SEQUENCING AND BIOCOMPUTATIONAL CHARACTERIZATION OF CODING REGION OF MAJOR FECUNDITY GENES (GDF9, BMP15 AND BMPR1B) IN BEETAL GOAT

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Submitted to the Guru Angad Dev Veterinary and Animal Sciences University
In partial fulfillment of the requirements for the degree of

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in
BIOTECHNOLOGY
(Minor Subject: Veterinary Microbiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"SEQUENCING AND BIOCOMPUTATIONAL CHARACTERIZATION OF CODING REGION OF MAJOR FECUNDITY GENES (GDF9, BMP15 AND BMPR1B) IN BEETAL GOAT"** submitted for the degree of **M.Sc.,** in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Ramandeep Kaur (L-2019-BT-07-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

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

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CERTIFICATE – II

This is to certify that the thesis entitled, **"SEQUENCING AND BIOCOMPUTATIONAL CHARACTERIZATION OF CODING REGION OF MAJOR FECUNDITY GENES (GDF9, BMP15 AND BMPR1B) IN BEETAL GOAT"** submitted by **Ramandeep Kaur (L-2019-BT-07-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in the partial fulfilment of the requirements for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Veterinary Microbiology**) has been approved by Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

GDF9, BMP15, and *BMPR1B* genes belong to the transforming growth factor β (TGF β) superfamily. They are oocyte-derived growth factors and are mainly expressed in ovaries. The *BMPR1B* is located on granulosa cells (GC) which interact with *GDF9* and *BMP15*. *BMPR1B* has a great influence on cumulus cells expansion, ovulation cycle, and skeleton system development. The present work focused on, the sequencing and biocomputational characterization of *GDF9*, *BMP15*, and *BMPR1B* coding region in Beetal goat. The ovaries were collected from slaughter house and total RNA was isolated by the Trizol method. Single strand cDNA synthesis was done by kit as per manufacturer's protocol and coding regions of these target genes were amplified using gene-specific primers and custom sequenced through Sanger sequencing. Sequence analysis revealed that these target genes could have evolved due to negative selection over the period. Phylogenetic analysis of these genes by MEGA7 indicated that breeds of goat, ruminants, marine, and non-ruminants form different clusters within the phylogenetic tree, non-ruminants showing that they are evolutionarily divergent from the different breeds of the goat. 3D protein structure prediction showed that all amino-acids are in the favorable region in the Ramachandran plot.

Keywords: *GDF9, BMP15, BMPR1B*, cDNA, Sequencing, Phylogenetic

Signature of Major Advisor **Signature of the student**

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LIST OF ABBREVATIONS

°C	:	Degree Celsius
µl	:	microliter
AICc	:	Akaike Information Criterion, corrected
BICc	:	Bayesian Information Criterion
BLAST	:	Basic Local Alignment Search Tool
BMP-15	:	Bone Morphogenetic Protein 15
BMPR1B	:	Bone Morphogenetic Protein Receptor 1B
Cdna	:	complementary DNA
DEPC	:	Diethylpyrocarbonate
DNA	:	Deoxyribo-nucleic acid
dNTP	:	deoxyribonucleotide triphosphate
FSH	:	Follicular Stimulating Hormone
GDF 9	:	Growth Differentiation Factor 9
GH	:	Growth hormone
LB	:	Luria Bertani
LH	:	Luteinizing Hormone
M	:	Molar
MAFFT	:	Multiple Alignment using Fast Fourier Transform
mg	:	Milligram (s)
min	:	Minute (s)
ml	:	Milliliter (s)
mM	:	Milimolar
NCBI	:	National Centre for Biotechnology Information
ng	:	nanogram (s)
OD	:	Optical Density
OD	:	Optical Density
PCR	:	Polymerase Chain Reaction
Pmol	:	pico mole
pmole	:	Picomole(s)
TAE	:	Tris Acetate EDTA

CHAPTER – I

INTRODUCTION

India ranks second in the world in terms of goat's population with about 135.17 million goats, representing 26.4 percent of the country's total livestock (Skapetas & Bampidis, 2016). In India, goats are mostly kept by landless farmers and small landholders, which provide a reliable source of income to the majority of the rural population who are below the poverty line. The total population of goats in India was 148.88million and was increased by 10.17% from 2016 to 2019, the population of Beetal goats in Punjab was 3.19 lakhs (Livestock Census, 2019).

Goat farming is an important part of the agricultural production system, and in some locations, especially in rural areas, goat milk is the primary source of animal protein. The goat is known as a Poor Man's Cow because of its capacity to provide the farmer with meat, milk, fiber, as well as a small surplus for sale. It is less expensive to purchase, easier to procure, and reproduction is more frequent and starts at an early age. It generates manageable amounts of meat, milk, and fiber for consumption or sale within the household (Bhattarai, 2014).

Between 2000 and 2013, global goat-milk production climbed by 39.2 percent (Skapetas & Bampidis, 2016). With 5.18 Metric ton of goat milk produced per year, India is the world's biggest goat milk producer and meat consumption maintains below five kilograms per capita per year (FAOSTAT, 2014). Goat milk, including its products such as powder, cheese, and curd has more calcium, magnesium, and phosphorus than cow and human milk. It also contains fatty acids and precursors of vitamin A. Goat milk fat globules are smaller, have a larger surface area (Getaneh et al., 2016), and have lipases that can specifically target lipids earlier in the gut. Lipases attack the organic acid of short-chain fatty acids (Arora et al., 2013) and this difference leads to significantly faster digestion, therapeutic value, and alkalinity (Lad et al., 2017).

Goats are one of the most prevalent meat-producing animals in India, and their flesh (chevon) is one of the most popular and in great demand throughout the country (Singh et al., 2016). Meat production output climbed by 41.66 percent. Meat is a rich source of high-quality proteins, vitamins, minerals, micronutrients, and lipids. It contains 76.8% water, 2.6% fat, 19.6% protein, 1% mineral, and is considered particularly nutritious among animal meals. It has become an integral part of the human diet. Meat consumption leads to the production of omega-3 fatty acids and conjugated polyunsaturated linoleic acid (fatty acids), which provide a variety of nutrients beneficial to human health (Devi et al., 2014).

Successful reproduction is the result of gene-environment interactions that allow genetic information to be passed down to the next generation. The purpose of reproductive biology is to learn about the main events that control the reproductive axis event and performance. Apes and many ruminants often only release one oocyte per cycle, whereas mice and pigs have consistently higher ovulation rates. Ovulation rate and litter size in animals are the results of planned interactions between endocrine and paracrine mediators (Polley et al., 2010).

GDF9 and *BMP15* are two closely related growth factors known to be important for optimal follicular development, are produced by the oocyte. The superfamily TGFβ includes over 30 proteins, including TGFβ, inhibin/activin, BMP, GDF, anti-Mullerian hormone, and others, that share shared structural motifs. TGFβ superfamily members are essential in controlling differentiation and cellular growth during adult and fetal life (Heldin et al., 1997).

There are three primary fecundity genes: (1) Growth Differentiation Factor 9 on chromosome 7 (*GDF9* as FecG); and (2) Bone Morphogenetic Protein 15 on chromosome X (*BMP15* as FecX) in goats (Hanrahan et al., 2004). (3) Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*, activin-like kinase 6, ALK6 as FecB) on chromosome 6 (Souza et al., 2001); These fecundity genes may show several mutations that can result in sterility in homozygous mutant animals and higher ovulation rates in heterozygous animals (Fabre et al., 2006).

GDF9 and *BMP15* are two genes that play important role in folliculogenesis. In goats, the *GDF9* gene has a 1362 bp mRNA and contains a pair of exons. Exon 1 is 397bp and Exon 2 is 965 bp long whereas the *BMP15* gene has 1230bp mRNA long. Exon 1 is 328bp and the other is 1187bp and the *BMPR1B* gene has a 1789bp mRNA long sequence (Wang et al., 2017). These genes are members of the TGF-β class, which code for different proteins that are mainly expressed in the oocyte of the developing follicle in ovarian tissue and are important for mammalian fertility (Hanrahan, 2001).

During the oestrus and menstrual cycle, *BMP15* has a physiological role in the monotropic rise of FSH secretion. It stimulates FSH but not LH, implying that *BMP15* may have an autocrine regulatory mechanism that selects FSH synthesis (Otsuka et al., 2011).

No work has been reported until now on the study of mRNA sequences in Beetal goat. Therefore, the current plan of work investigates the reproduction performance of fecundity genes. The present study was designed with the following two objectives:

1. To sequence coding region of GDF9, BMP15 and BMPR1B genes
2. To *in silico* characterize GDF9, BMP15 and BMPR1B cds

CHAPTER – II

REVIEW OF LITERATURE

Beetal goat is the native breed of Punjab. Goats have high fecundity rates and also there is an increased demand for goat meat and milk due to higher nutritional values thus it is essential to improve breeding methods that will contribute to the rapid development of goat rearing industries. The present chapter discusses the literature on fecundity genes *GDF9*, *BMP15* and *BMPR1B* gene and their brief role in the biological world.

2.1 Importance of goat farming in livestock

Goats are an important part of the livestock industry, and their hardiness makes them ideal for landless and marginal farmers (Belewu et al., 2009). Goats have an advantage over sheep and cattle in that they can adapt to a variety of diets. They can move for low forage attributable to grazing and digest them accordingly. A goat's reproductive capacity is doubled every year, with some goats capable of producing twins and triplets (Ndossi, 2003) Goats simply adapt to their surroundings. These animals are believed to be the earliest farmers' helpers because they provide manure for farms. Aside from that, they supply skins and hairs for clothing (Fabre, 2000)

In the United Kingdom, goat milk is referred as "Wet Nurse of Infant." For a baby's rapid growth, goat milk is a must-have in their diet. Due to reduced caseins, primarily alpha s-1-casein, it is readily digested and less allergenic than cow milk (Belewu et al., 2009). Sheep and goat milk fats are known to be rich in short-chain and medium-chain triacylglycerols (TAGs). Free long-chain saturated fatty acids, such as palmitic acid Most of the long-chain palmitic acid present in human milk (>70%) is located in the sn-2 position of the TAG structure; this position is considered to be suitable for the digestion and absorption of this fatty acid as well as other nutrients the location of saturated fatty acids, such as long-chain palmitic acid on the sn-2 position of TAGs, makes both the sn-1 and the sn-3 position fatty acids easily hydrolyzable by pancreatic lipases into free fatty acids, and produces sn-2 monoacylglycerols, which are easily absorbed in the small intestine; this also makes the milk calcium completely available and absorbable (Roy et al., 2020). When compared to cow milk, goat milk is higher in palmitic acid, oleic acid, lauric acid, capric acid, caproic acid, and saturated fatty acid (Belewu & Adewole, 2009).

Meat has a distinct flavor and taste, similar to goat milk and cheese, and is thinner and less tender than a variety of red meats. Unlike pig in some cultures, goat meat has no religious roots. Goat meat is low in fat and includes 20% protein, 2% mineral salt, vitamins A, B, and C, as well as are easily digestible (Hazel, 2000).

Goats are seasonally estrous, having a 20-to-21-day estrous cycle. Because tropical goat breeds mature around 5 to 9 months, it is not recommended to mate a goat until it is an adult or one year old (Hamilton, 2002).

Litter size (LZ) refers to the number of kids a goat can have in one kidding. Goats can have single, twin, or triplets. Goat breeders are very interested in increasing kid production as prolificacy is an economically important attribute. The goat's gestation period is 150 days in total. If the gestation period was shorter, litter size can be increased, which can have a substantial effect (Peaker, 1978).

Amongst the native breeds, Beetal is the dual-purpose breed for their milk and meat production. Pure Beetal breeds are mainly found in Gurdaspur, Amritsar, and Ferozepur districts of Punjab (Tantia et al., 2001).

2.1.1 Morphological character of the Beetal goat

Beetal is mostly black in color but sometimes white spots are also observed. The skin color is predominantly grey. The hair is of medium length and straight. Lips, eyes, hooves, and tail switch are black. The horns are either brown or grey in color, 85% of males and 93% of females are horned. The ears are long and hanging. The forehead is convex with a typical Roman nose. Wattles are mostly present in 15% of males. Teats are either funnel or tube-shaped. The teat tips are mostly pointed or rounded (Sharma et al., 2006) The average body length of the male is about 85 and female is about 70 (Acharya, 1982) Birth weights for male and female kids were 3.48 ± 0.06 and 3.29 ± 0.06 kg, respectively. Single-born kids were heavier 3.69 ± 0.06 kg than twins 3.37 ± 0.06 kg and triplets 3.08 ± 0.08 kg. The adult buck has 50-60kg weight and the adult doe has 35-40kg weight (Afzal et al., 2004)

2.2 Signaling pathway for activation of genes

BMP15 follows a similar BMP signaling pathway, binding to the *BMPRIIB* type II receptor and a particular type *BMPRIB* receptor ALK6, which activates RS mads 1, 5, and 8. *GDF9* activates RS mads 2 and 3 through the TGF activin signaling pathway, binding to the same *BMPRIIB*, type II receptor, and a specific type I receptor, ALK5 (TRI). ALK5 mRNA was found in oocytes from humans, sheep, and mice at all follicular stages. ALK5 expression in granulosa cells was found in the developing stage of mice in follicles, in humans primordial to primary stage follicles, and preantral follicles in all species. In ovine species, ALK6 is expressed at granulosa cells from the primary to late preantral follicle stages, with theca cells in a lower extension, and in antral follicles in bovines. In ruminants, the *BMPRIIB* receptor is expressed in the granulosa cells of primordial follicles, while in rodents, it is found in preantral follicles, and it is expressed in all phases of folliculogenesis. The *BMPRII* receptor is required for *GDF9* signaling in granulosa cells, and the extracellular domain of *BMPRII* blocks the cooperative effects of *GDF9* and *BMP15* on granulosa cell proliferation in sheep (Edwards et al., 2008)

