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Characterization of Leptin (*LEP*) gene in Indian dromedary camel (*Camelus dromedarius*)

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ABSTRACT

Information on the camel leptin gene when compared to other domestic livestock species is very limited. Hence, the present study was undertaken to characterize the leptin gene of the Indian dromedary camel (*Camelus dromedarius*). Five overlapping primer pairs were designed utilizing the available *Lama Glama* leptin gene sequence. Through the assembly, alignment, and editing of the five overlapping nucleotide sequences, a 2425 bp long leptin gene sequence (Accession no. MT103545.1) of dromedary camel covering partial exon-2, intron-2, exon-3, and partial-3' UTR region was characterized. The study successfully amplified the leptin gene, including intron-2 and 3'UTR of camel leptin gene for the first time using genomic DNA samples. The Indian dromedary camel leptin gene at the nucleotide level shared more than 97% similarity with llama and more than 80% similarity with major domesticated species like cattle, buffalo, sheep, and goat. The evolutionary relationship study based on amino acid sequences showed a close relationship between dromedary and bactrian camel species followed by llama. The high level of similarity between camel leptin gene amino acid sequence and other species showed the conserved nature of leptin protein.

Keywords: Bikaneri camel, Characterization, Leptin gene, Phylogeny

Camel is an integral part of the desert ecosystem and has unique bio-physiological characteristics. The camel is considered a draught species but is also used for racing, desert safari, milk, meat, leather, and hair fibre production. Camel milk is gaining importance these days because of its therapeutic properties in many diseases. The Bikaneri breed of camel is the heaviest breed among nine breeds of Indian dromedary camel. The specific characteristics of the Bikaneri breed is dark brown coat colour with dense hair growth over eyes and ears commonly referred to as *Zipra*.

Despite its enormous economic, cultural, and biological importance, the camel genome has not been widely studied (Ali *et al*. 2019). This can be realized by the fact that there are more than four lakhs nucleotide sequence entries for cattle and only a few known sequences for camels. The genetic improvement in the camel for different production and reproduction traits is very slow due to its large generation interval, seasonal breeding pattern, etc. Profitable economic return from camel can be possible by increasing its productivity. The molecular markers are useful to define the genetic makeup (genotype) and predict the performance of an animal.

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The leptin (*LEP*) gene is a candidate gene with known physiological effects. Leptin protein is synthesized by adipose tissue and is involved in the regulation of feed intake, energy balance, growth, fertility, production traits, and immune functions. Leptin is a product of the LEP gene which is a 16-kDa protein hormone related to the class-1 helical cytokine family of proteins (Akumbugu and Zanwa 2017). Leptin inhibits feed intake, decreases fat tissue deposition, and is negatively related to the quantity of non-esterified fatty acids, which reflects the amount of fat mobilization (Ashok 2008). Leptin also affects the immune system, reproductive efficiency, and growth (Fruhbeck *et al.* 1998). Leptin mediates miscellaneous physiological functions such as hematopoiesis, renal functions, and angiogenesis. Different studies stated that the food consumption of animals is regulated by the body fat content which led to the discovery of Leptin (Hossner 1998). Leptin is primarily synthesized by adipose tissues of the body (Kulig *et al*. 2009).

The leptin gene is comprised of three exons and two introns of which only exons 2 and 3 are translated into protein. The sequence pattern of the leptin gene is known for many livestock species. However, scanty information about the sequence structure of the camel leptin gene is available. In the NCBI nucleotide database repository, only a few entries are available for the camel leptin gene of which the majority of the nucleotide data is putative transcript variants of the leptin gene derived by automated

computational analysis using gene prediction methods. Other GenBank accession for camel leptin gene nucleotide sequences are RNA-derived sequences having accession no. AY247404.1 and JQ359756.1 and genomic DNA derived unverified sequence with accession no. KC571191.1. Considering the limited information available for the camel leptin gene, the objective of this study was to characterize the leptin gene of Indian dromedary camel.

