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Identification of Polymorphism in LEP Gene by Single Strand Conformation Polymorphism (SSCP) in Rathi Cattle

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A B S T R A C T

In present examination, Rathi cows were chosen to identified leptin gene qualities for polymorphism. Complete 160 lactating Rathi cows from livestock research station (LRS), Nohar and Bikaner were chosen. Blood samples were collected and DNA isolated by the spin column method. For exon-2 and exon 3 of LEP gene primers were constructed based on available sequences of LEP gene in the NCBI GenBank database. Fragments of 326 and 454 bp of Exon-2 and exon 3 regions of LEP gene, respectively, were amplified by a polymerase chain reaction in a final reaction volume of 25μl. The PCR-SSCP was utilized for amplification and detection of polymorphism in LEP gene. In exon-2 of LEP gene shows monomorphism. In exon 3 of the LEP gene, two SSCP patterns were observed and it shows the polymorphic nature of this region. In exon 3 of the LEP gene two alleles and two genotypes with frequency 0.915, 0.085, 0.832 and 0.168, respectively, were observed. In present examination, exon 2 of LEP gene noticed monomorphic nature and exon 3 of LEP quality uncovered polymorphic nature in studied population of Rathi cattle.

Keywords
Leptin (LEP) gene, SSCP, Polymorphism and Rathi cattle

Introduction

Milk is characterized as secretion from mammary glands in mammals and it meets the complete requirement of infants (Fox, 2009). Total milk production in-country is 187.75 million tonnes which are increased by 6.5% than the previous year. In Livestock census 2019, the total number of cattle in the country is 192.49 million that indicate a 0.8% higher value than the previous Census. The number of female cattle is 145.12 million, which increased by 18.0% over the previous census (Livestock census, 2012). India has great diversity in livestock population and possesses 50 registered, well-recognized breeds of cattle (NBAGR, 2020). The indigenous cattle contribute 10% and non-descript cattle contribution 11% of total milk production in the country (BAHFS, 2019). According to BAHFS (2019), Rajasthan produced 12.6% of total milk production and holds the second rank in India. In Rajasthan average milk yield for indigenous cows is 5.1 kg. per day. Indigenous cow milk contains A2 type milk which is good for human consumption (Behera et al., 2018). Rathi has a breeding tract in Hanumangarh,
Loonkaransar tehsil of Bikaner, Shriganganagar districts of the state (Anonymous, 2018). Rathit cattle contribute 0.83 percentage to the total population of indigenous cattle in India (Breed Survey, 2013). Rathit cattle breed is known for its hardiness to withstand the harsh agro-climatic conditions in the arid and semi-arid zone of Rajasthan Dhaka et al., (2015). Nowadays, breeding policies emphasized the conservation and improvement of the indigenous pure breed of cattle for improving milk production and productivity in a scientific manner (Anonymous, 2014). The product of the Leptin gene is a 16-kDa protein secreted by adipose tissue and regulates adiposity. Leptin, a hormone has many roles in body energy balance, tissue growth, body composition, reproduction, immunity and feed intake of an animal through a feed-back mechanism (Saleem et al., 2015). The bovine Leptin gene includes its promoter region, three exons and two introns and spanned about 18.9 kb (Taniguchi et al., 2002). The Leptin gene has its role in appetite, metabolism, growth and milk production in cattle. Exon 2 and exon 3 part of the LEP gene analyzed among three breeds: Frieswal, Ongole and Sahiwal cattle (Singh et al., 2014). Polymorphism in the leptin gene was identified by Liefers et al., (2002); Fontanesi et al., (2014).

**Materials and Methods**

An overall 160 Rathit animal were selected from Livestock Research Station Nohar (LRS), Hanumangarh and Bikaner (80 animals from each farm). Only milking cows with a minimum of 120 days of lactation were included in the study. After approval of the ethical committee, Blood samples were collected aseptically from jugular vein puncture into the anticoagulant EDTA containing vacutainers tube and were transported to Molecular Genetics Laboratory in an icebox. Genomic DNA from the whole blood sample was extracted through the spin column method as per standard protocol (Sambrook et al., 2001). Two primers according to the exon-2 and exon 3 of LEP gene were constructed based on available sequences of LEP gene in the NCBI GenBank database. The sequences of primers, the accession number of the reference sequence and expected fragment length of the different selected regions are represented in Table 1. In PCR reaction mixture (25μl) contents used for amplification of genomic DNA were 5X PCR buffer (5μl), 1.5mM MgCl2 (1.5 μl), 10 Mm dNTP’s mix (1μl), forward and reverse primer 70 pmol/μl (1.5 μl), Genomic DNA 25 ng/μl (5μl), Taq DNA polymerase (5U/μl) (0.25μl) and DNAase free water (9.25 μl) for both regions of LEP gene. PCR programming for amplification of both exons of LEP gene was as following:-