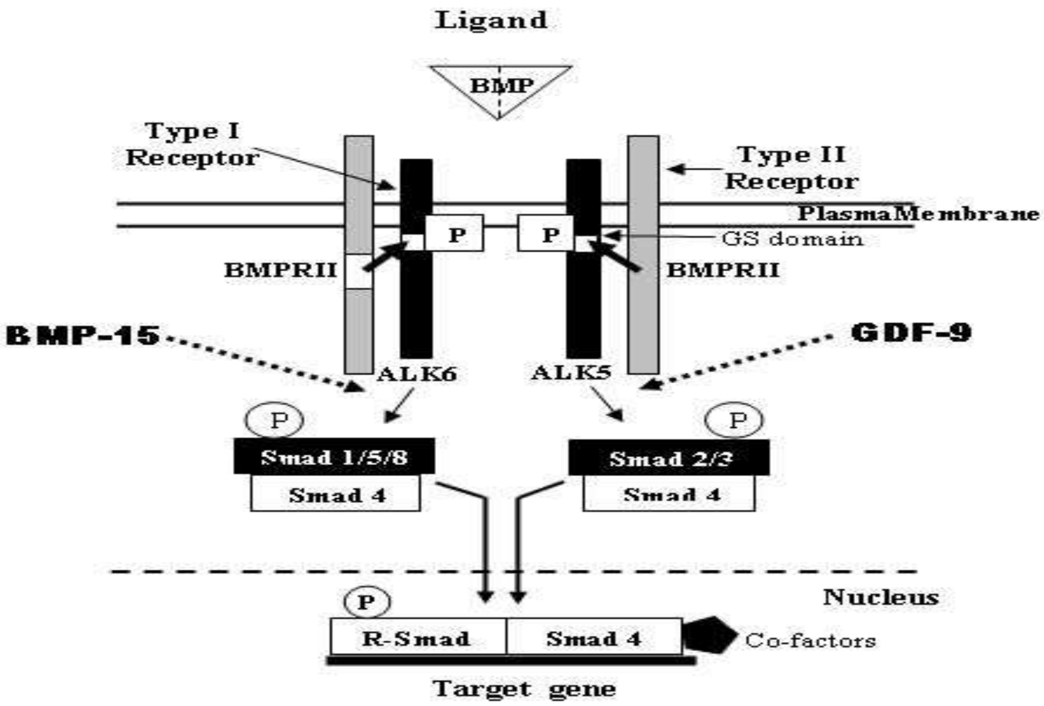


Figure 2.1: Signaling pathway for activation of genes

https://www.researchgate.net/Fig./Fig-1-Receptors-and-signaling-pathways-of-BMP-15-and-GDF-9-The-GDF-9-or-BMP-15_fig1_228736248

2.3 Mutation in Fecundity *(Fec)* gene

When the BMP signaling pathway is less active in the ovary of mutant Fec gene carriers (right) compared to non-carrier (left) ewes, both the positive and inhibitory effects of BMP on granulosa cell (GC) mitosis are diminished. As a result, smaller antral follicles with a lower number of granulosa cells with increased FSH sensitivity, indicating that follicular maturation has advanced as seen by precocious LH receptor expression. The smaller matured follicles found in mutant Fec gene carriers generate less estradiol (E2) and inhibin individually, but they produce the same amount as a large single wild-type follicle. As a result, both genotypes' ovaries and central nervous systems can create the same endocrine dialogue, resulting in the selection and ovulation of multiple smaller follicles in the mutant Fec gene carrier (Fabre et al., 2006)

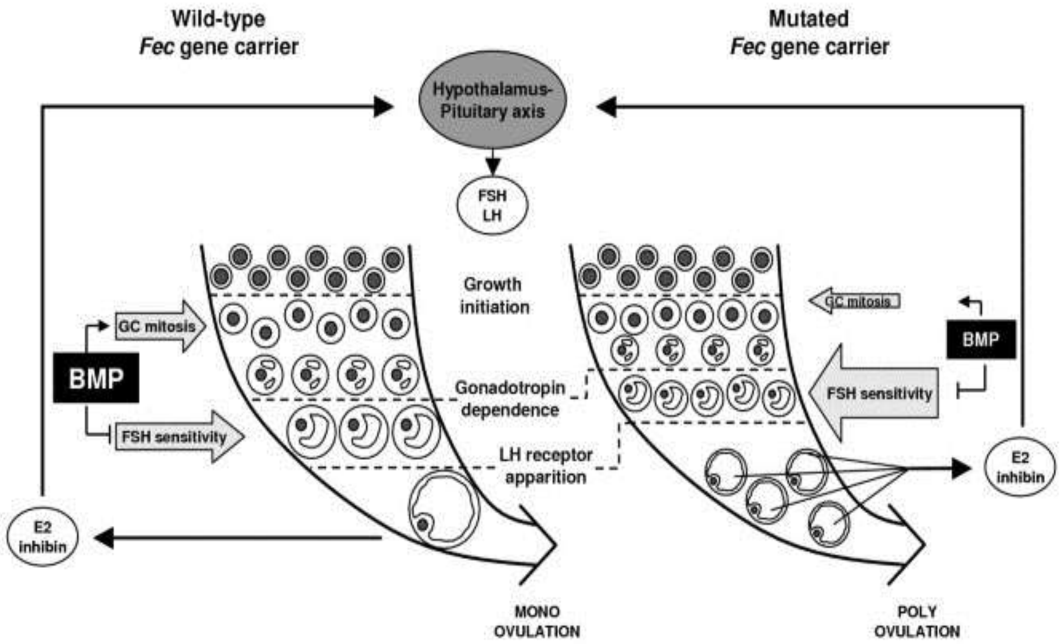


Figure 2.2: Mutation in Fecundity *(Fec)* gene

2.4 Relationship between BMP system activity and ovulation rate in sheep working hypothesis

Loss of function mutations in the BMP system is linked to increased ovulation rates, with additive or multiplicative effects of the mutant FecX, FecG, or FecB genes. As a result, the association is founded on the idea that "the lower the BMP system activity, the higher the ovulation rate". In homozygous FecX mut (mut for alleles I, H, B, G, or L) or homozygous FecGH ewes when BMP activity is low, folliculogenesis is stopped at an early stage, resulting in eventual sterility. Overactivity of the BMP system, on the other hand, may result in anovulation and subsequently sterility due to intense suppression of gonadotropin action *(*Fabre et al., 2006)

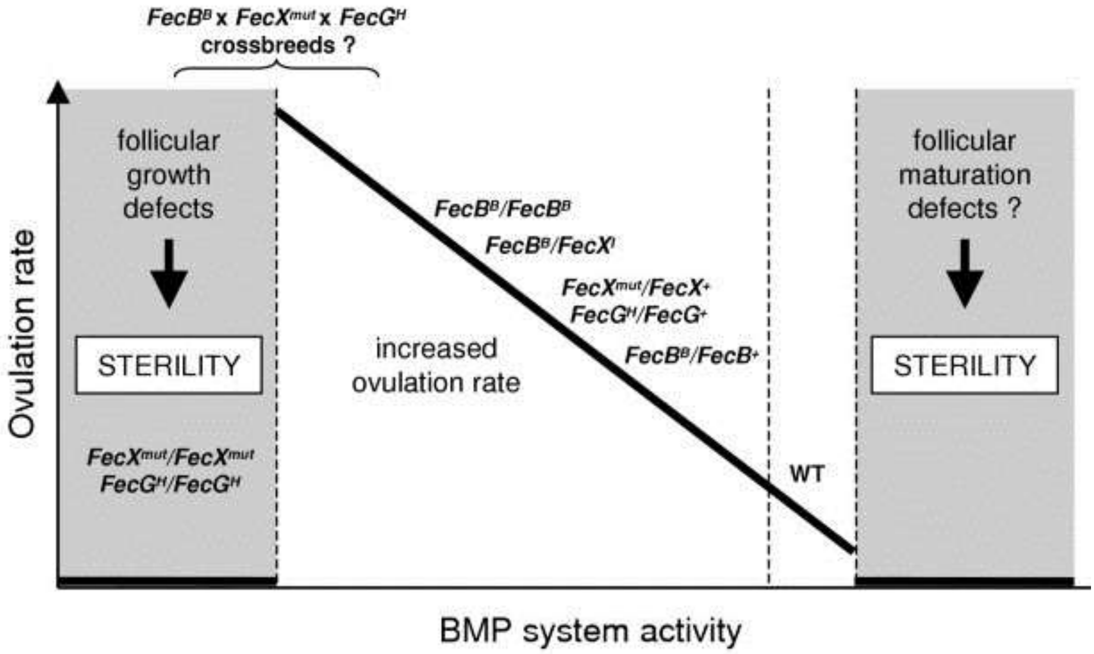


Figure 2.3: Relationship between BMP system activity and ovulation rate in sheep

2.5 Genes affecting ovulation rate in sheep

MacNatty et al. (2005) observed several breeds have been discovered with point mutations in two genes (*GDF9* and *BMP15*) and a related receptor (ALK6) are highly expressed in oocytes, based on hereditary patterns of ovulation rate in sheep. There are five distinct point mutations in the *BMP15* gene, one in *GDF9*, and one in ALK6. Animals heterozygous for all these mutations, heterozygous for two mutations, as well as homozygous for the ALK6 mutation exhibit higher ovulation rates (+0.6–10) over their wild-type counterparts. Because follicular development from the primary stage of growth is stopped in animals homozygous for the *BMP15* or *GDF9* mutations, they are infertile. Reduced quantities of the mature protein or altered binding to cell-surface receptors are thought to be the outcome of the *BMP15* and *GDF9* mutations. *GDF9* mRNA is found in germ cells before and after ovarian follicular formation, and during follicular growth in sheep, whereas *BMP15* mRNA is only detectable in oocytes from the initial stage of development. At most, if not all, phases of follicular growth, ALK6 and associated cell-surface receptors like ALK5 and BMPRII mRNA are present in oocytes. The presence of *GDF9* and *BMP15* proteins in follicular fluid indicates that they are secreted products. Both growth factors are required for follicular development, ovulation, and/or corpus luteum formation, according to sheep immunized with *GDF9* or *BMP15* peptides. Ovarian follicles in mice with the ALK6 mutation mature prematurely, resulting in three to seven follicles ovulating at lower diameters, with no increase in ovarian steroid or inhibin productions compared to wild-types. One key effect of the ALK6 mutation appears to be a reduction in the capacity of some BMPs to limit follicular cell development. *BMP15*, *GDF9*, and ALK6 may be targeted for new techniques of fertility management in several mammals, according to recent discoveries in sheep.

McNatty et al. (2005) studied animals homozygous for the *BMP15* or *GDF9* mutations are a genetic trait, but animals heterozygous for *BMP15* or *GDF9*, or heterozygous or homozygous for ALK6, ovulate at a higher rate than usual. Immunization of sheep with *BMP15* or *GDF9* demonstrates that both are required for optimal follicular growth and ovulation rate regulation. Changes in oocyte development during early preantral follicular growth, quicker maturity of granulosa cells, and ovulation of mature follicles with reduced diameters are common hallmarks of fertile animals with the *BMP15*, ALK6 mutations.

Table 2.1: Location of *GDF9* gene in different species

Chromosomal location, number of exons, Coding Region, and Accession no of *GDF9* gene in different species

S. No.	Species	Chromosomal location	No of Exons	Coding Region	Accession no	Reference
1.	Goat *(Capra hircus)*	7	2	1362bp	NM_001285708.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001285708.1
2.	Cattle *(Bos Taurus)*	7	2	1396bp	NM_174681.2	https://www.ncbi.nlm.nih.gov/nuccore/NM_174681.2
3.	Sheep *(Ovis aries)*	5	2	1362bp	NM_001142888.2	https://www.ncbi.nlm.nih.gov/nuccore/NM_001142888.2
4.	*Mice* *(Mus musculus)*	11	2	1446bp	NM_008110.2	https://www.ncbi.nlm.nih.gov/nuccore/NM_008110.2
5.	Pig *(Sus scrofa)*	2	1	1335bp	NM_001001909.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001001909.1

Table 2.2: Location of *BMP15* gene in different species

Chromosomal location, number of exons, Coding Region, and Accession no of *BMP15* gene in different species

S. No.	Species	Chromosomal location	No of Exons	Coding Region	Accession no	Reference
1.	Goat *(Capra hircus)*	X	2	1185bp	NM_001285588.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001285588.1
2.	Cattle *(Bos Taurus)*	X	2	1185bp	NM_001031752.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001031752.1
3.	Sheep *(Ovis aries)*	X	2	1182bp	NM_001114767.2	https://www.ncbi.nlm.nih.gov/nuccore/NM_001114767.2
4.	*Mice* *(Mus musculus)*	X	2	1540bp	NM_009757.5	https://www.ncbi.nlm.nih.gov/nuccore/1060604636
5.	Pig *(Sus scrofa)*	X	2	1262bp	NM_001005155.2	https://www.ncbi.nlm.nih.gov/nuccore/NM_001005155.2

Table 2.3: Location of *BMPR1B* gene in different species

Chromosomal location, number of exons, Coding Region, and Accession no of *BMPR1B* gene in different species

S. No.	Species	Chromosomal location	No of Exons	Coding Region	Accession no	Reference
1.	Goat *(Capra hircus)*	6	11	1578bp	NM_001285575.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001285575.1
2.	Cattle *(Bos Taurus)*	6	10	1521bp	NM_001105328.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001105328.1
3.	Sheep *(Ovis aries)*	6	12	1665bp	NM_001009431.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001009431.1
4.	*Mice* *(Mus musculus)*	3	11	1720bp	NM_001355043.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001355043.1
5.	Pig *(Sus scrofa)*	8	13	1913bp	NM_001039745.1	https://www.ncbi.nlm.nih.gov/nuccore/NM_001039745.1

2.6 Integral role of genes

The oocyte plays a crucial role in regulating and promoting follicle growth, and thus its development, by producing oocyte growth factors that primarily act on supporting granulosa cells through paracrine signaling. *GDF9* and *BMP15* both derived from oocytes and transforming growth factor (TGF) superfamily, were found to play critical roles in ovarian function in genetic studies in mice. The identification of *BMP15* and *GDF9* gene mutations because the causal mechanism underlying the highly prolific or infertile nature of several sheep strains during a dosage-sensitive manner also highlighted the important role these two genes play in ovarian function. Numbers of mutations in the *GDF9* and *BMP15* genes have been identified in women with premature ovarian failure and mothers of dizygotic twins (Otsuka et al., 2011).

