

Molecular Analysis of Leptin Gene Polymorphism in Achai, Sahiwal Cattle and Nili-ravi Buffalo Breeds of Pakistan

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Abstract: Pakistan is blessed with rich sheep genetic resources and being a source of milk, meat and wool sheep breeds are reared across the country. Despite its great economic importance very limited work has been done in Pakistan on sheep genetic exploration. Leptin hormone plays important role in milk yield, body weight, energy balance, feed intake, immune function and fertility performance in animals. The current study was carried out to investigate genetic polymorphism in exon III of *Leptin* gene (*LEP*) in indigenous animal breeds (Nili-ravi buffalo, Sahiwal and Achai cattle) of Pakistan using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). PCR-RFLP analysis revealed that all the three studied animals breeds were polymorphic for *LEP* gene (having all the three genotypes AA, AB and BB) with frequency of 60%, 33% and 7% in Nili-ravi buffalo, 55%, 41% and 4% in Achai cattle and 52%, 45% and 3% in Sahiwal cattle breed. The genotype BB is highly desirable in cattle and buffalo breeds but its frequency is very low in our indigenous population. Studying genetic polymorphism of milk protein is important due to its application in dairy industry and these in formations could further be utilized for future breeding programs particularly for the enrichment of the rare genotype.

Keywords: Leptin, Sahiwal, Achai, Nili-Ravi, Polymorphism, PCR-RFLP

1. Introduction

Cattle and buffalo are kept mainly for the production of milk and meat. The two riverine buffalo breeds (Nili-ravi and Kundi buffalo) are the most popular indigenous breeds of Pakistan. The Nili-ravi buffalo is famous for high milk yield, resistance to diseases and milk protein content [1]. Among the various indigenous cattle breeds, Sahiwal cattle and Achai breeds are well recognized and are used in breeding practices to produce quality breeds [2].

Milk quality and quantity are always remained as traits of great interest for the human. The yield and composition of milk vary greatly in different animal breeds. This variation in milk quantity and quality is caused by genetic and

environmental factors which make them multi-factorial polygenic traits. The polymorphism of milk protein is of particular interest due to its possible role in selection and genetic classification of different animal breeds [3]. The identification of genotypes of dairy cattle milk proteins could provide a distinctive role in molecular genetics to study quantitative traits [4-5].

Leptin, a 16kDa polypeptide hormone, is predominantly produced in the white adipose tissue and secreted in to the blood stream and milk (colostrums). *Leptin* performs important roles in controlling body weight, milk production, feed intake, immune function and reproduction [6]. Several reports mentioned that leptin gene influences milk performance in cattle [7-8]. The leptin gene is highly

conserved and has been mapped to chromosome 4 in cattle [9] and consists of 3 exons and two introns [10]. Among the three exons, exon 1 is a noncoding [11] while exon 2 and 3 are translated to polypeptide [12]. Four polymorphisms have been reported so far in *leptin* gene including R25C (C305T, R4C, C73T, *LepKpn2I*) and Y7F(A252T) [13-14] in exon II, while C963T and A80V (*Lep HphI*, A59V) were reported in promoter and in exon III respectively [13-15].

The current study was performed on c.177 C>T, p. A59V SNP, and it is believed that this mutation has role in milk production [16]. Animal can either be wild type normal with AA genotype or homozygous mutant with BB genotype or it can be heterozygous AB with one normal and one mutant allele. Genetic studies have shown that *HphI* site does not exist in normal homozygous animals, so digestion with *HphI* restriction enzyme gives a single band of 331 bp while in homozygous mutant animals C>T mutation creates *HphI* recognition sequence so digestion with *HphI* yields two bands of 311 bp and 20 bp. In case of heterozygous animals as both the normal and mutant alleles are present, restriction with *HphI* enzyme gives three bands of 331 bp, 311 bp and 20 bp size.

During lactation when energy intake is limited, body fat reserves play a vital role to sustain high milk production. Animals with homozygous TT allele tend to produce more milk as compared to other two genotypes [11], without affecting milk protein and fat contents during the whole lactation period. *HphI* polymorphism has also been found to have a considerable effect on milk production [8].

Conventional breeding strategies have been practiced since years to select animals with desired traits. However, MAS (Marker assisted selection) are now potentially used to select animals with desired traits [17]. Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) is one of the widely used and recommended methods to make the distinction between the genotypic variants [5-18].

