MOLECULAR GENETIC CHARACTERIZATION OF CERTAIN GROWTH RELATED GENES AND ANALYSE THEIR ASSOCIATION WITH SOME GROWTH TRAITS IN GADDI GOATS

THESIS

BY

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CERTIFICATE - I

This is to certify that the thesis entitled, **"Molecular genetic characterization of certain growth related genes and analyse their association with some growth traits in Gaddi goats"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science** in the discipline of **Animal Genetics and Breeding** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Gitanjali (V-2017-30-001)** daughter of Smt. Sudesh Kumari and Shri Srinagesh under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

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CERTIFICATE-II

This is to certify that the thesis entitled **"Molecular genetic characterization of certain growth related genes and analyse their association with some growth traits in Gaddi goats"** submitted by **Gitanjali (V-2017-30-001)** daughter of Smt. Sudesh Kumari and Shri Srinagesh to CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in the discipline of **Animal Genetics and Breeding** has been approved by Advisory Committee after an oral examination of student in collaboration with an External Examiner.

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Date:

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Abbreviations

%	Percentage
@	at the rate
°C	Degree Celsius
A	Adenine
AFLP	Amplified fragment length polymorphism
$AgNO_3$	Silver nitrate
AICRP	All India coordinated research project
AMSL	Above mean sea level
approx.	Approximately
APS	Ammonium per sulphate
BH	Body height
B_j	Fixed effect associated with i^{th} herd
BL	Body length
BLAST	Basic local alignment search tool
BLUP	Best linear unbiased prediction
bp	Base pair
BW	Body weight
BWG	Body weight gain
C	Cytosine
ChD	Chest depth
ChW	Chest width
Conc.	Concentration
CSKHPKV	Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya
dATP	Deoxy adenosine triphosphate
dCTP	Deoxy cytidine triphosphate
dGTP	Deoxy guanosine triphosphate
DNA	Deoxyribo nucleic acid
dNTP	Deoxyribo nucleotide triphosphate
Doc	Documentation
dTTP	Deoxy thymidine triphosphate
E	East
EDTA	Ethylene diamine tetra acetic acid
e_{ijkl}	Random residual effect
eSNPs	Expression single nucleotide polymorphisms
et al.	and others
etc.	Et cetera
FAO	Food and Agriculture Organization
F_{IS}	Fixation Index
FISH	Fluorescence in situ hybridization
g	Grams
G	Guanine
G_1	Fixed effect of 1^{th} genotype (1= 1,2,3)

GBW	Average daily gain from birth to weaning
GH	Growth hormone
GHR	Growth hormone receptor
GNY	Weight gain from 9 to 12 months
GSN	6 to 9 month weight gain
HaeIII	*Haemophilus aegypticus*
H_{exp}	Expected heterozygosity
HG	Heart girth
H_{obs}	Observed heterozygosity
HP	Himachal Pradesh
HPLC	High performance liquid chromatography
HWE	Hardy-Weinberg equilibrium
ICAR	Indian Council of Agricultural Research
IDT Inc.	Integrated device technology, Inc.
IGF-1	Insulin-like Growth Factor-1
IGFBP3	Insulin-like Growth Factor Binding Protein- 3
J&K	Jammu and Kashmir
Kb	Kilo base pairs
Kg	Kilogram
LD	Longissimus dorsi
M	Molar
MAS	Marker assisted selection
Mg^{++}	Magnesium ions
$MgCl_2$	Magnesium dichloride
min.	Minutes
ml	Millilitre
mM	Millimolar
MoDAD	Measurement of domestic animal diversity
MSTN	Myostatin gene
N	North
n	Total alleles
Na_2CO_3	Sodium bicarbonate
NCBI	National Centre for Biotechnology Information
N_e	Effective allele number
ng	Nanogram
NGS	Next generation sequencing
NSILA	Non suppressible insulin like activity
OD	Optical density
p mol	Picomoles
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	potential of hydrogen
P_i	Frequency of i^{th} allele

PIC	Polymorphic information content
PIT-1	Pituitary Specific Transcription Factor-1
P_j	Frequency of j^{th} allele
QTL	Quantitative trait locus/loci
RAPD	Random amplification of polymorphic DNA
RBC	Red blood cell
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SAS	Statistical analysis system
sec	Seconds
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
STAT5A	Signal transducer and activator of transcription 5A
T	Thymine
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	Tetramethyl ethylene diamine
Tm	Melting temperature
ul	Microlitre
UTR	Untranslated region
UV	Ultra voilet
V/cm	Volts per centimetre
v/v	Volume/volume
ver.	Version
viz.	Videlicet (namely)
VNTRS	Variable number tandem repeats
w/v	Weight/volume
W9	Weight at 9 months
WH	Wither height
WW	Weaning weight
X_k	Fixed effect associated with sex and season
Y_{ijkl}	Phenotypic value of growth trait
YW	Yearling weight
μ	Population mean
μM	Micromolar
χ^2	Chi-square

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ABSTRACT

This study was undertaken for molecular genetic characterization of *IGF-1* gene and four fragments of *GH* gene in Gaddi goats and analyse their association with growth traits. Blood samples from 63 genetically unrelated animals of Gaddi goat breed were taken from the migratory flocks under AICRP and Gaddi goat unit of CSKHPKV, Palampur and subjected to DNA isolation using Phenol Chloroform extraction technique. 363, 422, 116, 389 and 181 bp amplicons were generated on amplification of target loci of *IGF-1* and *GH* gene. *IGF-1* gene, *GH1* and *GH2* fragments were analysed using PCR-RFLP (*HaeIII* RE) that revealed two variants (AB and BB) for *IGF-1* and *GH2* whereas, three variants (AB, BB and AA) were revealed for *GH1* fragment. *GH3* and *GH4* fragments were subjected to PCR-SSCP that detected three genotypes (AB, BB and AA) for *GH3* however, *GH4* was found to be monomorphic. Significant association ($P \leq 0.05$) was detected for *GH1* with 9-month body weight, *GH2* with 9 and 12 months' heart girth and *GH3* with 6-month body weight, body height and body length, respectively. Allele frequencies for 'A' allele were 0.25, 0.53, 0.62 and 0.67 and 0.75, 0.47, 0.38 and 0.33 for 'B' allele. The PIC values revealed median level of polymorphism in studied population. From the present study, it was concluded that SNPs and their association with some body measurements may be useful in selecting goats for higher growth traits.

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CHAPTER-1

INTRODUCTION

India is one of the major livestock biodiversity centres of the world with livestock population of 512.05 million and poultry population of 729.2 million (19^{th} Livestock Census, 2012). At present, the country has 184 well documented and registered livestock and poultry breeds. India possesses rich diversity among indigenous goat genetic resources as reflected by its large population of 135.17 million (19th Livestock Census, 2012) and 34 recognised breeds as listed by ICAR-National Bureau of Animal Genetic Resources, Karnal.

The domestic goat (*Capra hircus*), also called as "poor man's cow", is an important livestock species reared for meat, milk, fiber, skin/hides and manure. Goats belong to the family *Bovidae* (hollow-horned ruminants) and are members of genus *Capra*. They are known for their capacity to flourish on paltry fodder and to withstand tough and harsh conditions.

The goats are believed to be domesticated about 10,000 years ago in the Zagros Mountains of the Fertile Crescent (Zeder and Hesse 2000). Domesticated goats (*C. hircus*) are descendants of wild goat or bezoar (*C.aegragus*), of South-west Asia, represented in Europe by the Cretan and Cyclades races. Goats are significant component of animal genetic resource diversity of India and play significant role in economy and nutritional security, particularly of resource poor farmers. Goats play a crucial role in fulfilling the ever increasing demand for animal origin protein foods and in sustainability of livestock production and overall farming frameworks.

Goat production is a predominant livestock activity in harsh climatic regions of the country particularly in hilly areas and arid zones with little arable agricultural land. Goats are well adapted to wide variation of agro-climatic conditions and extensive pasture grazing system. Gaddi, also known as White Himalayan goat is a prominent goat breed of mid to high hilly regions of the state of H.P. constituting 60-65% of total goat population of 11.20 lakh (19^{th} Livestock Census, 2012) with its true home tract in H.P. but distribution extending to adjoining hilly areas of Jammu & Kashmir and Uttarakhand (Acharya 1982).

'Gaddi goat' is a medium-sized animal usually with white coat colour, long horns, long drooping ears and convex nose line. It is mainly a meat breed with sturdy built and highly suitable to migratory production system. It is traditionally reared by nomadic

"Gaddi" shepherds throughout the hilly region of the state and is considered the best suited goat breed for the prevalent migratory sheep and goat production system. The breed is amply described in terms of its geographical distribution, morphological characteristics and production attributes but very less information is so far available on its molecular genetic characteristics including polymorphism of important genes regulating growth & reproduction and association of certain genes with performance traits.

Rapid growth as reflected by faster body weight gain, is important in meat goats as it influences net economic returns from sale of animals. Physiological regulation of body growth in animals is under the control of several genes like Growth Hormone (*GH*), Growth Hormone Receptor (*GHR*) and Insulin-like Growth Factor-1 (*IGF-1*) which are regulating specific physiological activities in growth process. Among the genes that operate in the somatotropin axis, the *GH* gene plays an important role in post-natal growth and development, lactation, tissue growth, reproduction as well as protein, lipid and carbohydrate metabolism (Pereira et al. 2005; Akers 2006). The *GH* gene and its receptors have been identified as major genes in regulating growth. *GH* is an anabolic hormone synthesized in anterior lobe of pituitary gland. This hormone aids in body's immune response, wound healing and haematopoiesis. *GH* also stimulates production of *IGF-1*, encoded by *IGF-1* gene and is also known as somatomedin C. It is a hormone which plays an important role in growth and has anabolic effects. Consequently, selection based on high level of endogenous growth hormone helps to explore and exploit superior growth potential.

The current knowledge in production biology indicates that genetically superior animals in growth differ from inferior animals in regulation of their nutrient utilization and *GH* plays a key role in nutrient use. Polymorphism in such genes may have association with certain important growth traits and thus, can serve as useful markers in marker assisted selection (MAS) for higher growth. Genetic diversity is vital for long-term survival of the species and populations since it provides the raw material for adoption, evolution and sustainable utilization particularly when habitat and environmental conditions are fast evolving (Rajora and Mosseler 2001). Genetic characterization can be done by different techniques like cytogenetic, biochemical and molecular. Recent trend in genetic characterization is to utilize molecular procedures which are based on DNA sequence polymorphisms and investigating molecular markers identifying genetic variation at DNA level.

The present study is therefore, proposed to genetically characterize and study polymorphism in certain growth related genes viz. some regions of *GH* gene and *IGF-1* gene in Gaddi goats and analyse their association with some growth traits for planning future genetic improvement strategy for higher body weight and meat yield in this goat breed. The study will also analyse the existing genetic diversity within breed population, a pre-requisite for undertaking future genetic improvement and breed conservation program. The broad objectives of the study are:

1. To study polymorphism of some growth related genes in native Gaddi goats of Himachal Pradesh.
2. To determine allele and genotypic frequencies for studied genes in Gaddi goats.
3. To analyse the association of identified genes with some growth traits.

CHAPTER-2

REVIEW OF LITERATURE

Genetic variation between and within the breeds also described as genetic diversity is the raw material for animal breeders to act upon for bringing genetic improvement in different livestock species. The population structure and genetic variation among breeds have been studied using different markers including detection of polymorphism at DNA level. Molecular genetic techniques are among the newer technologies that are being used efficiently in livestock conservation across the world (Joshi et al. 2012). Several advances have been made with the advent of molecular genetic technology, including the discovery of DNA based markers that have contributed immensely towards gene mapping, which would facilitate the identification of genes which control part of variability of phenotypic traits. A genetic marker is a DNA sequence or a gene with specific location on a chromosome that can be used to identify various individuals or species. It can also be termed as a variation (due to mutation or alteration in the genomic loci) that can be observed (Muhammad et al. 2018). An ideal genetic marker for population and evolutionary studies should be distributed widely across the genome (Sunnucks 2000). Since preservation of unique and precious richness is important for addressing emerging demands, variation at genetic level in native breeds is an important thrust area. To preserve the maximum amount of genetic diversity and further set conservation priorities, genetic characterization of animal genetic resources is essential to determine uniqueness of different breeds from other native populations (Eding et al. 2002).

2.1 Genetic Polymorphism

Genetic polymorphism is a difference in DNA sequence among individuals, populations or groups. It is also described as the inheritance of a trait controlled by a single genetic locus with two alleles, in which the least common allele has a frequency of about 1 % or greater to classify that locus as polymorphic. At the genomic level, polymorphisms can occur in the coding as well as non-coding regions of the gene with majority of them to be silent, meaning that they occur in the non-coding regions and therefore do not alter the expression or function of a gene (Hrdlickova et al. 2014), whereas that occurring in the coding regions can alter the expression of the gene and produce variation within a given population. Genetic polymorphisms may be the result of chance processes or have been induced by external agents. Sources of polymorphism include single nucleotide polymorphism (SNP), sequence repeats, insertions, deletions and recombination. Detection of these polymorphisms can be carried out by a wide variety of genetic tools like allele specific polymerase chain reaction (PCR), the microarray technique, restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP), whole genome sequencing, allozymes, tandem repeats (mini and microsatellite), that have proved to be useful in different research areas like population genetics, evolutionary genetics, molecular phylogeny, human genetics, systematics, agricultural genetics and forensics (Singh 2001; Pool et al. 2010; Yin et al. 2017).

Two experimental strategies presently being used in study of variability are the candidate gene approach and linkage studies (Zhu and Zhao 2007). The candidate gene approach is the analysis of genetic variability of candidate genes on basis of the physiological and biological information of the traits to be implicated for the portion of the trait variability whereas, the linkage studies are engaged with pursuit of quantitative trait loci (QTL) based on the knowledge of genetic maps in comparison with the segregation pattern of the genetic marker and the traits of interest. Consequently, association studies are undertaken to test whether a specific genotype is stably associated with the target trait.

2.2 Types of DNA Polymorphisms

DNA polymorphisms were first described in 1970's and early 1980's and till now their numbers and types have expanded rapidly. Polymorphism occurs when one of the two or more alternative forms (alleles) of a specific chromosomal region vary in nucleotide sequences. SNPs are the most common form of genetic differences. A specific location in the DNA sequence is defined as SNP if it exists in at least two types and rare allele being present in more than one percent of the population (Daniel 2011). Other types of polymorphisms include tandem repeat polymorphisms, insertions or deletions of a segment of the DNA, which result in repeated sequences (mini and microsatellites), gross genetic losses and rearrangement of nucleotides in the sequence. Due to deletion, duplication and rearrangement of the large portion of DNA, gross alterations in the sequences, chromosomal number and chromosomal translocation occur which are generally detected through various cytogenetic techniques. Different techniques such as southern blot, microsatellite and fluorescent in-situ hybridization (FISH) can be employed in the analysis of fragments of implicated chromosomal regions.

2.2.1 Polymorphism detection by using molecular markers

Detection of polymorphism refers to the identification of change in the DNA sequence. Direct sequencing is the most precise method to detect exact mutation, but for large samples, it is not cost effective that has put a limitation on its use. Cost effective studies on DNA polymorphism become possible with the advent of recombinant DNA technology. Before the discovery of PCR, cloning of desired fragment was necessary to get enough DNA to reduce the complexity of sample but with the discovery of PCR, detection of point mutation at large scale has become possible (Mullis and Faloona 1987).

For many years, use of gene mapping was limited to identify organisms by traditional phenotype markers that prompted the development of genetic markers which could identify genetic characteristics. Any stable and inherited variation that can be measured or detected by a suitable method and can be utilized to identify a specific genotype or phenotype, that is otherwise not measurable or very strenuous to detect is described as a genetic marker (Bhattacharya and Gandhi 1997).

The very first attempt of QTL mapping through linkage analysis of a marker locus was done by Sax (1923). Since then many attempts have been made to explore suitable markers for genome analysis. Molecular markers allow detection of variation for different genomic regions at population level that has immense application in constructing genetic maps. They are also used to analyse differences between markers in the phenotypic expression of certain traits that may point towards a substantial effect of observed differences in terms of genetic determination of the trait that can prove some degree of linkage of the QTL affecting the trait and the marker (Stein et al. 1996).

With the advancement in molecular biology, there is discovery of several ‘molecular/genomic markers’ that resulted in generation of huge database since the first demonstration of RFLP by Grodzicker et al. (1974). RFLPs are utilized in human genetics (Botstein et al. 1980), in genetic improvement programmes of animals (Soller and Beckmann 1983) and plants (Soller and Beckmann 1983; Burr et al. 1983). The use of PCR (Saiki et al. 1988) to exponentially amplify a specific DNA sequence and subsequent restriction enzyme analysis of the amplicon (Pinder et al. 1991) represents a milestone in this attempt. Genetic polymorphisms at the DNA level have provided a large number of genomic markers viz., RFLPs (Botstein et al. 1980), variable number tandem repeats (VNTRs) (Jeffreys et al. 1985; Nakamura et al. 1987), random amplification of polymorphic DNA (RAPD) (Welsh and Mc Clelland 1990; Williams et al. 1990), microsatellites (Litt and Luty 1989; Weber and May 1989; Fries et al. 1990; Tautz 1990; Yang et al. 2008), SNPs and amplified length fragment polymorphism (AFLP) markers.

2.2.2 Single Nucleotide Polymorphism

SNP is a substitution of a single nucleotide occurring at a specific location in the genome, where each variation is present to some substantial degree within a population (Brookes 1999). SNPs may arise due to single base substitution or point mutation that causes replacement of a single base nucleotide with another at the polymorphic site. Substitutions can be transitions where the replacement of one purine nucleotide by another purine or one pyrimidine nucleotide by another pyrimidine occurs. Substitutions can also be transversions, where replacement of purine base with pyrimidine base or vice-versa occurs. A SNP may also be a single base insertion or deletion which is referred as ‘indel’ (Weber et al. 2002). SNPs may fall within coding, non-coding or in the intergenic regions/sequences of genes. SNPs in the coding region are either synonymous or non-synonymous. Synonymous SNPs do not alter amino acid sequence of a protein, whereas non-synonymous SNPs alter the amino acid sequence of a protein (Brown 2002). The non-synonymous SNPs are further of two types: missense (code for different amino acids), silent (code for same or similar amino acids) and nonsense (sense codon changes to chain terminating codon) leading to protein truncation. SNPs also affect genetic processes of splicing, messenger RNA degradation, and transcription factor binding which are present outside the protein coding regions of the genome. These SNPs lying outside the coding region, when affect expression of genes are described as an expression SNPs (eSNPs). SNPs have characteristics of an ideal genetic marker and are the most common type of polymorphisms (Teneva 2009). SNPs can be used for biodiversity analysis, population structure studies, paternity testing and selection of suitable genotypes (Pariset et al. 2006). Although SNPs can be bi-allelic, tri-allelic or tetra-allelic, majority of them are usually bi-allelic and thus, can be easily assayed. Analytical methods to discover novel and detect known SNPs include DNA sequencing, SSCP, RFLP, hybridization analysis etc.

2.2.3 Single Strand Conformation Polymorphism

SSCP, first reported by Orita et al. (1989) in study of human DNA is one of the simple, appropriate and widely employed techniques to screen mutations (Bastos et al. 2001; Malveiro et al. 2001) which are randomly distributed throughout the genome. PCR-SSCP analysis is also used to detect point mutations, deletions and insertions in structural/regulatory genes. SSCP is based on the concept of differential migration of single stranded molecules through polyacrylamide gels due to effect of sequence variation on intra stranded loop formation. After the amplification of target gene or region of interest by PCR, amplicons are denatured using denaturing dye and subsequently polyacrylamide gel electrophoresis is performed. The folding confirmation of the sequence is altered in the presence of mutation that further affects electrophoretic mobility thus, wild and mutant type alleles produce differential migration patterns in polyacrylamide gel. Silver nitrate staining has been developed for relative ease of visualization of gel. Electrophoresis conditions such as temperature and ionic strength of buffer, gel concentration, voltage etc. require optimization and standardization for better sensitivity and resolution of the technique. Amplicon size also affects SSCP resolution which is better generated by fragments between 150 to 350 bp. The use of PCR-SSCP analysis for detection and genotyping of SNPs has been widely applied in major livestock species including goats.

2.2.4 Restriction Fragment Length Polymorphism

Nucleotide alterations in a DNA molecule like deletions, insertions or rearrangements that eliminate, create or translocate the cleavage sites of restriction enzyme (RE) result in RFLP (Botstein et al. 1980). An insertion, deletion or point mutation can create or eliminate the recognition site for a specific RE at a given locus and thus, there is change in the size of subsequent restriction fragments, in contrast, inversion causes changes in size of restriction fragments by changing the distance between a pair of RE sites. On gel electrophoresis, these changes in a DNA sequence associated with an allelic change at a particular locus will be visualized by the different mobility of restriction fragments and different band patterns will be revealed by individuals carrying different allelic variants of the gene. These differences in number and location of bands that result from changes in fragment size are defined as RFLPs, which are inherited in Mendelian fashion and since gene expression is not required for analysis of RFLPs, variation in the flanking regions or introns of genes may also be identified. For SNP genotyping, PCR-RFLP is a simple, rapid and precise technique that proved to be extremely useful.

2.3 Genes associated with regulation of growth in goats

Animals showing high genetic merit for growth (body weight, body measurements, weight gain and carcass characteristics) receive high priority in breeding programmes for meat purpose. Small ruminants, sheep and goats have enormous contribution to economy and livelihood of asset poor farmers in Asian countries (Dixit et al. 2010). Growth is a complex process regulated by coordination of a wide variety of neuro-endocrine pathways along with coordinated activity of various hormones (Falaki et al. 1997). Growth is influenced by several genes viz., Growth Hormone (*GH*), Growth Hormone Receptor (*GHR*), Insulin-like Growth Factor-1 (*IGF-1*) and Myostatin (*MSTN*) gene that have been identified as the major genes in the biological process (Hossner et al. 1997; Supakorn 2009).

2.3.1 Growth Hormone

Growth hormone (*GH*), also known as somatotropin is a polypeptide hormone of 191 amino acids and of molecular weight 22000 Daltons that is synthesized and secreted by somatotrophs in the anterior pituitary. It belongs to a family of somato-lactogenic hormones that also include prolactin and placental lactogen. *GH* is evolutionarily homologous to chorionic somatotropin and prolactin and has similar structure to their receptors and signal transduction pathways (Arkins et al. 1993; De Vos et al. 1993). *GH* has varying degree of lactogenic, somatotropic and metabolic effects most across mammalian species with following functions in the body.

2.3.1.1 Effect on growth

The pituitary growth hormone is important in postnatal growth and development. The major role of *GH* in body growth is to stimulate liver and other tissues to secrete *IGF-1*. It also helps in proliferation of chondrocytes resulting in bone growth.

2.3.1.2 Metabolic effects

GH also plays an important role in fat, protein and carbohydrate metabolism. *GH* enhances the utilization of fat by breakdown and oxidation of adipocytes. *GH* stimulates protein synthesis and decreases oxidation of proteins. It also helps to maintain the blood sugar level in normal range and has an anti-insulin activity as it inhibits insulin to uptake glucose in peripheral tissues and enhances glucose synthesis in liver.

2.3.2 Growth Hormone Gene

GH is encoded by a single gene about 2.5 kb in length and located on chromosome 19. It comprises of five exons with four intervening introns. Exon-1 is the shortest and spans from 432 to 444 bp, exon-2 spans from 692 to 852 bp, exon-3 spans from 1080 to

1196 bp, exon-4 spans from 1426 to 1587 and exon-5 has the longest span from 1864 to 2064 bp. *GH* gene encodes a total of 217 amino acids. Intron-1 spans from 445 to 691 separating exon 1 and 2. Intron-2 spans from 853 to 1079 separating exon-2 and 3. Intron-3 spans from 1197 to 1425 separating exon-3 and 4. Intron-4 spans from 1864 to 2064 separating exon-4 and 5 (Aradhana 2016).