Growth factors regulate the migration of germ cells to the gonadal ridge, which is critical during early ovarian development and folliculogenesis. Follicle recruitment, oocyte maturation, ovulation, granulosa cell proliferation/atresia, theca, steroidogenesis, and luteinization are all affected. *GDF9* and *BMP15*, both of which are members of the transforming growth factor-beta (TGF-β) superfamily, have been suggested as crucial for follicular development. *GDF9* and *BMP15* play a crucial role in the progression of the primordial follicle to the main follicle, boosting the expression of steroidogenic acute regulatory proteins, luteinizing hormone receptors, and plasminogen activator in the final stages of follicular development and maturation (LHR). These proteins also play a role in the interconnections between the cumulus cells and oocytes, where they regulate amino acid absorption, cholesterol production, and glycolysis. In vitro observations indicate that the factors *GDF9* and *BMP15* stimulate the growth of ovarian follicles and proliferation of cumulus cells by inducing mitosis in cells and granulosa and theca expression of genes linked to follicular maturation, although the mode of action has not been fully established (De et al., 2016).

GDF9 and *BMP15* are oocyte-secreted proteins that influence the cell fate of somatic granulosa cells, and also the quality and developmental competence of the egg, to control ovarian function in female reproduction. In women who have dizygotic twins, mutations in the *GDF9* and *BMP15* genes have serious consequences (Belli & Shimasaki, 2018).

The function of oocyte-secreted factors in the production and differentiation of ovarian follicles has long been recognized, but the factors involved in folliculogenesis have yet to be fully characterized. Studies on *GDF-9*, *BMP-15,* and two novel superfamily members TGF β, have dramatically changed our view of ovarian physiology. Loss-of-function experiments in female mice revealed that *GDF-9* is required for fertility, and in vitro studies revealed that *GDF-9* is required at multiple stages of female reproduction. *BMP-15*, which has a high degree of homology with *GDF-9*, does not appear to be as important in female mouse fertility as *GDF-9*, given the fact that its expression pattern is very similar to *GDF-9*. Studies on sheep having natural mutations in the *BMP15* gene, FecXI, and FecXH, show that *BMP-15* is associated with infertility and super-fertility in a dosage-sensitive manner. Evidence from in vitro experiments shows that *BMP-15* inhibits major FSH actions that are obligatory for follicle development and ovulation by suppressing FSH receptor expression in rats. In a species-dependent manner, *GDF-9* and/or *BMP-15* can play dominant roles in female fertility (Xuemei & Matzuk, 2002).

2.7 Status of fecundity genes in different species

2.7.1 Goats

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR were used to detect the genes' mRNA expression in prolific Jining Grey goats and non-prolific Liaoning Cashmere goats. *GDF9* and *BMPR1B* were reported to be extensively expressed in 20 tissues, however, the *BMP15* gene was only found in the ovary and pituitary. *GDF9* and *BMPR1B* genes have the highest levels of expression in the ovary. *GDF9* and *BMPR1B* genes have the highest expression levels in the ovary. The expression level of the *BMP15* gene in the ovary of the Jining Grey goat was 4.96-fold greater than that of the Liaoning Cashmere goat (P 0.05), however, the expression levels of *GDF9* and *BMPR1B* genes were not different ($P > 0.05$). the study is to analyze the tissue expression patterns of the *GDF9*, *BMP15*, and *BMPR1B* genes in goats, and *BMP15* appears to be a key gene for Jining Grey goat prolificacy (Pan et al., 2015).

The amounts of *BMPRIB* and *BMPRII* mRNA in caprine preantral follicles, as well as small and big antral follicles, were determined using real-time polymerase chain reaction (PCR). Preantral follicles (≥150 µm) from goat ovaries were extracted and cultured in α-MEM+ supplemented with or without *BMP-15* (10, 50, or 100 ng/ml) for 18 days. Some follicles were fixed for ultrastructural examination at the end of the culture. When granulosa/theca cells from large antral follicles were compared to those from small antral follicles, higher amounts of *BMPRIB* mRNA were found. Furthermore, cumulus-oocyte complexes from big antral follicles produced more *BMPRII* mRNA than their corresponding granulosa/theca cells. After 18 days in culture, 50 ng/ml *BMP-15* promoted antral cavity formation and follicle growth while also maintaining follicular integrity. As a result, *BMPRIB* and *BMPRII* mRNAs can be found in all follicular types. After 18 days of culture, BMP-15 (50 ng/ml) increases proliferation, antrum development, and ultrastructural integrity of isolated caprine preantral follicles (Lima et al., 2012).

2.7.2 Ewes

Davis et al. (2005) studied in sheep the *GDF9* gene, *BMP15*, and *BMPR-1B* genes the ovulation rate increases when mutations have been found and others are known to exist from the expressed inheritance patterns although the mutations have not been located. In *BMP15*, four different mutations have been found but each produces the same phenotype. Autosomal dominant genes with additive effects on ovulation rate (*BMPR-1B*; Lacaune), autosomal over-dominant genes with infertility in homozygous females, are all modes of inheritance for prolificacy genes. *GDF9*, X-linked maternally imprinted genes FecX2 and X-linked over-dominant genes with infertility in homozygous females *BMP15*. The effect of one copy of a mutation on ovulation rate varies, ranging from an extra 0.4 ovulations per oestrus for FecX2 to an extra 1.5 ovulations per oestrus for *BMPR-1B*. DNA tests of these mutations can be used in genetic modification programs based on marker assisted selection.

Sejian et al. (2015) studied in ewes the effect of a FecB gene mutation on the growth and reproduction of Garole Malpura ewes. The study lasted five months and involved 97 adult Garole Malpura (GM) ewes. These 97 animals were separated into three groups based on their FecB genotype: group I (FecB BB; n = 13), group II (FecB B+; n = 65), and group III (FecB ++; n = 19). All of the animals were kept under a semi-intensive management scheme that included grazing during the day and lodging at night. Aside from grazing, ewes were fed a concentrate combination at a rate of 150 grams per day per ewe. The ewes in estrus, mostly in morning and evening, with a ram of proven fertility after 12 hours after estrus start. Real-time B-mode transabdominal ultrasonography verified the pregnancy. The ewes' development and reproductive characteristics were evaluated under different FecB genotype treatments. Body weight, heart girth, pin shoulder-length, height at wither, lambing percentage, litter size, twinning percentage, and birth weight of lambs were all affected by the FecB genotype (P 0.05). It may be stated from this study that breeding the Galore and Malpura breeds will assist in ensuring an economically sustainable future.

2.7.3 Humans

Persani et al. (2014) studied *BMP15* and *GDF9* generated from oocytes, is a key regulator of folliculogenesis and GC activity. Variations in *BMP15* gene dosage have a significant impact on ovarian function and can cause a variety of female reproductive problems. *BMP15* maps to the X locus, which is implicated in determining the ovarian deficiency in Turner syndrome and contributes considerably to ovarian reserve determination.

Four *BMP15* gene variants were identified in 6 patients in heterozygous conditions. Out of these 4 variants, 3 variants namely, c.538 G>T (p. Aln180 Ser), c.165A>T (p. Glu55Asp) and c. 510_512 delT were novel. In silico analysis using SIFT, Provean, and Polyphen 2 score predicted the non-deleterious effect of c.165A>T and c.538 G>T variant. 788insTCT variant was identified in 3 patients (Kumar et al., 2017).

2.7.4 Zebrafish

Clelland et al. (2006) studied the mature protein shares 46–51 percent of its sequence with BMP-15 from fugu, chicken, and mammals. It also has 38–46 percent similarity with fish, poultry, and mammals' growth and differentiation factor-9. The zfBMP-15 is most closely linked to *BMP15* from other species, according to a phylogenetic study, although the *GDF9* peptides from fish to mammals form a separate branch. When zfBMP-15 cDNA was compared to the zebrafish genome database, it was discovered that zfBMP-15 is encoded by a gene on chromosome 6 that has two exons and one intron. *BMP15* mRNA is found in the ovary and testis, as well as the brain, liver, gut, heart, and muscle, to a lesser level. The results of real-time PCR were revealed. BMP-15 plays a role in regulating gonadal functions in fish, in particular oocyte maturation.

Recent zebrafish studies have provided preliminary evidence that BMP-15 is also a key regulator of ovarian function. During follicle growth and maturation, the zebrafish ovary produces BMP-15. *In vitro* studies using zebrafish, follicles show that incubation with recombinant human BMP-15 or overexpression of BMP-15 in oocytes inhibits gonadotropin- and maturation-inducing hormone (MIH)-induced oocyte maturation. Immunoneutralization with BMP-15 antiserum or suppressing BMP-15 expression with morpholino antisense oligonucleotides, on the other hand, promotes oocyte maturation. The sensitivity of follicles to MIH is an important stage in BMP-15 activity. In vivo injection of BMP-15 antiserum reduces maturation-incompetent (MIH-insensitive) smaller early growth phase follicles while increasing mature follicles. These data suggest that BMP-15 has a role in preventing premature oocyte maturation in zebrafish. BMP-15's regulation of early oocyte maturation may be critical for oocyte quality, ovulation, and fertilization, according to our findings (Peng et al., 2009).

2.7.5 Hen

Elis et al. (2007) studied *BMP15* expression in the ovaries of hens. In hen GCs, *BMP15* might activate the Smad1 (mothers against decapentaplegichomolog1) signaling pathway. Furthermore, they discovered that *BMP15* inhibits gonadotropin-induced progesterone synthesis in hen GCs. This inhibitory action was linked to a drop in the amount of the steroidogenic acute regulatory protein (STAR). The research shows that *BMP15* may play an important role in avian female fertility.

The ideal ovulation rate in the hen is determined by the placement of a single follicle in the pre-ovulatory hierarchy. In preparation for ovulation, follicle selection is connected with enhanced oocyte development and changes in gene expression in the granulosa cells surrounding the oocyte. The expression, function, and regulation of bone morphogenetic protein-15 (*BMP15*) during follicle development in the hen were examined in this work. The expression of *BMP15* mRNA in the ooplasm and granulosa cells of 3 mm follicles was examined, and it was shown to be predominantly in the ooplasm. By culturing granulosa cells from 3–5 mm and 6–8 mm follicles with recombinant human *BMP15*, the function of *BMP15* was investigated (rh*BMP15*). In the hen, *BMP15* boosted the expression of follicle-stimulating hormone receptor (FSHR) mRNA while decreasing anti-Müllerian hormone (AMH) mRNA and occludin (OCLN), both of which are involved in follicle maturation and growth. Whole follicle culture with estradiol (E2), which elevated *BMP15* mRNA expression, was used to examine hormone regulation of *BMP15*. The fact that *BMP15* and its receptors have a different expression pattern, as well as the fact that *BMP15* increases FSHR mRNA while decreasing AMH mRNA and OCLN mRNA and protein production, suggests that the oocyte may play a part in the process (Stephens et al., 2016).

2.7.6 Frog

Unexpectedly, this is discovered during the early stages of Xenopus (frog) development. *BMP15* is both required and sufficient for the definition of dorso-anterior structures, and new mechanisms of *BMP15* function have been discovered, pointing to a reconsideration of its role in the ovaries, particularly in the context of ovarian failure (Di et al., 2009).

The *grem1* gene codes a protein that inhibits the action of multiple members of a growth factor family known as bone morphogenetic proteins (BMPs). Certain members of this BMP family can regulate both fecundity and fertility in mammals via their action on oocyte (egg) development, and *grem1* has been identified as a marker of oocyte quality in humans. The model amphibian *Xenopus laevis* is far more fecund than mammals, producing thousands of eggs in a clutch. However, female transgenic frogs carrying *grem1* under the control of a stress-inducible *hsp70* promoter ("G" frogs) produce very few viable offspring. Here, we show that this is not due to reduced fecundity or fertilization rate, but results from a significant reduction in subsequent survival over the first day of development. Embryos that successfully survive for the first day were found to go on to develop normally when compared to their peers. Both the morphology and stage distribution of oocytes from G females appear normal, and oocytes develop at expected rates, although stage VI oocytes were found to have a lower response to in vitro progesterone treatment. Unexpectedly, levels of *grem1* mRNA were found to be consistently lower in the female ovaries from four independent G transgenic lines than in wild-type ovaries. Both transgenic and wild-type offspring were equally affected, confirming a maternal effect. Our study shows that transgenic females with the lowest levels of *grem1* transcripts in the ovary have the lowest rates of survival past the first day of amphibian embryogenesis, equivalent to pre-implantation staged mammalian embryos. The reduced expression of *grem1* in the oocytes of transgenic females suggests transgene suppression of an endogenous locus may occur in the *Xenopus* female germline, an unexpected finding (Beck et al., 2018).

2.8 Evolutionary divergence studies in various species

Divergent evolution allows species with common ancestral origin to be able to adapt to their habitats. Thus, it is likely to ease competition among them since they evolve traits that make them explicitly suited to their habitat and ecological niche.

Rajput et al. (2018) studied the *in-silico* characterization of the coding region of buffalo, CXCR1 (Interleukin-8 Receptor A) that is one among the 2 high-affinity receptors (IL-8RA and IL-8RB), present on the surface of neutrophils that binds to the IL-8 ligand, which ends up in the activation of immune reaction and allows the migration of immune cells to the site of inflammation. CXCR1 is associated with inflammatory responses against most Gram-negative bacteria. The amplified target gene of buffalo CXCR1 was custom-sequenced, and therefore the annotated sequence of 1130bp having a coding region of 1083bp submitted to DDBJ with accession number LC384988. Pairwise sequence alignment of the buffalo CXCR1 and the reference cattle CXCR1 matched 95% similarity. CXCR1 Sequence analysis and integrative analysis in 36 divergent species showed that 7 codon positions have undergone positive selection pressure, indicating that the gene has evolutionary changed over the time. Evolutionary analysis of the CXCR1 gene (Data monkey and MEGA 6 online server) showed that ruminant, avian, fish CXCR1 formed different clusters within the phylogenetic tree, showing evolutionary divergence.