- **First cycle-Initial Denaturation (95°C, 4min., 1 cycle),**
- **Second cycle- (35 cycles)**
  - Denaturation (95°C, 5 min.)
  - Annealing (59°C for exon-2 and 60°C for exon 3)
  - Synthesis (72°C, 1min),
- **Final extension (72°C, 10 min, 1cycle),**
  - Hold (4°C, 5 min., 1 cycle)

The quality and size of the PCR amplicons for different studied locus were assessed on 1.5% agarose gel containing ethidium bromide (1% solution) by electrophoresis method. The genetic variation in the selected genomic regions of LEP gene was identified through SSCP methods. SSCP analysis (Zhang et al., 2007) is considered as a more economic method for the initial screening of a large number of samples to detect polymorphism in...
sequences of amplified fragments. In our study, for SSCP analysis took aliquots of 5μL PCR products mixed with 5μL denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene cyanole and 0.025% bromophenol blue), denatured for 10 min at 95°C followed by a rapid chill on ice for 10 min. Denatured PCR products were subjected to 8% polyacrylamide gel electrophoresis in Tris-Borate-EDTA buffer and constant voltage (120 V) for 15 h at a constant temperature of 4°C, and then gels were stained with 1% ethidium bromide solution and visualized with under UV light and documented by gel documentation system. Individual genotypes were defined according to band patterns. The frequencies of different electrophoretic patterns were recorded under each group.

The genetic structure of the studied population at two locations (LRS, Nohar and Bikaner) for gene and genotypic frequencies, observed heterozygosity (Ho), expected heterozygosity (HE) and expected unbiased heterozygosity (HE unbiased), an effective number of alleles and Nei’s genetic distances were analyzed through POPGENE program (version 3.1) of Yeh (1997).

**Results and Discussion**

**Variation in regions of LEP gene**

One type of SSCP band pattern (‘P1’) was observed for the 326-bp fragment of exon-2 of the LEP gene in all analyzed samples of Rathi cattle and is suggestive of the single allelic nature of this region. Thus monomorphism observed in the 326-bp fragments of the LEP gene in Rathi cattle was revealed through the SSCP method (Fig. 1).

In Pasundan cattle (49), Hil mia et al., (2019) investigated SNP on exon 2 of the leptin gene. They observed three alleles- A (31.64%), C (42.86%), and T (25.50%), with six different genotypic patterns –CC (24.49%), CT (32.65%), CA (4.08%), TT (8.16%), TA (2.05 %) and AA (28.57%). In Japanese black cattle, Aierqing et al., (2020) reported three genotypes-CC, CT, TT and two types of allele-C, T with frequencies 0.55, 0.30, 0.15, 0.71 and 0.29, respectively. Haruna et al., (2020) analyzed many breeds of cattle by PCR-SSCP method to detect SNPs in three regions of the leptin gene.

In Rathi cattle, through SSCP analysis was reported the presence of sequence variation in ‘A’ allele of LEP gene exon-3 locus and is suggestive of multiple allelic nature of this region. Thus polymorphism contained in the 454-bp fragments of the LEP gene in Rathi cattle was revealed more clearly through the SSCP method. The genotypic frequencies of different patterns by SSCP have been represented in Table 2. Two SSCP band patterns (‘P1’ and ‘P2’) were observed for the 454-bp fragment of exon-3 of the LEP gene in Rathi cattle (Fig. 2). Rambachan et al., (2019) also observed polymorphic nature in exon 3 of leptin gene in Hariana cows.

**Gene and genotypic frequency of exon-3 of LEP gene**

The genetic structure of different breeds in terms of gene and genotypic frequency for exon-3 of the LEP gene as detected through the SSCP marker are presented in Table 2.