The aim of the present study was to investigate genetic polymorphism of the *leptin* A59V in the selected indigenous buffalo and cattle breeds of Pakistan. The information will provide a data for the animal breeding strategies especially marker assisted selection for better milk producing animals and other improvements strategies of the animals.

2. Materials and Methods

The current research study was conducted in National Institute for Genomics and Advanced Biotechnology (NIGAB), Islamabad with the main objective to identify A59V *leptin* gene polymorphism in indigenous Pakistani bovine breeds. All the procedure adopted in the present study was approved by the ethical and review board of the National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre Islamabad, Pakistan.

2.1. Experimental Animals and Blood Sample Collection

The present study was conducted to investigate the *leptin* gene polymorphism in Nilli-ravi buffalo, Sahiwal and Achai cattle breeds of Pakistan. A total of 300 animals (100 from each breed) were randomly collected from livestock production and research institute, (LPRI), Bahadurnagar and breeding and dairy farm Harri-Chand, Charsadda of Pakistan. A total of 5ml blood sample were collected from the jugular vein puncture in ethylenedi-amine tetraacetic acid (EDTA) coated vacutainer tubes and stored at 4°C for genomic DNA extraction.

2.2. Genomic DNA Extraction

Genomic DNA from whole blood was extracted using standard DNA extraction method described by Sambrook *et al.* [19] and the quality was checked on 1% agarose gel under ultra violet (UV) light.

2.3. PCR Amplification

The PCR amplification of 331bp DNA fragment of *leptin* gene (exon III) was performed in veriti 96 wells thermocycler (Applied Biosystem CA, USA) by using specific set of primer previously used by Haegeman *et al.* (2000). Forward primer 5'- GGAAGGGCAGAAAGATAG-3' and Reverse Primer 5'- TGGCGAACTGTTGAGGATC-3'. The PCR reaction was carried out in a total volume of 25 µl containing 50ng of template DNA, 10X (2.5 µl) PCR buffer, 1.5 mM (2.5 µl) MgCl₂, 0.25 mM (0.5 µl) dNTPs, 0.5 U (0.25 µl) Taq DNA polymerase (Fermentas) and 50 pmol (2.5 µl) of each of the forward and reverse primers. The thermal cyclic profile was started with initial denaturation at 94.0°C for 2 minutes followed by 35 cycles of denaturation at 94.0°C for 30 seconds, primer annealing at 56.0°C for 60 seconds and extension at 72.0°C for 30 seconds while the final extension was carried out at 72°C for 14 minutes. The amplified fragments were checked on 2% agarose gel and compared with the standard DNA marker.

2.4. Analysis of Restriction Fragment Length Polymorphism (RFLP) Analysis

The desired PCR product of 331 bp were digested with *HphI* restriction enzyme, in order to find out the RFLP in the given amplicon. The restriction digestion was carried out in a total volume of 20ul consisting of buffer R (2µl), PCR water (2.5µl), *HphI* restriction enzyme 0.5µl of (Fermentas) and 15µl of PCR products were added to eppendorf tube and incubated overnight at 37°C. The resulting restriction digested products were visualized on 3% agarose gel containing ethidium bromide under UV light in gel documentation system and was compared with 50 bp DNA marker (Fermentas) which was run in parallel.

2.5. Statistical Analysis

The banding pattern of restriction product gave a clear picture to calculate the genotypic and allelic frequencies.

Expected allelic and genotypic frequencies were calculated using the following equations Rosner [20].

$$\text{Expected (AA)} = p^2 n$$

$$\text{Expected (AB)} = 2pq.n$$

$$\text{Expected (BB)} = q^2 n$$

Whereas,

‘p’ represents allele ‘A’

‘q’ represents allele and

‘n’ is the number of individuals.

However, observed heterozygosity (H_o) was calculated by finding the ratio between ‘total heterozygous animals’ to ‘total number of alleles’ as shown,

$$\text{Observed heterozygosity (H}_o\text{)} = \frac{\text{Total number of heterozygous animals}}{\text{Total number of alleles}} \quad (1)$$

Total number of alleles

However, expected heterozygosity (H_e) was calculated using the following formula;

$$\text{Heterozygosity expected (H}_e\text{)} = 2 \times p \times q \quad (2)$$

To find the Hardy and Weinberg equilibrium for observed and expected heterozygosity and to find whether it is statistically similar or dissimilar, Chi-square test (χ^2) was calculated using the following equation.

$$\chi^2 = \sum \frac{(O-E)^2}{E} \quad (3)$$

Where ‘ χ^2 ’ represents chi square, ‘O’ represents observed heterozygosity (H_o) and ‘E’ represents expected heterozygosity (H_e).