Bhardwaj et al. (2015) reported *in silico* characterize the TLR4 gene of India water buffalo (Bubalus bubalis). Primers were (viz. TLR4-2B, TLR4-3B, and TLR4-5B) designed from TLR4 gene complete cds sequences available on NCBI database after thorough Clustalomega analysis. The blood sample was collected by a jugular vein of healthy Murrah buffalo and was processed to isolate peripheral blood mononuclear cells (PBMCs). Total RNA extracted from PBMCs, cDNA synthesized, and TLR4 cDNA amplified. PCR amplification resulted in amplicons of 863bp, 871bp, and 899bp, respectively. The amplified product was cloned in TA cloning vector. Blue-white screening on a selective medium was used to select recombinant clones (LB agar containing X-gal, IPTG, and ampicillin). Six white colonies were chosen at random and grown in LB broth with ampicillin (100g/ml). Plasmids were isolated from clones using a standard protocol, and the presence of the insert was tested using PCR and *EcoRI* restriction digestion. Sequencing was performed on positive clones, and the results were analyzed. The deduced sequence was submitted to NCBI. The gene has been shown to be evolutionary conserved, according to phylogenetic analysis. The present study suggested that TLR4 cds are highly conserved among the distant species.

Singh et al. (2015) reported the primary coding sequence of bubaline dicer, which was 5778bp long, and used it for further bioinformatics analysis. Divergence studies through evolutionary tree revealed that all the transcript variants of Dicer1 belonging to the specific species were within the same node and the sequences belonging to primates, rodents, and lagomorphs, avian, and reptiles formed independent clusters. The bubaline Dicer1 is seen to be closely related to that of cattle and other ruminants and significantly divergent from dicer of lower species such as tapeworm, sea urchin, and fruit fly. MEGA 6 software was used for evolutionary divergence studies conducted that dicer has undergone purifying selection over time. Seventeen divergent sequences were used to study the specific regions of positive as well as negative selection using different models like single likelihood ancestor counting, fixed effects likelihood, and random effect likelihood of Datamonkey server. Further through comparative analysis of domain structure, it was revealed that the dicer1 protein is conserved across mammalian species while variation both term of length of Dicer enzyme and presence or absence of domain is evident in the lower organism.

Bhardwaj et al. (2016) reported through phylogenetic analysis and multiple sequence alignment that host-specific PRRs i.e., TLRs forms entirely different clusters, with the active domain of NLR (NACHT) evolved earlier in comparison to the active domains of TLRs. The heat map analysis showed that evolutionary divergence was seen in the TIR NACHT domains of TLRs with respect to each others.

Hussain et al. (2016) reported *in silico* characterized the cloned bubaline cathelicidin3 (Cath3) peptide and studied the evolution of bubaline caths. Multiple sequence alignment and the homologous peptide sequence of Cath3 revealed an insertion of 6 amino acids in the cathelin domain of the bubaline Cath3 peptide when compared with that of cattle. Further biocomputational analyses of the Cath3 coding sequence (cds) as well as the amino acid sequence by using MEGA6 software & Datamonkey webserver revealed that different types and transcript variants of cathelicidin varied considerably within the same species which indicated the role of natural selection during the evolution of caths. The cathelicidin cds belonging to divergent species were analyzed using different models like SLAC, FEL, and REL (Datamonkey webserver) and it was con cluded from the REL model that bubaline cathelicidin3 antimicrobial peptides have undergone episodic positive selection (in up to 36 codons) conferring selective advantage in the evolution of the peptide.

Dhaliwal et al. (2015) reported the *in-silico* analysis of functional divergence of two ovine cathelicidin coding sequence (cds) variants of Indian sheep ie Cath1 and Cath2. The reported that Evolutionary analysis of the Cath2 and Cath1 found that the mammalian cathelicidins clustered separately from avian fowlicidins based on the evolutionary differences between them. Amino acid sequence analysis revealed that Cath1, Cath2, and Cath7 of different ruminant species (including our Cath1 and Cath2 variants) forms individual clads, suggesting that these types have evolved differently to the target specific types of microbes. Further *in silico* analysis of Cath1 and Cath2 peptide sequence indicated that the C-terminal antimicrobial peptide domain of Cath2 is more immunogenic than that of the ovine Cath1 because of its higher positive antigenic index, making Cth1 a promising antigen for the production of monoclonal antibodies.

2.9 Polymorphism study of fecundity genes

After Garole in India, Kendrapada's second prolific breed of sheep was screened for a mutation in three fecundity genes: FecG, FecX, and FecB. Samples were genotyped using the tetra primer amplification refractory mutation system-PCR, and 11 SNP sites, one on FecB, and five each on *BMP15* and *GDF9* were evaluated. Ewes with non-carriers, heterozygous, and homozygous carriers of the FecB locus mutation had litter sizes of 1.61, 1.80, and 2.06 respectively. According to Dash and his colleagues' research, Kendrapada sheep are the sixth sheep breed to have a coexisting polymorphism in two separate fertility genes BMPRIB and *GDF9* genes (Dash et al., 2017).

Zhu et al. (2013) investigated the relationship between the genetic polymorphism of Growth Differentiation Factor 9 (*GDF9*) genes and the litter size in 384 individuals of five breeds of Black goats. Four pairs of primers were designed to detect SNPs of the *GDF9* gene in goats by PCR-SSCP. The least-square was used to analyze the relationship between different genotypes and the litter size. Three genotypes (AA, AB, and BB) were identified polymorphic with primer pair 1 in Big Foot (BF) and Jin Tang (JT) Black goats. For primer pairs of P2, P3, and P4, there was no polymorphism. The sequencing results revealed a single nucleotide mutation (A792G) in exon 2 of the *GDF9* gene in BF and JT Black goats. This mutation resulted in an amino acid change: valine to isoleucine. *GDF9* gene could be therefore considered as a candidate gene for marker-assisted selection of litter size traits in goats.

Shokrollahi and their colleagues investigated the polymorphism of *GDF9* and *BMPR1B* genes and their relation with markhoz goats. The polymorphism of these genes in sheep and goats was well documented with the help of RFLP-PCR and tetra primer amplification mutation system-PCR (T-ARMS-PCR). The 164 blood samples were tested. PCR was performed using two pairs of primers to detect polymorphism in *GDF9* and *BMPR1B* genes, respectively. PCR products were digested with *PvuII* (G423A), *SspI* (G3288A), *MspI* (G1189A), and *MvaI* (A959C), restriction enzymes. Results show that the mutation is present in the tested animals and has no significant effect on litter size (Shokrollahi & Morammazi 2018).

The association study between FecB and high prolificacy in rather goats was undertaken and three genotypes of the *BMPR1B* gene due to allele A and G with the heterozygous mutant. FecB was screened for polymorphism studies through T-ARMS-PCR and electrophoretogram. All the tested animals showed amplification of outer PCR product that had amplicon size 1100bp and this product size was a wild homozygous genotype. There was no inner band of amplicon size 136bp and it described that there was no FecB mutation. If the sample showed both bands of 1100bp and 136bp then the genotype of heterozygous mutant type (Palai et al., 2013).

Goat farming in Bangladesh is primarily centered on indigenous Black Bengal goat, a highly prolific breed. Searching for genetic markers associated with prolificacy in this breed is vital for the country's goat breeding industry. However, there are no reports on polymorphisms associated with the fertility of Bangladeshi Black Bengal goats. This study investigated two major fecundity genes- *BMP15* and *GDF9* to detect any possible mutations in these two genes associated with litter size in Black Bengal goats. Blood samples were collected from 40 raised goats in Hathazari Government Goat Farm, Bangladesh. Genomic DNA was extracted; PCR amplification was performed, and sequencing of PCR products was performed to detect polymorphism loci in the target genes. Five SNPs viz. C735A, C743A, G754T, C781A, and C808G were detected in exon 2 of *BMP15* gene. In *GDF9* exon 2, an SNP (T1173A) was discovered. SNPs at the 735, 754, and 781 nucleotide positions of *BMP15* exon 2 were found to have a significant relationship with litter size in Black Bengal goats. The influence of parity on litter size was also highly significant (P 0.001). This study looked at SNP loci in fecundity genes in Bangladeshi productive Black Bengal goats for the first time. Further research with a large number of genetically unrelated animals to investigate the connection of these and other fecundity genes with litter size may be beneficial (Das et al., 2021)

CHAPTER – III

MATERIALS AND METHODS

The protocols followed to conduct the cloning, sequencing and biocomputational characterization of the molecular sequences along with the standardization processes have been present in this chapter.

3.1 Instruments

The important instruments used in the present study includes Horizontal Electrophoresis assemblies (Bio-Rad, USA), Thermocycler (Bio-Rad), Minicentrifuge, Laminar Air Flow, Refrigerated centrifuge, UV Transilluminator (WEALTEC USA), Gel documentation system (SYNGENE G: Box Singapore), Spectrophotometer (Thermo Scientific NanoDrop One USA), Dry bath (Labnet AccuBlock USA), Shaker Incubator, Water bath (Stuart swbd UK), Vertical Deep Freezer, Digital pH meter, Autoclave, Microwave oven (Panasonic China), Electronic balance (AND GF-300 Japan), Distillation Assembly (Micropore Q3 France), etc. available at College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

3.2 Chemicals, glassware, plastic ware, and media

Molecular Biology grade chemicals were procured from Amresco (USA), G biosciences (USA), Invitrogen (USA), Thermo Scientific (USA), Qiagen (Japan), SRL (Mumbai), and Restriction Endonucleases enzymes from Thermo Scientific (USA). Glassware was procured from Borosil, India while plastic ware from Tarsons, India.

Dehydrated Bacteriological Media viz. LB (Luria Bertani) broth, LB (Luria Bertani) agar, and other media ingredients viz. tryptone, yeast extract, etc. were obtained from HiMedia Laboratories, Mumbai.

3.3 Methods

Isolation of total RNA from ovaries of goat

The Total RNA isolation was carried out by the method described by Sambrook and Russell (2001) with slight modifications, as mentioned below:

1. Ovaries samples (n=4) were collected in a sterile 50ml tube containing RNA later.
2. Samples were washed twice with 0.9% saline or by 75% ethanol, then homogenized using sterilized pestle and mortar, followed by addition of Trizol reagent and triturated completely (Ambion by life technology).
3. Transferred homogenate to 2ml DEPC treated microcentrifuge tubes.
4. The supernatant was collected following centrifugation @ 12,000rpm at 4°C for 10 min.
5. The supernatant was collected and transferred into new sterile 1.5ml MCTs.
6. 600µl supernatant harvest was taken in 1.5ml centrifuge tubes.
7. To this, 200µl chloroform (HiMedia) was added and mixed well by gentle vortexing.
8. Again, centrifuged @ 12,000rpm at 8°C for 15 min.
9. The supernatant was collected and transferred to fresh 1.5ml tubes
10. Added 500µl of isopropanol (Amresco) and mixed by inverting.
11. Centrifuged @ 12,000rpm at 4°C for 10 min.
12. The supernatant was discarded; the RNA pellet was washed with 500µl of 75% ethanol and centrifuged @ 8,000rpm at 4°C for 5 min.
13. Again, the supernatant was discarded and the RNA pellet was air-dried for 30-45min.
14. The pellet was dissolved in 30 µl Nuclease Free Water.

3.4 Quality check of RNA by agarose gel electrophoresis

RNA samples were diluted and subjected to agarose gel electrophoresis for quality check.1.2% agarose in 1X TAE buffer (pH 8.0) was used for gel electrophoresis. Agarose 1.2 g was dissolved in 1X TAE buffer to a final volume of 100 ml and was heated in a microwave oven. Melted agarose was taken out carefully. Ethidium bromide was added 5µl/100 ml of gel solution. Melted agarose was poured into a gel casting tray with the comb positioned appropriately and allowed to solidify.

Once the gel got solidified, the gel was placed in the tank, and then comb taken out carefully without disturbing the wells and it was transferred to a horizontal electrophoresis tank filled with 1X TAE buffer. The level of the buffer was kept at least 1cm above the gel. The wells were carefully loaded with 5µl RNA mixed with 1µl 6X gel loading dye using a micropipette. Electrophoresis was carried out at 85V for 45-55 min. On completion of electrophoresis, the gel was visualized under the gel documentation system to judge the quality of RNA.

3.5 Quantification of RNA using NanoDrop spectrophotometer

1) The RNA samples, stored at -20^{o}C, were properly thawed at room temperature.
2) To begin, the upper and lower optical surfaces of the micro volume spectrophotometer sample retention system were cleaned by pipetting 1µl of clean nuclease-free water onto the lower optical surface.
3) The lower arm was closed, ensuring that the upper pedestal comes in contact with the nuclease-free water and the arm lifted to wipe off both optical surfaces with a clean, dry, lint-free lab wipe.
4) NanoDrop software was opened and selected the RNA option from the screen.
5) Lowered the lever arm once the blank measurement was completed both optical surfaces were cleaned with a clean, dry, lint-free lab wipe.
6) The appropriate amount of the sample was loaded in the lower optical surface and lowered lever arm then automatically measured the RNA concentration and purity.

3.6 Synthesis of complementary DNA (cDNA) by reverse transcription

The RNA samples qualifying the standard were converted to cDNA by using Takara PrimeScript™ first-strand cDNA synthesis kit.

1. The template RNA was thawed on ice. The components of the Takara cDNA synthesis kit (5x prime script Buffer, Primescript Reverse Transcriptase, 10Mm dNTPs, Random hexamer primer, RNAse inhibitor, and Nuclease free water were thawed on ice.
2. Following reagents were added into a sterile, nuclease-free tube on ice in the indicated order.
3. The reaction mixture was prepared in the total volume of 20µl.