It is well established that animals with high level of *GH* and its receptors show superior growth therefore, for enhanced growth performance, animal selection based on high endogenous levels of growth hormone is widely investigated. This superiority may be a result of more active variants in *GH* gene (ThidarMyint et al. 2008).

2.3.3 *IGF-1* (Insulin like growth factor-1)

IGF-1 and *IGF-2* were identified by Salmon and Daughaday (1957) and designated as "sulphation factors" by their ability to stimulate sulphate incorporation into rat cartilage. Froesch et al. (1963) described the non-suppressible insulin-like activity (NSILA) of two soluble serum components (NSILA I and II). In 1972, the labels sulphation factor and NSILA were replaced by the term "somatomedin", denoting a substance under control and mediating the effects of *GH*. In 1976, Rinderknecht and Humbel isolated two active substances from human serum, which owing to their structural resemblance to proinsulin were renamed *IGF-1* and *2*. *IGF-1* is the mediator of the anabolic and mitogenic activity of *GH* (Laron 2001).

2.3.3.1 Chemical structure of *IGF-1* gene

The *IGF gene* is a member of the family of insulin related peptides that include relaxin and several peptides isolated from lower invertebrates. *IGF-1* is a small peptide consisting of 70 amino acids with a molecular weight of 7649 Daltons. Similar to insulin, *IGF-1* has A and B chains connected by disulphide bonds. The C peptide region has 12 amino acids. The structural similarity to insulin explains the ability of *IGF-1* to bind (with low affinity) to the insulin receptor (Laron 2001).

2.3.4 *IGF-1* gene

The *IGF-1* gene is on the long arm of chromosome 12q23–23. The human *IGF-1* gene consists of six exons, including two leader exons, and has two promoters (Laron 2001). *IGF-1* gene encodes the protein that is structurally and functionally similar to insulin and is involved in regulation of growth and development. *IGF-1* is produced from a precursor peptide and is secreted into the bloodstream after binding to a specific receptor. It is produced as an endocrine hormone primarily by the liver but is also produced in paracrine/autocrine fashion in target tissues. Usually, *IGF-1* is bound to one of the 6 receptors of *IGFBP3* and only 2% is in the free form. *IGF-1* plays an important role in a variety of physiological processes such as growth, reproduction, lactation and embryogenesis (Adam et al. 2000). *IGF-1* participates in the growth and regulates function of almost every organ in the body (Daughaday and Rotwein 1989). Apart from insulin-like effects, *IGF-1* also regulates cellular synthesis of DNA as well as cellular growth and development, especially in neurons and also mediates the effect of *GH* gene.

2.4 Candidate gene polymorphism for growth related genes

Gupta et al. (2007) studied SNP in exon-4 and exon-5 of *GH* gene using PCR-SSCP in Black Bengal goats. SSCP showed 7 and 5 haplotypes in caprine *GH* gene and all the haplotypes revealed novel sequences. In exon-4, codons 6, 36 and 54 were polymorphic. At codon 6, AA arginine (R) changed to histidine (H) and proline (P), showing 6RR, 6HH and 6PP genotypes. At codons 36, three genotypes DD, VV and DV were observed due to SNP and at codon 54, 54RR and 54WW genotypes were observed. SNPs were also observed at codon 23 (serine to threonine) and at 37 (arginine to proline) in 8% of goats. In exon-5, nucleotide substitution (G/A) at codon 10 and (A/G) at 14, respectively changed AA from glycine (K) to glutamic acid (E). Silent mutations were also observed. The findings indicated that the Black Bengal goats had high genetic variability and PCR-SSCP is a simple and efficient technique for the detection of single base substitutions and can be employed for evaluating genetic variability in large populations.

Lan et al. (2007) determined polymorphism in *GH* gene in seven goat populations in China. To amplify *GH* gene, five pairs of primers i.e. *GH1* and *GH2* (for 5' UTR region), *GH3* and *GH4* (for exon-2 and flanking regions) and *GH5* (for exon-5 and flanking 3' UTR region) were utilized and in all the amplified regions, polymorphic SSCP patterns were detected. Significant association was found only between SSCP in exon-5 and milk yields in the third and fourth lactations, respectively. 1651T-to-G mutation in exon-5 and flanking 3' UTR region was used for PCR-RFLP genotyping of different goat breeds with *KpnI* that identified two alleles GH-T and GH-G and two genotypes (TT/TG). Frequencies of GH-G allele were 0.142, 0.092, 0.032, 0.131, 0.059, 0.050 and 0.000 for Xinong Sannen dairy, Laoshan dairy, Guanzhong dairy, Shaannan White, Guizhou White, Angora and Boer populations reared in China. The study revealed that association of TT and TG genotype with litter size, body weight and milk performance may be useful for studying breed characteristics.

Zhang et al. (2008) studied polymorphism of *IGF-1* gene and its association with growth traits in 592 Nanjiang Huang goats in the complete coding sequence, part of introns and the 5′ regulatory region of the *IGF-1* gene by PCR-SSCP technique. A new SNP (G to C transversion) was identified at intron-4 which revealed two alleles and three genotypes. The statistical analysis showed that polymorphism of *IGF-1* gene had a significant association with birth weight, heart girth, body length and wither height at different ages that was higher for genotype CC than those with GC and GG genotypes. Therefore, CC may be the most advantageous genotype for growth traits in the Nanjiang Huang goat. However, no significant association between SNP genotypes and other growth traits was observed. The results indicated that SNP marker of *IGF-1* gene may be a potential molecular marker for studying growth traits in Nanjiang Huang goats.

Gupta et al. (2009) studied SNP in *GH* gene of Jakhrana goat breed. PCR-SSCP patterns on non-degenerative PAGE revealed 6 amplicons in caprine *GH* exon-4 and three alleles viz., A4, B4 and C4 were identified. In exon-5, six SSCP variants revealed three alleles A5, B5, and C5. Out of 54 AA sites of *GH-4* coding region, six codons were polymorphic. At codon-6, nucleotide substitution of G/A resulted into genotypes 6RR, 6HH and G/C into genotypes 6PP, 6RP. At codon-36, A/G nucleotide substitution resulted into a new genotype of 36GG and at codon-54, C/T nucleotide substitution resulted into a new genotype of 54WW. Synonymous mutations were present at codons 25, 31 and 62 in all the animals of Jakhrana goats. The high genetic variability in exon-4 and exon-5 of *GH* gene may be useful in exploring their associations with milk and growth traits in goats for further genetic improvement.

Hua et al. (2009) studied *GH* gene polymorphism and its association with growth traits in Boer goat bucks, amplifying two regions of *GH* gene from caprine genomic DNA. Two SNPs were identified by gene sequencing and PCR-RFLP analysis. AA genotype resulted in a significant decrease in birth chest girth (P=0.03) and weaning weight (P=0.014) comparing to AB genotype, while CC genotype contributed to weaning height (P= 0.04) more than CD genotype. The quantified results revealed that *GH* gene is an important growth regulating gene in goats.

Mousavizadeh et al. (2009) studied genetic polymorphism at *GH* locus in 90 Iranian Talli goats by PCR-SSCP. Genotyping revealed nine conformational patterns in exon-4 of the *GH* gene, with frequencies of 27.7% for the homozygous pattern (AA) and 72.2% for all other heterozygous patterns. The study revealed that exon-4 of *GH* gene in Talli goats is highly polymorphic and can be exploited as a candidate gene for MAS.

An et al. (2010) determined novel polymorphisms of the *GH* gene and their effect on growth traits in 686 individuals from four populations of Chinese goats. Three haplotypes (A, B and C) and three observed genotypes (AA, AB and AC) were detected at the P2 locus, and three haplotypes (E, F and G) and three observed genotypes (EE, EF and EG) were also detected at the P4 locus. In addition, five SNPs viz., A112G, C142T, C214T, C266A and C214T were identified by *GH* gene sequencing and PCR-SSCP analysis. The SNPs loci were in Hardy–Weinberg disequilibrium in three goat populations. Association of polymorphisms with growth traits was done which were shown to be associated with growth traits in three goat populations. The SNPs in the goat *GH* gene had significant effects on growth traits, suggesting that the *GH* gene is a strong candidate gene that affects growth traits in goats.

Deng et al. (2010) studied association of *IGF-1* gene polymorphisms with yield and body size in 708 individuals of two Chinese dairy goat breeds (Guanzhong and Xinong Saanen). PCR-SSCP and DNA sequencing methods were employed in screening for genetic variation that detected 2 novel mutations in 5'-flanking region and in intron-4 of *IGF-1* gene, viz., g.1617 G > A and g.5752 G > C, respectively. In Xinong Saanen goats with CC genotype, milk yield was significantly higher during the first and second lactations ($p < 0.05$). Hence, g.5752 G > C mutation can facilitate association analysis and serve as a genetic marker for selection of goats.

Wickramaratne et al. (2010) studied use of *GH* gene polymorphism in selecting Osmanabadi and Sangamneri goats. DNA samples of 240 goats were subjected to SSCP and DNA sequencing techniques, to detect SNPs in *GH* gene and their association with growth traits. 23 and 18 nucleotide changes were observed in Osmanabadi and Sangamneri goats, respectively out of which, twelve were seen unique to both goat breeds indicating considerable deviation from exotic goat sequence. The study revealed point mutations of G200T, A815G, A1753, C1763T and A1789G in *GH* gene sequence of both goat breeds.

In contrast, A497G, A499G, C500G, C501-2, C730T, C781T and C2055T were observed specific to Osmanabadi and Sangamneri breeds, respectively indicating the possibility of using them as breed specific markers.

Kumar et al. (2011) studied genetic variability in caprine *GH* gene by SSCP analysis of 8 amplified fragments covering almost the entire gene (approx. 2.5 kb) in 188 Sirohi goats and carried association studies with body weights at birth, 3, 6 and 9 months of age. SSCP analysis revealed 4 to 8 unique banding patterns across 8 studied fragments of *GH*. The promoter and region having exon-3 showed higher level of polymorphism with 8 variants. The fragments consisting of exon-1, exon-4 and exon-5 revealed 6 variants. SSCP patterns in the promoter region had significantly influenced the birth weight. The SSCP variants in fragments consisting of exonic regions had also influenced the body weight at different ages. SSCP analysis has indicated the possibility of marker assisted selection for higher body weight at different ages in Sirohi goat breed.

Tahmoorespur et al. (2011) determined variation of exon-5 of *GH* gene and exons-7 & 8 of *STAT5A* gene and their association with estimated breeding values of growth traits in 190 lambs of Iranian purebred Baluchi sheep by using PCR-SSCP analysis. *GH* gene revealed three (G1, G2, and G3) conformational patterns however, *STAT5A* loci were not polymorphic. Breeding values for growth traits including birth, weaning, 6 months, 9 months and yearling weights were estimated by using Best Linear Unbiased Prediction (BLUP) based on an animal model with a relationship matrix. The findings suggested that animals with G2 genotype had highest breeding value for 6-month weight, while these animals had lowest breeding value for pre-weaning traits. Higher performance of G2 animals in adult ages may be related to the role of growth hormone in puberty. The other traits showed no relationship to the genotypes examined.

Wang et al. (2011) analysed association between polymorphisms of *IGF-1* gene and cashmere production and body weight traits in goats. PCR-SSCP and DNA sequencing were used to detect polymorphisms of *IGF-1* gene using 776 animals of three Xinjiang local goat breeds (Xinjiang, Bogeda Cashmere and Nanjiang Cashmere). The results showed that the frequencies of genotype AA were 0.414, 0.591 and 0.319; genotype AB were 0.000, 0.126 and 0.029; genotype BB were 0.586, 0.241 and 0.597 and genotype AC were 0.000, 0.042 and 0.055 for Xinjiang, Bogeda Cashmere and Nanjiang Cashmere goat breeds, respectively. The mutation was detected at *IGF-1* P1 locus, a novel SNP revealed in exon-4 that belongs to silent mutation. In addition, this SNP at *IGF-1* P1 locus was significantly associated with cashmere production traits. In Nanjiang Cashmere goat population, the cashmere fineness of AA genotype was significantly ($p<0.05$) lower than that of AB genotype. The body weight of AC genotype was significantly ($p<0.05$) higher than that of BB genotype which indicated that the *IGF-1* gene may be regarded as candidate gene for cashmere production traits.

Alakilli et al. (2012) studied polymorphisms of 5 candidate genes including *GH* and *IGF-1* in four Egyptian and Saudi goat breeds (Barki, Zaribi, Ardi and Masri) using PCR-RFLP technique. The analysis for *GH* gene using *HaeIII* restriction enzyme revealed four banding patterns corresponding to genotypes AA, AB, CC and CD and the frequencies of 'A' allele varied from 0.410 to 0.620. The *IGF-1* gene revealed three fragments after digestion with *HaeIII* RE with genotypes AA, AB and BB and the frequencies of allele 'A' varied from 0.432 to 0.731. PCR-RFLP method employed for polymorphism analysis in five genes in the investigated breeds successfully detected polymorphism that indicated that different genotypes observed for respective genes could be used as molecular markers for growth traits in MAS.

Liu et al. (2012) studied DNA polymorphism by PCR-RFLP of *IGFBP-3* gene and its association with fibre traits in 444 Chinese Inner Mongolian Cashmere goats. A 316 bp fragment of *IGFBP-3* gene in exon-2 was amplified and digested with *HaeIII* restriction enzyme that revealed 3 patterns of restriction fragments. Nucleotide sequencing revealed a C>G transition in exon-2 region of *IGFBP-3*, resulting in R158G change which caused the polymorphism. Least square analysis revealed a significant effect of genotypes on cashmere weight ($p<0.0001$), cashmere fibre length ($p<0.001$) and hair length ($p<0.05$). The effect of genotypes on cashmere fibre diameter was not statistically significant ($p>0.05$). The AB and BB genotypes showed higher fibre length and hair length than AA genotype. The study suggested that polymorphisms in caprine *IGFBP-3* gene can be potential molecular markers for cashmere weight in cashmere goats.

Dettori et al. (2013) studied variability in each of the five exons of caprine *GH* gene in order to establish the possible relationships with milk traits in Sarda goats. The linear model procedure was used to analyse the effects of SSCP patterns on milk traits of 100 lactating goats. Analysis of conformational polymorphism at exons 1 to 5 revealed a total of 25 differing banding patterns and sequencing revealed 21 nucleotide changes. The association analysis revealed that each exon, except exon-1, affected milk yield, exons-1 and 3 influenced milk fat percentage, and all exons, except exon-2, had an effect on protein percentage, supporting previous results in livestock. The variability detected at the caprine *GH* gene might provide useful information for the phylogeny of ruminants and more importantly, have implications on the biological function of the growth hormone and on those traits resulting from its physiological action, including milk production and composition. The caprine *GH* gene may become a useful molecular marker for a more effective genetic selection for milk production traits in goats.

Hajihosseinlo and Negahdary (2013) studied SNP in exon-4 of *GH* gene using PCR-SSCP in 100 animals of Iranian indigenous Makooei sheep breed. A non-radioactive method for detection of SSCP was optimized, starting from genomic DNA and PCR amplification of exon-4 of the growth hormone gene that revealed five conformational patterns. The findings determined that Iranian indigenous sheep breed has immense genetic variability, which opens remarkable prospects for future selection programs and preservation strategies. The study concluded that PCR-SSCP is an appropriate tool for evaluating genetic variability.

Moradian et al. (2013) investigated polymorphism at exon-1 of *IGF-1* gene in Makooei sheep using PCR-SSCP. *IGF-1* was amplified by PCR, using the locus specific primers and produced 265 bp amplicon. Three different patterns (AA, AG and GG) were detected using PCR-SSCP analysis of 5' flanking region (exon-1) of *IGF-1* gene. The frequencies of the observed genotypes were 0.52, 0.42, and 0.06 and allele frequencies were 0.73 and 0.27 for A and G, respectively. The allele A with frequency of 0.73 was the most frequent allele while AA genotype was the predominant genotype with frequency of 0.52. Observed heterozygosity (H_{obs}) value was 0.39. The genotypic distribution was within the expectations under the assumption of HWE. The study concluded that *IGF-1* may be used as a candidate gene for MAS in sheep breeding programs.

Negahdary et al. (2013) detected PCR-SSCP variation of *IGF-1* and *PIT-1* (Pituitary Specific Transcription Factor-1) genes and their association with estimated breeding values of growth traits in Makooei sheep. PCR-SSCP analysis of the exon-1, a part of intron-2, exon-3 and a part of intron-3 of *IGF-1* gene and *PIT-1* gene revealed three (AA, AG, GG) and four AA (p1), AB (p2), CC (p3), CD (p4) banding patterns, respectively. Results demonstrated higher performance of AA genotype in BW and GBW (average daily gain from birth to weaning), and AG genotype in WW (weaning weight) and weight at 6 months that may be associated to the role of *IGF-1* at the pre-puberty and puberty stages. Also higher performance of p3 genotype in W9, YW (yearling weight) and GSN (6 to 9 month weight gain), and p1 genotype in GNY (weight gain from 9-12 months) may be related to the role of *PIT-1* in post-puberty. The study concluded that polymorphisms contributed to variation in the growth traits that emphasized the possibility of utilizing these polymorphisms in molecular MAS and breeding programs.

Sharma et al. (2013) determined the effect of genetic polymorphisms in *IGF1*, *GHR* and *IGFBP-3* genes on body weight of Sirohi and Jamnapari goat breeds. A total of 80 samples from nine Indian goat breeds (Sirohi 187, Jamnapari 154, Barbari 12, Beetal 6, Osmanabadi 6, Jakhrana 6, Black Bengal 6, Changthangi 6 and Gaddi 6) were utilized for identification of SNPs, out of which 18 SNPs in *IGF1*, 5 SNPs in *GHR* and 8 SNPs in *IGFBP-3* genes were detected. All the detected SNPs were genotyped in 309 goats. Statistical analysis revealed that two SNPs of *IGF-1*, one SNP of *GHR* and one SNP of *IGFBP-3* genes have significant association with body weight in Sirohi goats at different ages and only 1 SNP of *GHR* gene was found to be associated with body weight at birth in Jamnapari goats. The findings indicated that studied genes can be used for selection of animals with superior growth performance.

Dayal et al. (2014) studied SSCP typing of *GH* gene and its association with birth weight in Black Bengal goats. For detection of polymorphism, a 245 bp fragment (partial intron-1, exon-2 and partial intron-2) of growth hormone gene was analyzed. SSCP typing revealed 5 genotypes AA, AB, AC, AD and CC and consequently, 4 alleles A, B, C and D were identified. Least square analysis revealed that genotypes had significant effect on birth weight. Animals having AC genotype had highest birth weight whereas animals having CC genotype had lowest birth weight. Therefore, the study indicated that the growth hormone gene may possibly be used as a marker for improving birth weight in Black Bengal goats.

Gharedaghi et al. (2014) studied polymorphism at exon-4 of *IGF-1* gene and its association with milk production and growth traits in Mahabadi goats using PCR–SSCP. PCR was used to amplify 326 bp fragment of exon-4 of *IGF-1* gene. The results revealed two banding patterns when stained with silver nitrate method.

Kumari et al. (2014) studied genetic polymorphism of *GH* gene at locus A1575G and A781G for 9 sheep breeds of different agro-ecological regions of India, analysing 324 samples of Indian sheep breeds by PCR-RFLP technique. Restriction digestion analysis at A781G locus revealed two allelic variants (A= 0.6016 and B= 0.3983) and two genotypes (AA= 0.2032, AB= 0.7968) whereas, at A1575G locus, all individuals were homozygous (CC) in all 9 breeds. The allelic frequency differences for both alleles across the Indian breeds, irrespective of their geographic distribution, colour pattern and utility traits, were statistically non-significant. The average heterozygosity was 0.4712. The findings revealed substantial level of genetic variation at A781G locus and adds substance to search for QTL for growth traits in Indian sheep breeds.

Malewa et al. (2014) studied *GH* gene polymorphism using PCR-RFLP in fat tailed sheep breeds (42 Donggala and 28 East Java) and its relationship with birth weight, growth rate and weaning weight. A fragment of 934 bp of *GH* gene, comprising of a part of intron-2, complete exon-3, complete intron-3, complete exon-4, and a part of intron-4 was amplified and was digested with *HaeIII* restriction enzyme that showed polymorphism with three genotypes (AA, BB and AB). In Donggala sheep, AA and AB genotypes had the same frequency of 0.357, whereas BB genotype had frequency of 0.286. In East Java sheep, AA genotype is the most frequent genotype (0.464), whereas BB and AB had frequency of 0.286 and 0.250, respectively. *GH/HaeIII* polymorphism affected growth rate and weaning weight both in Donggala and East Java sheep. In Donggala sheep, AA was observed to have significantly higher growth rate than BB. AB, as a heterozygote, did not show significant differences in growth rate compared to both homozygotes (AA and BB). Genotype AA showed significantly higher weaning weight than genotype BB, both in Donggala (11.6 kg vs. 9.68 kg) and East Java (10.83 kg vs. 9.37 kg) sheep breeds. Genotype AB did not show significant differences in weaning weight as compared to genotypes AA and BB, both in Donggala and East Java sheep. The findings concluded that *GH/HaeIII* polymorphism can be used as a genetic marker in sheep selection program for improvement of growth rate and weaning weight.

Nejhad et al. (2014) studied genetic polymorphism of *GH* gene in 34 Alpine and 42 Saanen goats. 365 bp region of exon-5 of the *GH* gene was amplified by PCR. PCR products were analyzed using SSCP method on polyacrylamide gel which resulted in four (G1, G2, G3 and G4) conformational patterns with frequencies 0.38, 0.21, 0.23 and 0.18

for Alpine and 0.48, 0.21, 0.17 and 0.14 for Saanen goats, respectively. The study revealed that *GH* gene was polymorphic and showed that PCR-SSCP is an appropriate tool for detecting polymorphism and evaluating genetic variability.

Sharma et al. (2014) studied sequence characterization and genetic variability analysis of *GHR*, *IGF-1* and *IGFBP-3* genes in 80 animals of nine Indian goat breeds. The comparative gene sequence analysis in all goat breeds revealed 31 SNPs across different loci of *GHR*, *IGF-1,* and *IGFBP-3* genes. Out of 31 SNPs detected, 18, 8, and 5 SNPs were present in *IGF-1*, *IGFBP-3* and *GHR* genes, respectively. All the mutations observed in *GHR* and *IGF-1* genes were found to be synonymous and 6 of 8 SNPs in *IGFBP-3* were found to be non-synonymous. Across breeds, the average frequency of the least frequent alleles ranged from 0.02 to 0.17.

Marini et al. (2015) studied the inheritance of *GH* gene and analysed the relationship of *GH* gene polymorphism and pre-weaning growth in Savanna goats. A total of 133 Savanna goats were used, from which 63 does with *GH1* and *GH2* combinations were selected for breeding based on body condition score of 2.5 and above, history of kidding and reproductive soundness. F1 progeny were evaluated for their genotypic patterns and growth performance that identified two variants *GH1* and *GH5*. *GH1* gene revealed polymorphisms with two genotypes AA and AB and *GH5* gene with three genotypes GH, GG and HH. The four parental combinations of *GH1* and *GH5* genes produced F1 genotypes which followed the Mendelian inheritance. The birth weight of ABGG-F1 Savanna kids was higher ($p<0.05$) than ABGH genotype. The study revealed that the genetic polymorphism of *GH* gene can be used as a potential marker in genomic selection and breed improvement of the goats in line with good management and environmental conditions.

