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Studies on growth hormone gene polymorphism in EXON-5 and 3' UTR in Nellore sheep through PCR-SSCP

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Abstract
Sheep are one of the most essential livestock species for resource-poor farmers in rural areas of arid and semi-arid climates, especially where crop and dairy farming are not economically viable. Polymorphisms in the sheep GH gene have been studied to better understand the population's genetic diversity and its utility in improving economic features. If this polymorphism is linked to economic qualities, it can be used as a foundation for improving animal output through selection. A total of about 50 unrelated animals of Nellore brown sheep belonging to different flocks including, Livestock Research Station (LRS), Mamnoor, Instructional Livestock Farm Complex (ILFC), Rajendranagar, Hyderabad and farmers flocks from Shamshabad region of Ranga Reddy district were screened to explore the polymorphism of exon-5 and 3' UTR of growth hormone (GH) gene using PCR-SSCP technique. The PCR-SSCP yielded two conformational patterns 11 and 12 in exon-5 (480 bp) corresponding two alleles 1 and 2. The PCR-SSCP of 3' UTR (320 bp) yielded two SSCP patterns 12 and 13 corresponding to three allelic variants 1, 2 and 3. No significant differences were observed between different SSCP genotypes for the morphometric and body weights at 12 months age.

Keywords: growth hormone gene, morphometric traits, Nellore sheep, polymorphism, PCR-SSCP

Introduction
Nellore sheep is a popular and tallest mutton breed found in the northern Andhra Pradesh.
It can withstand harsh climate conditions and is known for disease resistance and heat tolerance. Growth is influenced by growth hormone (GH), which is a 191-amino acid, single polypeptide chain that is synthesized, stored, and secreted by somatotropic cells within the lateral wings of the anterior pituitary in vertebrates. This hormone is regulated by the neurosecretory nuclei of the hypothalamus. Being an anabolic hormone, promotes body growth by enhancing muscle growth, bones, and organs in the body. The ovine GH (oGH) gene has been mapped to chromosome 11 (11q25). The GH gene has a direct effect on the synthesis and secretion of growth hormone and plays an important role in animal growth. The GH gene spans 2.6 to 3.0 kbp in most mammals and comprises a 5' regulatory region, five exons, four introns, and a 3' untranslated region (UTR).
Single-Strand Conformation Polymorphism (SSCP) is the simplest and most sensitive and appropriate method for mutation detection in the amplified DNA fragments (Orita *et al.* 1989) [7]. PCR-SSCP analysis is a valuable tool for the establishment of allelic variants in the genes. The present study was carried out to decipher the Single-Strand Conformation Polymorphism (SSCP) in exon-5 and 3' UTR of GH gene and its association with body weight and morphometric traits i.e body length (BL), height at withers (HAW) and heart girth (HG).

Materials and Methods
Blood samples were collected from 50 Nellore sheep breed which were maintained at Livestock Research Station (LRS), Mamnoor, Instructional Livestock Farm Complex (ILFC), Rajendranagar, Hyderabad and farmers flocks from Shamshabad region of Ranga Reddy district.
The DNA was isolated from blood through a standard phenol chloroform method. Quality of the DNA was checked by running the sample in 0.8% agarose gel and quantity by spectrophotometry. The DNA was amplified by using different set of primers as presented in table.1 and PCR master mix composition given in table 2.

Table 1: Description of primers used and the amplified product of different loci

S.no	Region	Primer sequence	Amplified product	Authors
1	Exon-5	F: 5' AGCAGAGTCTTCACCAACAGC 3' R: 5' TAGTTCTTGAGCAGCGCATC 3'	480 bp	Jia *et al*. (2014) [4]
2	3'UTR	F: 5' TGGCAGGAGCTGGAAGATGT 3 'R: 5' CCTACTCAGACAATGTGATGCAA 3'	320 BP	Jia *et al*. (2014) [4]

