such as marbling, 12th rib back fat thickness, kidney pelvic heart fat and quality grade in beef (Geary *et al*., 2003). The polymorphism in the Exon3 of Leptin gene in sheep has also been reported by Zhou *et al*. (2009) using PCR-SSCP technique. The reported five unique SSCP patterns corresponding to five alleic sequences using the combination of two different electrophoresis conditions in six common New Zealand sheep breeds. One or two of these patterns for each sheep was recorded reflecting the presence of homozygous or heterozygous genotype at the ovine leptin locus. Boucher *et al*. (2006) has reported leptin gene polymorphism and its association with skeletal muscle growth and meat quality using single nucleotide polymorphism (SNP) analysis. Almeida *et al*. (2003) reported that RFLP marker genotypes in the leptin gene were significantly associated with calving date in a Brangus herd in Brazil.

Very little information is currently available to compare different sheep population. Leptin gene polymorphism exon 3 was studied and found that with growth traits are significantly affected by the genotypes in Kermani sheep by Shojaei *et al* in 2010 and suggested that polymorphism in Leptin gene loci may be used as a selective marker to improve growth traits in future. The exon 3 of leptin a gene was also studied in Iranian Makoei sheep was studied and Five SSCP patterns were identified by Hashemi *et al*. 2011. Maitra *et al*., 2014 found SNPs in Exon 2 (1029T>C), Intron 2 (1621G>A) and 3'UTR (3968T>C, 3971C>T, 4026G>A, 4105G>A and 4225T> C) in Indian goats that may be screened in large population for association studies with meat quality traits and marker-assisted selection. The present study may be regarded as beginning of the attempt to understand the genetic diversity of local sheep breeds in Pakistan. Further studies are suggested to investigate the relationship between leptin gene polymorphism, serum concentration level and the performance traits in local Pakistani sheep breeds.

Conclusion: The goal of the present study was to determine genetic polymorphism of the leptin gene in selected Pakistani sheep breeds (Lohi, Kajli and Sipli) mostly used for meat, milk and wool purposes. We are able to generate the basic data in Pakistani sheep breeds and open interesting aspects for future selection programs, especially marker assisted selection of animals.

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