MATERIALS AND METHODS

Animals and blood samples: To characterize the leptin gene three Bikaneri breed camel were randomly selected from camel herd of ICAR-National Research Centre on Camel, Bikaner, Rajasthan, India, and about 10 ml of blood were collected aseptically through the jugular vein and stored at -20°C till isolation of genomic DNA. Approval of the Institutional Animal Ethics Committee (IAEC) was taken for the collection of blood from selected animals.

DNA extraction: Genomic DNA was isolated from the blood samples by the 'Phenol: Chloroform extraction' method as described by Sambrook and Russel (2001) with slight modification. The integrity and the quality of extracted DNA was checked on 0.8% agarose under horizontal gel electrophoresis. The concentration and purity of DNA were estimated through a spectrophotometer (Bio-Rad, USA). Only the DNA samples having a concentration greater than 50 ng/µl with an optical density of 1.6-1.8 were used further for amplification of desired DNA fragment by Polymerase Chain Reaction (PCR).

Designing of primers: The leptin gene sequence of *Lama glama* available online in the GenBank (www.ncbi.nlm.nih.gov) (Acc. No KC295539.1) was used for designing the primers. Five pairs of overlapping primers were designed. Designing of primers was done with the help of Primer 3 software (Untergasser *et al.* 2012). The genomic region coverage of five overlapping primers is depicted in Fig.1. The sequence of primers and expected fragment length of the different selected regions are presented in (Table 1).

Amplification of camel leptin gene from genomic DNA: The gradient PCR program was used to find out the appropriate annealing temperature. The appropriate annealing temperature identified for each fragment was further used for the amplification of the samples. The

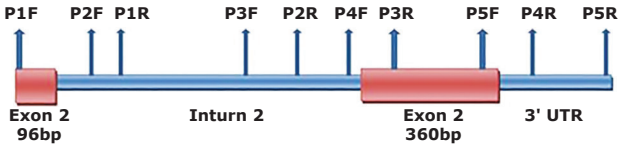

Fig. 1. Genomic region covered by five pairs of overlapping leptin primers.

reactions were carried out in a programmable gradient thermocycler (Applied Biosystems, USA) using GoTaq® Green Master Mix (Promega, USA). The PCR reaction conditions for 5 amplified amplicons are presented in Table 2. Except for annealing temperature same thermal cycling profile was used for all five amplicons, which consisted of an initial denaturation at 95°C for 5 min, 34 cycles of denaturation at 94°C for 1 min, annealing for 45 s, and extension at 72°C for 45 s followed by a final extension at 72°C for 10 min and hold at 4°C for 5 min. The quality and size of the PCR amplicons for different studied leptin gene regions were assessed on 2% agarose gel (Himedia Laboratories, India), containing ethidium bromide (1% solution) in parallel with 100 bp DNA marker (Thermo Fisher Scientific, USA).

Sequencing and evolutionary analysis: After purification of the amplified PCR products bidirectional sequencing using forward and reverse primers was done by the Sanger Dideoxy Chain termination method (Eurofins Genomics, India). The forward and reverse sequences obtained for each fragment were edited using Codon Code Aligner software (Codon Code Corporation, USA) and contigs were prepared for different amplified regions. These contigs were assembled and edited to deduce the desired camel leptin genomic sequence. To study the variation in camel leptin gene nucleotide and amino acid sequence and their relationship with different leptin gene sequences available at the National Center for Biotechnology Information (NCBI) database, multiple sequence alignment of the whole nucleotide sequence and deduced amino acid was done using a BLAST software program (http://www.ncbi.nlm.nih.gov/). BLAST search was applied with those nucleotide sequences which covered more than 70% of our sequence (Accession No. MT103545.1). The evolutionary relationship between different species was inferred by the neighbor-joining method using Molecular Evolutionary Genetics Analysis software, MEGA X (Kumar *et al.*