F: Forward, R: Reversed

Table 2: Composition of PCR reaction mix for amplification

S. No	Components	Volume	Final concentration
1	10x Taq buffer	1.25 µl	1 X
2	dNTPs (10mM)	0.5 µl	0.4 mM
3	Primer-Forward (50 pM)	1 µl	10 pM
4	Primer-Reverse (50 pM)	1 µl	10 pM
5	$MgCl_2$(25 mM)	0.5 µl	1 mM
6	Taq Polymerase(1unit/µl)	0.5 µl	0.5 unit
7	Autoclaved Mille Q water	6.75 µl	

An aliquot of 11.5 µl of master mix per sample was drawn into thin-walled PCR tubes and 1 µl (50-100 ng) of template DNA was added for making 12.5 µl of PCR mix. The PCR tubes were marked for the identification, then spinned briefly for proper mixing and mounted in PCR machine. The PCR reaction condition for each exon is varies and presented in table.3 and table.4

Table 3: PCR reaction conditions of Exon-5

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	5 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	62.9	30 sec
4	Cyclic extension	72	30 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	10min
7	Hold	4	Forever

Table 4: PCR reaction conditions of 3' UTR

Step	Process	Temperature (°C)	Time
1	Initial denaturation	94	5 min
2	Cyclic denaturation	94	30 sec
3	Primer annealing	62.9	30 sec
4	Cyclic extension	72	30 sec
5	Steps 2 to 4 were repeated for 36 cycles		
6	Final extension	72	10min
7	Hold	4	Forever

The PCR products were confirmed by agarose gel electrophoresis and visualized in gel documentation system. For SSCP, 5 µl of PCR product was mixed with 12 µl formamide dye in a 0.2 ml PCR tube. This mixture was denatured at 95 °C for 5 min in hot water and snap cooled on ice for 15-20 min. The product was loaded in the PAGE and electrophoresis performed at 4 °C for 10-12 h at 110 V. The gel was silver stained to visualize the specified banding patterns. The most common band pattern identified was named as 1. If there are more bands, in addition to the common bands, they were marked as 2, 3, 4 etc., depending on the band pattern.

Univariate GLM model of SPSS 20 was used to study the association of each genotype on body weight and different morphometric traits with the statistical model:

$$Yij = \mu+Gi+Sj+eij$$

Yij = dependent variable µ = overall population mean, Gi = fixed effect of genotype, Sj = fixed effect of sex, eij = random error. Significant differences between least square means of different genotypes were tested by Duncan's method (DMRT). Values were considered significant at $P \leq 0.05$ and presented as least square means ± standard errors.

Results and Discussion

A 480 bp of exon-5 (Fig.1) of growth hormone gene was amplified and was subjected to SSCP. Two different SSCP patterns 11 and 12 (Fig.3) were observed corresponding to two allelic variants 1 and 2. Among 50 samples, thirty five samples showed pattern 11 and fifteen samples showed pattern 12. Similar SSCP patterns (two) for Exon 5 of the GH gene were reported by Farag *et al.* (2016) [3] in two pure breeds (Barki and Rahmani) and one crossbred (Rahmanix Awase) sheep. Several authors like Tahmoorespur *et al.* (2011) [8] in Baluchi sheep,