Dubey et al., (2008) investigated polymorphism in 202 Sahiwal cattle by PCR-SSCP method and indicates high genetic variability in the entire leptin gene. Aslaminejad et al., (2010) analyzed the genetic variability of exon 3 of the leptin gene in four indigenous cattle of Iran (Golpayegani, Najdi, Sarabi and Sistani). They found two alleles and three genotypes in all four cattle breeds. Yadav et al., (2020)
analyzed the genomic region of the leptin gene and found an association of production traits with all three genotypic patterns - AA, AG and GG in Hardhenu cattle.

**Table 1** Primer sequences and expected fragment sizes of PCR products of selected genomic regions

| Selected Region | Primer Sequences | GenBank Accession No. | Expected Fragment Length | References |
|-----------------|-------------------|-----------------------|--------------------------|------------|
| *LEP* Exon 2    | Forward 5’-TGGCAGACAGCAATCTTGT-3’ | JQ711179.1 | 326 | Ranjan *et al.*, (2011) |
|                 | Reverse 5’-CCACGGTTCTACCTCGTCTC-3’ |            |      |                        |
| *LEP* Exon 3    | Forward 5’-GGGAAGGGCAGAAAGATAG-3’ | JQ711179.1 | 454 | Kumar *et al.*, (2018) |
|                 | Reverse 5’-CCAAGCTCTCTCAGCTC-3’ |            |      |                        |

**Table 2** Gene and genotypic frequencies of exon 2 of *LEP* gene detected through SSCP analysis

| Group | N | Genotypic Pattern | Gene frequency |
|-------|---|-------------------|----------------|
|       |   | P1                | P2 A           | B             |
| G1    | 80 | 0.788 (63)        | 0.212 (17)     | 0.895         | 0.105 |
| G2    | 80 | 0.875 (70)        | 0.125(10)      | 0.937         | 0.063 |
| Overall | 160 | 0.832 (133)      | 0.168 (27)     | 0.915         | 0.085 |

Note: G1=LRS, Nohar; G2 =LRS, Bikaner; Number in parenthesis are number of observations

**Table 3** Hardy-Weinberg equilibrium for exon-3 of *LEP* gene

| Group | N | Chi2 | p value | Significance | G2 | p value | Significance |
|-------|---|------|---------|--------------|----|---------|--------------|
| G1    | 80 | 0.318| 0.572   | NS           | 0.600 | 0.438 | NS           |
| G2    | 80 | 1.058| 0.303   | NS           | 1.906 | 0.167 | NS           |
| Overall | 160 | 1.304 | 0.253   | NS           | 2.399 | 0.121 | NS           |

Note: G1=LRS, Nohar; G2 =LRS, Bikaner; N= number of samples, * = significant (p≤0.05), NS= non –significant

**Table 4** Within-population heterozygosity estimates, PIC and FIS values of Rathi cattle for exon-3 of *LEP* gene

| Group | Sample size (N) | Observed Heterozygosity (Ho) | Expected Heterozygosity (He) | Nei’s unbiased Heterozygosity (Hu) | PIC | Fixation index (Fis) |
|-------|-----------------|-----------------------------|-----------------------------|-----------------------------------|-----|----------------------|
| G1    | 80              | 0.1250                      | 0.1179                      | 0.1172                            | 0.1536 | -0.0667              |
| G2    | 80              | 0.2125                      | 0.1911                      | 0.1899                            | 0.1536 | -0.1189              |
| Overall | 160         | 0.1688                      | 0.1550                      | 0.1545                            | 0.1536 | -0.0922              |

Note: G1=LRS, Nohar; G2 =LRS, Bikaner
Both the Chi-square and G square test values observed for the LEP exon 3 gene in both studied populations of Rathi cattle revealed non-significant deviation from Hardy-Weinberg equilibrium (p 0.05) which indicates that animals similar in their genotypic distribution concerning gene frequency (Table 3). The Shannon index value indicates heterozygosity in the studied population. For exon-3 of the LEP gene was 0.2338 and 0.3386 (Table 4). This indicates the low to intermediate heterozygosity.

The present study concluded the polymorphic nature of exon 2 and exon 3 of the LEP gene. Monomorphism was observed in exon2 of leptin gene while exon 3 of leptin gene showed polymorphism in studied animals of Rathi cattle.

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