Table 1. Primer sequences used for amplification of camel leptin gene

Primer	Primer sequences (5' to 3')	Amplicon size	Region covered
P1	P1F-TATGTTGAAGCCGTGCCCAT P1R- GAGTTCAGCTCCAACCACGA	441 bp	Partial exon-2 and partial intron-2
P2	P2F-CGTGGTTGGAGCTGAACTCT P2R-AGGTCCTCCATAAGGTCCCC	1071 bp	Partial intron-2
P3	P3F-TGGATCCAGAGTGTGCCAAC P3R-TAGAGGGAGGCTTCCAGGAC	844 bp	Partial intron-2 and partial-exon-3
P4	P4F-TGATGTGGAAGGGAGAAAGG P4R-AGACACCTGGAAGCTCAGGA	540 bp	Partial intron-2, exon-3, and partial 3' UTR
P5	P5F-CCTGGAAGCCTCCCTCTACT P5R-CCAGATCTAGTCCGCTGCTG	342 bp	Partial exon-3 and partial 3'UTR

2018). The evolutionary distances were computed using the Poisson correction method (Kumar *et al.* 2018) and are shown in the units of the number of amino acid substitutions per site.

RESULTS AND DISCUSSION

The optimal annealing temperature for amplification of the different leptin gene fragments of the dromedary camel ranged from 53 to 56ºC (Table 2). The clear single DNA bands were observed when the PCR products of different leptin gene fragments were checked for amplification by electrophoresis on 2% agarose gel in parallel with 100 bp DNA marker (Fig. 2 and 3). After bidirectional sequencing of PCR products of different amplified fragments and their editing, five different contigs of 441, 1071, 840, 544, and 342 bp length were confirmed. The five contigs were aligned and after removal of the overlapping segments and joining of the different fragments a contiguous camel leptin gene sequence of 2425 bp was obtained. The 2425 bp leptin gene sequence covered 93 bp of exon-2 (nucleotide position 1 to 93), 1733 bp of intron-2 (nucleotide position 94 to 1826) and 599 bp of exon-3 (nucleotide position 1827 to 2425). The 599 bp of exon-3 comprised 360 bp of the coding region and 239 bp of 3' UTR. First-time complete camel intron-2 and partial 3' UTR region were sequenced for dromedary camel. The UTRs are known to play crucial roles in the post-transcriptional regulation of gene expression and their role in regulating gene expression has been underlined by the finding that mutations that alter the UTR lead to serious pathology (Conne *et al.* 2000). The nucleotide sequence was submitted to the GenBank database with accession no. MT103545.1. The nucleotide sequence of exon-1 of the gene is not translated and was not covered in the present study. The amplified partial exon-2 and complete coding region of exon-3 translated into 150 amino acids sequence in this study.

Table 2. PCR reaction composition (25 µl reaction volume) for different leptin primer sets

DNA (50 ng conc.) (µl)	GoTaq® Green Master Mix (µl)	Primers (each) (µl)	Nuclease free water (µl)	Annealing temperature (ºC)	Primer
2.0 µl	13 µl	1.5 µl	7.0 µl	55.0	P1
2.5 µl	13 µl	1.5 µl	6.5 µl	55.5	P2
2.2 µl	13 µl	1.3 µl	7.2 µl	53.0	P3
2.0 µl	13 µl	1.5 µl	7.0 µl	56.0	P4
2.3 µl	13 µl	1.5 µl	6.7 µl	54.0	P5