Table 3.1: Components for cDNA synthesis

S. No.	Component	Volume / reaction
1.	Oligo dT Primer	1µl
2.	dNTP Mixture (10 mM each)	1µl
3.	Template RNA	1.5µl
4.	RNase free H_2O	6.5µl
	Total Volume	10µl

4. The above mixture was incubated at 65^{o}C for 5minute
5. The tubes were again kept on ice.

Table 3.2: components and volume of reverse-transcription master mix

S. No.	Component	Volume / reaction
1.	Template RNA mixture	10µl
2.	5X PrimeScript Buffer	4µl
3.	RNase Inhibitor	0.5µl
4.	Prime Script RTase	1µl
5.	RNase free H_2O	4.5µl
	Total Volume	**20µl**

The above components were mixed gently.

1. It was then incubated at 30^{o}C for 10minute followed by an incubation of 42^{o}C for 45 minutes.
2. The reaction was further stopped by incubating at 72^{o}C for 15minute.
3. The cDNA was used either immediately or stored at -20^{o}C.

a. Primer Designing

To amplify the *GDF9*, *BMP15*, and *BMPR1B*genes in goat, a set of specific primers was designed by primer blast using the mRNA coding sequences of *GDF9* (NM_001285708.1), *BMP15* (NM_001285588.1), and *BMPR1B* (NM_001285575.1).

Table 3.3: Primer details for target gene amplification

S. No.	Primer Name	5′<-Sequence->3′	Length	Amplicon
1.	*GDF9* F *GDF9* R	GCTTCCCAACAAATTCTTC TCTTTATAAGCGATTGAGCC	19 20	1317bp
2.	*BMP15* F *BMP15* R	TCTTCTTTGGGGACTGGTGC CTGCCGTTCGACGATTTCAC	20 20	1185bp
3.	*BMPR1B*F *BMPR1B*R	ACTTCCCTGATAACATGCTTTTGCG GCAGTTTCTCCCGCCTACAGAC	25 22	1734bp

3.8 Primers Dilution

Oligos supplied as freeze-dried powder form were reconstituted in nuclease-free water to the volume in µl equivalent to the mass (g/mol) of the primer to create 100pmoles/µl of stock solution that was further diluted to give a final concentration of 10pmoles/µl.

Table 3.4: Dilution of primers in 100pmol

Gene	Sequence	Concentration of Primer in pmoles/ µl	Nuclease-free water
GDF9	Forward	172.85	345.7µl
	Reverse	174.2	348.4µl
BMP15	Forward	174.53	349.06µl
	Reverse	175.68	351.36µl
BMPR1B	Forward	174.29	348.58µl
	Reverse	172.25	344.5µl

3.9 PCR composition for amplification

PCR was carried out in a final reaction volume of 25µl. The master-mix reaction contains buffer, $MgCl_{2,}$ Taq Polymerase (GoTaq® Flexi DNA Polymerase, Promega), Primers (Eurofins), dNTPs (Promega), and nuclease-free water. This master-mix was prepared in 0.2 ml thin-wall PCR tubes. PCR tubes containing the mixture were capped tightly and quickly spin for a few seconds. The tubes were placed in a thermal cycler and subjected to PCR.

Table 3.5: PCR reaction mixture for *GDF9* and *BMP15* gene

PCR components	**Final Concentration**
10X PCR buffer	1x
25mM $MgCl_2$	2mM
dNTPs	0.2mM
100pmolForward Primer	10pmol/µl
100pmol Reverse Primer	10pmol/µl
Taq Polymerase	1U/µl
Template cDNA	2µl
Nuclease free water	
Total	**25µl**

Table 3.6: Amplification conditions for *GDF9* and *BMP15* gene

Steps	**Temperature**	**Time**	**Cycles**
Predenaturation	95 °C	3min	1
Denaturation	95 °C	1min	35
Annealing	52 °C	1min	
Extension	72 °C	2min	
Final extension	72 °C	10min	1

Table 3.7: PCR reaction mixture for *BMPR1B* gene

PCR components	**Final Concentration**
10X PCR buffer	1x
25mM $MgCl_2$	2mM
dNTPs	0.2mM
100pmolForward Primer	10pmol/µl
100pmol Reverse Primer	10pmol/µl
Taq Polymerase	1U/ µl
Template cDNA	2µl
Nuclease free water	
Total	25µl

Table 3.8: Amplification conditions for *BMPR1B* gene

Steps	Temperature	Time	Cycles
Pre-denaturation	95 °C	3min	1
Denaturation	95 °C	1min	35
Annealing	62 °C	1min	
Extension	72 °C	2min	
Final extension	72 °C	10min	1

3.9.1 Agarose gel electrophoresis

1. The PCR product was analyzed by running on the agarose gel as per the protocol given below
2. 1.2% of agarose was dissolved in 100ml of 1X TAE buffer melted, allowed to cool at room temperature, and finally 5μl of ethidium bromide was added and then mixed completely.
3. As the mixture comes to normal temperature, this was poured into a gel casting tray fitted with the acrylic comb and then allowed to solidify.
4. Once this gel was solidified, the comb was removed carefully, and then the gel was immersed into an electrophoresis tank containing 1X TAE buffer.
5. Samples were mixed with 6X loading dye (Bromophenol blue dye) and then loaded into the wells so that the final concentration of the dye becomes 1X.
6. In the adjacent well, a 1Kb ladder was loaded.
7. Electrophoresis was carried out @ 85 volts for 50minutes.
8. The bands were visualized using the Gel Documentation System.

3.9.2 Gel purification of PCR product

Electrophoresis was done with PCR product in 1% agarose gel and the specific band was cut carefully with minimum exposure to the low-intensity UV light to avoid damage of the DNA and was collected in a 1.5 ml microcentrifuge tube. PCR product was purified by Qiagen Gel Purification Kit.

3.10 Cloning of coding region of *GDF9* gene

PCR product was cloned into pGEMT - easy vector following the TA cloning protocol. Gel purified PCR product was ligated with pGEMT- easy Vector (Promega).

A 10 of reaction was set up using 1:3 vector: insert ratio for ligation which is given below:

Table3.9: Composition of the mixture for the ligation reaction of *GDF9* gene

S. No.	Components	Volume
1.	2X rapid ligation buffer	5µl
2.	Vector (50ng/µl)	1µl
3.	PCR product	3µl
4.	T4 DNA ligase	1µl
	Total	**10µl**

The reaction mixture was spin; incubated at room temperature for 1 hour after that kept for overnight incubation at 4°C.

3.10.1 Preparation of competent cells by Calcium Chloride method

1. The Top10 cells were revived by growing overnight in 5ml of LB broth.
2. Following day 500µl culture was inoculated into fresh 25ml SOB medium in 150ml conical flask and grown under vigorous shaking (180 rpm) until subscript of 0.6 is achieved (about 3 hr.)
3. The culture was taken in a centrifuge tube and kept for 15-25 minutes on ice.
4. The cells were pelleted @ 8,000rpm for 10min at 4°C. The supernatant was discarded and the pellet was resuspended in 1ml chilled 100mM $CaCl_2$ solution with cooled microtip and incubated on ice for 10minutes.
5. Again, the cells were centrifuged @ 6,000 rpm for 10minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 400µl chilled $CaCl_2$ solution with cooled microtip and incubated on ice for 40minutes.
6. Competent cells were ready for use.
7. A volume of 50µl of the cells was used for one transformation.

3.10.2 Transformation

Following protocol was used for transformation of the ligated product into Top10 competent cells:

1. 50µl of freshly prepared Top10 competent cells were added to 10µl of ligated product in a 2ml centrifuge tube with the cooled tip
2. It was mixed gently and left on ice for 30minutes.

3. Heat shock was given at 42°C for 45-50 seconds followed by immediate cooling on ice for 10minutes.
4. 300μl of SOC medium was added and incubated at 37°C for 2hours under vigorous shaking at 180 rpm for 90minutes.
5. 150μl of the broth was plated by sterile L- spreader on two LB agar plates containing a final concentration of ampicillin (100μg/ml).
6. Plates were incubated at 37°C for 15-18hr for the development of the colonies.

3.10.3 Streaking and colony PCR

The following day, the plates were observed for the development of colonies. The colonies were picked by sterile toothpick/tip and streaked onto another LB agar plate and mark numbers. The plates were incubated at 37°C for 15-18hr.

Following day, for the conformation of positive clones set up PCR tubes (Buffer, $MgCl_2$, dNTPs, Primer, Taq Polymerase and nuclease-free water) with the final concentration of 25μl each, touch a fresh toothpick onto a colony, dip it into a PCR tube gently mixes and spin

Table 3.10: Colony PCR reaction mixture for *GDF9* gene

PCR components	Final Concentration
10X PCR buffer	1x
25mM $MgCl_2$	2mM
dNTPs	0.2mM
100pmolForward Primer	10pmol/μl
100pmol Reverse Primer	10pmol/μl
Taq Polymerase	1U/ μl
Colonies	
Nuclease free water	
Total	**25μl**

After the conformation of positive clones, the colonies were picked by sterile toothpick and inoculated to 5ml of LB broth containing ampicillin (100μg/ml) and incubated at 37°C overnight in a shaker incubator, next day the plasmid was isolated.

3.10.4 Plasmid isolation

The bacteria were pelleted for plasmid isolation using protocol given by (Green & Sambrook, 2012), which has been adumbrated below:

1. 4ml of overnight grown LB culture was taken in a centrifuge tube and centrifuged @ 12,000 rpm for 10minutes at 8°C.
2. The pelleted cells from 4ml LB culture were resuspended completely by pipetting up and down in 200 µl of P1 solution on ice.
3. 200µl of P2 solution was added at room temperature and mixed thoroughly by inverting the tubes 3-4 times and kept for 5minutes.
4. 250µl of P3 solution was added and kept on ice for 5minutes and centrifuged @ 12,000 rpm for 10minutes to pellet cell debris and chromosomal DNA.
5. The supernatant was transferred to 2ml tube and an equal amount of PCI (25:24:1) was added, mixed thoroughly, and centrifuged @ 12,000 rpm for 10minutes.
6. The aqueous layer was collected and 0.7 volume of isopropanol and 10^{th} part of 3M sodium acetate were added to it and kept at -80°C for 20minutes to precipitate plasmid DNA.
7. The microcentrifuge tubes were centrifuged @ 12,000 rpm for 10minutes at 4°C.
8. The supernatant was discarded and the pellet was washed with 70% ethanol and the tubes were centrifuged @ 12,000 rpm for 10minutes at 4°C.
9. The supernatant was discarded and 50 µl nuclease-free water was added.
10. RNase treatment was given and incubated @ 37°C for 1hour**.**
11. The purified plasmid DNA was then stored at -20°C.

Table 3.11: components of Plasmid PCR for *GDF9* gene

PCR components	**Final Concentration**
10X PCR buffer	1x
25mM $MgCl_2$	2mM
dNTPs	0.2mM
100pmolForward Primer	10pmol/µl
100pmol Reverse Primer	10pmol/µl
Taq Polymerase	1U/ µl
Plasmid	1µl
Nuclease free water	
Total	**25µl**

Table 3.12: Restriction digestion of plasmid isolated from positive clones

S. No.	Components	Volume
1.	Plasmid	3µl
2.	10X *EcoRI* buffer	2µl
3.	*EcoRI* enzyme	1.5µl
4.	Nuclease free water	13.5µl
	Total	20µl

1. The contents were mixed by tapping the tubes and the tubes were incubated at 37°C for 2hours in a water-bath
2. The digested product was run on 0.8% agarose gel by electrophoresis at 85 volts
3. Ethidium bromide was added @ 0.5µl/ml of melted agarose.
4. The digested products were visualized under a gel documentation system.

3.11 Storage of clones

Stab culture with positive clones was sent to the University of Delhi, South Campus (UDSC) for sequencing by primer walking method. The clones were also stored in glycerol stock (50% glycerol) 600µl culture and 400µl glycerol in cry-preservative tubes at -80°C.

3.12 *In Silico* Characterization of *GDF9*, *BMP15*, and *BMPR1B* genes

3.12.1 Homology Search

The final cDNA sequences were subjected to BLAST (Altschul et al., 1990) Divergent species were downloaded in FASTA format from the nucleotide database of NCBI (https://www.ncbi.nlm.nih.gov/nucleotide?emd=search). The *in silico* translated complete amino acid sequences of *GDF9*, *BMP15*, and *BMPR1B* from divergent species were retrieved in FASTA format.

3.12.2 Pairwise Sequence Alignment

The obtained nucleotide sequence of goat *GDF9*, *BMP15*, and *BMPR1B* was compared with the reference *GDF9* (NM_001285708.1), *BMP15* (NM_001285588.1), and *BMPR1B* (NM_001285575.1) of goat (from where primers were designed), by using Clustal W online tool (http://www.genome.jp/tools-bin/clustalw) and MEGA7 software. The coding sequence of *GDF9*, *BMP15*, and *BMPR1B* was translated into amino acid sequence by using the ExPASy (https://web.expasy.org/translate/). The obtained amino acid sequence of goat *GDF9, BMP15,* and *BMPR1B* was compared with the reference amino acid sequence of *GDF9, BMP15,* and *BMPR1B* by using the Clustal online tool and MEGA7 software in order to determine the similarity between them.

3.12.3 Multiple Sequence Alignment

The coding sequences of *GDF9*, *BMP15,* and *BMPR1B* gene collected and aligned by using Molecular Evolutionary Genetic Analysis MEGA7 version software (Kumar et al., 2016) and Multiple Alignment using Fast Fourier Transform (MAFFT) online tool (https://mafft.cbre.jp/alignment/software/) was used for multiple sequence alignment of these sequences. The sequences were further analyzed for their group-wise alignment. The alignment between groups was used for the determination of evolutionary distance.

3.12.4 Phylogenetic Tree Construction

The 36 nucleotide sequences of *GDF9*, 34 nucleotide sequences of *BMPR1B,* and 25 nucleotide sequences of *BMP15* were retrieved from NCBI. The best evolutionary model, based on the lowest Bayesian Information Criterion, corrected (BICc), and Akaike Information Criterion, corrected (AICc) values were determined for each of the models scores using the MEGA7 software. The best model obtained for analyzing the nucleotide data was K2+G for *GDF9* and *BMPR1B*, K2+I for *BMP15*. The matrix-based model MEGA7 software was used for the construction of the phylogenetic tree, estimation of evolutionary divergence. The evolutionary tree was constructed using the maximum composite likelihood method, considering the K2+G and K2+I substitution model and 5 discrete Gamma categories for rates of substitution among sites. The reliability of the branching of the tree was checked by 1000 bootstrap resampling.