Othman et al. (2015) studied the genetic polymorphism in exon-2 and 3 of *GH* gene in Egyptian sheep and goat breeds utilizing PCR-RFLP. 422 bp PCR amplified fragments were digested with *HaeIII* endonuclease that detected two genotypes, GG and AG. Frequencies were 43.56 and 56.44% for GG and AG genotypes in 101 tested sheep whereas in 48 tested goats, the frequencies were 12.5 and 87.5% for GG and AG genotypes, respectively. The sequence analysis revealed the presence of a SNP (G6A) at position 55 in the amplified fragment which was responsible for the destruction of the restriction site GG^CC and consequently the presence of two different alleles G and A.

The association studies showed significant effect of heterozygous genotypes on some growth traits. Due to association between A/G genotype with growth traits, selection was recommended to increase the frequency of A/G genotype to improve production efficiency and concluded that detected SNP could be of potential application in carrying out MAS and bring genetic improvement in studied breeds.

Singh et al. (2015) studied polymorphisms of exon-2 and exon-3 of *GH* gene and their association with growth traits in Sirohi and Barbari goat breeds. An amplified product of 422 bp size was observed in both breeds. *HaeIII* digestion revealed two genotypic variants viz., AB and BB but none of the two breeds was in HWE for these variants. The least squares analysis of variance revealed non-significant effect of *GH* genotype and breed × genotype interaction on chest girth and paunch girth from birth to 180 days of age. The effect of breed was highly significant ($p<0.01$) at all ages.

Ilham et al. (2016) studied genetic polymorphisms of *GH* gene in 168 Kacang goats based on PCR-RFLP method, using *HaeIII* restriction enzyme that revealed two genotypes; AA and AB with frequencies of 0.095 and 0.904. The frequencies of allele A (0.547) and B (0.452) indicated a polymorphism in *GH* A781G locus in these goats. The observed (Ho) and expected (He) heterozygosity values were 0.0904 and 0.496. *GH* alleles distribution in Kacang goat populations were not in HWE. Based on statistical analysis, there was no difference between body size of genotypes AA and AB. The results of the study can be used as preliminary information in formulating a strategy for Kacang goat breeding by utilizing MAS.

Khichar et al. (2016) studied molecular characterization of exon-3 of caprine Myostatin gene in Marwari goats. The genetic variability in exon-3 of Myostatin gene in 120 Marwari goats was assessed on PAGE to detect SSCP patterns and revealed two types of conformational patterns. Two genotypes were revealed with frequency of 0.90 for AA and 0.10 for AB, respectively. Low level of polymorphism was observed at exon-3 region of *MSTN* gene in Marwari goats through SSCP analysis which might be utilized in future breeding plan to exploit the unique characteristics of Marwari goats.

Ncube et al. (2016) assessed the genetic diversity in *GH1* gene within and between South African goat breeds. PCR together with next-generation sequencing (NGS) was used to generate the full length (2.54 kb) of the *GH1* gene and screening for SNPs in three goat populations. A range of 27 to 58 SNPs per population were observed. Mutations resulting in amino acid changes were observed at exons-2 and 5. Higher within-breed diversity of 97.37% was observed within the population category consisting of SA village ecotypes and the Tankwa goats. Highest pairwise F_{st} values ranging from 0.148 to 0.356 were observed between SAB and both the South African village and Tankwa feral goat populations. Phylogenetic analysis indicated nine genetic clusters, which reflected close relationships between the South African populations and the other international breeds with the exception of the Italian Sarda breeds. The results implied greater potential for within population selection programs, particularly in SA village goats.

Radhika et al. (2016) studied the polymorphism of exon-2 and 3 of *GH* gene and presence of a rare genotype in native goat breeds of Kerala. A polymorphism at 781A > G of *GH* gene was screened in 343 goats of two native breeds of Kerala viz., Malabari and Attappady Black, along with Malabari crossbreds. Restriction digestion with enzyme *HaeIII* resulted in three genotypes AA, AB and BB. Frequency for genotype AB was higher for all the populations indicating its selective advantage, but no association between genotypes and growth traits could be established. The BB genotype had a low frequency of 0.02 and appeared to be a rare genotype, which had not been reported in other similar studies with *GH* gene. This genotype was seen mostly in Attappady Black goat population which comes under 'insecure' category of conservation and hence cannot afford to undergo strict selection measures, that might be the reason for the presence of this rare genotype.

Depison et al. (2017) studied association of *GH* gene polymorphism with quantitative characteristics (wither height WH, body length BL, chest depth ChD, chest width ChW, body weight BW and body weight gain, BWG) of thin-tailed sheep using PCR-RFLP with the *MspI* and *AluI*. Quantitative characteristics of thin-tailed sheep, both males and females in highlands were better than in the lowlands. Studied locus was polymorphic in highlands as well as in lowlands. The *GH* gene diversity was associated with quantitative traits of thin-tailed sheep both in the highlands and lowlands of Jambi province.

Gorlov et al. (2017) studied association of the *GH* gene polymorphism with growth traits in Salsk sheep by PCR-RFLP using *HaeIII* enzyme. AA, AB, and BB genotypes were identified with frequencies of 57, 36, and 7%, respectively. The values of weight at weaning, at the age of 9 months and the average daily weight gain of the animals with AB genotype exceeded the values of these parameters in animals with AA genotype by 0.92

kg, 10.67 kg and 47.3 g, respectively. AB genotype also caused a greater weight of heart and kidney by 75.21 and 75.44 g, respectively. Thus, the presence of a heterozygous AB genotype in Salsk sheep breed had a positive effect on the growth traits. AB genotype significantly exceeded AA genotype and were found to have the best meat productivity.

Grochowska et al. (2017) studied the effect of the *IGF-1* gene polymorphism on growth, body size, carcass and meat quality traits in 305 coloured Polish Merino sheep by PCR-RFLP method. In total 78 live and post mortem traits were investigated. In association studies, traits of interest were analysed by using MIXED and GENMOD procedures of the SAS statistical package that revealed two alleles, A and B and two *IGF-1* genotypes, AA and AB. The A allele and the AA genotype were predominant with frequencies of 91.6 and 83.3 %, respectively. The *IGF-1* genotype was found to have a highly significant effect on fore shank weight, kidney fat class and it significantly affected external fatness of carcass class, drip loss and it tended to be associated with longissimus dorsi (LD) muscle width and flavour. The study concluded that the *IGF-1* gene can be considered as a candidate gene for selecting carcass and meat quality traits in sheep.

Naicy et al. (2017a) studied association of *Cac81* polymorphism in *IGF-1* gene with growth traits in two indigenous goat breeds of Kerala viz., Malabari and Attappady Black. Using PCR-RFLP, a total of 277 goats were genotyped, using restriction enzyme *Cac8I*. One SNP, A224G was detected in the 5´ non-coding region of the *IGF-1* gene, and accordingly two genotypes were revealed, GG and AG. This SNP was significantly associated with growth traits in Attappady Black goats, which is maintained as meat breed in Kerala. The study demonstrated higher performance of GG animals for growth traits. The association of *IGF-1* gene with these traits emphasized the importance of caprine *IGF-1* as a candidate gene for growth traits in goat breeding.

Rasouli et al. (2017) analysed polymorphism in *IGF-1* and *IGFBP-3* genes and their relationship with twinning rate and growth traits in Markhoz goats. Two sets of specific primers were used to amplify a 249 bp fragment of *IGF-1* gene and a 316 bp fragment of *IGFBP-3* gene. Three banding patterns for each gene were revealed using PCR-SSCP analysis that confirmed presence of a mutation at position 1617 of *IGF-1* and at position 58 of *IGFBP-3*. The genotype frequencies for *IGF-1* gene were 0.81 (GG), 0.16 (GA) and 0.03 (AA) and for *IGFBP-3*, genotype frequencies were 0.79 (TT), 0.17 (TC) and 0.04 (CC). The study revealed that different genotypes of these genes had no significant effect on birth weight, weight at 6 months, at 9 months and at 12 months but the interactions between different genotypes of *IGF-1* and *IGFBP-3* genes were significant for weaning weight and average daily gain from birth to weaning which suggested that twinning rate in Markhoz breed is statistically affected by these genes and may be considered in breeding programs.

Susilorini et al. (2017) studied the genetic polymorphism of *GH* gene in 94 Etawah crossbred goats using PCR-RFLP. The PCR amplified fragments were digested with *HaeIII* endonuclease. The results showed the presence of two genotypes CC and CD. The genotype frequencies were 0.47 (CC) and 0.43 (CD). Statistical analysis showed that CD genotype had higher birth weight and weaning weight than CC genotype. The study concluded that the *GH* gene may be a major gene or linked to the major gene to affect the body weight traits and could be used in MAS for selecting goats.

Bayan et al. (2018) studied genetic polymorphism of exon-4 of *GH* gene in Surti and Mehsani goats by PCR-RFLP. *GH* gene exon-4 region was found to be monomorphic on restriction digestion with *HaeIII*, which revealed only one genotype CC in both Surti and Mehsani goat breeds. The allelic frequency of C was 1.00 in both the breeds of goats with absence of D allele. Since the growth hormone locus studied was monomorphic in both breeds, indicating that monomorphism at this locus may be a species characteristic of goats probably due to absence of any mutation and high degree of sequence conservation. As goat *GH* exon-4 studied was monomorphic, it cannot be used as genetic marker for selection purpose.

Mahdi et al. (2018) determined genetic variability of the *GH* gene at sites A781G (*GH1*) and A1575G (*GH2*) for three Iraqi sheep breeds (Awassi, Hamdani, Karadi) by using PCR-RFLP technique. *HaeIII* enzyme digestion revealed two loci of *GH* gene in the sheep genome. The first region was located in the second and third exons with 422 bp (*GH1*), while the second region was in the fourth exon with 116 bp (*GH2*). The genotypes BB and AB of *GH1* were found in both Karadi (0.70, 0.30) and Awassi (0.60, 0.40). The genotypes AA, BB and AB were found in Hamdani (0.10, 0.50 and 0.40). The frequency of alleles A, B and C in *GH1* and *GH2* was 0.45, 0.55 and 1.00 in Hamdani, for alleles A, B and D was 0.15, 0.85 and 1.00 in Awassi and for alleles A, B, C and D was 0.20, 0.80, 0.90 and 0.10 in Karadi. The results showed that the *GH* gene can be used as a marker assisting for selection by relying on genetic diversity in the studied genetic sites.

Sankhyan et al. (2019) studied genetic polymorphism in *IGF-1* gene in four sheep and goat breeds along with its association with biometrical traits in Gaddi goat breed using PCR-RFLP. A total of 63, 68, 197 and 73 animals belonging to Gaddi sheep, Rampur-Bushair sheep, Gaddi goats and Chegu goats, respectively were used. A 363 bp amplicon was yielded and PCR-RFLP digestion with *HaeIII* revealed three fragments of 363 bp, 264 bp and 99 bp. The frequency of AA, AB and BB genotypes ranged from 0.38 to 0.55, 0.30 to 0.42 and 0.14 to 0.19, respectively in different breeds studied. The Ne, H_{obs} and H_{exp} ranged from 1.80 to 1.99, 0.30 to 0.43, 0.42 to 0.48, and 0.33 to 0.37, respectively in different populations. The PIC values revealed median level of polymorphism in studied breeds and alignment of sequences also confirmed presence of C/G substitution at 264 bp. Nucleotide variability was found at 17 places between different breeds. Genetic variability was detected among all four breeds studied, however no significant association could be detected in Gaddi goats.

CHAPTER-3

MATERIALS AND METHODS

The present study was carried on “Gaddi”, a distinct goat breed of high altitude areas of Western Himalayan region, for molecular characterization of certain growth related genes and further analyse their association with growth traits along with analyzing the existing intra population genetic variability. The breed derived its name from the local nomads known as “Gaddi”, who are the customary stakeholders of this breed, patronizing the breed since long. For this, true to breed animals maintained under prevalent migratory production and management system in its natural habitat in Himachal Pradesh were utilized. The breed is also called as “White Himalayan” in certain pockets of its distribution.

3.1 Background/ General information about the Gaddi goat breed:

3.1.1 Habitat & distribution: Gaddi goats, primarily raised for meat and fibre by transitory nomads “Gaddis”, is native to H.P, with its distribution reaching out to adjoining regions of Jammu and Kashmir and Uttarakhand. H.P is the north-western Himalayan state of Indian sub-continent which is geographically situated south of Jammu and Kashmir, north east of Punjab, north west of Haryana and parts of Uttarakhand, west of Tibetan plateau of autonomous Chinese province, between latitude 30º22’ N to 34º12’N and longitude 75º47’E to 79º40’E. The elevation varies from 350 meters (low hills) to 6975 meters (higher mountains) AMSL. The breeding tract of this goat breed lies in districts of Chamba, Kangra, Kullu, Shimla, Sirmaur, Kinnaur & Lahul and Spiti in H.P. These goats are also found in hilly regions of Tehri, Garhwal and Chamoli in Uttarakhand and in Bhadarwah, Doda and Kishtwar areas of Jammu and Kashmir. Migratory tracts of different adopted flocks are presented in Figure 3.1.

3.1.2 Morphological features of Gaddi goats: Gaddi is a medium to large sized goat usually with white coat color but black, brown or tan markings are also found in few animals. It has long horns, long drooping ears, long hair (ranging from 20 to 25cm) and convex nose line. Animals of both sexes of the breed have long spiral upward and backward directed horns with pointed tips. Prolificacy is less and twinning is hardly 10 to 15 percent. It is mainly a meat breed with sturdy built. The breed is highly suitable to extensive migratory production system in hilly areas.

Plate 3.1 Adult female animal of Gaddi goat breed

Plate 3.2 Adult male animal of Gaddi goat breed

Plate 3.3 Gaddi goat flock in its breeding tract

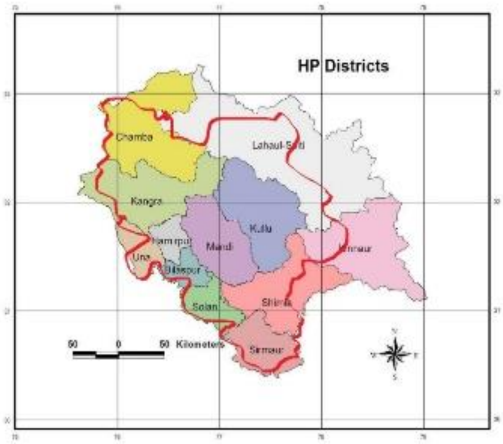


Plate 3.4 Breeding tract of Gaddi goats in H.P. depicted by red colour

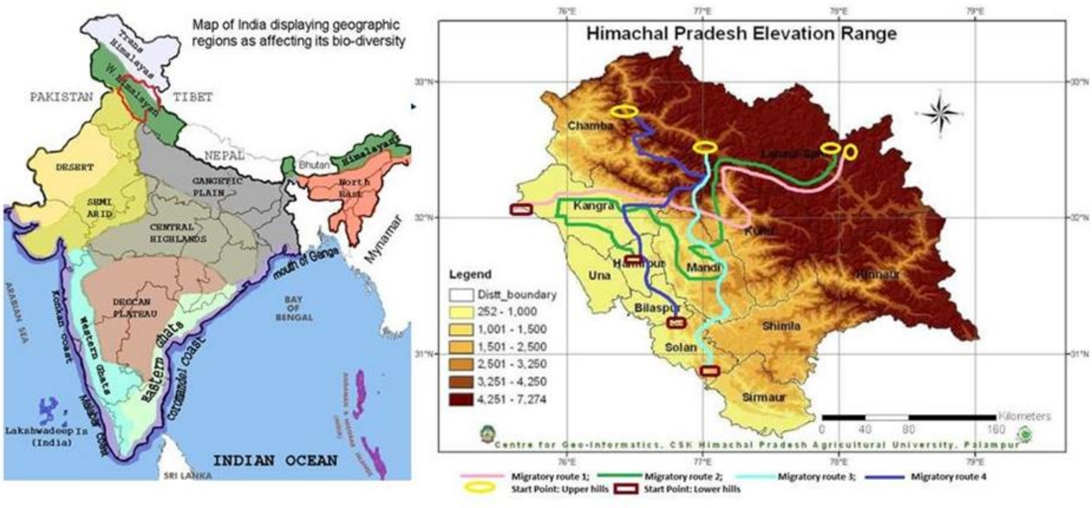


Figure 3.1: Map depicting seasonal migratory routes of adopted Gaddi goat flocks considered for association studies

3.1.3 Production system and breed utility: "Gaddi", breed of goats is exceptionally suited to the prevalent transitory sheep and goat production system in high altitude regions of H.P. These goats are raised under migratory/pastoralist framework, although some of small sized flocks (4 to 5 animals) are reared under stationary, semi intensive system. The flock size is highly variable ranging from 20 to 500 animals. The smaller flocks are stationary but, large flocks move in summer to high altitude alpine pastures and lower regions and valleys during winters. In migratory system, only grazing with no supplementary feeding is practiced. The major forage resource accessible during migration include smooth brome grass, *Bromus mimis, Dactylis glomerata and Festuca alpinus*, while under semi intensive, some supplementation of grains, concentrates, salt etc. is given. During winters, the animals are fed stored feed and fodder. Gaddi goats are mainly utilized for chevon production and as pack animals especially males for transportation of material in hilly terrain. The hair are utilized for making floor rugs, ropes and other items of household utility.

3.1.4 Population Status: The goat population of the state of Himachal Pradesh is 11.20 lakh (19^{th} livestock census, 2012) with Gaddi goats constituting 60 to 65% percent of the total goat population. These goats are raised by traditional "Gaddi" tribe as migratory flocks on sub mountainous pastures during winters and high altitude alpine pastures during summers.

3.2 Molecular genetic characterization of Gaddi goat breed: The molecular genetic characterization of Gaddi goats as envisaged in the present study involved the following steps:

- Selection of experimental animals for the study.
- Collection of blood samples.
- Isolation of genomic DNA from the blood samples.
- Qualitative and quantitative analysis of the isolated DNA samples.
- PCR amplification of genomic DNA.
- Visualizing PCR product on agarose gel electrophoresis with Gel Doc apparatus.
- Polymorphism detection of PCR amplified product using RFLP and SSCP techniques.
- Association studies and statistical analysis of data.

3.2.1 Experimental animals and source of data: The experimental animals for the present investigation were taken from various sources in the breeding tract of Gaddi goats in Himachal Pradesh. Blood samples for the study were gathered from adopted migratory flocks under AICRP on goat improvement and Buck rearing center of CSKHPKV, Palampur. Blood samples were collected from 63 genetically unrelated, randomly selected animals from the breeding tract as per the guidelines of FAO (1995) for measurement of domestic animal diversity (MoDAD).

Biometrical measurements on Gaddi goats were obtained for association studies from the data maintained on animals under the said project. The information on growth records at different ages for each animal that was considered for association studies was collected (body weight, body length, body height and heart girth).

3.2.2 Collection of blood samples: 5 to 10 ml of venous blood was collected from the jugular vein of 63 genetically unrelated animals of different sex and age groups in sterile vacutainers containing ethylene-diamine tetra acetic acid (EDTA) as anticoagulant. After collection, the tubes were firmly capped and shaken delicately to facilitate thorough mixing of blood with the anti-coagulant. The tubes containing blood samples were transported to the laboratory in an icebox containing ice packs and kept in the deep freeze at –20°C for further storage under frozen conditions for subsequent analysis.

3.2.3 Isolation of genomic DNA: Genomic DNA is an essential test material for undertaking the molecular genetic characterization. The results of such experiments to a great extent depend upon the quality of extracted DNA. The quality of genomic DNA implies that it should be of high molecular weight (>100 kb) and free from impurities like protein, RNA, organic solvents or salt. Any biological material, for example, blood, hair follicles, tissue, semen can be utilized for extraction of genomic DNA. The most favored material is the peripheral blood from live animal as DNA of high quality can be effectively obtained from it.

Genomic DNA was isolated by utilizing phenol chloroform extraction technique, as described by Sambrook and Russel (2001), with minor modifications. Generally, the procedure for genomic DNA isolation from mammalian samples includes lysis of cells, digestion of proteins which are released by cell lysis with Proteinase K, extraction of DNA with phenol and precipitation of DNA with alcohol as per the methodology presented underneath.

Phenol-chloroform extraction technique for genomic DNA isolation:

(a) Lysis cycle:

1. 5 to 10 ml of frozen blood samples were thawed in water bath at room temperature (25°C to 30°C), then transferred to the Oakridge tubes and two volumes of ice cold 1X RBC lysis buffer was added (Appendix-I) to the whole blood sample and mixed well by vortexing and kept on ice for 10 min.
2. It was then subjected to centrifugation at 10,000 rpm at 4°C for 10 min.
3. The supernatant containing the lysed RBC's was decanted and these steps were repeated two to three times for complete lysis of RBCs until a white DNA pellet was obtained.

(b) Proteinase-K digestion:

1. Pellet was resuspended in 5 ml of extraction buffer (Appendix-I) and mixed well by vortexing.
2. SDS (final conc. 0.5%) @ 200 µl per 10 ml was added and Proteinase-K (final conc. 100µg /ml) @ 40 µl (20 mg/ml stock solution) per 10 ml of blood sample was added.
3. It was mixed gently and incubated at 56°C overnight.

(c) Phenol extraction:

1. After incubation, equal volume of Tris saturated phenol (pH 8.0) was added to the above mixture and was mixed well by repeated inversions for 5 to 10 min. to form a uniform suspension.
2. The mixture was centrifuged at 10,000 rpm for 15 min. at 25^0C and the upper aqueous phase (containing DNA) was aspirated gently by using sterile pasture pipette and transferred to the fresh Oakridge tube and a mixture containing equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and mixed well by repeated inversions followed by centrifugation at 10,000 rpm for 15 min. at 20^0C.
3. Again, the upper aqueous phase was carefully aspirated using sterile pasture pipette and transferred to the fresh Oakridge tube and equal volume of chloroform: isoamylalcohol (24: 1) was added. The contents were then mixed well for 5 to 10 min. and centrifuged as in the previous step.
4. Upper aqueous phase was collected in the fresh Borosil glass tube.

(d) Precipitation and washing of DNA:

1. To this aqueous phase, 1/10th volume of Sodium Acetate (3 M, pH 5.2) to a final concentration of 0.33 M was added (Appendix-I) and double volume of ice cold ethanol was added. The contents were mixed well and tilted gently to obtain the precipitated DNA which was visible as white stringy threads.
2. DNA was spooled out with bent pasture pipette and transferred to an eppendorf tube. The precipitated DNA was washed twice to thrice with 70% ethanol and air dried at room temperature.
3. DNA pellet was dissolved in 500 µl of TE buffer (pH 8.0) and kept at 65°C for 20 to 30 min. and finally stored at -20°C for future use.

3.2.4 Qualitative and quantitative assessment of DNA: For evaluating the qualiy of extracted DNA, agarose gel electrophoresis was carried out. Quality was evaluated through 0.7 percent horizontal agarose gel electrophoresis (Hoefer HE 33) as mentioned underneath:

1. Agarose (0.7% w/v) was boiled in 1X TBE buffer (Appendix-II). After boiling, it was cooled to 50 to 55^{o}C and afterward ethidium bromide @ 2µl/100ml was added to it. The gel was delicately poured into the casting tray, avoiding bubble formation and was allowed to solidify at room temperature.
2. The comb was removed after the solidification of the gel, the gel casting plate was expelled from the gasket and submerged in gel electrophoresis unit containing 1X TBE buffer.
3. Samples for loading were set up by mixing 3µl of genomic DNA, 7µl of distilled water and 2µl of 6X loading dye. Samples were carefully stacked in the wells.
4. Electrophoresis was performed at 2 to 5 volts/cm for 60 to 90 min. until the satisfactory migration of samples and ladder occurred and then gel was visualized and captured by Gel Documentation System (Bio-Rad).

The genomic DNA samples having sharp bands without smearing were considered of good quality and used in further experimental work.

3.2.5 Estimation of DNA concentration and purity: Quantitative analysis of DNA (concentration) was done by Nano Drop method, spectrophotometrically. 1µl of DNA was used for evaluating the concentration. The ratio between OD-260 and OD-280 was used to evaluate DNA samples by using the formula:

DNA concentration (ug/ml) = OD-260 x Dilution factor x 50

The samples with OD-260/280 ratio between 1.7 to 1.9 were used in subsequent experiments.