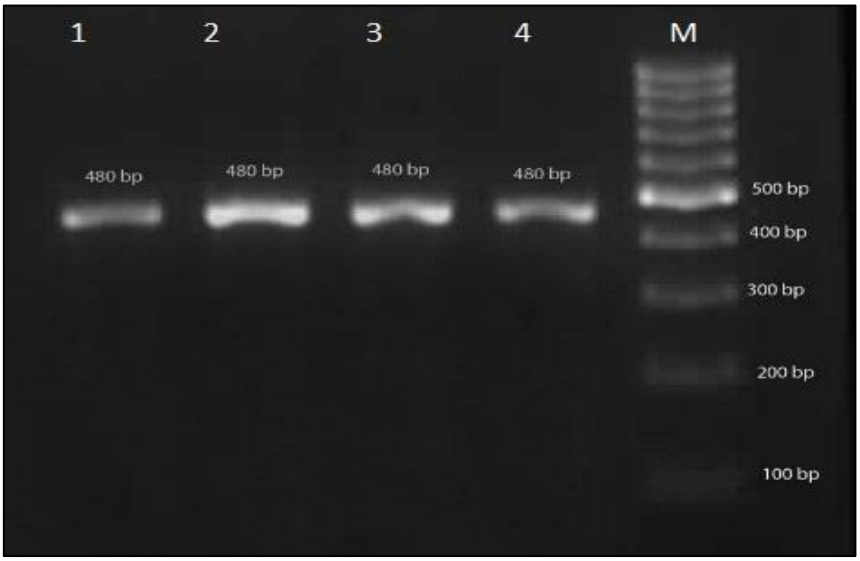

Fig 1: Exon-5(480bp) PCR amplified product of GH gene Lane 1-4: PCR prodcut, Lane M: 100 bp ladder

Azari *et al.* (2011) [1] in Dalagh sheep and Yousefi and Azari (2012) [10] in Zel sheep found three conformational patterns, while Marques *et al.* (2006) [6] observed five different conformational patterns in "Serra da Estrela sheep" in exon-5.

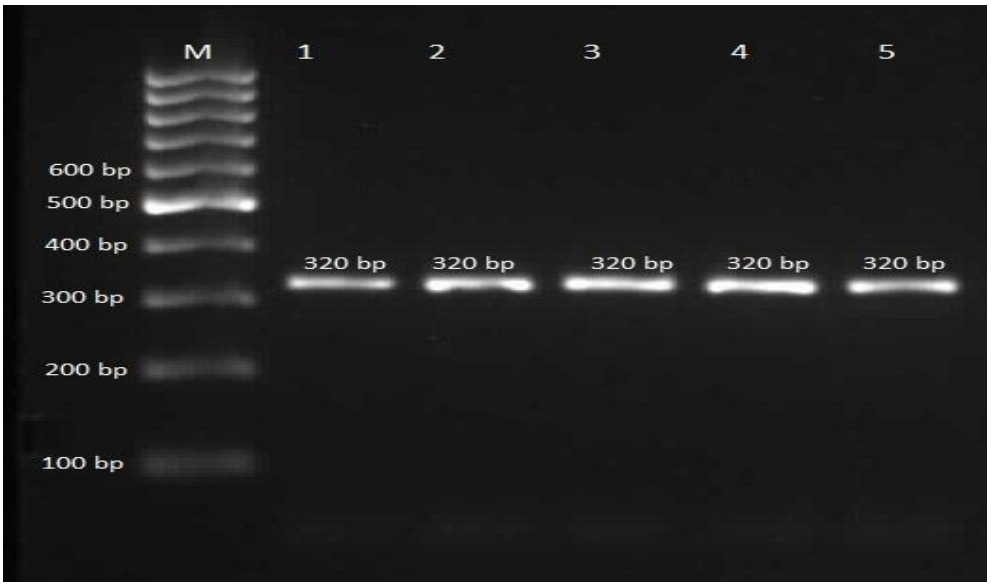

Fig 2: 3' UTR (320bp) PCR amplified product of GH gene Lane 1-4: PCR product, Lane M: 100 bp ladder

A 320 bp (Fig. 2) 3' UTR of growth hormone gene was amplified and 5 µl of PCR product was used for SSCP. The SSCP yielded two different patterns 12 and 13 (Fig. 4) corresponding to three allelic variants 1, 2 and 3. Among 50 samples, thirty one samples showed pattern 12 and nineteen samples showed pattern 13. In contrary to these findings Jia *et al*. (2014) [4] and Marques *et al.* (2006) [6] found four conformational patterns in four sheep breeds (Small Tail Han, Tibetan, German Merino and Polled Dorset sheep) of China. Bahrami *et al.* (2015) [2] reported five genotypes in the gene portion covering exon-5 and part of 3' UTR of this gene in Mehraban sheep, while Wicramaratne *et al*. (2010) [9] reported six distinct patterns in Osmanabadi and four patterns in Sangamneri goats. The observed over all mean body weight (BW), body length (BL), height at withers (HAW) and heart girth (HG) in Nellore sheep were 25.93 ± 0.20 kgs, 71.46 ± 0.43 cm, 74.17 ± 0.17 cm and 75.70 ± 0.53 cm respectively.