3.12.5 Evolutionary Divergence

The analysis for estimating the evolutionary divergence between nucleotide sequences was conducted using the K2+G and K2+I substitution model. The sequences were distributed into groups on the basis of similarity between them. Pair-wise distance analysis was done between different taxa and groups and then the evolutionary divergence was estimated between the sequence pairs between groups and the sequences of different taxa. The analysis was conducted using the maximum composite likelihood method (Tamura et al., 2004). The rate variation among sites was modeled with a gamma distribution of 5. The position containing gaps was eliminated (Tamura et al., 2013). Mean evolutionary divergence for the entire population and interpopulation was determined. The analysis was conducted by using the maximum composite likelihood method (Tamura et al., 2004), and the rate variation among sites was modeled with a gamma distribution of 5. Standard error was estimated using a bootstrap of 1000 replicates. The differences in the composition bias were considered in evolutionary divergence (Tamura et al., 2002).

3.14 Protein structure prediction

The coding sequence of *GDF9*, *BMPR1B*, and *BMP15* gene in goat was translated to protein sequence by using ExPASy translated tool (http://web.expasy. org/translate/). The amino acids of *GDF9*, *BMPR1B*, and *BMP15* were further subjected to 3D structure prediction by using the *ab initio* approach using the online available tool RaptorX (http://raptorx.uchicago.edu) and Swiss model software (https://swissmodel.expasy.org/). Further, the 3D structure obtained from RaptorX was subjected to structural validation by using Ramachandran's plot analysis using the RAMPAGE online tool (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php)

CHAPTER – IV

RESULTS AND DISCUSSION

The goat *GDF9* gene coding sequence was cloned, sequenced, and bio-computationally characterized. The *BMPR1B* and *BMP15* coding sequences were amplified, sequenced and bio computationally characterized. The results obtained and the relevant discussions have been presented in this chapter.

4.1 RNA isolation from Ovaries

Total RNA from goat's ovaries (Fig. 4.1) was extracted using a standard protocol (Green & Sambrook, 2012). The subscript ratio of the extracted total RNA was ~1.99-2.04, indicating the purity of the preparation. The concentration of the total RNA ranged from 9000-12,000ng/ul (Fig. 4.2). The extracted RNA produced intact bands of 28S and 18S RNAs (Fig. 4.3) was observed in gel electrophoresis on 1.5% agarose prepared in 1X TAE

4.2 PCR Standardization using primers designed for *GDF9*, *BMP15*, and *BMPR1B* genes

The cDNA reverse transcribed from the total RNA was used for PCR amplification of *GDF9*, *BMP15*, and *BMPR1B* genes coding region by sequence designed a set of primer pair (namely, forward and reverse primers). The primers showed single expected bands with amplification size (Figures. 4.4, 4.5, and 4.6 respectively) on gel electrophoresis in 1.2% agarose gel prepared in 1X TAE buffer and 1 Kb ladder was used to check the amplicon size.

4.3 Cloning, Sequencing and analysis of *GDF9* coding region

4.3.1 Cloning and sequencing of *GDF9* gene

Ligation and transformation of PCR product corresponding to the goat *GDF9* (gel-purified target gene product) in TOP10 cells resulted in the development of colonies on the selective media (LB agar supplemented with ampicillin). Colonies were picked up randomly from the plate by using a sterile toothpick and transferred to 10ml of LB broth supplemented with ampicillin and the plasmids were isolated from overnight growth culture. Plasmids were amplified by using plasmid PCR and restriction digestion was carried out using the *EcoR1* enzyme that recognizes the restriction sites in the pGEMT-easy cloning vector. Plasmids isolated from white colonies gave positive results in plasmid PCR and restriction digestion. Restriction digestion was kept at 37°C for overnight followed by gel electrophoresis using 1.2% agarose gel. Restriction digestion by suitable enzymes showed the release of target insert of 1317bp approximately (Fig. 4.7). The positive clones were sent for commercial sequencing through Sanger sequencing.

(a) **(b)**

Figure 4.1: Sample collection from slaughterhouse (Beetal goat's ovaries)

Figure 4.2: quantity analysis: -OD260/280 ratio-1.99-2.04 Concentration of 9000ng-12,000ng/µl

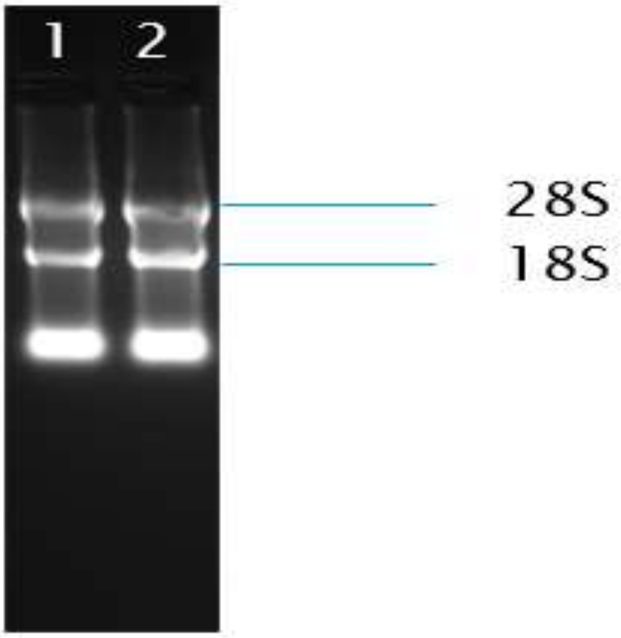


Figure 4.3: Extracted RNA intact bands showing 28S and 18S RNAs

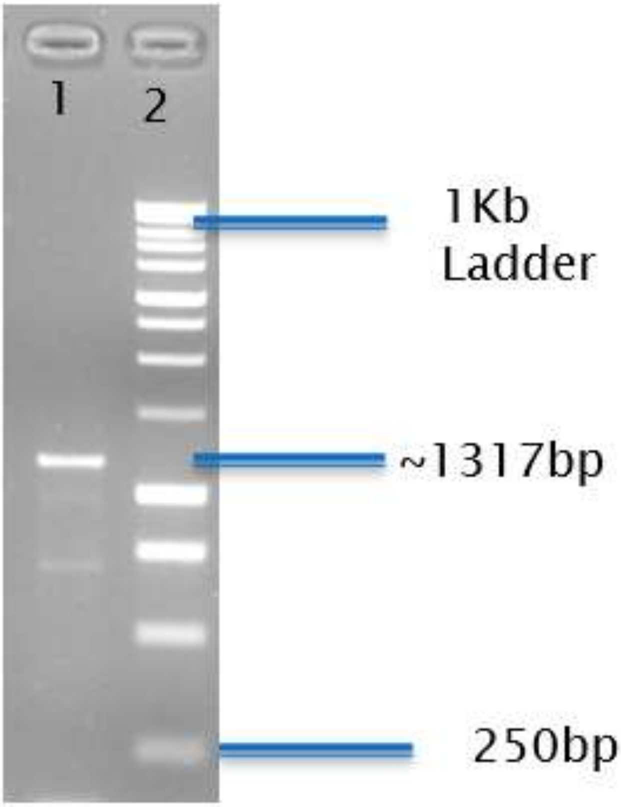


Figure 4.4: Lane 1: positive amplification of *GDF9* gene, Lane 2: 1Kb ladder (Promega)

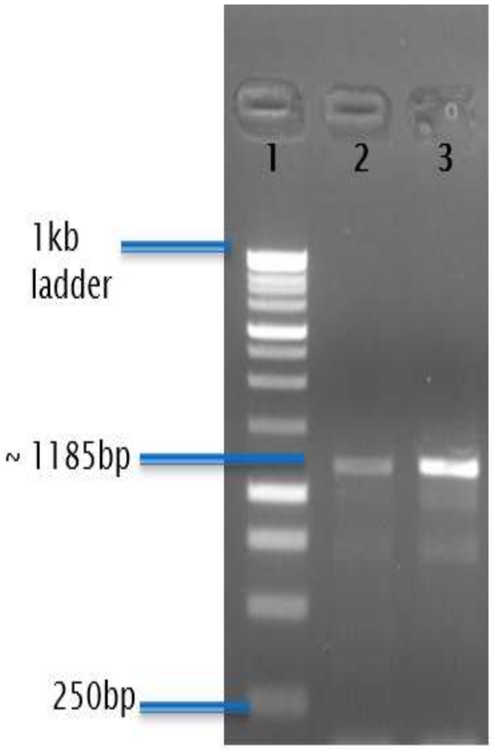


Figure 4.5: Lane 1: 1Kb ladder (Promega), Lane 2,3: positive amplification of *BMP15* gene

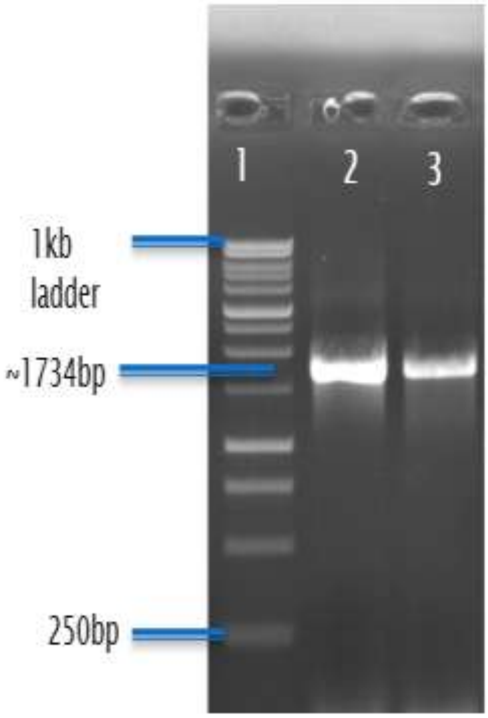
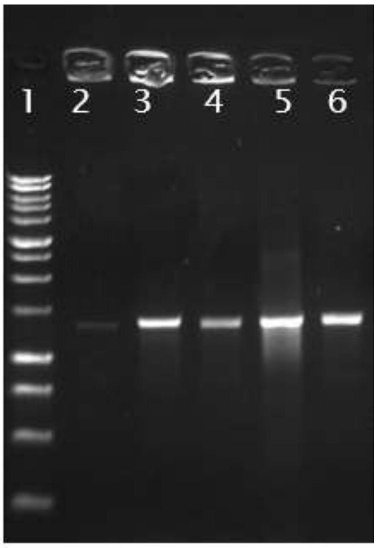


Figure 4.6: Lane 1: 1Kb ladder (Promega), Lane 2,3: positive amplification of *BMPR1B* gene

a. 

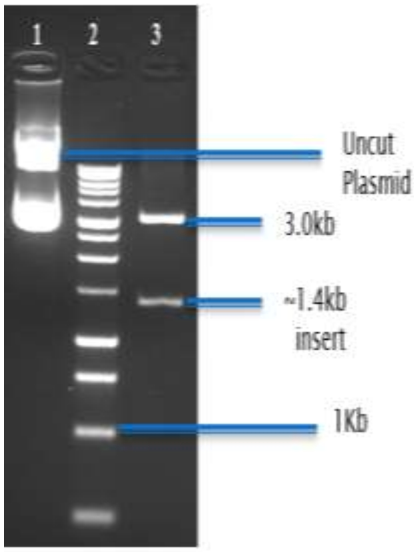
b. 

Figure 4.7.a.: Lane 1 1Kb ladder (Promega) Lane 2,3,4., Negative, Lane 5,6: positive

Figure 4.7.b.: Lane 1: Uncut Plasmid containing desired insert, Lane 2: 1Kb ladder (Promega), Lane 3: EcoR1 digested pGEMT Plasmid

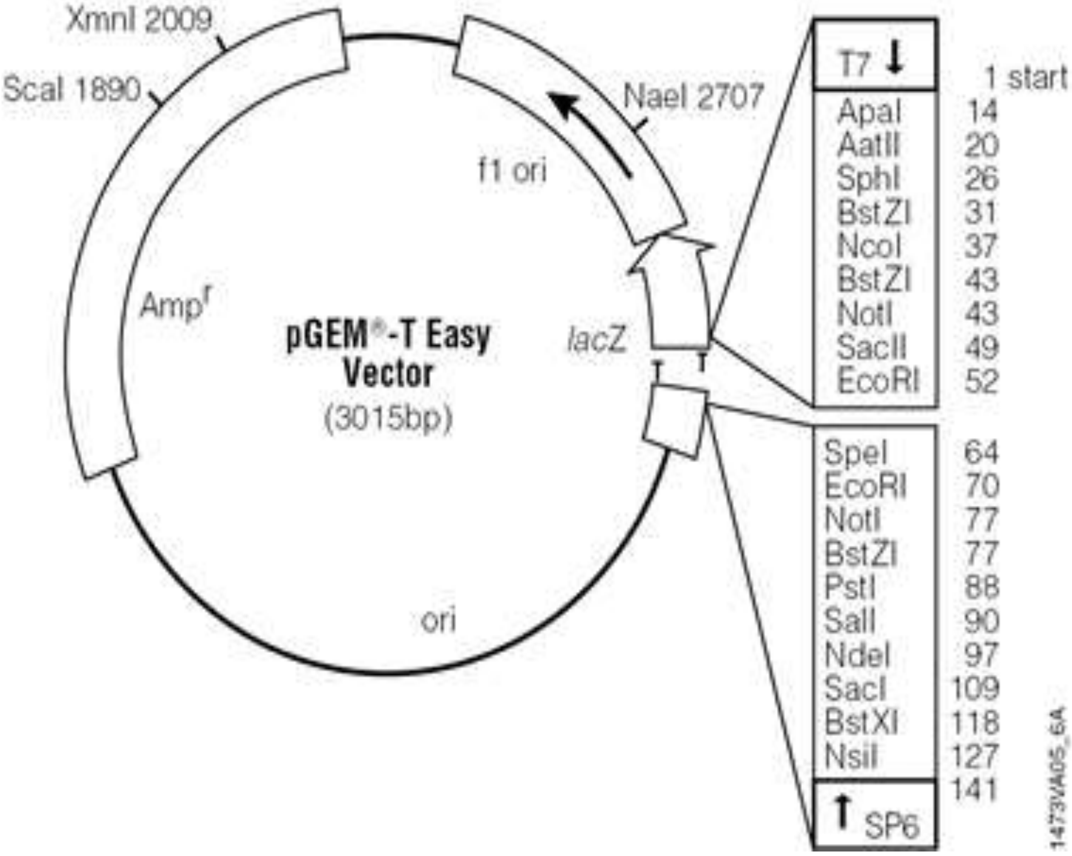


Figure 4.8: Restriction map of pGEMT-easy vector

The sequence showed similarity with a homology of the goat reference *GDF9* gene. No study is reported in the Beetal goat of Punjab till now. The sequence of mRNA coding region of *GDF9, BMP15,* and *BMPR1B* gene is not reported and the detailed bioinformatics analysis on these genes is limited in the Punjab region.