3.2.6 Dilution of DNA: After assessing the quality of DNA at 0.7% agarose gel electrophoresis and evaluating the concentration, dilution was carried out on each DNA sample to obtain the final concentration of 100ng/µl by adding molecular biology grade nuclease free water.

3.2.7 Primers utilized for amplification of growth related genes and PCR conditions: For amplification study of *IGF1, GH1, GH2, GH3 and GH4* genes, details of selected primer sequences, product size and amplification conditions are depicted in Table 3.1. Product size of amplicon, restriction digest conditions are given in Table 3.2. The primers were synthesized from IDT Inc., based on the literature review for selected genes. PCR conditions for different genes studied are presented in Table 3.3.

Table 3.1: Details of Primer sequences and annealing temperatures for the amplification of *IGF-1* gene and *GH* gene fragments

Gene/ Fragment	Primer sequence (5'-3')	Product Size (bp)	Annealing temperature	Reference
IGF1	F-CACAGCGTATTATCCCAC R-GACACTATGAGCCAGAAG	363	56°C (35 cycles for 2 min.)	Liu et al. (2012)
GH1	F-CTCTGCCTGCCCTGGACT R-GGAGAAGCAGAAGGCAACC	422	54°C (30 cycles for 1 min.)	Singh et al. (2015)
GH2	F-TCAGCAGAGTCTTCACCAAC R-CAACAACGCCATCCTCAC	116	65°C to 52°C (35 cycles for 30 sec.)	Hua et al. (2009)
GH3	F-CTAGCAGCCCAGTCTTGACC R-GGGGAGGGGTAACAACAGAT	389	56°C (29 cycles for 30 sec.)	Wickramaratne et al. (2010)
GH4	F-GCACATCCCCTTCTCTGTGA R-CTTCCCACTCTTGGAGGCTA	181	56°C (29 cycles for 30 sec.)	Wickramaratne et al. (2010)

Table 3.2: Product size and PCR-RFLP parameters for amplification of selected genes

Gene/ Fragment	**Product size (bp)**	**Restriction enzyme used**	**Digest conditions**	**Expected fragments after digestion (bp)**
IGF1	363	RFLP-*Hae III*	37°C for 6-12 hrs	363, 264 and 99
GH1	422	RFLP-*Hae III*	37°C for 6-12 hrs	422, 366 and 56
GH2	116	RFLP-*Hae III*	37°C for 6-12 hrs	116, 88 and 28

Table 3.3: PCR conditions of different growth related genes

Gene/ Fragment	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final Extension**
IGF-1	94°C, 5 min	94°C, 1 min	56°C (35 cycles for 2 min.)	72°C, 1 min	72°C, 5 min
GH1	94°C, 10 min	94°C, 1 min	54°C (30 cycles for 1 min.)	72°C, 1 min	72°C, 10 min
GH2	94°C, 5 min	95°C, 30 sec	65°C to 52°C (35 cycles for 30 sec.)	72°C, 45 sec	72°C, 7 min
GH3	94°C, 5 min	94°C, 1 min	56°C (29 cycles for 30 sec.)	72°C, 1 min	72°C, 5 min
GH4	94°C, 5 min	94°C, 1 min	56°C (29 cycles for 30 sec.)	72°C, 1 min	72°C, 5 min

3.2.8 PCR reaction mix:

The following reaction mix was utilized for amplification (Promega, USA)

Template DNA	:	1µl
Forward primer	:	1µl
Reverse primer	:	1µl
5X Flexi buffer	:	10µl
dNTP mix (10mM)	:	1µl
$MgCl_2$ (25mM)	:	4 µl
Taq DNA polymerase (5 units/µl)	:	0.25µl
Autoclaved distilled Water	:	31.75µl
Total	:	50µl

3.3 Techniques utilized:

3.3.1 Polymerase Chain Reaction:

PCR has turned out to be one of the widely utilized techniques in molecular science to make multiple copies of particular DNA segment. Using PCR, a specific DNA sequence is exponentially amplified by the concurrent primer extension of complementary strands of DNA. Although the principle of PCR had been discussed by Khorana and his colleagues a decade earlier (Kleppe et al. 1971; Panet and Khorana 1974), the actual method was developed and named by Karry Mullis and coworkers of the Cetus corporation in 1983 (Mullis and Faloona 1987). Inspite of its high sensitivity, application of PCR was restricted until the thermostable DNA polymerase such as *Taq* polymerase, isolated from *Thermus aquaticus* became widely accessible (Chien et al. 1976).

After optimization of standard protocol, selected genes for the present study were amplified in programmable thermal cycler. The amplicon size and optimized annealing temperatures for amplification of different genes utilized is given in Table 3.1.

3.3.1.1 Components of PCR reaction mixture:

3.3.1.1.1 Template DNA: Usually, the amount of template DNA is in the range of 0.01 to 1 ng for plasmid or phage DNA and 0.1 to 1 µg for genomic DNA for a total 50µl reaction mix. Higher quantity of template DNA usually leads to increment in the yield of non-specific PCR products but if the fidelity of synthesis is essential, maximal suitable template DNA amounts, together with a predetermined number of PCR cycles should be utilized to increase the level of "correct" PCR products. Nearly all routine techniques are efficient for template DNA purification. Since, even small amounts of reagents used in DNA purification methods (phenol, EDTA, proteinase K etc.) strongly restrain *Taq* DNA polymerase so ethanol precipitation of DNA and repetitive treatment of DNA pellets with 70% (v/v) ethanol is generally successful in removing traces of contaminants from DNA samples.

3.3.1.1.2 Primers (Guidelines for selection of primers):

a. PCR primers are generally 15 to 30 nucleotides long. Higher specificity is provided by primers of longer lengths.
b. In order to maintain a strategic distance from primer-dimer and hairpin formation, the primers should not be self-complementary or complementary to any other primer in the reaction mixture.
c. The G and C nucleotides should be distributed uniformly all through the primer and the GC content should be 40 to 60%. More than three G or C nucleotides at 3’ end

of the primer should be avoided, as non-specific priming may happen.

d. The melting temperature of flanking primers should not vary by more than 5°C, so the GC content and length must be chosen accordingly.
e. All possible complementary sites between template DNA and primers should be noted.
f. If primers are degenerated, at least 3 conservative nucleotides must be located at the primer 3’end.

3.3.1.1.3 Estimation of the melting and annealing temperatures of primers:

The approximate melting temperature (Tm) is determined utilizing the following formula, if the primers are shorter than 25 nucleotides in length:

Tm = 4 (G + C) +2 (A + T)

G, C, A and T are the nucleotides present in the primers.

If the primers are longer than 25 nucleotides, the melting temperature should be determined using specialized PC programs where the interactions of adjacent or nearby bases and the impacts of salt concentration etc. are assessed. Annealing temperature should be around 3 to 5°C lower than melting temperature.

3.3.1.1.4 $MgCl_2$ concentration: The ideal concentration of $MgCl_2$ must be chosen because Mg^{++} ions form structure complex with dNTPs, primers and DNA templates. Trace amounts of Mg^{++} ions result in a low yield of PCR product and too many ions promote mis-incorporation and lead to increment in the yield of non- specific products. Lower Mg ion concentration is desirable when fidelity of DNA synthesis is critical. Under the specified standard conditions of PCR, recommended range of $MgCl_2$ is 1 to 4 µl. If the DNA samples contain EDTA or other chelaters, the $MgCl_2$ concentration in the reaction mixture should be raised relatively.

3.3.1.1.5 dNTPs: It is critical to have equal concentration of each dNTP (dATP, dCTP, dGTP & dTTP) as any inaccuracy in concentration of even a single dNTP increases the mis-incorporation percentage. The concentration of each dNTP in the reaction mixture is usually 200 mM. At the point, when maximum fidelity of the PCR procedure is essential, the final concentration of dNTPs should be 1050 µl, since the fidelity of DNA synthesis is maximal in this concentration range. Likewise, the concentration of $MgCl_2$ should be chosen empirically, starting from 0.1 mM and further expanding in 0.l mM steps, until adequate yield of PCR product is obtained.

3.3.1.1.6 *Taq* DNA polymerase: Typically, the amount of *Taq* DNA polymerase utilized in 50 µl reaction mix ranges from 1 to1.5µl. Higher concentration of *Taq* DNA polymerase may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g. if the template DNA used is not highly purified), higher quantities of *Taq* DNA polymerase (2 to 3 µl) may be desirable to get a superior yield of amplification products.

3.3.1.1.7 Cyclic conditions and reaction overlay: Parameters of amplification depend significantly on template DNA, primers and amplification operators utilized in the PCR process. If necessary, the reaction mixture can be overlaid with mineral oil or paraffin (melting temperature 50°C to 60°C) of special PCR grade. One half of the total reaction volume is normally adequate.

3.3.1.2 PCR product documentation by agarose gel electrophoresis: The amplified PCR products were checked on 2% (w/v) agorose gel (2g agarose in 100 ml 1X TBE buffer) , to verify the amplification of target sequences. 5 µl of PCR product well mixed with 2µl of 6X gel loading dye were loaded in separate wells and 100 bp DNA ladder was loaded along with the products to determine the optimized size. The horizontal electrophoresis chamber was closed with the lid and power supply turned on to 100 volts and 200 mA for 45 minutes (2 to 5 V/cm). Power supply was then turned off at the end of the run and after placing the gel carefully on transilluminator, the amplified products were visualized under UV light and photographed with Gel Doc system.

3.3.2 Gradient PCR: Problems with amplification of a particular DNA fragment using PCR is a normal event in the laboratory. Non-specific secondary bands may appear after the PCR reaction, which hinder or even counteract the further experimental analysis (cycle sequencing, mutation detection, etc.) or an obvious appraisal of the PCR results. To avoid such instances, PCR conditions must be optimized. Gradient PCR is a method that permits the exact determination of an optimal annealing temperature using minimal number of steps. This optimization can frequently be accomplished in one experiment. To determine the best annealing temperature for each primer set, the gradient PCR was carried out on the Takara mastercycler.

Procedure: 1. PCR was performed in a total reaction mix of 50 µl containing approximately 100 ng genomic DNA, 5X PCR buffer, 2.0 to 3.0 mM MgCl2 (depending on each primer set), 200µM of each dNTPs, 100 pmol (optimized concentration) of each primer and 1.5 U of Taq polymerase (Promega, USA).

2. The test parameters selected were an initial denaturation at 95 °C for 7 min, 35 cycles of final denaturation at 95 °C for 45 sec, optimized annealing temperature which is specific for different genes used with gradient±2, 1 min at 72°C for extension followed by final extension of 10 min at 72°C.

3. Finally, 8-10 µl PCR products were loaded on 2% standard agarose gel and was run at 70 V for 60 min. The fragments were visualized by staining with ethidium bromide under a UV transilluminator (Gel Doc, Bio-Rad).

3.3.3 PCR-RFLP: RFLP is a method in molecular biology which exploits variations in homologous DNA sequences, known as polymorphisms, in order to differentiate individuals, populations and species or to illustrate the location of genes within a sequence. In RFLP analysis, a DNA sample is digested into fragments by one or more restriction enzymes and the restriction fragments obtained are then separated by agarose gel electrophoresis according to their size. The basic procedure for the detection of RFLPs involves fragmenting a DNA sample with the application of a restriction enzyme, which can selectively cleave a DNA molecule wherever a short, specific sequence is recognized in a process known as restriction digest. The DNA fragments produced by the digest are then resolved on 2.5 to 3% agarose gel horizontal electrophoresis and visualized by ethidium bromide staining.

The agarose gel electrophoresis was performed in 1X TBE buffer at 100 volts for 60 to 90 min. till complete separation. The restriction enzyme digested gene fragments were examined under UV transilluminator and photographs were taken with gel documentation system. Depending on the number of digested fragments visualized on the gel, the genotypes were recorded accordingly.

RFLP analysis: PCR products (15 µl) for specific genes were digested by adding 1 unit of respective restriction enzyme (Fermentas, Germany) specific for each gene along with distilled water and 10X digest buffer, making a final reaction volume of 25µl. The reaction mixture was incubated in water bath at 37°C as per the recommendations for digest conditions of each enzyme. After restriction digestion, the restricted fragments were analyzed by 2.5% agarose gel electrophoresis and stained with ethidium bromide to visualize under UV illuminator.

3.3.4 Single strand conformation polymorphism:

SSCP is a conformational distinction of single-stranded nucleotide sequences of indistinguisable length as induced by differences in the sequences under certain experimental conditions. This property enables sequences to be recognized by gel electrophoresis, which separates fragments as per their diverse conformations. A single nucleotide difference in a specific sequence, as found in a double-stranded DNA, can't be recognized by electrophoresis, since the physical properties of the double strands are almost identical for both alleles. After denaturation, single-stranded DNA undergoes a 3-dimensional folding and may assume an exceptional conformational state based on its DNA sequence. The difference in shape between two single-stranded DNA strands with different sequences can make them relocate distinctively on an electrophoresis gel, despite the fact that the quantity of nucleotides is the same.

PAGE is utilized for DNA analysis with specific buffers, without urea. In non-denaturing PAGE, the components that form the matrix are acrylamide monomers, N, N-methylene bisacrylamide (Bis), ammonium persulphate (APS) and tetramethylethylenediamine (TEMED). The acrylamide and bisacrylamide was used in the ratio of 49:1, utilizing autoclaved HPLC water. Acrylamide-bisacrylamide solution (49:1) was dissolved completely using magnetic stirrer and kept in the refrigerator for further use. Free radicals are generated when ammonium persulphate (APS) is dissolved in water. These free radicals activate acrylamide monomers inducing them to react with other molecules of acrylamide forming long chains, which form cross-links with Bis. As a catalyst for gel formation, TEMED was used in light of its capacity to exist in free radical form.

Procedure: The procedure of PCR-SSCP includes the following steps:

Amplification of specific gene fragments

Resolution in non-denaturing PAGE

Visualization using silver staining technique

The standard protocol for PCR amplification of all the SSCP primers utilized is same with the exception of the annealing temperatures, which are specific for various primers.

Different buffer systems and chemicals needed for SSCP analysis are depicted in Appendix-I.

Preparation of glass plates: The SSCP analysis of PCR amplified gene fragments was carried out by using Hoefer vertical gel electrophoresis apparatus. Two thoroughly cleaned and grease free glass plates were used.The glass plates, after washing were wiped with 70% alcohol and then air-dried. The cleaning treatment was similar for comb and spacers too.

Assembling and preparation of SSCP gel: The gel sandwich was assembled on a clean surface by laying down the long rectangular plate first, then two spacers of equivalent thickness, along the long edges of plate were placed. Finally, the short plate was placed over the long rectangular plate. The two glass plates with spacers between them were aligned properly by fixing the bulldog clamps and the sandwiched gel plates were fitted in the stand with the help of screw clamps. The comb with 20 wells was embedded from the top of the gel sandwich and quickly bulldog clamps were connected over the plates containing comb to make sharp wells. The gel sandwich was kept in inclined position and the filtered solution of acrylamide, distilled water, glycerol, 10X TBE buffer well mixed with 80 µl APS and 200 µl TEMED infused between the two glass plates and allowed to polymerize for 20 to 30 min. 12% non-denaturing PAGE solution with a volume of 100 ml is prepared by mixing 12 ml of Acrylamide-bisacrylamide, 5ml 10 X TBE, 3ml glycerol and 30 ml molecular biology grade water.

Gel loading in vertical assembly: After the gel was sufficiently polymerized, the comb was removed and wells were infused with 1X TBE buffer with the help of sterile syringe. The gel sandwich was placed in the vertical electrophoresis unit, containing 1X TBE buffer. After proper placement, the gel was given pre-run at 200 volts at constant temperature for a period of 45 min. or so. Cool water circulation should be maintained to avoid excessive heating of the core of the assembly and to maintain a constant temperature.

Sample preparation for gel loading: 4 to 8 ml of PCR products were taken in different reaction tubes, after the assessment of quality of PCR amplified products and each reaction tube was diluted with denaturing dye. Denaturing dye was added twice the amount of the PCR product. Samples were denatured for 10 min. at 95°C in the thermal cycler. After denaturation, the samples were immediately placed in ice-chilled box and kept at -20°C for 8 to 10 min. After thawing, the denatured samples were loaded in the wells of the gel. Electrophoresis was performed in 1 X Tris borate-EDTA buffer (pH 8.3) for 12 to 16 hours at 10 to 12.5 volts/cm, depending on the optimal conditions for each primer used. After the electrophoresis was completed, the glass plates were removed carefully from the assembly and the gel was subjected to silver staining to visualize the SSCP band patterns

Silver staining technique:

a. **Nucleic acid fixation:** To stain the gel, it was immersed in clean plastic staining tray of appropriate size filled with fixer solution (add 7.5% v/v dilute glacial acetic acid in distilled water to make final volume of 500ml) for 20 to 30 min. for fixing DNA bands in gel so as to avoid diffusion of the DNA bands and subsequent image blurring. The gel was agitated slowly by placing it on platform rocker. Fixation of DNA is crucial for stain sensitivity and to make DNA molecules immobile in gel. It also helps in removing unwanted chemicals.

b. **Gel washing:** Following fixation, glacial acetic acid was decanted and sufficient quantity (500 ml) of distilled water was poured in the staining tray and rinsed thoroughly by placing the tray on platform rocker. Wash steps were repeated 2 to 3 times for a total period of 15 to 20 min. Washing removes trace substances and acid that may otherwise, hinder the staining process and also provides clear background to the final stain.

c. **Preparation of fresh chemicals:** Meanwhile, various reagents used in the staining process were prepared: formaldehyde solution (15ml formaldehyde to 85 ml distilled water) and 500 ml of 0.1% silver nitrate solution (0.1g $AgNO_3$ in 100 ml distilled water) were prepared. Developer solution (3g sodium carbonate in 100 ml distilled water), sodium thiosulphate stock solution (0.2g sodium thiosulphate in 50ml distilled water) and developer stop solution (7.5% dilute glacial acetic acid in distilled water) were also prepared.

d. **Formaldehyde pre-treatment:** Sufficient formaldehyde solution was poured over the gel to cover it completely and placed on rocker for 20 min. It is essential for maximum image contrast and stain sensitivity.

e. **Silver impregnation:** Sufficient silver solution was added to cover the gel properly and placed on rocker for 60 to 90 min., after which it was decanted and gel was rinsed briefly with distilled water to remove residual silver solution.

f. **Image development:** Chilled developer solution was added to the staining tray and agitated continuously throughout the image development. Developer solution should be kept cold to control the rate of image development. Image development takes about 3 to 5 min., depending upon the gel thickness.

g. **Stopping the reaction:** After decanting developer solution, developer stop solution was added and gel was allowed to sit in developer stop solution for 5 to 10 min.

h. The developer solution was decanted and gel was rinsed with distilled water and finally the gel was photographed.

3.4 Determination of banding patterns/genotypes: After amplification of target fragments, banding patterns or genotypes of selected genes were determined by utilizing the methodology as below:

PCR: Genotyping was performed by visualizing the amplified products on agarose gel (horizontal electrophoresis).

SSCP: Recognition of different patterns was accomplished by visualizing the amplified products after running SSCP gel (vertical electrophoresis) and staining with silver nitrate. The most widely recognized banding pattern was identified and named as AB, if there are more bands in addition to the common bands, they were marked as AA, BB and so on, depending on the band pattern. Representative samples portraying unique band patterns in comparison to other common pattern were chosen for subsequent sequence analysis.

3.4.1 Sequencing of selected samples: Representative samples, with unique band patterns after RFLP/ SSCP analysis were sent for sequencing. Sequencing was performed with both forward and reverse primers. The sequences received were subjected to editing and adjusted to identify the position of SNPs.

3.5 Statistical Analysis of data:

Genetic diversity analysis and test for Hardy-Weinberg equilibrium, population genetic indices, gene and genotypic frequencies, gene heterozygosity (He), polymorphic information content (PIC), effective allele numbers (Ne) and inbreeding estimates were calculated using Popgene 32 (Ver. 1.32), Microsoft Windows-based freeware for population genetic analysis (Yeh et al. 1999). Chi square (χ^2) analysis was applied to test population for genetic equilibrium utilizing Popgene 32 software. Polymorphic Information Content (PIC) value was determined by the formula given by Botstein et al. (1980) as:

$$PIC = 1 - \sum_{i=1}^{n} P i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2P_j^2$$

where, P_i and P_j are the frequencies of i^{th} and j^{th} alleles respectively at a particular locus with n alleles in a population.

3.6 Association studies

3.6.1 Source of data

For Gaddi goats, the data for the study was collected from adopted migratory flocks under AICRP (Gaddi field unit) at CSKHPKV, Palampur. Biometrical measurements of Gaddi goats were collected for association and statistical analysis.

3.6.2 Association of growth related genes in Gaddi goats

The following fixed effects model was used for analysis of growth traits in Gaddi goats and least square means were used for multiple comparison of body weights and body measurements for different genotypes.

$$Y_{ijkl} = \mu + b_i + Bj + X_k + G_l + e_{ijkl}$$

where, Y_{ijkl} is the phenotypic value of growth traits (body weight, body length, heart girth and body height) measured on $ijkl^{th}$ animal.

μ is population mean

B_j is fixed effect associated with i^{th} herd

X_k is fixed effect associated with season and sex

G_l is the fixed effect of the l^{th} genotype (l = 1, 2, 3)

e_{ijkl} is the random residual effect of each observation

Analysis was performed using the general linear model procedure of SAS (ver. 9.3) (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were conducted using a least significant difference test.

Chapter-4

RESULTS AND DISCUSSION

The present study was aimed at molecular genetic characterization of certain growth related genes in Gaddi goats and study their association with growth traits. The *IGF-1* gene and four fragments of *GH* gene (*GH1, GH2, GH3* and *GH4*) were analysed for genetic diversity. The genomic DNA, isolated from blood samples collected at random from 63 genetically unrelated animals of Gaddi goat breed were subjected to PCR amplification of studied growth genes. All the amplified genes were resolved on 2% agorose gel (w/v) and the product size was assessed by comparing either with 50 or 100 bp DNA ladder (co-migrated in the same gel). RFLP analysis of *IGF-1, GH1* and *GH2* was done by *HaeIII* RE and *GH3* and *GH4* gene fragments were subjected to SSCP analysis and were resolved on 12% PAGE. As per the number of bands observed, the genotypes were designated as AA, BB and AB and the representative samples were sent for sequencing. Many a times, these bands appeared associated with 'stutter' (shadow) bands, such samples were improved by optimization of PCR conditions and protocol. The samples were then analyzed for total number of alleles for a particular gene, their frequency, heterozygosity variables, F-stat, PIC and to determine HWE within the population.

4.1 Polymorphism of *IGF-1* gene

Somatotropic axis is one of the major hormonal systems regulating growth, which is a complex process that requires the coordinated activity of various hormones in different neuro- endocrine pathways and further expressed as a phenotypic trait. The somatotropic axis, mainly consisting of *GH*, *IGFs* (*IGF-1* and *IGF-2*) and their associated receptors and carrier proteins, plays a significant role in postnatal growth and development in mammals including different livestock species (Shoshana et al. 2000; Burkhard et al. 2005). Insulin-like growth factor-1 (*IGF-1*) is one of the essential components of the somatotropic axis that participates in the growth and function of almost every organ in the body (Daughaday and Rotwein 1989) and has a key role in a variety of physiological and biological processes such as protein synthesis, embryogenesis, skeletal growth, reproduction and lactation (Adam et al. 2000). In goats, *IGF-1* gene is located on chromosome-5, consisting of six exons and five intervening introns (Mikawa et al. 1995).