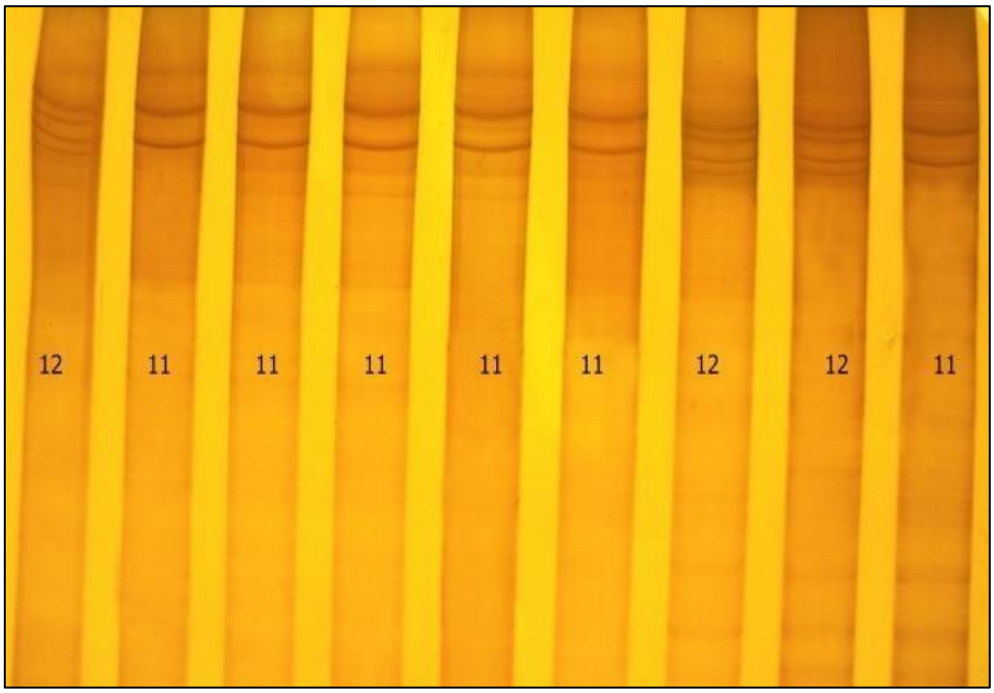

Fig 3: PAGE showing SSCP patterns in exon-5 (480 bp) of GH gene

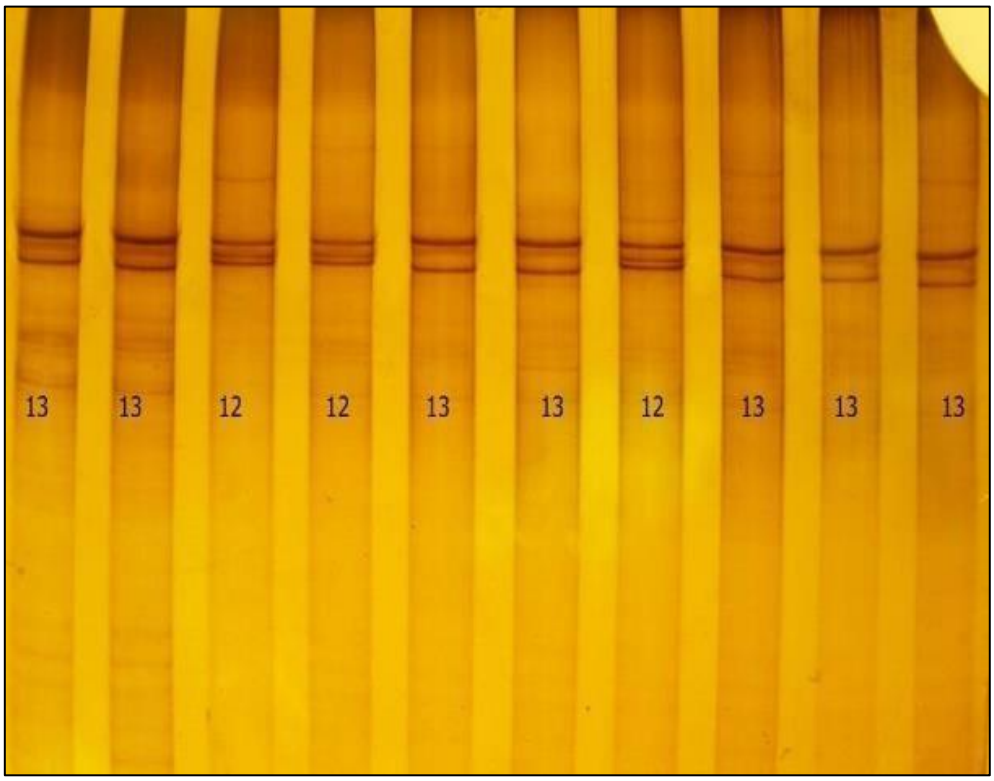

Fig 4: PAGE showing SSCP patterns in 3' UTR (320 bp) of GH gene

The SSCP patterns of gene segments, exon 5 and 3' UTR of GH gene were associated with body weight and other morphometric traits, analyzed and tabulated in tables 5 and 6 respectively.

Table 5: Association of SSCP patterns of exon-5 with BW, BL, HAW and HG at 12 months age

Pattern	Body weight (kg)	Body length (cm)	Height at withers (cm)	Heart girth (cm)	No of animals
11	26.11±0.27	71.68±0.53	74.24±0.21	75.52±0.67	35
12	25.50±0.25	70.94±0.74	74.00±0.30	76.13±0.89	15

Table 6: Association of SSCP patterns of 3' UTR with BW, BL, HAW and HG at 12 months age

Pattern	Body weight (kg)	Body length (cm)	Height at withers (cm)	Heart girth (cm)	No of animals
11	25.75±0.36	71.16±0.61	74.24±0.23	75.10±0.63	31
13	26.22±0.56	74.53±0.53	74.06±0.27	76.69±0.93	19

In case of SSCP in exon 5, Lan *et al.* (2007) [5] study in exon-5 reported little influence of different patterns of growth hormone gene on weight in one-year-old animals in 5 indigenous sheep breeds, Tahmoorespur *et al.* (2011) [8] studied growth hormone gene polymorphism in exon-5 and reported a significant effect of polymorphism on 6 months body weight. However, Yousefi and Azari (2012) investigated the association between SSCP genotypes of exon-5 of GH gene and body weights in Zel sheep. The statistical analysis revealed no significant differences between the genotypes studied.

In case of SSCP in 3' UTR, Jia *et al.* (2014) [4] observed statistically significant differences in the scores of body weight, body length, wither height and heart girth in Polled Dorset and Tibetan sheep and non- significant differences in Small Tail Han and German Merino sheep among the SSCP patterns. As discussed above, the differences were observed between the SSCP variants in the different regions of GH gene in body weight, body length, height at withers and heart girth.

But these differences were not found to be statistically significant ($P \leq 0.05$).

Conclusion

The present investigation to decipher the genetic polymorphism of growth hormone gene revealed limited polymorphism. Further studies with large samples need to be carried out to elucidate allelic variants of the gene for marker assisted selection for body weights and other morphometric traits. No significant differences among SSCP genotypes for body weights and other morphometric traits at 12 months age were noticed.

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