4.3.2 Sequence analysis of *GDF9, BMP15,* and *BMPR1B* coding region

The final sequence of *GDF9, BMP15,* and *BMPR1B* was subjected to BLAST (Altschul et al., 1990) and the similarity of this sequence with other species was determined. The BLAST results with expectation value (E value) less than 10 were downloaded in FASTA format. Overall, 36 nucleotide sequences of divergent species related to *GDF9* gene, 34 nucleotide sequences of divergent species related to *BMPR1B,* and 24 nucleotide sequences of divergent species related to *BMP15* were selected and downloaded in FASTA format and used for further bioinformatics analysis. The coding region of *GDF9*, *BMPR1B,* and *BMP15* gene in goat was *in silico* translated to amino acid sequence by using ExPASy Translate Tool (http://web.expasy.org/translate/)

Table 4.1: *GDF9* sequence retrieved from NCBI Gen-Bank

S. No	Accession No.	Species	Breed	Sequence Type
1.	JN100109	*Capra hircus*	lezhi black	mRNA Cds
2.	NM_001285708	*Capra hircus*	Reported	mRNA
3.	JN100108	*Capra hircus*	Tibetan	mRNA Cds
4.	FJ712709	*Capra hircus*	Black Bengal	Cds
5.	KY780296	*Capra hircus*	Tibet Cashmere	Cds
6.	EU883989	*Capra hircus*		Cds
7.	EF446168	*Capra hircus*		Cds
8.	KF758766	*Capra hircus*		Cds
9.	JN587295	*Capra hircus*	Jhakhrana	Exon 2 partial
10.	FJ472652	*Capra hircus*	Markhoz	Exon 2 partial
11.	MK421573	*Capra hircus*	Isolate Nnandidurga 4	Exon 2 partial
12.	MK421572	*Capra hircus*	Isolate Nnandidurga 3	Exon 2 partial
13.	MK421567	*Capra hircus*	Bidril	Exon 2 partial

S. No	Accession No.	Species	Breed	Sequence Type
14.	MK421570	*Capra hircus*	Isolate Nnandidurga 1	Exon 2 partial
15.	LC586428	*Capra hircus*		Partial Cds
16.	KR063137.1	*Ovis Aries*	Sheep	mRNA Cds
17.	AB058416.1	*Bos Taurus*	Cattle	Cds
18.	EU267798.1	*Bos Grunnies*	Yak	Cds
19.	FJ529501.2	*Bubalus Bubalis*	Cattle	Cds
20.	GQ227503.1	*Moschus berezouskii*	Deer	Cds
21.	XM040240121.1	*Oryx Dammah*	Oryx	Cds
22.	XM005890431.2	*Bost Mutus*	Wild yak	Cds
23.	XM022589005.2	*Delphinapterus Leucas*	Beluga whale	Cds
24.	XM007461344.1	*Lipotes Vexillifer*	Baiji	Cds
25.	XM029217693.1	*Monodon Monoceros*	Narwhale	mRNA Cds
26.	XM036847515.1	*Balaenoptera Musculus*	Whale	mRNA Cds
27.	XM007188566.2	*Balaenoptera acutorostrata scammoni*	whale	mRNA Cds
28.	XM004420206.2	*Ceratotherium Simum Simum*	White rhinoceros	mRNA Cds
29.	XM001504427.4	*Equus Caballus*	Horse	mRNA Cds
30.	XM006212821.3	*Vicugna Pacos*	Alpaca	mRNA Cds
31.	XM014856804.2	*Equus Asinus*	Asinus	mRNA Cds
32.	XM010981399.2	*Camelus Dromedarius*	Camel	mRNA Cds
33.	XM010971602.1	*Camelus Bactrianus*	Camel double hump	mRNA Cds
34.	XM008571635.1	*Galeopterus Variegatus*	Sunda flying lemur	mRNA Cds
35.	XM039879918.1	*Pteropus Giganteus*	Flying Fox	mRNA Cds

Table 4.2: ***BMP15*** **sequence retrieved from NCBI Gen-Bank**

S. No	Accession No	Species	Breed	Sequence Type
1.	JF824148	*Capra hircus*	Tibetan	Mrna cds
2.	JF824149	*Capra hircus*	Lehzi black	mRNA cds
3.	EU847289	*Capra hircus*	Boer	mRNA cds
4.	MK421566	*Capra hircus*	Isolate Nandidurga4	Exon 2 partial
5.	MK421564	*Capra hircus*	Isolate Nandidurga2	Exon 2 partial
6.	EU847284	*Capra hircus*	Yunling black	mRNA
7.	EU888137	*Capra hircus*	Black Bengal	mRNA
8.	NM001114767	*Ovis aries*	Sheep	mRNA
9.	KT853038	*Ovis aries*	Pelibuey	mRNA cds
10.	KT013295	*Ovis aries*	Afshari	Complete cds
11.	XM040265988	*Oryx dammah*	Oryx	mRNA
12.	JQ326273	*Bubalus bubalis*	Water buffalo	mRNA
13.	EF375880	*Bubalus bubalis*	Water buffalo	mRNA
14.	XM005898488	*Bos mutus*	Wild yak	mRNA
15.	DQ463368	*Bos taurus*	Cattle	mRNA
16.	XM020901080	*Odocoileus virginianus*	White tailed deer	mRNA
17.	XM043897208	*Cervus elaphus*	Red deer	mRNA
18.	XM043459547	*Bos grunniens*	Yak	mRNA
19.	XM030846710	*Globicephala melas*	Long-finned-pilot-whale	mRNA
20.	XM004325009	*Delphinus*	Dolphin	mRNA
21.	XM007450928	*Lipotes vexillifer*	Baiji	mRNA
22.	XM029240827	*Monodon monoceros*	Narwhale	mRNA
23.	XM022563769	*Delphinapterus leucas*	Beluga-whale	mRNA
24.	XM006754222	*Chiroptera*	Bat	mRNA
25.	XM038448952	*Canis lupus*	Wolf	mRNA

Table 4.3: ***BMPR1B*** **sequence retrieved from NCBI Gen-Bank**

S. No	Accession No	Species	Breed	Sequence Type
1.	NM_001285575	*Capra hircus*	Reported	mRNA
2.	DQ060443	*Capra hircus*	*BMPR1B*	Complete Cds
3.	FJ712710	*Capra hircus*	Black Bengal	mRNA Cds
4.	JN100106	*Capra hircus*	Tibetan	mRNA Cds
5.	EU847285	*Capra hircus*	Yunling Black	mRNA Cds
6.	EU847290	*Capra hircus*	Boer	mRNA Cds
7.	JN100107	*Capra hircus*	Lezhi Black	mRNA Cds
8.	JN049449	*Capra hircus*	*BMPR1B*	mRNA Cds
9.	GU359045	*Capra hircus*	*BMPR1B*	mRNA Cds
10.	DQ666418	*Capra hircus*	Goat	Cds
11.	KF758765	*Capra hircus*	Goat	Cds
12.	AF298885	*Ovis Aries*	Sheep	mRNA Cds
13.	AF357007	*Ovis Aries*	Sheep	Complete Cds
14.	EU183347	*Ovis Aries*	Sheep	Complete Cds
15.	XM040237828	*Oryx dammah*	Oryx	X6 transcript Variant mRNA
16.	XM040237823	*Oryx Dammah*	Oryx	X1
17.	XM019962427	*Bos Indicus*	Zebu Cattle	X1
18.	XM019962436	*Bos Indicus*	Zebu Cattle	X9
19.	XM019962431	*Bos Indicus*	Zebu Cattle	X5
20.	XM027544025	*Bos Indicus X Bos Taurus*	Zebu Cattle X Cattle	X4
21.	XM027544029	*Bos Indicus X Bos Taurus*	Zebu Cattle X Cattle	X8
22.	XM027544034	*Bos Indicus X Bos Taurus*	Zebu Cattle X Cattle	X13
23.	NM001105328	*Bos Taurus*	Cattle	mRNA
24.	BC134547	*Bos Taurus*	Cattle	Clone mRNA
25.	XM005907860	*Bos Mutus*	wild yak	X2
26.	XM005907859	*Bos Mutus*	wild yak	X1
27.	XM010848331	*Bison Bison*	Bison	X3
28.	XM044946207	*Bubalus bubalis*	Buffalo	X4
29.	XM044946214	*Bubalus bubalis*	Buffalo	X13
30.	XM043871281	*Cervus Elephus*	Red deer	X1
31.	XM043871289	*Cervus Elephus*	red deer	X9
32.	XM043438645	*Cervus Canadensis*	Elk	X6
33.	XM043438647	*Cervus Canadensis*	Elk	X8

4.3.3 Pair-wise sequence alignment of *GDF9*, *BMP15*, and *BMPR1B* gene

The nucleotide sequence and the *in silico* translated amino acid sequence of *GDF9*, *BMP15*, and *BMPR1B* gene were aligned with the reference sequence by using Clustal Omega online tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) in order to determine the percentage of similarity between them.

4.3.4 Multiple Sequence Alignment

The multiple sequence alignment was performed on the nucleotide sequence of *GDF9*, *BMP15*, and *BMPR1B* gene of different divergent species by using Multiple Alignment using Fast Fourier Transform (MAFFT). The conserved region in the aligned sequences of *GDF9*, *BMP15* and *BMPR1B* gene of divergent species was determined as shown in (Figures 4.9, 4.10, and 4.11). Similarity studies showed that *GDF9*, *BMP15* and *BMPR1B* gene has shown conserved and variable region at the nucleotide level.

Figure 4.9: Visual representation of MSA of *GDF9* gene sequence, aligned by Clustal ω and visualized on Jalview software on the basis of nucleotides. sequences in this study along with *GDF9* reference sequences downloaded from NCBI-GenBank

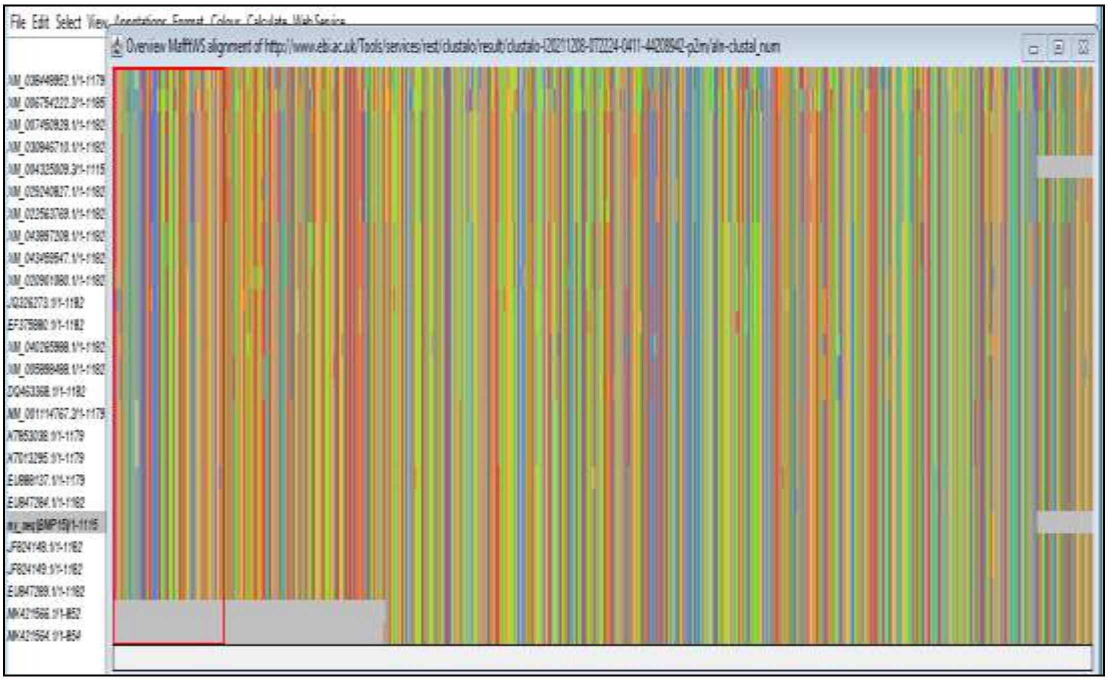


Figure 4.10: Visual representation of MSA of *BMP15* gene sequence, aligned by Clustal ω and visualized on Jalview software on the basis of nucleotides. sequences in this study along with *BMP15* reference sequences downloaded from NCBI-GenBank

Figure 4.11: Visual representation of MSA of *BMPR1B* gene sequence, aligned by Clustal ω and visualized on Jalview software on the basis of nucleotides. sequences in this study along with *BMPR1B* reference sequences downloaded from NCBI-GenBank

4.4 Best Model Selection

The best model i.e., K2+G and K2+I was chosen for further evolutionary analysis. The selected coding sequence of ***GDF9*** gene showed that K2+G (Kimura-2 parameter) was the best model among the rest with a minimum BICc score of 7636.027267 and AlCc score of 7044.852897 (Fig. 4.12)

Non uniformly evolutionary rates among sites were modeled by using discrete Gamma distribution (G) with 5 rate categories.