The *IGF-1* gene has been considered as a candidate marker associated with growth traits in different livestock species (Thue and Buchanan 2002; Zhang et al. 2008; Wu-Jun et al. 2010). Association between *IGF-1* gene variability and various traits of economic importance in goats had been determined by some researchers utilizing PCR-RFLP method. In the present study, a 363 bp amplicon was yielded by PCR amplification of *IGF-1* locus (Plate 4.1).

4.1.1 PCR-RFLP analysis

PCR-RFLP analysis of amplified products with *HaeIII* RE exhibited three fragments of 363 bp, 264 bp and 99 bp (Plate 4.2) that revealed two genotypes viz., AB and BB. The allele and corresponding genotype frequencies for the analysed population of Gaddi goats are presented in Table 4.1. The allele frequencies for A and B alleles were estimated as 0.25 and 0.75, respectively. The frequency of AB and BB genotypes were 0.51 and 0.49. Similarly, Deng et al. (2010) observed genotype frequencies of AG and GG for P1 locus as 0.15 and 0.85 in Guanzhong goats and 0.21 and 0.79 in Xinong Saanen goats. For P4 locus, genotype frequencies for CC, CG and GG genotypes were 0.10, 0.16 and 0.74 in Guanzhong breed and 0.08, 0.43 and 0.49 in Xinong Saanen breed. In another study of *IGF-I* gene in Nanjiang Huang goats, Wang et al. (2011) reported genotype frequencies of GG (0.59), GA (0.372) and AA (0.038) and allele frequencies of G (0.78) and A (0.22). Alakilli et al. (2012) also reported similar findings in Egyptian goat breeds with allele frequencies of A and B as 0.731 and 0.269 in Barki, 0.615 and 0.385 in Ardi, 0.473 and 0.527 in Masri and 0.432 and 0.568 in Zaribi goat breeds.

Similar to the present investigation, this polymorphism had been reported by Kurdistani et al. (2013) in Iranian Kurdish goats with no AA genotype and genotype frequencies of GA and GG were 0.04 and 0.96, respectively. Othman et al. (2016) also observed similar *IGF-1* gene polymorphism in Egyptian small ruminant breeds. Allele frequencies for C and G alleles varied from 0.69 to 0.72 and 0.28 to 0.31, respectively while genotype frequencies for CC, GC and GG genotypes varied from 0.09 to 0.18, 0.22 to 0.38 and 0.53 to 0.59 in Egyptian sheep and goat breeds, respectively. Rasouli et al. (2017) documented polymorphic pattern in *IGF-1* gene in Markhoz goats. The allele frequency for G was 0.89 with GG pattern as the most frequent genotype (81%). Consequently, the GG and AA genotypes were detected as wild and mutant types in Markhoz goats. Many other studies by Zhang et al. (2008) in Chinese goat breeds, Tahmoorespur et al. (2009) in Iranian Baluchi sheep, Qiong et al. (2011) in Chinese goats, Wang et al. (2011) in Nanjiang Huang goat breed and Sharma et al. (2013) in Indian some goat breeds also reported polymorphic patterns at *IGF-1* locus.

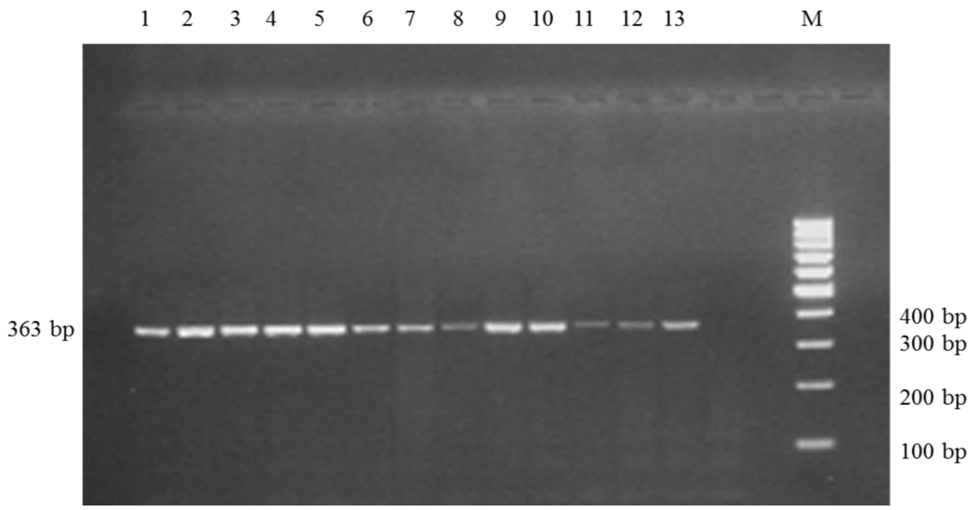


Plate 4.1: Electrophoretic profile of amplified *IGF-1* in 2% agarose gel. Lanes 1-13 represented amplicon size of 363 bp. M represented 100 bp DNA marker

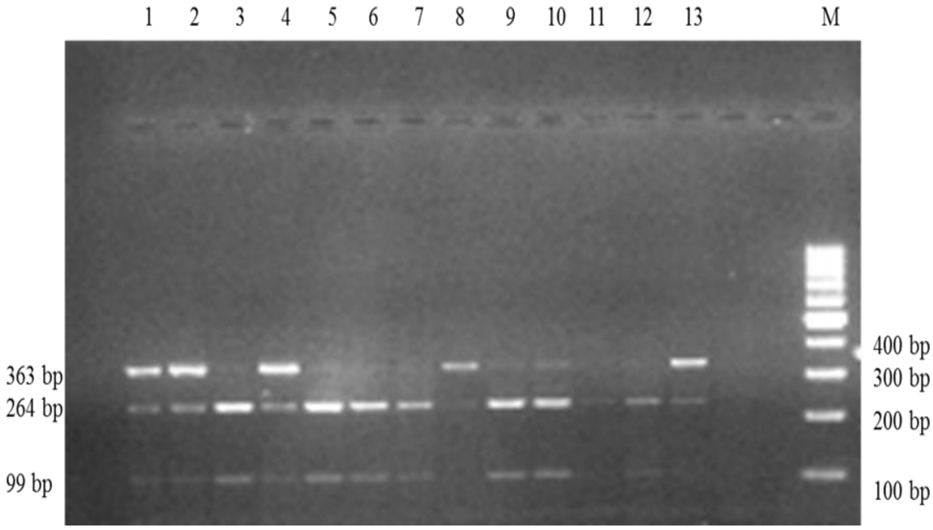


Plate 4.2: Electrophoretic pattern obtained after *HaeIII* digestion of *IGF-1* gene. Lanes 1,2,4 and 8-13 represented AB genotype. Lanes 3 and 5-7 represented BB genotype. M represented 100 bp DNA marker

Table 4.1: Allele and genotype frequencies for *IGF-1* gene in Gaddi goats

N	Allele frequencies		Genotypic frequencies	
	A	**B**	**AB**	**BB**
63	0.25	0.75	0.51 (32)	0.49(31)

Numbers in brackets represent the number of animals belonging to the respective genotype

4.1.2 Genetic diversity analysis and test for Hardy-Weinberg Equilibrium (HWE)

The *IGF-1* gene showed polymorphism in the studied population of Gaddi goat breed. The measures of genetic diversity are represented in Table 4.2, indicating considerable variation at the studied locus. The estimates obtained for Ne, H_{obs}, H_{exp} and PIC were 1.61, 0.51, 0.38 and 0.31, respectively. In bi-allelic marker system, the maximum value for PIC is 0.375 (Shete et al. 2000). Therefore, PIC value of 0.31 implies the effectiveness of the marker in population studies and also revealed median level of polymorphism in investigated Gaddi goat population. The absence of heterozygous deficiency at studied locus for Gaddi goats is indicated by negative F_{IS} measure of -0.34. Wu-Jun et al. (2010) reported H_{obs}, H_{exp} and PIC values of 0.52, 0.47 and 0.36 in Chinese goats which were almost similar with the present study. The test for HWE indicated that Gaddi goat population was deviating significantly from equilibrium (P<0.01). Wu-Jun et al. (2010) in Chinese goats and Negahdary et al. (2013) in Makooei sheep breed of Iran reported genotypic distribution at *IGF-1* loci that were in agreement with HWE as seen in the present study.

Table 4.2: Measures of genetic diversity in *IGF-1* gene in Gaddi goats

Locus	No	Ne	H_{obs}	H_{exp}	PIC	I	F_{IS}	Chi-Square
IGF1	2	1.61	0.51	0.38	0.31	0.57	-0.34	7.03(<0.01) *

No: observed number of alleles, Ne: effective number of alleles, He: Heterozygosity, PIC: Polymorphic information content, F_{IS}: Fixation index. *P< 0.01 indicated significant deviation from HWE.

4.1.3 Sanger sequencing of PCR products

Purified PCR products of different genotypes obtained for *IGF-1* gene were got sequenced. The sequences thus, generated were subjected to BLAST analysis so as to ascertain whether they belong to *IGF-1* gene and to check the accuracy of PCR-RFLP detection. The chromatogram representing the substitution is depicted in Figure 4.1.

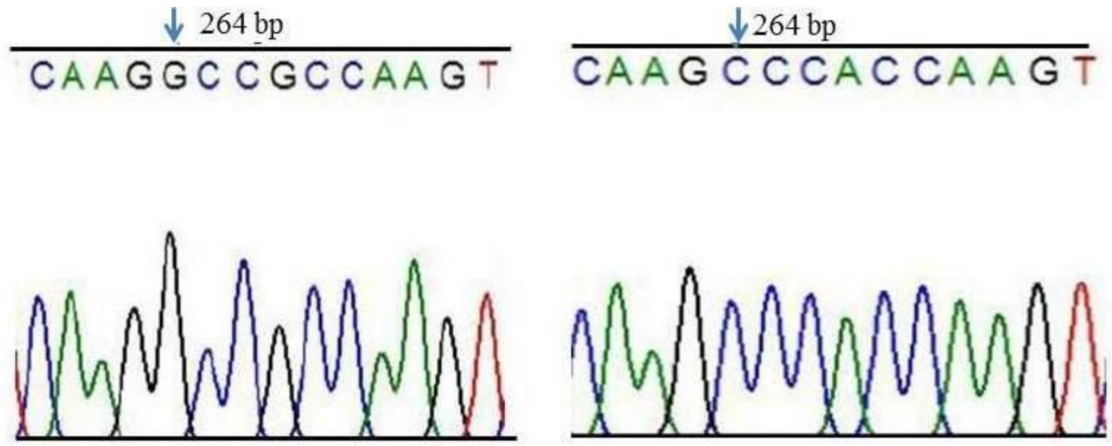


Figure 4.1: Chromatogram representing C/G substitution in *IGF-1* gene (*HaeIII* restriction digestion)

The sequence of amplified region was aligned with reference sequence from NCBI database using MegAlign and aligned sequences are presented in Fig. 4.2. The amplified region corresponding to exon-3 region of *IGF1* gene spans from 5489 bp to 5852 bp.

4.1.4 Association of *IGF-1* polymorphism with growth traits in Gaddi goat breed

The *IGF-1* genotypes of 63 animals belonging to Gaddi goat breed were screened along with the data on growth and body measurements traits (BW, BL, BH and HG) at different ages (3M, 6M, 9M, 12M). The association of *IGF-1* variants with growth traits is depicted in Table 4.3. The mean body weights (kg) at 3M, 6M, 9M and 12M were estimated as 17.34±0.42 and 17.44±0.48, 22.44±0.58 and 22.54±0.56, 25.54±0.50 and 26.73±0.57 and 28.26±0.59 and 29.87±0.59, respectively for the detected AB and BB genotypes. The corresponding body heights (cm) at 3M, 6M, 9M and 12M were 63.12±0.74 and 63.87±0.67, 66.48±0.59 and 66.72±0.40, 69.32±0.54 and 70.03±0.60 and 67.52±0.71 and 68.69±0.52, respectively. The body lengths (cm) at 3M, 6M, 9M and 12M were 65.25±0.74 and 65.74±0.78, 67.68±0.71 and 68.03±0.47, 71.35±0.49 and 72.69±0.53 and 68.74±0.65 and 69.09±0.75, respectively for different genotypes. The heart girth (cm) was 65.28±0.57 and 65.52±0.59, 67.00±0.64 and 67.97±0.48, 72.09±0.52 and 72.87±0.43 and 70.16±0.59 and 71.21±0.53 for 3M, 6M, 9M and 12M. No significant association could be observed between different variants of *IGF-1* gene and growth traits in Gaddi goats.

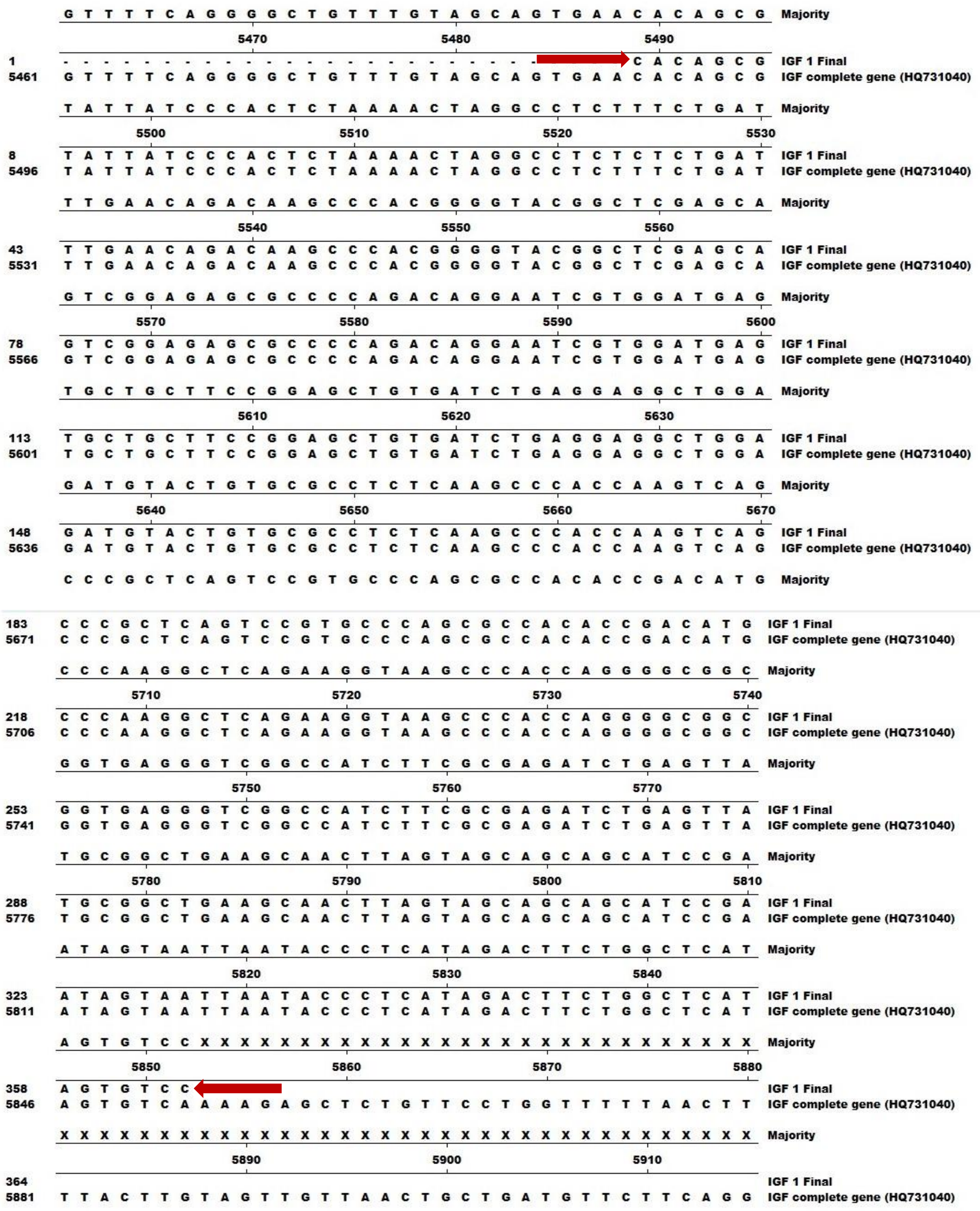


Figure 4.2: Sequence alignment using MegAlign for amplified region (5489 to 5852 bp) of *IGF-1 gene* corresponding to reference sequence of complete *IGF-1* gene

Table 4.3: Mean body weights and body measurements at different ages depicting association with observed *IGF-1* genotypes in Gaddi goats

Trait	*IGF1* genotype	
	AB	**BB**
Body weight (kg)		
3 month BW	17.44±0.48	17.34±0.42
6 month BW	22.44±0.58	22.54±0.56
9 month BW	26.73±0.57	25.54±0.50
12 month BW	29.87±0.59	28.26±0.59
Body height (cm)		
3 month BH	63.12±0.74	63.87±0.67
6 month BH	66.72±0.40	66.48±0.59
9 month BH	70.03±0.60	69.32±0.54
12 month BH	68.69±0.52	67.52±0.71
Body length (cm)		
3 month BL	65.25±0.74	65.74±0.78
6 month BL	68.03±0.47	67.68±0.71
9 month BL	72.69±0.53	71.35±0.49
12 month BL	69.09±0.75	68.74±0.65
Heart girth (cm)		
3 month HG	65.28±0.57	65.52±0.59
6 month HG	67.97±0.48	67.00±0.64
9 month HG	72.87±0.43	72.09±0.52
12 month HG	71.21±0.53	70.16±0.59

Table 4.4: F values for effect of *IGF-1* locus on body weights and body measurements at different ages in Gaddi goats

Trait	F- value	P>F
Body weight (kg)		
3 month BW	0.02	0.88
6 month BW	0.02	0.90
9 month BW	2.45	0.12
12 month BW	3.66	0.06
Body height (cm)		
3 month BH	0.55	0.46
6 month BH	0.11	0.74
9 month BH	0.76	0.38
12 month BH	1.77	0.19
Body length (cm)		
3 month BL	0.21	0.65
6 month BL	0.17	0.68
9 month BL	3.43	0.07
12 month BL	0.13	0.72
Heart girth (cm)		
3 month HG	0.08	0.77
6 month HG	1.49	0.23
9 month HG	1.32	0.25
12 month HG	1.79	0.19

The earlier studies carried on various breeds also reported that the results differ in association between *IGF-1* polymorphism and growth traits. The findings of the present study were similar to those reported by Wang et al. (2011) with no association between birth weight and *IGF-1* polymorphism in three indigenous Chinese goat breeds. Similarly, Gholibeikifard et al. (2013) did not observe the effect of SNPs at exon-3 of *IGF-1* gene on growth traits in Baluchi sheep. The results of present investigation were in agreement with those reported by Rasouli et al. (2017) in Markhoz goats with no significant effect of *IGF-1* polymorphism on birth weight and body weight at 6, 9 and 12 months of age.

However, Zhang et al. (2008) observed significant ($P<0.05$) association of *IGF-1* gene polymorphism with birth weight and body weights at 6 and 12 months in Nanjing Huang goats. Wu-Jun et al. (2010) also reported similar *HaeIII* digestion patterns as in the present study along with significant association of *IGF-1* genotypes with body weight, cashmere yield and fiber diameter in cashmere goats.

Sharma et al. (2013) reported significant association of SNPs of *IGF-1* gene with growth traits in Sirohi goats. Naicy et al. (2017b) also reported significant association of *IGF-1* genotypes with Attappady Black goats and differences in body weight, body height and chest circumference between various genotypes were significant ($P< 0.05$). The literature reviews further showed that the results differ in association of *IGF-1* polymorphism and growth traits in different breeds and the varied results could be due to linkage disequilibrium of the *IGF-1* gene with QTLs. Quantitative traits are regulated by polygenes and are also affected by the interaction between these genes consequently, varied effect of a candidate gene associated with a particular trait in a population is observed.

4.2 Polymorphism of *GH* gene

Growth hormone or somatotropin is an anabolic protein hormone that is synthesized and secreted by the somatotrophs in anterior lobe of the pituitary gland in a circadian and pulsatile manner (Ayuk and Sheppard 2006). It is a major participant of a wide variety of physiological processes for instance, reproduction (Scaramuzzi et al. 1999), lactation (Baldi 1999) and growth and metabolism (Breier 1999). *GH* gene, with its positional and functional potential, has been extensively used as a marker in several livestock species, including goats (Sodhi et al. 2007).

To amplify the four fragments of *GH* gene, four pairs of primers were utilized in accordance with the published literature. The fragment-1 of *GH* gene amplified exons-2 and 3, fragment-2 amplified exon-4, fragment-3 amplified I4-E5-D and fragment-4 amplified 3´-D, respectively.

4.2.1 PCR amplification of *GH* fragments

PCR products of 422 bp, 116 bp, 389 bp and 181 bp, respectively were detected on PCR amplification of various fragments (*GH1, GH2, GH3* and *GH4*) of *GH* gene as depicted in Plates 4.3 to 4.6. Earlier investigations by Singh et al. (2015) in Sirohi and Barbari goat breeds of India, Hua et al. (2009) in Boer goats and Wickramaratne et al. (2010) in Osmanabadi and Sangamneri goat breeds also reported similar amplicon sizes. Various fragments of *GH* gene were also screened for variability in small ruminants including goats by other researchers (Gupta et al. 2007; Zhang et al. 2011; Hajihosseinlo and Negahdary. 2013; Kumari et al. 2014; Singh et al. 2015; Gorlov et al. 2017; Susilorini et al. 2017). The amplified Growth hormone regions corresponding to different fragments of *GH* gene, amplification techniques utilized and their corresponding amplicon size are presented in Table 4.5

Table 4.5: Details of fragment location, amplicon size and techniques used for *GH* gene amplification

GH gene fragment	Region amplified	Amplicon size (bp)	Technique used
GH fragment-1 (*GH1*)	exons-2 and 3	422	PCR-RFLP
GH fragment-2 (*GH2*)	exon-4	116	PCR-RFLP
GH fragment-3 (*GH3*)	I4-E5-D	389	PCR-SSCP
GH fragment-4 (*GH4*)	3´-D	181	PCR-SSCP

4.2.2 PCR-RFLP analysis

PCR-RFLP analysis of amplified products of *GH* fragment-1 with *HaeIII* RE revealed two fragments of 422 bp and 366 bp (Plate 4.7) that exhibited three genotypes viz., AB, BB and AA. Amplicons of *GH* fragment-2 on digestion with *HaeIII* RE revealed two genotypes AB and BB (Plate 4.8).

4.2.3 PCR-SSCP analysis

Two fragments representing distinct regions (*GH3* and *GH4*) of *GH* gene, after PCR amplification were subjected to SSCP analysis. In total, three different SSCP patterns were detected for *GH* fragment-3 and one genotype was detected for fragment-4. The observed SSCP patterns are represented in Plates 4.9 and 4.10. Fragment-4 of *GH* gene was found to be monomorphic for investigated goat population.