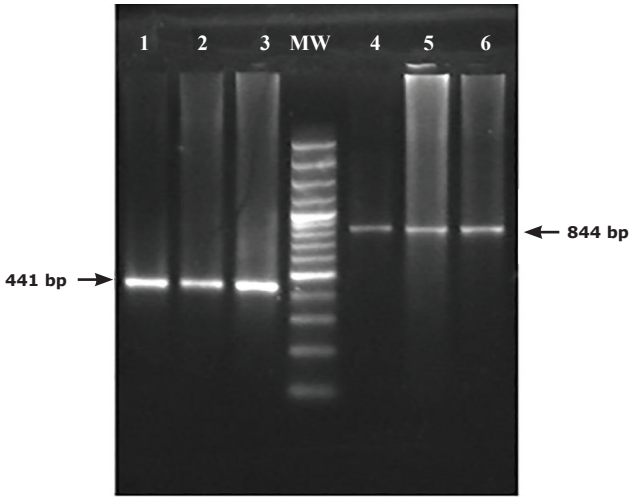

Fig. 2. Amplification of camel leptin gene, Partial exon-2 and partial intron-2 (441 bp, lane 1-3) and Partial intron-2 and partial-exon-3 (844 bp, lane 4-6). MW, Molecular weight marker (100 bp).

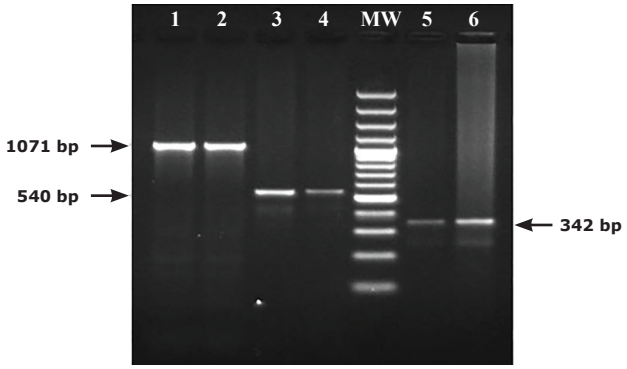

Fig. 3. Amplification of Partial intron-2, exon-3, and partial 3' UTR (540 bp, lane 3-4), partial intron-2 (1071 bp, lane 1-2) and partial exon-3 and partial 3'UTR (342 bp, lane 5-6) region. MW, Molecular weight marker (100 bp).

The BLAST search showed that at the nucleotide level, camel leptin gene shared more than 97% similarity with llama and more than 80% similarity with other domesticated species like cattle, buffalo, sheep, and goat (Table 3). The differences observed in nucleotide sequences of different species were due to transition, transversion, insertion, and deletion (Table 3). The amino acid similarity was highest

Table 3. Nucleotide sequence similarity of Indian dromedary leptin gene (MT103545.1)

Accession No.	Scientific name (Common name)	Per cent identity	Mismatches	Gap opens
KC295539.1	*Lama glama* (llama)	97.073	67	4
JN833619.1	*Delphinapterus leucas* (Beluga whale)	82.714	159	53
JQ739233.1	*Capra hircus* (Goat)	81.981	160	29
HE605296.1	*Ovis aries* (Sheep)	81.944	159	26
EU642566.1	*Bos frontalis* (Gayal)	81.107	219	40
EU603265.1	*Bos grunniens* (Domestic yak)	80.965	222	40
JQ711179.1	*Bos taurus* (Cattle)	80.965	225	37
HE605297.1	*Bubalus bubalis* (Buffalo)	80.940	227	39
EU313203.1	*Bos indicus* (Zebu cattle)	80.900	223	40