3. Results

3.1. PCR-RFLP Analysis

The 331bp PCR product of *Leptin gene* (exon III) from all three mentioned bovine breeds (Figure 1A) was used for RFLP analysis which resulted into three genotypes as shown in Figure 1B. The genotype AA showed a single undigested band of 331bp which indicates homozygous animal for allele A, genotype BB showed two bands (311 and 20 bp) and are homozygous for allele B whereas genotype AB, showed three bands (331, 311 and 20bp) and are heterozygous (Figure 1B) through PCR-RFLP.

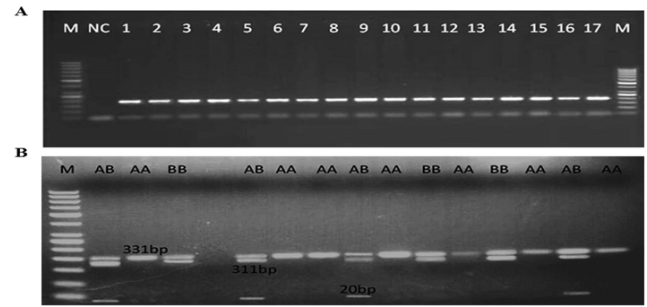


Figure 1. Polymerase chain reaction-restriction fragment length mutation (PCR-RFLP) assay of the *Leptin gene* in Achai, Nili-Ravi and Sahiwal cattle breeds. (A). Amplified 331bp PCR product of *Leptin gene* in Achai (lane 1-5), Nili- Ravi (lane 6-11) and Sahiwal (lane 12-17) cattle breeds. (B) Digested 331bp *leptin gene* product in Achai (lane 1-5), Nili- Ravi (lane 6-11) and Nili- Ravi (lane 6-11) and Sahiwal (lane 12-17) cattle breeds which shows three fragments of 331bp 311 and 20bp.

3.2. Genotypic and Allelic Frequencies of Sahiwal, Achai and Nili-Ravi Buffalo

All the three genotypes of leptin gene were observed in the studied population of above the mentioned breeds (Figure 1B). Sahiwal cattle breed studied were polymorphic for *Leptin gene*, showing all the three possible genotypes BB, AB and the most frequently observed AA genotype (Figure 1B) with 0.03%, 0.45% and 0.52% genotypic frequency respectively (Table 1). The allelic frequency of A and B alleles were found to be 0.745 and 0.255 respectively (Table 1). Achai cattle breed also showed all the three genotypes AA, AB and BB with 0.55%, 0.41% and 0.4% genotypic frequency respectively (Figure 1B, Table 1). The allelic frequency of A allele in Achai cattle was 0.755 and that of B allele was 0.245 (Table 1). Similarly the Nili -ravi buffalo also showed the presence of all the three genotypes but with different genotypic frequencies (Figure 1B). The most common genotype observed was genotype AA with frequency of 0.60% while frequency of AB and BB were 0.33% and 0.7% respectively. Frequency of allele A was 0.765 and that of allele B was 0.235 (Table 1). In general the homozygous genotype AA was observed in highest frequency in comparison to genotypes AB and BB in all the studied animal breeds. Over all the indigenous population showed that the genotype AA is most the frequent genotype with a frequency of 55.6% while the least frequent genotype observed was BB with frequency of 4.6% for *leptin gene* in Pakistani bovine breeds. The genotypic and allelic frequencies of the studied breeds are shown in Table 1.

Table 1. Genotypic and allelic frequencies, observed and expected heterozygosity and chi square values for Hardy –Wienberg equilibrium.

Breed	n	Genotypic frequencies			Allelic frequencies		H_o	H_e	χ^2
		AA	AB	BB	A	B			
Sahiwal	100	0.52	0.45	0.03	0.745	0.255	0.45	0.38	3.39 S*
Achai	100	0.55	0.41	0.04	0.755	0.245	0.41	0.37	1.17 ^{ns}
Nili-Ravi	100	0.60	0.33	0.07	0.765	0.235	0.33	0.36	0.69 ^{ns}

H_o ; Observed Heterozygosity H_e ; Expected Heterozygosity χ^2 ; chi-square value, ns; Non significant S*; significant value.