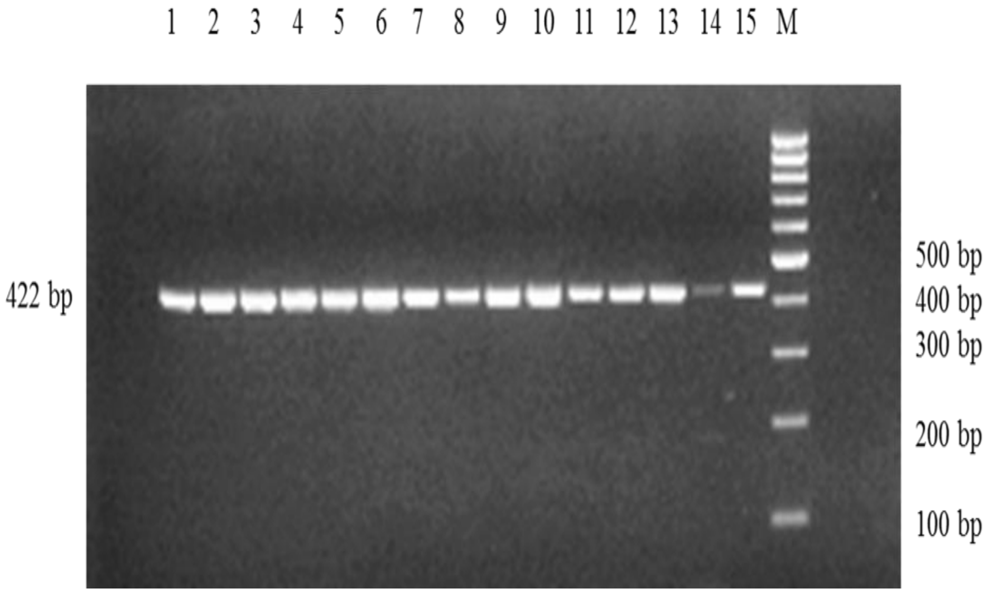


Plate 4.3: Electrophoretic profile of amplified *GH* fragment-1 in 2% agarose gel. Lanes 1-15 represented amplicon size of 422 bp. M represented 100 bp DNA marker

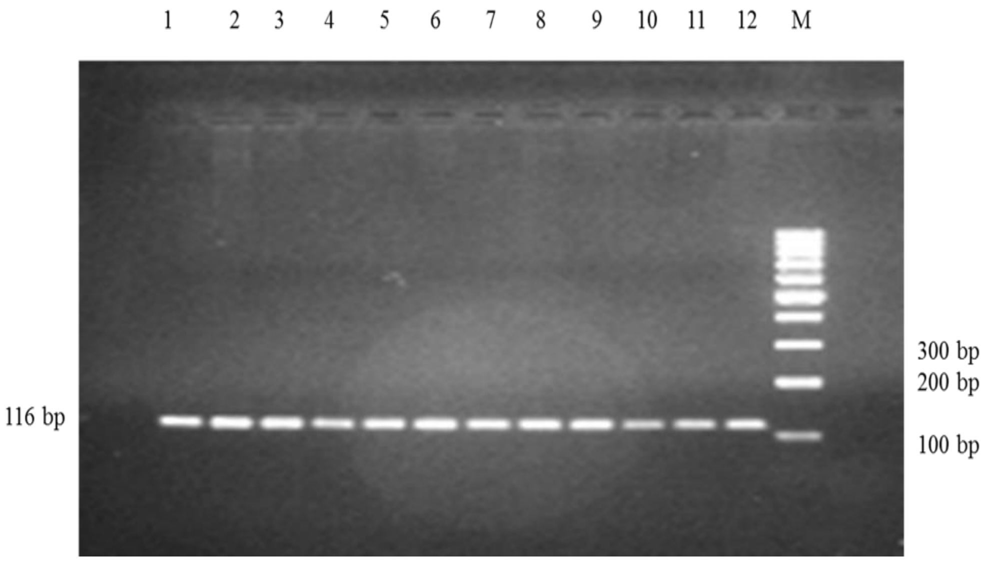


Plate 4.4: Electrophoretic profile of amplified *GH* fragment-2 in 2% agarose gel. Lanes 1-12 represented amplicon size of 116 bp. M represented 100 bp DNA marker

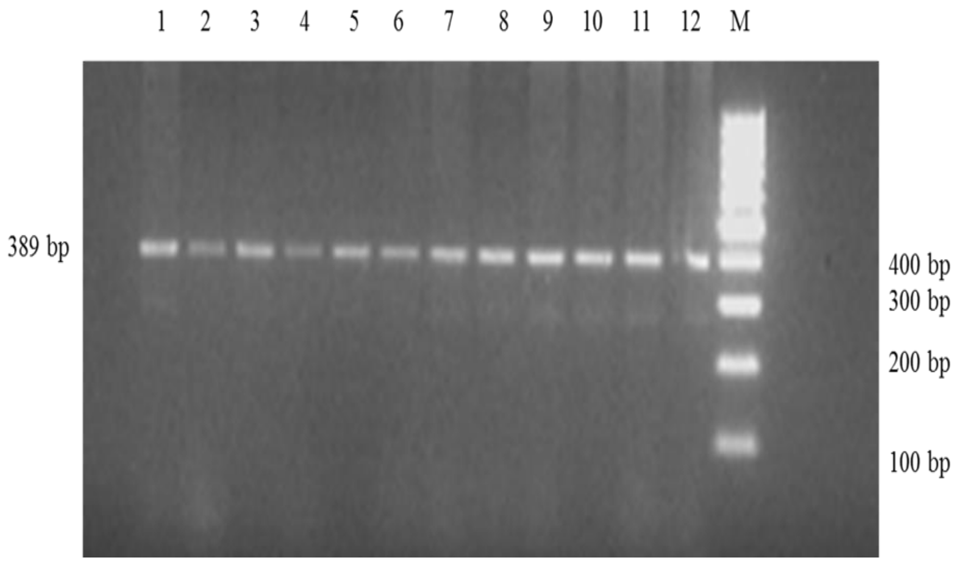


Plate 4.5: Electrophoretic profile of amplified *GH* fragment-3 in 2% agarose gel. Lanes 1-12 represented amplicon size of 389 bp. M represented 100 bp DNA marker

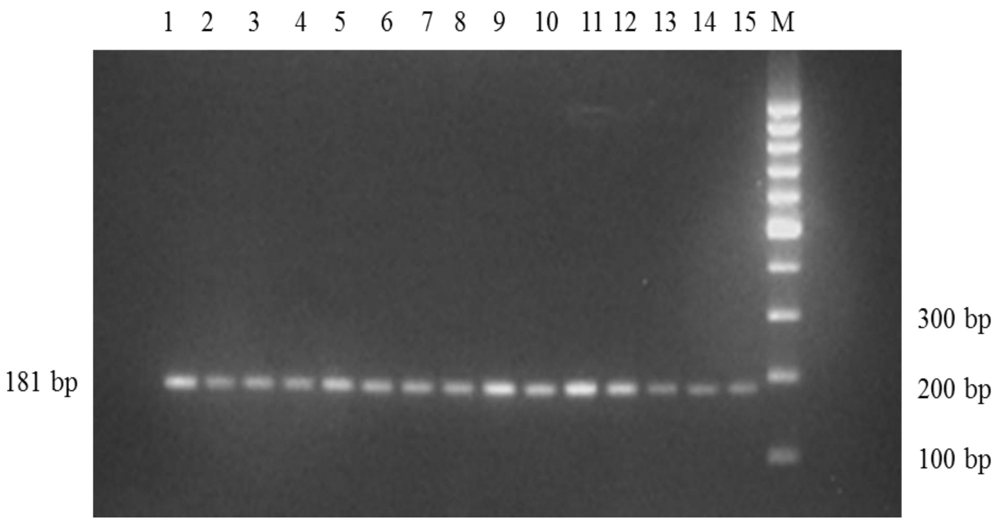


Plate 4.6: Electrophoretic profile of amplified *GH* fragment-4 in 2% agarose gel. Lanes 1-15 represented amplicon size of 181 bp. M represented 100 bp DNA marker

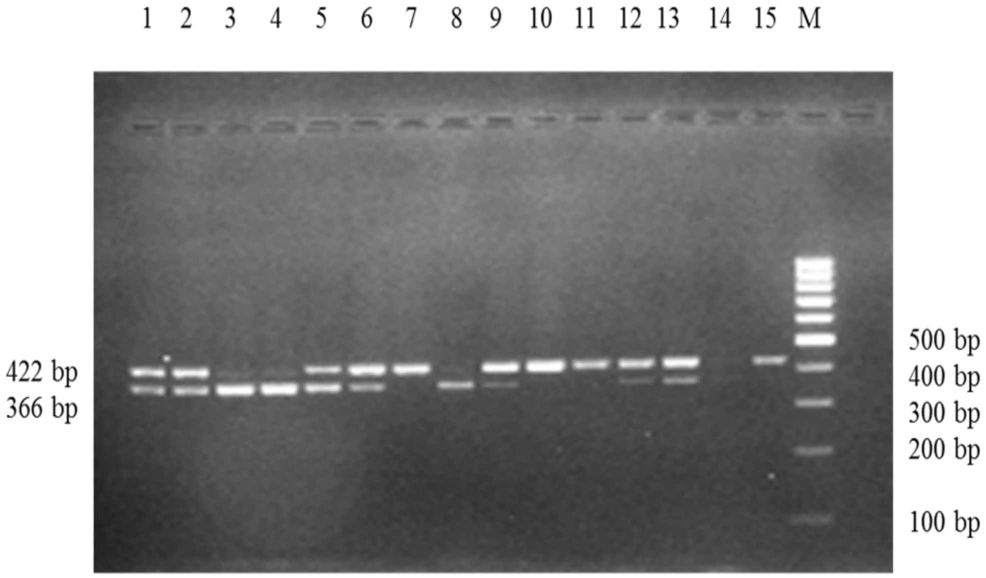


Plate 4.7: Representative genotyping of *GH1*. Strands with 422 bp and 366 bp for genotypes AB and BB appeared. Genotype AA represented uncut strands. M represented 100 bp DNA ladder

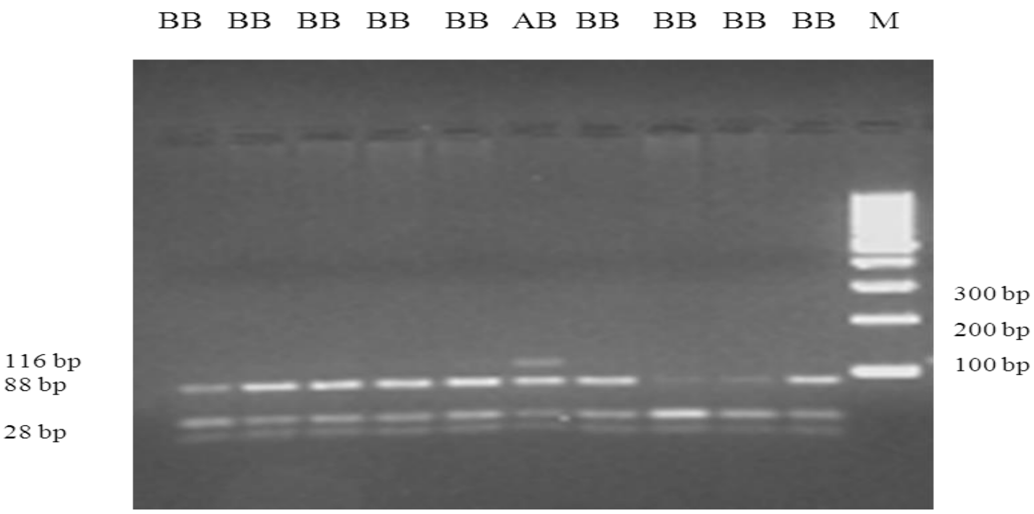


Plate 4.8: Representative genotyping of *GH2*. Strands with 116 bp, 88 bp and 28 bp for genotypes AB and BB appeared. M represented 100 bp DNA marker

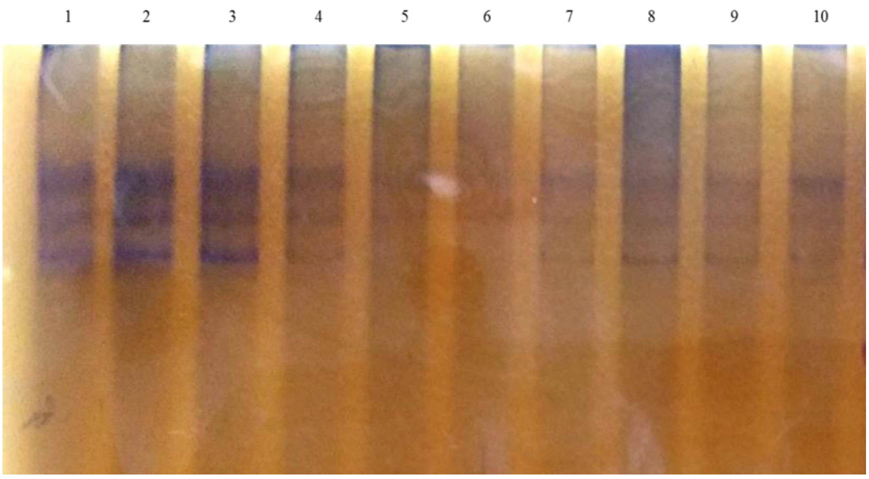


Plate 4.9: SSCP gel representing different patterns of *GH3*. Lanes 1-4 and 7-9 represented AB pattern, lane 5 represented AA and lane 10 represented BB pattern

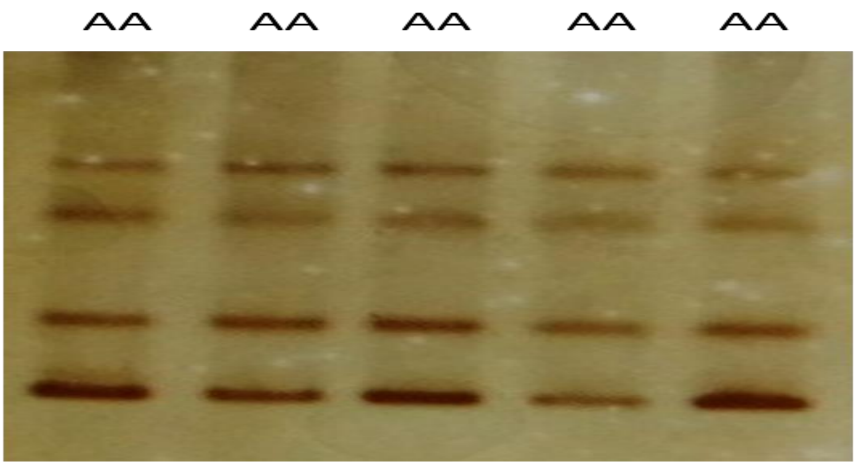


Plate 4.10: SSCP gel representing monomorphic pattern of *GH4*

4.2.4 Frequencies of different variants of *GH* gene

Three genotypes were detected for *GH* fragment-1 with frequency of variant AA as 0.27, AB as 0.52 and BB as 0.21 in Gaddi goats in the present study. AB variant had highest frequency (0.52) followed by AA (0.27) variant. The respective allele frequencies for A and B alleles were 0.53 and 0.47. For *GH* fragment-2, two genotypes were observed with frequencies of 0.76 (BB) and 0.24 (AB) and corresponding allele frequencies of A and B alleles were 0.62 and 0.38. *GH* fragment-3 had allele frequencies of 0.67(A) and 0.33(B), respectively. Three variants (AA, AB and BB) were recorded with genotype frequency of AA as 0.57, AB as 0.21 and BB as 0.22, respectively. *GH* fragment-4 was found to be monomorphic for the studied locus. The allele and corresponding genotype frequencies for the studied population of Gaddi goats for various fragments of *GH* gene are presented in Table 4.6.

Table 4.6: Allele and genotype frequencies of *GH* gene regions in Gaddi goats

Fragment	N	Allele frequencies		Genotype frequencies		
		A	B	AA	AB	BB
GH1	63	0.53	0.47	0.27 (17)	0.52 (33)	0.21 (13)
GH2	63	0.62	0.38	--	0.24 (15)	0.76 (48)
GH3	63	0.67	0.33	0.57 (36)	0.21 (13)	0.22 (14)
GH4				Monomorphic		

Numbers in brackets represent the number of animals belonging to the respective genotype

In earlier studies, Hua et al. (2009) in Boer goat bucks reported two patterns for the *GH* fragment-1 with frequency of 0.84 (AB) and 0.16 (AA) as well as for the exon-4 of *GH* gene, with frequency of 0.86 and 0.14, respectively which were in agreement to the present study. Wickramaratne et al. (2010) investigated different *GH* fragments and reported 4 and 3 SSCP variants for one *GH* fragment with frequency varying from 0.06 to 0.57 and 0.07 to 0.49 in Osmanabadi and Sangamneri goats, respectively and 3 and 4 SSCP variants for the other fragment with frequency of 0.71, 0.16, 0.13 in Osmanabadi goats and 0.12, 0.42, 0.16 and 0.30 in Sangamneri goats. Kumar et al. (2011) also detected 4 SSCP variants in one *GH* fragment with frequency of 0.45, 0.17, 0.13 and 0.22, respectively and 6 SSCP variants in another fragment in Sirohi goats with frequency ranging from 0.06 to 0.43.

Similarly, Kumari et al. (2014) in an investigation of 9 Indian sheep breeds for same *GH* gene region reported two variants with frequency of 0.80 and 0.20, respectively. Dayal et al. (2016) also reported 4 and 5 SSCP variants, respectively with frequency of 0.28, 0.34, 0.23 and 0.15 and 0.12, 0.40, 0.36, 0.07 and 0.05 in Black Bengal goats. Gorlov et al. (2017) observed three genotypes with frequency of 0.57, 0.36 and 0.07, respectively in Salsk sheep. Susilorini et al. (2017) observed two genotypes with frequency of 0.47 and 0.53, respectively in Etawah crossbred goats. Malverio et al. (2001) reported 4 conformational patterns in Portuguese Algarvia goats and Marques et al. (2003) documented 6 patterns in Serena goats. Other studies by Tahmoorespur et al. (2011) in Kurdi and Baluchi sheep breeds, Hajihosseinlo (2013) in Makooei sheep, and Malewa et al. (2014) in Donggala and East Java sheep breeds also reported polymorphism in *GH* gene. *GH* gene polymorphism was also reported by Jia et al. (2014) in Chinese sheep breeds, Othman et al. (2015) in Egyptian small ruminant breeds and Seevagan et al. (2015) in Indian sheep breeds. The SSCP analysis of *GH* gene revealed polymorphism at different fragments in studied breeds and indicated SSCP as a valuable tool for identification of genetic variants.

4.2.5 Measures of genetic diversity and test for genetic equilibrium for *GH* gene

The measures of genetic diversity and respective Chi-square values estimated for studied Gaddi goat population are presented in Table 4.7.

For *GH1*, the estimates for Ne, H_{obs} and H_{exp} were 1.99, 0.48 and 0.52, respectively. For *GH2*, 1.89, 0.24 and 0.53 values were obtained for Ne, H_{obs} and $H_{exp,}$ whereas 1.78, 0.21 and 0.44 values of Ne, H_{obs} and H_{exp} were obtained for fragment-3, respectively. These measures indicated substantial genetic variation across different regions of *GH* gene in studied Gaddi population. The PIC values for *GH1, GH2* and *GH3* were 0.37, 0.36 and 0.34, respectively which suggested the median level of polymorphism at studied loci and also indicated the effectiveness of the studied marker for population genetic studies. Similar to the present investigation, Wickramaratne et al. (2010) observed $H_{obs,}$ H_{exp} and PIC ranging from 0.00 to 0.55, 0.00 to 0.49 and 0.28 to 0.54, respectively in Osmanabadi and Sangamneri goats. The negative F_{IS} measures for *GH1* and *GH2* suggested the absence of heterozygous deficiency at studied loci for Gaddi goats.

The present Gaddi goat population were in HWE for *GH1* while for *GH* fragment-2 and fragment-3, the studied population revealed significant deviation from HWE. *GH4* revealed monomorphic pattern in investigated population.

Table 4.7: Measures of genetic diversity and test for genetic equilibrium for different fragments of *GH* gene in Gaddi goats

Locus	No	Ne	H_{obs}	H_{exp}	PIC	I	F_{IS}	Chi-Square
GH1	2	1.99	0.48	0.52	0.37	0.69	-0.05	0.12(0.72)
GH2	2	1.89	0.24	0.53	0.36	0.67	-0.61	23.28(P<0.01)*
GH3	2	1.78	0.21	0.44	0.34	0.63	0.53	18.31(P<0.01)*
GH4	Monomorphic							

No: observed number of alleles, Ne: effective number of alleles, He: Heterozygosity, PIC: Polymorphic information content, P* represents deviation from HWE.

4.2.6 DNA sequencing and analysis

Representative samples of different genotypes as revealed by PCR-RFLP and PCR-SSCP analysis were got sequenced after purification of PCR products of respective *GH* fragments. Sequence alignment with reference sequences from NCBI database confirmed the accurate amplification of various *GH* gene fragments. Chromatograms for *GH1*, *GH2* and *GH3* are presented in Figures 4.3, 4.4 and 4.5, respectively while sequence alignment for *GH1*, *GH2* and *GH3* are presented in Figures 4.6, 4.7 and 4.8 respectively. Sequence analysis revealed the accuracy of PCR-RFLP and PCR-SSCP detection.

For *GH* fragment-1, sequencing of representative PCR-RFLP genotype depicted SNP (G781A). This transition at *GH* fragment-1 resulted in amino acid change from Serine to Glycine at residue 35. Chromatogram highlighting G/A substitution for *GH1* is depicted in Figure 4.3. For *GH2*, SNP (G1536A) was revealed by sequencing of representative PCR-RFLP genotypes. Chromatogram highlighting C/A substitution for *GH2* is depicted in Figure 4.4. The results of present investigation for *GH1* and *GH2* fragments are in agreement with earlier findings of Hua et al. (2009) in Boer goats. The substitution in *GH2* had not resulted in any amino acid change. Sequencing of representative PCR-SSCP analysis revealed A/G substitution for *GH* fragment-3. Chromatogram highlighting A/G substitution for *GH* fragment-3 is depicted in Figure 4.5.