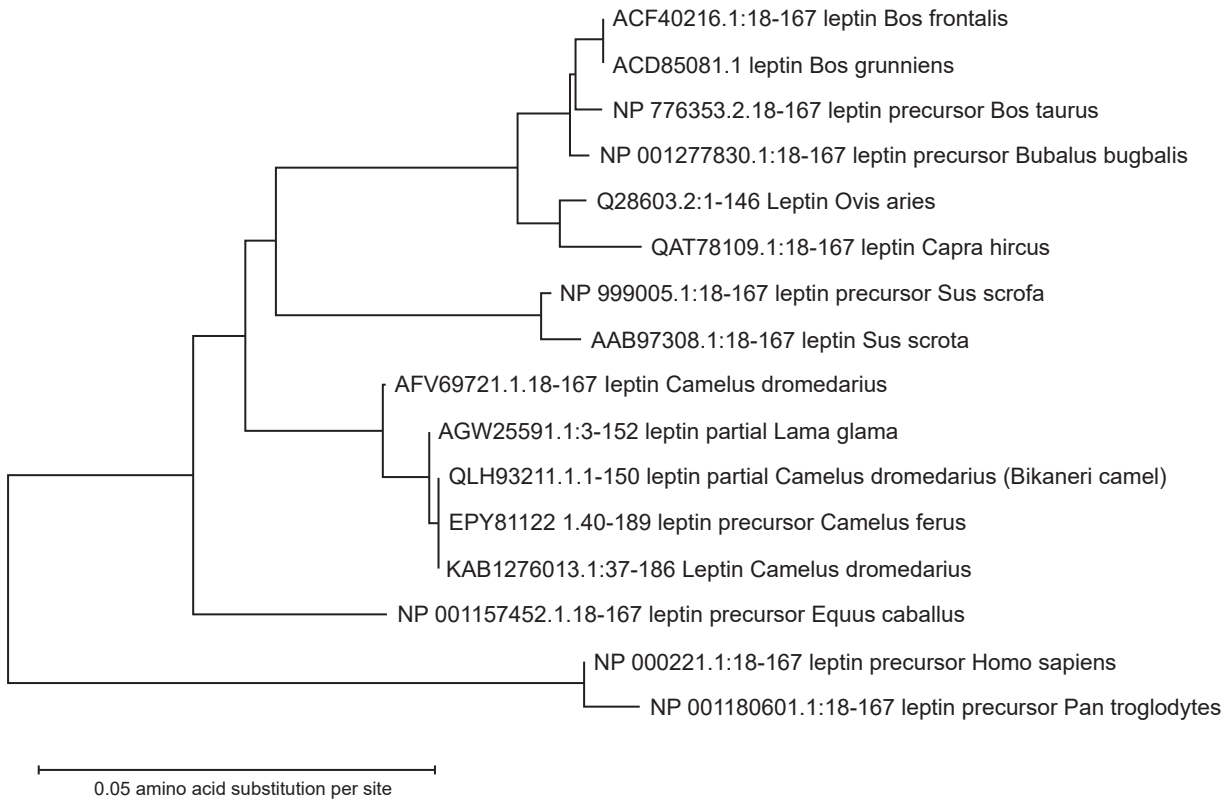

Fig. 4. Phylogenetic tree to analyse the evolutionary relationships of camel leptin gene.

with camel (99.33 to 100%), followed by llama (99.33%), pig (94%), cattle (93.33%), buffalo (92.67%), goat (93.15%), sheep (90%) and human (88%). Premraj *et al.* (2015) reported that the camel leptin gene amino acid has 97.6%, 96.4%, 97.0%, 91.6%, and 91% similarity with the porcine, equine, bovine, human, and murine orthologues. The proteins with high sequence identity and high structural similarity tend to possess functional similarity and evolutionary relationships.

The evolutionary relationship of camel leptin gene amino acid sequence was inferred using the Neighbor-Joining method. A total of 16 leptin amino acid sequences including camel leptin amino acid sequence (QLH93211.1) with 150 amino acids in each data set were analyzed in the present study. The sum of the branch length of the optimal phylogeny tree (Fig. 4) was 0.269. The evolutionary relationship between amino acid sequences showed a close relationship between dromedary and bactrian camel species followed by llama. The domesticated species like pig, goat, sheep buffalo, cattle, yak and mithun were distantly related to camel.

Thus, the present study successfully amplified the camel leptin gene, including maiden amplification of intron-2 and 3'UTR of the camel leptin gene. The Indian dromedary leptin gene shared greater than 80% similarity at the nucleotide level with other domestic species. The sequence differences (mismatches) observed were mostly in the intronic region. The high level of similarity between the leptin gene amino acid sequence of the dromedary camel and other species showed the conserved nature of leptin protein.

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