3.3. Statistical Analysis

Chi square value (χ^2), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated for the aforementioned breeds (Table 1). H_o value for Sahiwal cattle, Achai cattle and Nili -ravi buffalo were 0.45, 0.41 and 0.33 respectively. H_e value for Nili -ravi buffalo was 0.36 while that for Sahiwal cattle and Achai cattle breeds were 0.38 and 0.37 respectively. The χ^2 value at one degree ($p < 0.05$) of freedom was calculated which was found less than tabulated values which means that chi-square values of all the breeds are non-significant and all the studied population are under Hardy-Weinberg equilibrium.

4. Discussion

Studying genetic polymorphism of milk protein is particularly of great significance due to its practical application in the dairy industry. Thus keeping in mind the economics importance of the dairy industry the current innovation was started to explore the natural genetic potential of indigenous cattle and buffalo breeds of Pakistan.

To our knowledge this is the first report of *leptin* gene polymorphism in Nili-ravibuffaloe, Achai and Sahiwal cattle breeds of Pakistan. In current study, all the three genotypes AA, AB and BB for *leptin* gene were obtained. The frequency of AA genotype was 60%, 55% and 52% whereas for AB genotype it was observed 33%, 41% and 45% in Nili-ravi buffalo, Achai cattle and Sahiwal cattle breeds respectively. The observed frequency for BB genotype was 7%, 4% and 3% in Nili-ravi buffalo, Achai cattle and Sahiwal cattle breeds respectively. Allelic frequency of A allele was 0.76, 0.75 and 0.74 while for allele B it was observed 0.24, 0.25 and 0.26 in Nili-ravi buffalo, Achai cattle and Sahiwal cattle respectively. The overall frequency of A allele was higher than allele B in all the three studied animal breeds. Allelic and genotypic frequencies calculated were compared to the previous published data to find out possible co-relation.

Our results of genotypic and allelic frequency are in accordance with the observation of Kaygisiz *et al.* who worked on the polymorphism of *leptin* gene in East Anatolian, Red Anatolian and Anatolian Black and found T allele (52%) more frequent than allele C (48%) (Corresponds to A and B allele respectively) [21]. Similar pattern of genotypes were reported by Javanmard *et al.* who studied *leptin* gene in Iranian Sistani and Golpayegani cattle and observed genotypic frequencies of 55%, 37% and 8.3% for the AA, AB and BB genotypes respectively [12]. Likewise in Jersey and Ayrshire cattle, the frequency of T and C allele observed were in accordance to our observations which showed that T allele is more common than C allele [11]. A quite similar distribution of genotype was obtained in the study on Polish black and white cows by Kulig *et al.* who found that the occurrence of allele A (0.805) is more common than B allele (0.114) [22]. Kong *et al.* also reported similar findings that the dominant allele A (0.585) is more

frequent than allele B (0.415) in Korean cattle population [23]. Similar results were also observed by Liefers *et al.* [7]. Who reported genotypic frequencies of 0.83, 0.18 and 0.002 for the genotypes AA, AB, and BB while working on *leptin* gene polymorphism in Holstein Heifers. Yazdani *et al.*, further supported our observations, reported that allele A is dominant (0.76) over allele B (0.24) in Iranian Holstein [24].

In contrast, Nassiry *et al.* [18] worked on diversity of *leptin* gene in Iranian brown Swiss and Holstein cattle and identified two genetic variants T and C, representing C allele more frequent than T allele with frequency of 62.5% and 37.5% respectively. Similar findings were observed by Sadeghi *et al.* who found the occurrence of allele C (0.575) greater than allele T (0.425) [16].

For allelic frequencies, A allele was found dominant over the B allele. Sahiwal cattle showed maximum (45%) observed heterozygosity while it was minimum (33%) in Nili-ravi buffalo. This discrepancy in Nili-ravi buffalo might be explained by high inbreeding rate and genetic drift.

The current study is a first report on *leptin* gene polymorphism in native milch breeds of Pakistan. As *leptin* (*LEP*) gene influences milk production in cattle therefore further studies will be needed to establish association of A59V *LEP* gene polymorphism with the milk production trait.

5. Conclusion and Recommendation

This preliminary data will be helpful for future studies not only on these breeds but also on other bovine breeds to increase the frequency of the desirable homozygous BB allele in bovine breeds. It is therefore suggested that selection on the basis of BB genotype in studied breeds can bring improvement in milk production.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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