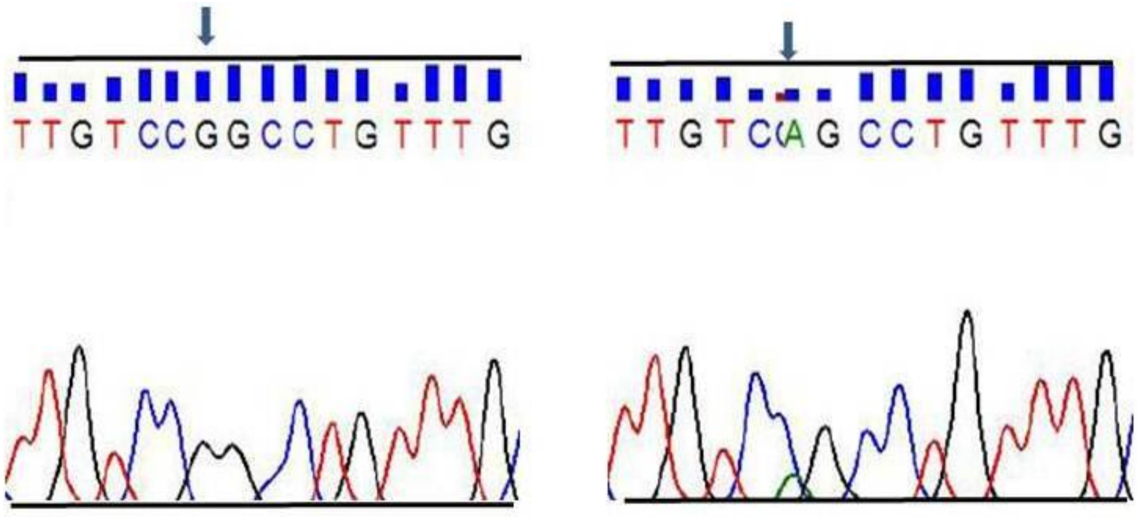


Figure 4.3: Chromatogram representing G/A substitution in fragment-1 of *GH* gene

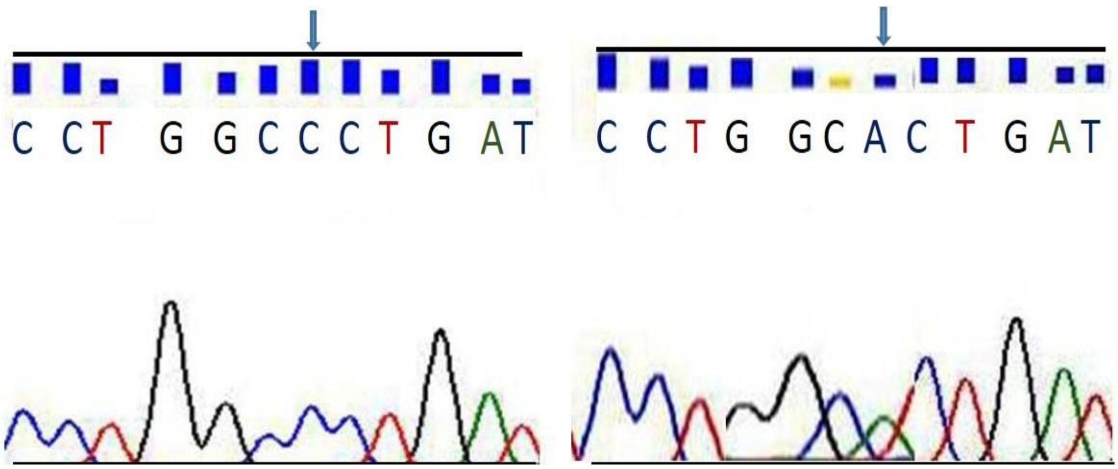


Figure 4.4: Chromatogram representing C/A substitution in fragment-2 of *GH*

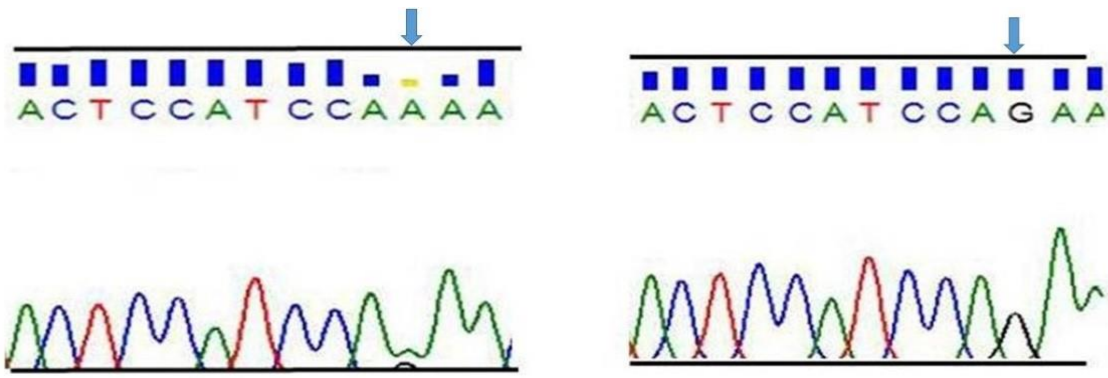
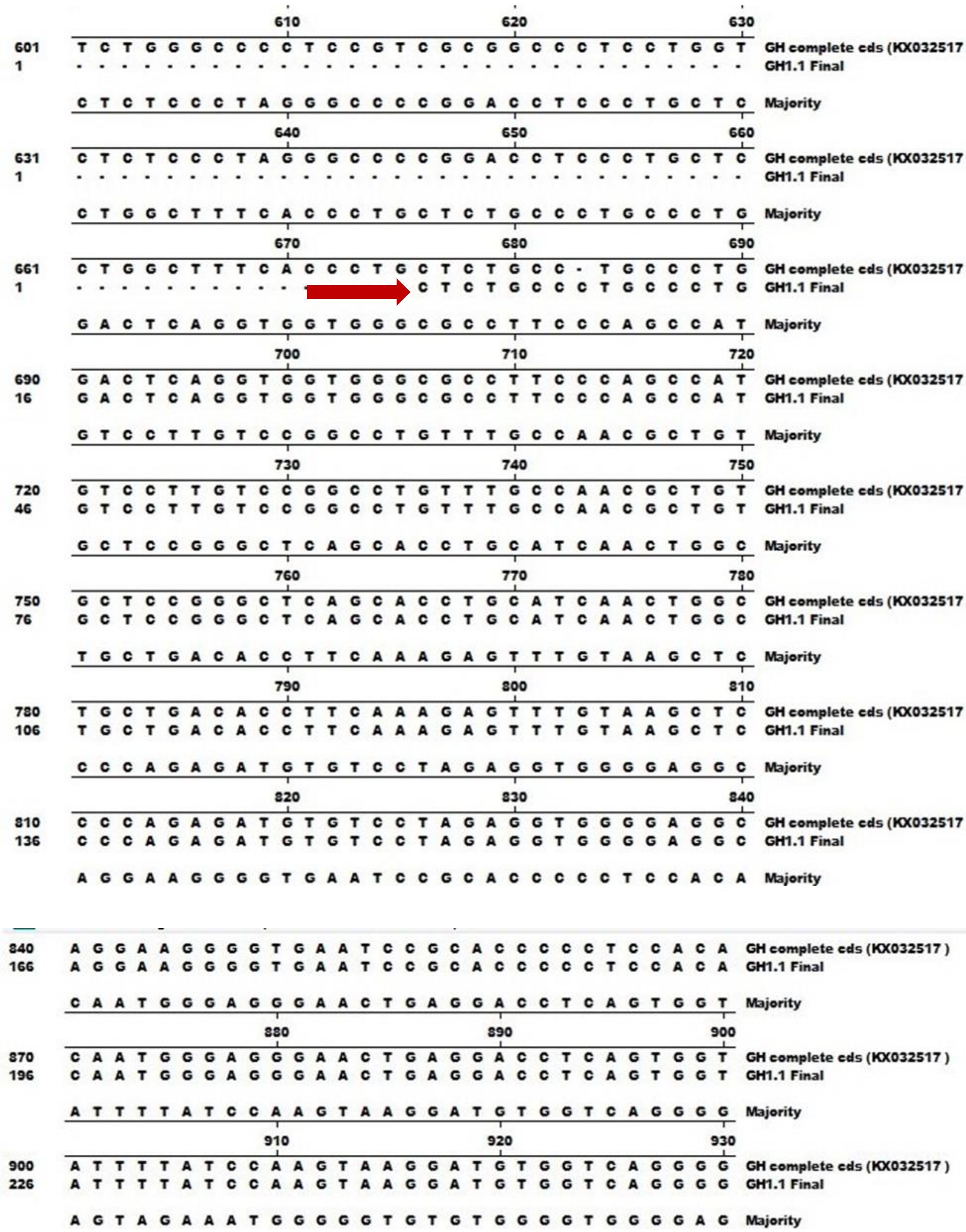


Figure 4.5: Chromatogram representing A/G substitution in fragment-3 of *GH*


610 620 630
601 TCTGGGCCCCTCCGTCGCGGCCCTCCTGGT GH complete cds (KX032517
1 .... GH1.1 Final
CTCTCCCTAGGGCCCCGGACCTCCCTGCTC Majority
640 650 660
631 CTCTCCCTAGGGCCCCGGACCTCCCTGCTC GH complete cds (KX032517
1 .... GH1.1 Final
CTGGCTTTCACCCTGCTCTGCCCTGCCCTG Majority
670 680 690
661 CTGGCTTTCACCCTGCTCTGCC-TGCCCTG GH complete cds (KX032517
1 .... CTCTGCCCTGCCCTG GH1.1 Final
GACTCAGGTGGTGGGCGCCTTCCCAGCCAT Majority
700 710 720
690 GACTCAGGTGGTGGGCGCCTTCCCAGCCAT GH complete cds (KX032517
16 GACTCAGGTGGTGGGCGCCTTCCCAGCCAT GH1.1 Final
GTCCTTGTCCGGCCTGTTTGCCAACGCTGT Majority
730 740 750
720 GTCCTTGTCCGGCCTGTTTGCCAACGCTGT GH complete cds (KX032517
46 GTCCTTGTCCGGCCTGTTTGCCAACGCTGT GH1.1 Final
GCTCCGGGCTCAGCACCTGCATCAACTGGC Majority
760 770 780
750 GCTCCGGGCTCAGCACCTGCATCAACTGGC GH complete cds (KX032517
76 GCTCCGGGCTCAGCACCTGCATCAACTGGC GH1.1 Final
TGCTGACACCTTCAAAGAGTTTGTAAGCTC Majority
790 800 810
780 TGCTGACACCTTCAAAGAGTTTGTAAGCTC GH complete cds (KX032517
106 TGCTGACACCTTCAAAGAGTTTGTAAGCTC GH1.1 Final
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820 830 840
810 CCCAGAGATGTGTCCTAGAGGTGGGGAGGC GH complete cds (KX032517
136 CCCAGAGATGTGTCCTAGAGGTGGGGAGGC GH1.1 Final
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196 CAATGGGAGGGAACTGAGGACCTCAGTGGT GH1.1 Final
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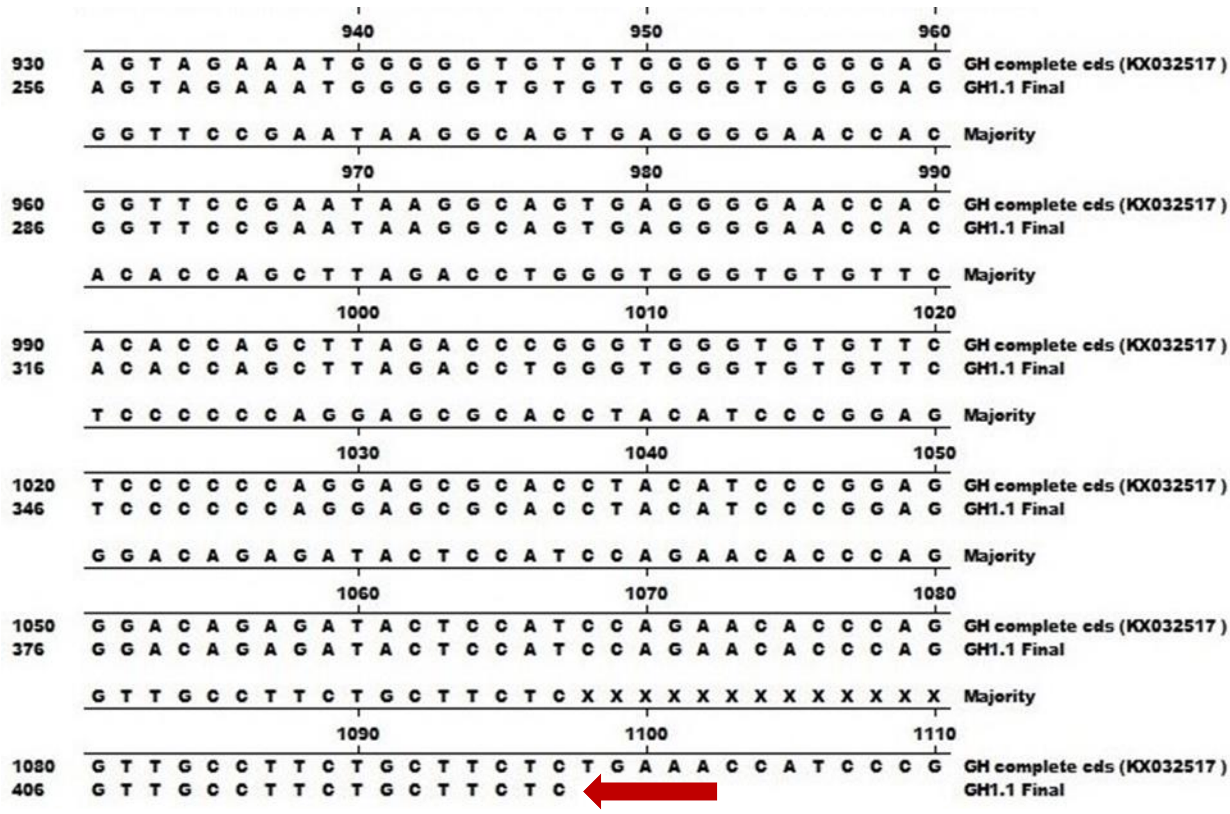

Figure 4.6: Sequence alignment using MegAlign for amplified region (676 to 1097 bp) of *GH* gene fragment-1 corresponding to reference sequence of complete *GH* gene

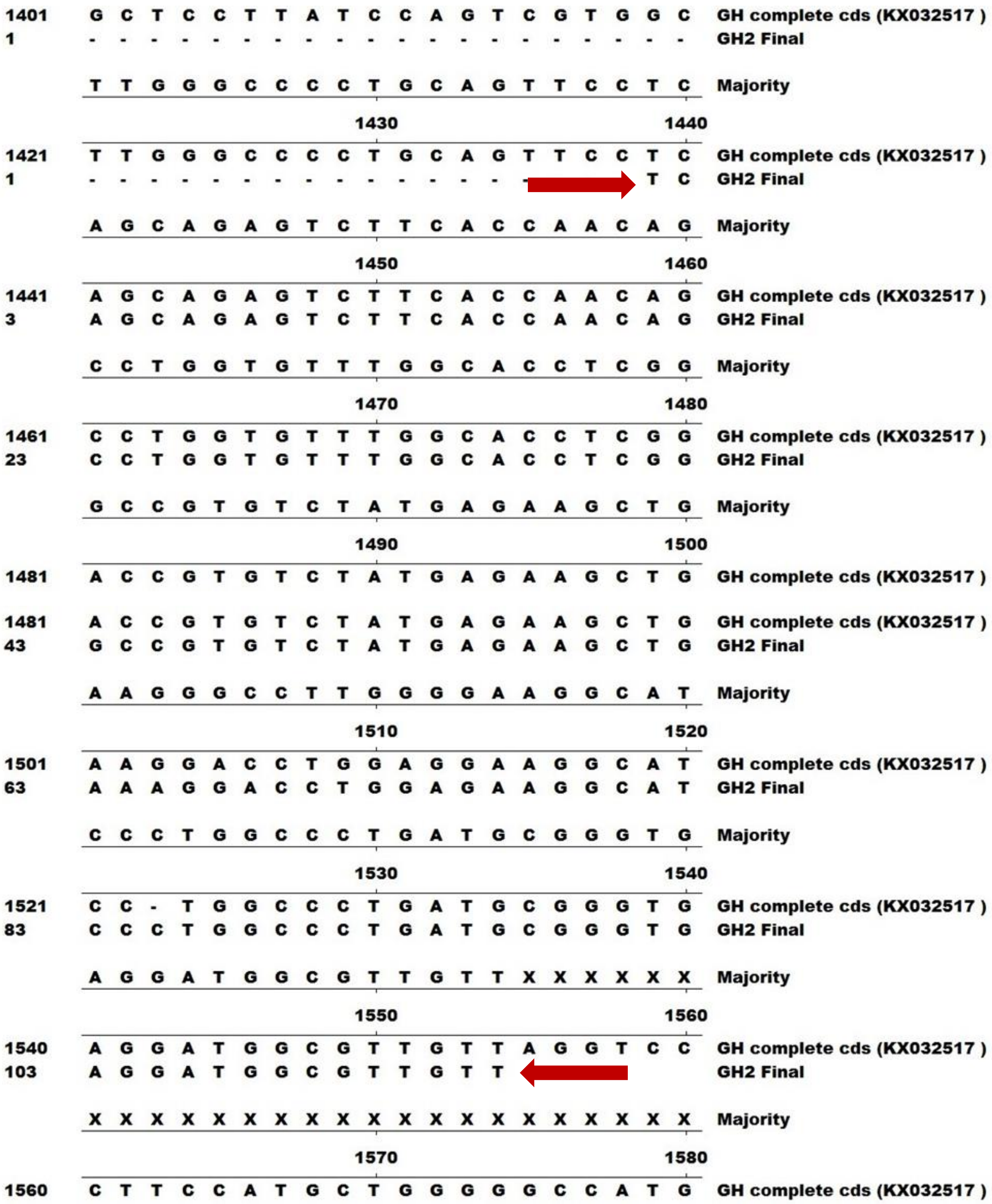

Figure 4.7: Sequence alignment using MegAlign for amplified region (1439 to 1554 bp) of *GH* gene fragment-2 corresponding to reference sequence of complete *GH* gene

```
1626   A G A G A G A T C C C T G C T C T C T C T C T C T   GH complete cds (KX03251
1      - - - - - - - - - - - - - - - - - - - - - - - - -   GH 3 Final

       C T T T C T A G C A G C C C A G T C T T G A C C C   Majority
                         1660                1670
1651   C T T T C T A G C A G C C C A G T C T T G A C C C   GH complete cds (KX03251
1              C T A G C A G C C C A G T C T T G A C C C   GH 3 Final

       A G G A G A A A C C T T T T T C C G T T T T G A A   Majority
                 1680                1690                1700
1676   A G G A G A A A C C T C T T C C C G T T T T G A A   GH complete cds (KX03251
22     A G G A G A A A C C T T T T T C C C C T T T G A A   GH 3 Final

       A C C T C C T T C C T T G C C C T T T T T C C A G   Majority
                         1710                1720
1701   A C C T C C T T C C T C G C C C T T C T C C A A G   GH complete cds (KX03251
47     A C C T C C T T C C T T G C C C T T T T T C C A G   GH 3 Final

       C C T G T A G G G G A G G G T G G A A A A T G G A   Majority
                 1730                1740                1750
1726   C C T A T A G G G G A G G G T G G A A A A T G G A   GH complete cds (KX03251

1726   C C T A T A G G G G A G G G T G G A A A A T G G A   GH complete cds (KX032517
72     C C T G T A G G G G A G G G T G G A A A A A G G A   GH 3 Final

       G C G G G C A G G A G G G A G C C G C T C C T G A   Majority
                         1760                1770
1751   G C G G G C A G G A G G G A G C C G C T C C T G A   GH complete cds (KX032517
97     G C G G G C A G G A G G G A G C C G C T C C T G A   GH 3 Final

       G G G C C C T T C G G C C T C T C T G T C T C T C   Majority
                 1780                1790                1800
1776   G G G C C C T T C G G C C T C T C T G T C T C T C   GH complete cds (KX032517
122    G G G C C C T T C G G C C T C T C T G T C T C T C   GH 3 Final

       C C T C C C T T G G C A G G A G G T G G A A G A T   Majority
                         1810                1820
1801   C C T C C C T T G G C A G G A G C T G G A A G A T   GH complete cds (KX032517
147    C C T C C C T T G G C A G G A G G T G G A A G A A   GH 3 Final

       G T T A C C C C C C G G G G C T G G G C A G A T C C   Majority
                 1830                1840                1850
1826   G T T A C C C C C C G G G G C T G G G C A G A T C C   GH complete cds (KX032517
```

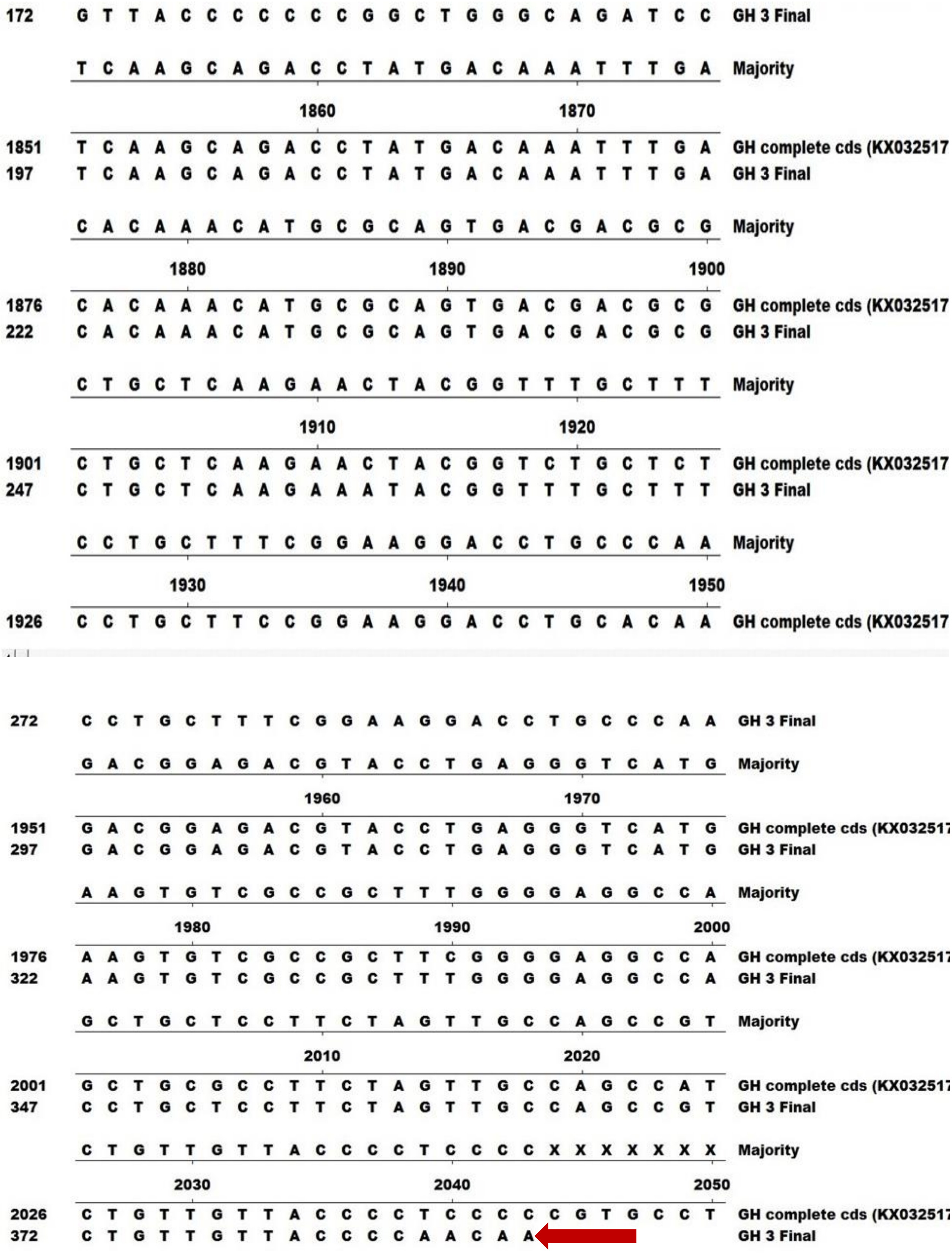

Figure 4.8: Sequence alignment using MegAlign for amplified region (1655 to 2043 bp) of *GH* gene fragment-3 corresponding to reference sequence of complete *GH* gene

The findings of association analysis of PCR-RFLP and PCR-SSCP variants with body weights (kg) and body measurement (cm) traits at different ages in Gaddi goats are described below.

4.2.7.1 Association analysis of *GH* fragment-1

The association of observed variants of *GH* fragment-1 with body weights and body measurements is presented in Table 4.8 and respective F values in Table 4.9. For different genotypes (AA, AB and BB) of *GH1*, body weights (kg) at 3M, 6M, 9M and 12M ranged from 16.05±0.34 to 17.35±0.49, 20.62±0.40 to 21.50±0.47, 25.15±0.48 to 27.43±0.48 and 28.17±0.53 to 30.48±0.73, respectively. The corresponding body heights (cm) at 3M, 6M, 9M and 12M ranged from 58.59±0.92 to 59.96±0.60, 66.18±0.74 to 66.85±0.49, 68.18±0.68 to 69.15±0.95 and 69.21±0.60 to 70.69±0.80, respectively for different genotypes. Body lengths (cm) at 3M, 6M, 9M and 12M ranged from 59.85±0.81 to 61.38±1.36, 67.54±0.79 to 68.00±0.64, 68.29±0.90 to 70.46±1.39 and 71.46±0.56 to 72.92±0.72, respectively while heart girth (cm) at 3M, 6M, 9M and 12M ranged from 62.55±0.63 to 62.94±0.69, 67.24±0.96 to 67.64±0.51, 70.24±0.79 to 71.54±0.63 and 72.01±0.78 to 73.31±0.44, respectively for different genotypes. Significant association was observed for *GH1* fragment with 9-month BW as depicted in Table 4.8. Similar to present study, Hua et al. (2009) also assessed the association between different *GH* genotypes and growth traits in Boer goat bucks and observed significant decrease in birth chest girth (P = 0.03) and weaning weight (P = 0.014) with AA genotype as compared to AB genotype, while CC genotype contributed to weaning height (P = 0.04) greater than CD genotype. Thomas et al. (2007) also reported that heterozygous genotypes for two *GH* polymorphisms appeared advantageous for traits of adiposity and muscularity in the cooperating breeding program. Similar to present findings, Mahdi et al. (2018) observed three genotypes (AA, AB and BB) for exon-2 of *GH* gene in three Iraqi sheep breeds with frequencies of 0.40, 0.10 and 0.50 in Hamdani sheep however, two genotypes (AB and BB) were observed in other two breeds with frequencies of 0.30 and 0.70 in Awassi and 0.40 and 0.60 Karadi sheep. These results were also consistent with findings of other researchers like Rajni *et al*. (2014) in native sheep breeds of India and Marini *et al*. (2012) in Savanna and Kalahari goat breeds.

