Original Research Article

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

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ABSTRACT

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In present examination, Rathu cows were chosen to identified leptin gene qualities for polymorphism. Complete 160 lactating Rathu 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 Rathu 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). Rathu has a breeding tract in Hanumangarh,

Loonkaransar tehsil of Bikaner, Shriganganagar districts of the state (Anonymous, 2018). Rathi cattle contribute 0.83 percentage to the total population of indigenous cattle in India (Breed Survey, 2013). Rathi 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 Rathi animals 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 (H_o), expected heterozygosity (H_e) and expected unbiased heterozygosity (H_e 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 Rathu 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 Rathu cattle was revealed through the SSCP method (Fig. 1).

In Pasundan cattle (49), Hilmi *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 Rathu 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 Rathu 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 Rathu cattle (Fig. 2). Rambachan *et al.*, (2019) also observed polymorphic nature in exon 3 of leptin gene in Haryana 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'-TGGCAGACAGCAAATCTTGT-3'	JQ711179.1	326	Ranjan <i>et al.</i> , (2011)
	Reverse 5'-CCACGGTTCTACCTCGTCTC-3'			
LEP Exon 3	Forward 5'-GGGAAGGGCAGAAAGATAG-3'	JQ711179.1	454	Kumar <i>et al.</i> , (2018)
	Reverse 5'-CCAAGCTCTCTCCAAGCTCTC-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

Exon-3 region of <i>LEP</i> 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 ($p \leq 0.05$), NS= non –significant

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

Group	Sample size (N)	Observed Heterozygosity (H_o)	Expected Heterozygosity (H_e)	Nei's unbiased Heterozygosity (H_e)	PIC	Fixation index (F_{IS})
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; G 2 =LRS, Bikaner

Fig.1 PCR-SSCP patterns of exon 2 of *LEP* gene in Rathu cattle

Lane 1-7: AA genotype

Fig.2 PCR-SSCP of exon 3 of *LEP* gene in Rathu cattle

Lane 1-6: AA genotype
7: AB genotype

Both the Chi-square and G square test values observed for the *LEP* exon 3 gene in both studied populations of Rathu 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 Rathu cattle.

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