Table 4.8: Mean body weights and body measurements at different ages depicting association with observed *GH1* genotypes in Gaddi goats

Trait	GH1 genotype		
	AA	AB	BB
Body weight (kg)			
3 month BW	16.40±0.50	16.05±0.34	17.35±0.49
6 month BW	20.62±0.40	20.76±0.33	21.50±0.47
9 month BW	$26.43±0.78^{ab}$	$25.15±0.48^{b}$	$27.43±0.48^{aa}$
12 month BW	29.18±0.85	28.17±0.53	30.48±0.73
Body height (cm)			
3 month BH	59.41±0.65	59.96±0.60	58.59±0.92
6 month BH	66.18±0.74	66.72±0.53	66.85±0.49
9 month BH	68.18±0.68	68.64±0.67	69.15±0.95
12 month BH	69.83±0.73	69.21±0.60	70.69±0.80
Body length (cm)			
3 month BL	60.35±0.76	59.85±0.81	61.38±1.36
6 month BL	67.82±0.78	68.00±0.64	67.54±0.79
9 month BL	68.29±0.90	68.64±0.61	70.46±1.39
12 month BL	72.47±0.58	71.46±0.56	72.92±0.72
Body girth (cm)			
3 month BG	62.94±0.69	62.55±0.63	62.77±0.74
6 month BG	67.24±0.96	67.64±0.51	67.46±0.75
9 month BG	70.24±0.79	70.60±0.60	71.54±0.63
12 month BG	72.01±0.78	72.42±0.48	73.31±0.44

Means with different superscripts differ significantly (P<0.05) for respective traits

Table 4.9: Mean Squares and F values for effect of *GH1* fragment on body weights and body measurements at different ages in Gaddi goats

Trait	SS	Mean Square	F- value	P>F
Body weight (kg)				
3 month BW	57.01	28.51	2.01	0.14
6 month BW	28.17	14.09	1.03	0.36
9 month BW	164.57	82.29	3.66	0.03
12 month BW	168.97	84.48	2.69	0.08
Body height (cm)				
3 month BH	59.89	29.94	0.75	0.47
6 month BH	15.90	7.95	0.27	0.76
9 month BH	29.66	14.83	0.84	0.44
12 month BH	69.60	34.80	1.01	0.37
Body length (cm)				
3 month BL	76.69	38.34	0.58	0.56
6 month BL	6.77	3.38	0.09	0.92
9 month BL	171.02	85.51	1.32	0.27
12 month BL	70.44	35.22	1.47	0.24
Heart girth (cm)				
3 month HG	4.82	2.41	0.09	0.92
6 month HG	5.06	2.53	0.09	0.91
9 month HG	56.77	28.38	0.65	0.53
12 month HG	55.65	27.83	0.89	0.42

4.2.7.2 Association analysis of *GH* fragment-2

The association of various observed variants of *GH2* with body weights and body measurements is depicted in Table 4.10. Two different genotypes (AB and BB) were revealed by RFLP analysis of *GH2* for which body weights (kg) at 3M, 6M, 9M and 12M were 16.24±0.29 and 16.97±0.50, 20.69±0.27 and 21.46±0.39, 25.93±0.43 and 26.07±0.63 and 28.79±0.84 and 28.96±0.46. The corresponding body heights (cm) at 3M, 6M, 9M and 12M were 58.20±0.74 and 59.98±0.47, 66.00±0.80 and 66.79±0.39, 66.80±0.88 and 68.52±0.50 and 69.00±0.77 and 69.89±0.47. Body lengths (cm) at 3M, 6M, 9M and 12M were 60.23±0.66 and 60.53±0.93, 67.64±0.46 and 68.53±0.97, 68.00±0.74 and 69.21±0.60 and 72.02±0.42 and 72.07±0.78, respectively while heart girth (cm) at 3M, 6M, 9M and 12M was 62.13±0.72 and 62.87±0.48, 66.33±1.00 and 67.85±0.41, 68.73±0.83 and 71.31±0.42 and 70.87±0.82 and 73.00±0.34, respectively. Significant association had been observed for *GH2* with 9-month HG and 12-month HG. Similarly, Dayal et al. (2014) also reported significant association of *GH* genotypes with birth weight. The AC genotype had 47% more weight than the CC genotype and order of performance for birth weight was AA, CC <AB, AD < AC in Black Bengal goats. Min *et al*. (2005) observed the significant effect of growth hormone gene on birth weight in Boer goats and found that AA genotype had significantly higher body weight than BB and AB genotypes. Hua *et al*. (2009) also detected a significant and positive association of growth hormone gene in Boer goats.

4.2.7.3 Association analysis of *GH* fragment-3

Association of various variants of *GH3* with body weights and body measurements is presented in Table 4.12. Three different genotypes were revealed by SSCP analysis. For different variants of *GH3*, body weights (kg) at 3M, 6M, 9M and 12M ranged from 16.92±0.67 to 18.71±0.60, 21.72±0.99 to 23.56±0.96, 25.82±0.44 to 27.36±1.06 and 28.48±0.49 to 30.83±1.09, respectively. The corresponding body heights (cm) at 3M, 6M, 9M and 12M ranged from 63.08±1.18 to 63.33±0.68, 65.78±0.47 to 68.21±0.73, 69.11±0.52 to 71.31±0.84 and 67.89±0.63 to 68.54±0.66, respectively for different genotypes. Body lengths (cm) at 3M, 6M, 9M and 12M ranged from 64.89±0.72 to 67.50±0.87, 67.39±0.63 to 69.43±0.66, 71.50±0.54 to 73.46±0.59 and 68.47±0.65 to 70.61±1.19, respectively while heart girth (cm) at 3M, 6M, 9M and 12M ranged from 64.54±0.93 to 66.57±0.61, 67.25±0.52 to 68.31±0.73, 72.00±0.87 to 73.38±0.54 and 70.43±0.95 to 71.46±0.86, respectively for different genotypes. Significant association had been observed for *GH3* with 6-month BW, 6 month BH and 6 month BL, respectively

Table 4.10: Mean body weights and body measurements at different ages depicting association with observed *GH2* genotypes in Gaddi goats

Trait	*GH2* genotype	
	AB	**BB**
Body weight (kg)		
3 month BW	16.24±0.29	16.97±0.50
6 month BW	20.69±0.27	21.46±0.39
9 month BW	25.93±0.43	26.07±0.63
12 month BW	28.96±0.46	28.79±0.84
Body height (cm)		
3 month BH	59.98±0.47	58.20±0.74
6 month BH	66.79±0.39	66.00±0.80
9 month BH	68.52±0.50	66.80±0.88
12 month BH	69.89±0.47	69.00±0.77
Body length (cm)		
3 month BL	60.23±0.66	60.53±0.93
6 month BL	67.64±0.46	68.53±0.97
9 month BL	69.21±0.60	68.00±0.74
12 month BL	72.02±0.42	72.07±0.78
Heart girth (cm)		
3 month HG	62.87±0.48	62.13±0.72
6 month HG	67.85±0.41	66.33±1.00
9 month HG	71.31±0.42^{a}	68.73±0.83^{b}
12 month HG	73.00±0.34^{a}	70.87±0.82^{b}

Means with different superscripts differ significantly (P<0.05) for respective traits

Table 4.11: F values for effect of *GH2* fragment on body weights and body measurements at different ages in Gaddi goats

Trait	F- value	P>F
Body weight (kg)		
3 month BW	1.55	0.22
6 month BW	2.10	0.15
9 month BW	0.03	0.87
12 month BW	0.03	0.86
Body height (cm)		
3 month BH	3.65	0.06
6 month BH	0.91	0.34
9 month BH	2.83	0.09
12 month BH	0.89	0.35
Body length (cm)		
3 month BL	0.06	0.81
6 month BL	0.80	0.37
9 month BL	1.09	0.30
12 month BL	0.00	0.96
Heart girth (cm)		
3 month HG	0.61	0.44
6 month HG	2.71	0.10
9 month HG	8.54	0.004
12 month HG	7.98	0.006

Table 4.12: Mean body weights and body measurements at different ages depicting association with observed *GH3* genotypes in Gaddi goats

Trait	*GH3* genotype		
	AA	**AB**	**BB**
Body weight (kg)			
3 month BW	17.04±0.43	16.92±0.67	18.71±0.60
6 month BW	22.72±0.47[ab]	20.69±0.99[a]	23.56±0.96[a]
9 month BW	25.82±0.44	25.87±0.84	27.36±1.06
12 month BW	28.48±0.49	28.98±0.98	30.83±1.09
Body height (cm)			
3 month BH	63.33±0.68	63.08±1.18	64.28±0.92
6 month BH	65.88±0.47[a]	66.84±0.54[ab]	68.21±0.73[a]
9 month BH	69.11±0.52	71.31±0.84	69.64±0.87
12 month BH	67.89±0.63	68.54±0.66	68.28±1.01
Body length (cm)			
3 month BL	64.89±0.72	65.00±1.25	67.50±0.87
6 month BL	67.39±0.63[a]	67.46±0.69[b]	69.43±0.66[a]
9 month BL	71.50±0.54	73.46±0.59	72.07±0.61
12 month BL	68.47±0.65	70.61±1.19	68.50±0.92
Heart girth (cm)			
3 month HG	65.25±0.57	64.54±0.93	66.57±0.61
6 month HG	67.25±0.52	68.31±0.73	67.36±1.01
9 month HG	72.36±0.45	73.38±0.54	72.00±0.87
12 month HG	70.53±0.51	71.46±0.86	70.43±0.95

Means with different superscripts differ significantly (P<0.05) for respective traits

Table 4.13: F values for effect of *GH3* fragment on body weights and body measurements at different ages in Gaddi goats

Trait	SS	Mean Square	F- value	P>F
Body weight (kg)				
3 month BW	126.16	63.08	2.57	0.08
6 month BW	274.33	137.17	3.08	0.04
9 month BW	96.48	48.24	1.30	0.28
12 month BW	193.09	96.55	2.39	0.10
Body height (cm)				
3 month BH	50.50	25.25	0.37	0.69
6 month BH	172.78	86.39	3.93	0.02
9 month BH	166.11	83.05	2.33	0.11
12 month BH	13.49	6.74	0.18	0.83
Body length (cm)				
3 month BL	274.56	137.28	2.09	0.13
6 month BL	169.00	84.50	3.07	0.04
9 month BL	128.07	64.03	2.26	0.11
12 month BL	189.68	94.84	1.56	0.22
Heart girth (cm)				
3 month HG	133.72	66.86	1.44	0.24
6 month HG	42.80	21.40	0.54	0.58
9 month HG	64.56	32.28	0.99	0.38
12 month HG	40.65	20.33	0.47	0.62

Wickramaratne et al. (2010) observed a significant association of various SNPs with growth traits in Osmanabadi and Sangamneri goat breeds and concluded that the association of SNPs with body weights and body measurements may be useful in selecting goats for higher growth traits. Kumar et al. (2011) in a study of Sirohi goats, reported significant association of *GH* gene with body weights at different ages. The association analysis of variants revealed that the AE variant had significantly less birth weight (2.227 kg) ($P<0.05$) as compared to the AH variant (2.567 kg) with an increase of 13.25% and concluded that *GH* mutation can be detected by PCR-SSCP, which can provide information for advancement of fast screening methods for further selection indicating the possibility of using *GH* gene as a marker in MAS for higher body weight. Dayal et al. (2016) also reported association of AC variant with higher body weight in Black Bengal goats and the order of performance for body weight at 6 months of age was CC< AA< AB< BB< AC whereas order of performance at 9 months of age was CC< BB< AA, AB< AC. In a similar way, other researchers (Min et al. 2005; Hua et al. 2009) had also reported the significant association of *GH* gene polymorphism with growth traits.

4.2.7.4 Association analysis of *GH* fragment-4

No polymorphism was detected in the amplified fragment of *GH4* by SSCP analysis. Similar to present investigation, Wickramaratne et al. (2010) also reported the absence of polymorphism in *GHR* gene in Osmanabadi and Sangamneri goats. Sahu et al. (2016) in a study of South Indian sheep breeds also reported monomorphic pattern of *GHR*. Although in cattle and other species, *GHR* polymorphism had been detected but studies in sheep and goats showed the scarcity of *GHR* polymorphism. Various investigations carried out in small ruminants including goats, revealed high conservation of *GHR* region. Absence of polymorphism doesn’t essentially indicate the absence of variation in the whole gene but it implied that the studied locus of *GHR* gene is highly conserved in the investigated population of Gaddi goats.

Chapter-5

SUMMARY AND CONCLUSIONS

India is a rich repository of goat genetic resources with 135.17 million goat population (19^{th} Livestock Census, 2012). Goats have distribution across the widest ecological ranges and are considered poor man's most reliable livelihood source since their domestication during Neolithic period. Goat farming is an inseparable part of predominant migratory production system prevailing in extreme climatic areas of the country particularly in hilly and arid regions with little arable agricultural land.

"Gaddi" (also known as *White Himalayan* goat), is a predominant goat breed of Western Himalayan region constituting 60 to 65% of total goat population in Himachal Pradesh. It is imperative to improve the production performance of this indigenous goat breed for enhanced growth, production, reproduction and survivability. In this purpose, phenotypic, quantitative and genetic characterization would provide comprehensive information on this indigenous goat breed and would pave way for its further improvement. Consequently, it becomes essential to study the genetic profile of Gaddi goat breed for important economic traits associated with growth, production and reproduction using modern molecular genetic tools, which are being used effectively worldwide for livestock improvement.

The aim of the present study was to investigate genetic polymorphism of some caprine growth related genes (Growth Hormone, Insulin-like Growth Factor-1) in Gaddi goats in order to analyse the possible association with some growth traits. To achieve the envisaged objectives of the study, blood samples were collected at random from genetically unrelated and adequate number of animals of Gaddi goats (63 animals) from adopted flock under AICRP on goat improvement as well as the flock maintained at university farm of CSKHPKV, Palampur.

Approximately, 10 ml of blood was collected aseptically from jugular vein and further subjected to DNA isolation using standard phenol chloroform extraction technique (Sambrook et al.2001). The extracted DNA, after its qualitative and quantitative evaluation, was subjected to PCR amplification of the studied genes using standard primers for their amplification. Polymorphism of the candidate genes viz., 4 fragments of *GH* gene and *IGF-1* gene was detected using PCR-SSCP and PCR-RFLP techniques. The homozygous and heterozygous genotypes for different genes under study were got sequenced by commercial service providers in both directions.

For association studies, data on some growth traits viz. pre-pubertal body weights and body measurements (body length, body height and body girth) at different ages (3 months, 6 months, 9 months and 12 months) were utilized. The genetic diversity data generated was subjected to analysis using Popgene Software to calculate gene, genotype frequency and Hardy Weinberg equilibrium. The allelic frequencies, heterozygosity (H_e) and polymorphic information content (PIC) were calculated. The association studies between observed variants of studied genes and growth traits were done using SAS software to investigate the effects of polymorphism in the studied genes.

PCR-RFLP analysis of *IGF-1* gene revealed two variants with allele frequency of 0.25 for A allele and 0.75 for B allele. The respective genotype frequencies were 0.51 and 0.49 for AB and BB genotypes. The estimates obtained for Ne, H_{obs}, H_{exp} and PIC were 1.61, 0.51, 0.38 and 0.31, respectively. PIC value (0.31) revealed median level of polymorphism which implied the effectiveness of the marker in population studies in Gaddi goat population. No significant association with various body growth and measurements was established for *IGF-1* locus.

Two fragments of *GH* gene (*GH1* and *GH2*) were also subjected to RFLP analysis that revealed five different patterns. The frequencies of A and B alleles were 0.53 and 0.47 in *GH1* whereas in *GH2*, the allelic frequencies were 0.62 and 0.38 for A and B alleles, respectively. The corresponding genotype frequencies were 0.21, 0.27 and 0.52 for BB, AA and AB in *GH1* whereas 0.76 and 0.24 genotype frequencies were obtained for two variants (AB and BB) of *GH2*. For *GH1*, 1.99, 0.48 and 0.52 values were obtained for Ne, H_{obs} and H_{exp} respectively. For *GH2*, the estimates for Ne, H_{obs} and H_{exp} were 1.89, 0.24 and 0.53 respectively.

The SSCP analysis of two other fragments of *GH* gene (*GH3* and *GH4*) revealed four different SSCP patterns. For *GH3*, two alleles A and B were observed with frequency of 0.67 and 0.33, respectively. The corresponding genotype frequencies were 0.21, 0.22 and 0.57 for AB, BB and AA variants. The values of Ne, H_{obs} and H_{exp} estimated were 1.78, 0.21 and 0.44 for *GH3*, respectively. These measures indicated substantial genetic variation across different regions of *GH* gene for studied Gaddi population. The SSCP analysis for *GH4* fragment revealed absence of polymorphism in studied population which indicated that *GH4* is highly conserved in investigated Gaddi goat population.

The association analysis of different fragments of *GH* gene viz. *GH1*, *GH2* and *GH3* revealed polymorphism but significant association with body weights and body measurements were observed only for 9-month body weight with *GH1* fragment, 9 and 12 months' body girth with *GH2* fragment and 6-month BW, BL and BH with *GH3* fragment.

Conclusions

Following salient conclusions can be drawn based on the findings of the present study:

1. *IGF-1* and *GH* gene fragments (*GH1*, *GH2* and *GH3*) revealed polymorphism in studied Gaddi goat breed, indicating existence of genetic variability in the population.
2. Two genotypic patterns were observed for *IGF-1* gene however, no association was determined between RFLP variants of *IGF-1* gene with body weights and body measurements in investigated population.
3. Three different patterns were observed for *GH1* fragment but significant association of RFLP patterns was observed only with 9-month body weight. In *GH2*, two variants were observed which were found to be significantly associated with heart girth at 9 and 12 months of age. Three SSCP variants were revealed for *GH3* that were observed to have significant association with 6-month BW, BH and BL.
4. The SSCP analysis of *GH-4* fragment was observed to be monomorphic which may be due to sampling effect, resolution of technique or actual absence of genetic variability in the population at studied locus.

Recommendations

1. Patterns/SNPs observed in the present investigation could be combined with traditional selection methods to select Gaddi goats for higher growth. However, reconfirmation of SNPs with increased sample size is needed to convincingly revalidate the results and include those SNPs in breeding programme for improvement in growth.
2. The information generated from the present investigation of growth genes in Gaddi goats could be a valuable input for conservation and future genetic improvement of this goat breed. Future studies may also be directed to study association of these SNPs with other traits like carcass quality.

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APPENDIX-I

DNA Isolation

RBC lysis buffer:

NH_4Cl	155 ml	
$KHCO_3$	10 ml	
EDTA	2 ml	
Distilled water	1000 ml	Autoclave and store at room temperature

Extraction /digestion (SE) buffer:

Tris (pH 8.0)	10mM
NaCl	400mM
EDTA (pH 8.0)	2mM

Tris EDTA:

Tris Cl (pH 8.0)	10mM
EDTA	1mM

0.5 M EDTA:

Dissolve 18.91g of EDTA in 80 ml distilled water by stirring the solution vigorously. Adjust the pH to 8.0 using 10 M NaOH pellets. Make the final volume to 100 ml.

10% SDS:

Dissolve 1g of Sodium dodecyl sulphate in 10 ml of distilled water in a sterile glass vial and keep at room temperature.

Sodium acetate (3 M, pH 5.2):

Sodium acetate	40.8 g
Distilled water	100 ml

Adjust pH with glacial acetic acid.

Proteinase K:

Dissolve 20 mg of proteinase K in 1 ml of double distilled water. Make 400 ul of aliquots per vial and store at -20^{o} C. Make a final volume of 100 ml, sterilize by autoclaving.

Ethanol 70%:

Ethanol 99.9%	70 ml
Distilled water	30 ml

TE buffer:

Tris (1M, pH 8.0)	1 ml
EDTA (0.5 M, pH 8.0)	200 ul

Make final volume to 100 ml with distilled water, autoclave and store at room temperature.

APPENDIX-II

Gel Electrophoresis and Silver Staining

Agarose gel:	0.7%	2%
Agarose	0.7g	2 g
TBE (1X)	100 ml	100 ml

Microwave for 2 minutes, cool for 5 minutes and add 2 µl EtBr.

Ethidium Bromide (10 mg/ml)

Ethidium Bromide	10mg
Distilled water	1 ml

Mix by stirring till dye mixes completely and wrap the container with aluminium foil. Store at 4^0C.

Molecular Weight Markers

50 bp	- 17 fragments	50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000.
100 bp	- 10 fragments	100, 200, 300, 400, 500, 600, 700, 800, 900, 1000.

10% APS:

Dissolve 1 g of Ammonium per sulphate in 10 ml of distilled water.

PAGE :

Acrylamide- Bisacrylamide solution	12ml	Mix thoroughly and filter.
TBE (10X)	5 ml	
Distilled water	30 ml	
Glycerol	3 ml	
APS (10%)	80 ul	Added just before casting.
TEMED	200 ul	

TBE buffer (10X)

Tris base	108 g
Boric acid	55g
EDTA (0.5 M, pH 8.0)	40 ml
Double distilled water	1000 ml

Store at room temperature. Filter and autoclave before use.

TAE buffer (50X):

Tris base `	242 g
$Na_2EDTA.2H_2O$	37.2 g
Glacial acetic acid	57.1 ml
Distilled water	1000 ml

Ethanol (10%)

Ethanol Absolute	50 ml
Distilled water	500 ml

Prepare before use.

Fixative solution:

Glacial acetic acid	37.5 ml
Distilled water	500 ml

Formaldehyde solution:

Formaldehyde	88.23 ml
Distilled water	500 ml

Silver Nitrate solution:

Silver nitrate	0.5 g
Distilled water	500 ml

Developer solution:

Na_2CO_3	15gm
Distilled water	500 ml

Developer Stop solution

Glacial Acetic acid	37.5 ml
Distilled water	500 ml

Store at 4^0 C

Brief Bio-data of the student

Name	:	Gitanjali
Father's name	:	Srinagesh
Mother's name	:	Sudesh Kumari
Date of birth	:	01-01-1994
Permanent Address	:	VPO- Dalogra, Distt.-Rajouri, J&K

Academic Qualifications

Qualification	Month/Year of passing	Name of the board/University	Marks(%) or OGPA
10^{th} class	2009	CBSE	89%
12^{th} class	2011	CBSE	80%
B.V.Sc & AH	2017	Sher-e-Kashmir University of Agricultural Sciences and Technology, Jammu (J & K)	7.04/10
M.V.Sc (AGB)	2019	COVAS, CSK HPKV, Palampur H.P.	8.19

Fellowship/Scholarship/Gold Medals/Award/Any other distinction

Year	Distinction
2012	Awarded ICAR seat in Masters of Animal Genetics and Breeding

Publications:

Total: Nil

Research papers (in peered journals): Nil

Scientific popular articles: Nil

Others: Nil

Visits Abroad with duration and purpose of visit: None

Any other remarks: None