4.4.1 *BMP15* gene showed that K2+I (Invariable sites) (Kimura-2 parameter) was the best model among the rest with a minimum BICc score of 5300.241079 and AlCc score of 4896.782141 (Fig.4.13).

4.4.2 *BMPR1B* gene showed that K2+G (Kimura-2 parameter) was the best model among the rest with a minimum BICc score of 6429.217668 and AlCc score of 5837.548944 (Fig. 4.14)

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R
K2+G	71	7636.027267	7044.852897	-3451.25939	n/a	0.77758325	2.458287172
K2+I	71	7643.671344	7052.496973	-3455.081428	0.433270032	n/a	2.436495198
K2+G+I	72	7646.356159	7046.860106	-3451.258283	0.019753922	0.814901582	2.45837102
T92+G	72	7648.339182	7048.843129	-3452.249794	n/a	0.770951754	2.464567937
T92+I	72	7655.960441	7056.464388	-3456.060424	0.434807353	n/a	2.442181195

Figure 4.12: Selection of Best Evolutionary model (Kimura-2+ Gamma) for the determination of the phylogenetic relationship between *GDF9* sequences using MEGA7 software

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R
K2+I	51	5300.241079	4896.782141	-2397.259796	0.419896036	n/a	2.675708617
K2+G	51	5300.774362	4897.315424	-2397.526438	n/a	0.906170307	2.686591075
K2	50	5305.382674	4909.829733	-2404.788647	n/a	n/a	2.537177325
K2+G+I	52	5310.161244	4898.796507	-2397.261825	0.418434177	200	2.676155023
T92+I	52	5310.239758	4898.875021	-2397.301082	0.421287424	n/a	2.678152693

Figure 4.13: Selection of Best Evolutionary model (Kimura-2+ Invariable) for the determination of the phylogenetic relationship between *BMP15* sequences using MEGA7 software

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R
K2+G	67	6429.217668	5837.548944	-2851.684479	n/a	0.05	3.693792981
K2+G+I	68	6434.606055	5834.109145	-2848.961891	0.678729194	0.62610396	3.748653865
T92+G	68	6436.938384	5836.441474	-2850.128055	n/a	0.05	3.705120749
T92+G+I	69	6444.066294	5834.741276	-2848.275229	0.68238682	0.624371865	3.766269155
K2+I	67	6450.948385	5859.279662	-2862.549838	0.468812877	n/a	3.605061989

Figure 4.14: Selection of Best Evolutionary model (Kimura-2+ Gamma) for the determination of the phylogenetic relationship between *BMPR1B* sequences using MEGA7 software

4.5 Phylogenetic tree construction

4.5.1 *GDF9*

The phylogenetic tree was constructed subjecting 36 nucleotide sequences of divergent species to a maximum likelihood model with 1000 bootstrap resampling. The phylogenetic analysis revealed that the sequences belonging to the same family or order were forming a cluster. The tree depicts the formation of different clad on the basis of the evolutionary changes between sequences. The *GDF9* sequence formed one clad with different breeds of goats (Nandidurga, Bidri, Jhakrana, Markhoz, Black Bengal, Lezhi black, Tibetan, and Tibet cashmere). A higher bootstrap value indicates the higher consistency of the given data. Other clad was formed between Ruminants, Marines, and non-Ruminants. Phylogenetic analysis showed that *GDF9* sequences of non-Ruminants (Horse, Donkey, White Rhino, Flying Fox, and Flying Lemur) are forming an independent clad show higher divergence.

4.5.2 *BMP15*

The phylogenetic tree was constructed subjecting 25 nucleotide sequences of divergent species to a maximum likelihood model with 1000 bootstrap resampling. The tree in (Fig. 4.15) depicts the formation of different clad on the basis of the evolutionary changes between sequences. The *BMP15* sequence formed one clad with different breeds of goats (Nandidurga, Boer, Yunling black, black Bengal, Lezhi black, and Tibetan). Phylogenetic analysis showed that *BMP15* sequences of non-Ruminant bat and Canis lupus familiaris (wolf) are forming an independent clad show higher divergence between goat and wolf.

4.5.3 *BMPR1B*

The phylogenetic tree was constructed subjecting 34 nucleotide sequences of divergent species to a maximum likelihood model with 1000 bootstrap resampling. The phylogenetic analysis revealed that the sequences belonging to the same family or order were forming a cluster. The tree in (Fig. 4.16) depicts the formation of different clad on the basis of the evolutionary changes between sequences. The *BMPR1B* sequence formed one clad with different breeds of goats (Boer, Yunling black, black Bengal, lezhi black, and Tibetan). A higher bootstrap value indicates the higher consistency of the given data. Phylogenetic analysis showed that *BMPR1B* sequences of cattle and hybrid zebu are forming an independent clad show higher divergence between goats and zebu

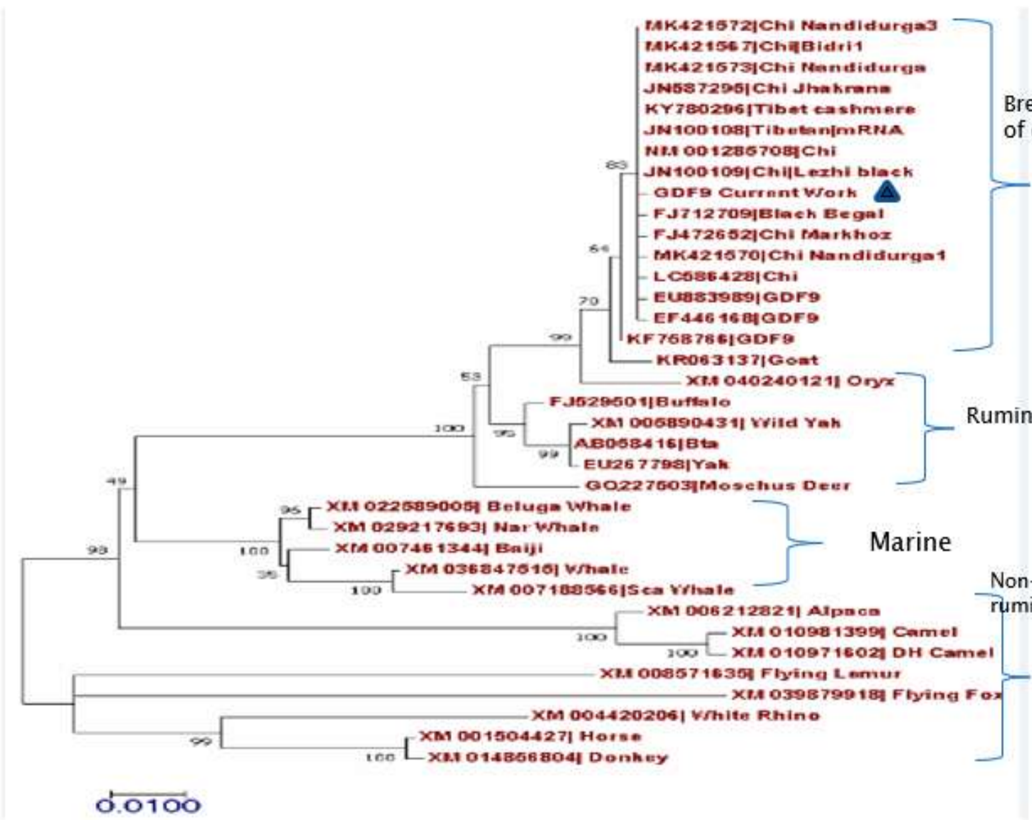


Figure 4.15: Phylogenetic tree of *GDF9* gene was constructed using MEGA 7. The maximum likelihood (ML) method was used as a statistical method K2+G model and 1000 bootstrap replications were used to assess the reliability of the tree. The gene *GDF9* in this study is denoted by a triangle

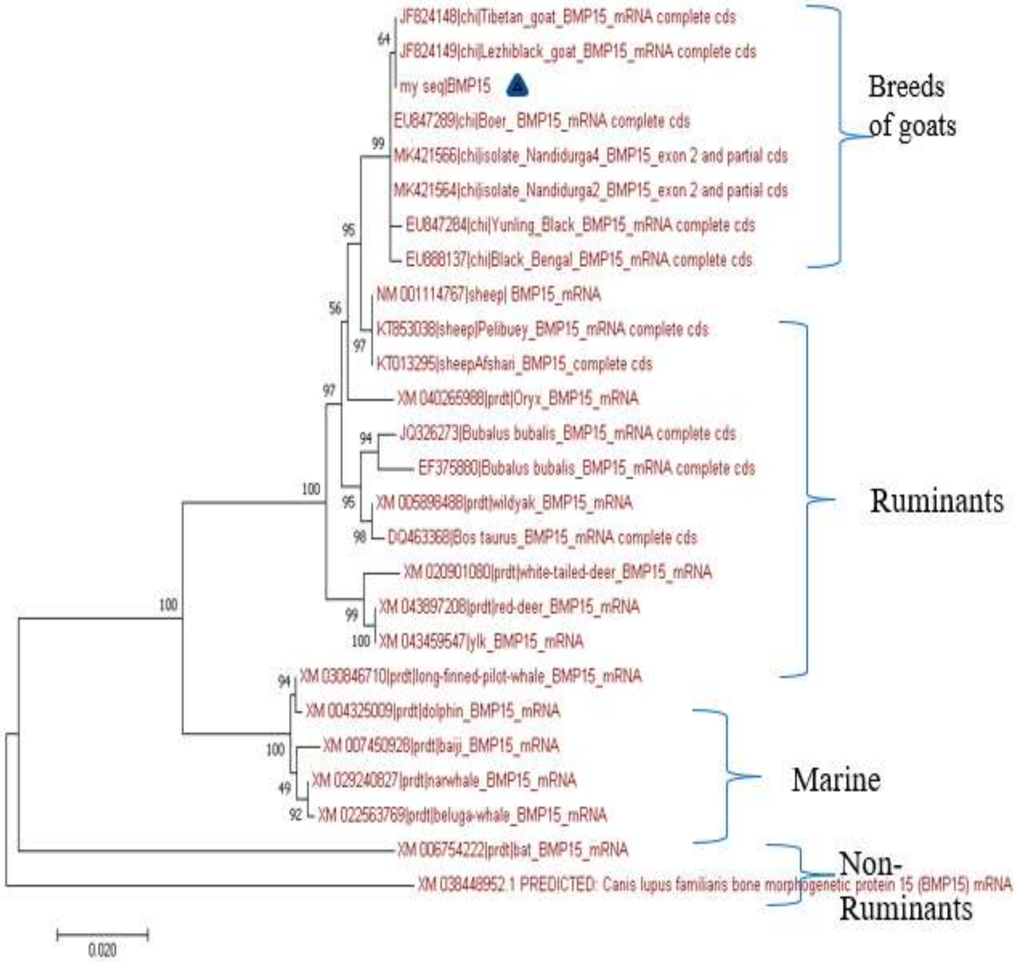


Figure 4.16: Phylogenetic tree of *BMP15* gene was constructed using MEGA 7. The maximum likelihood (ML) method was used as a statistical method K2+I model and 1000 bootstrap replications were used to assess the reliability of the tree. The gene *BMP15* in this study is denoted by a triangle

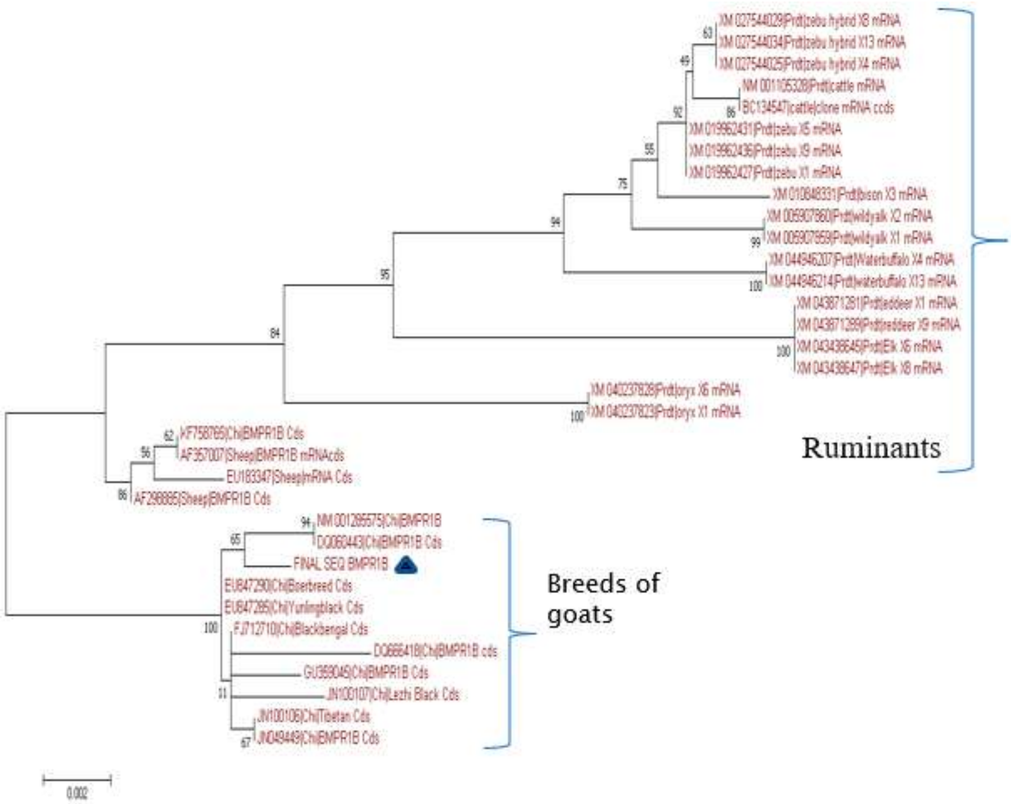


Figure 4.17: Phylogenetic tree of *BMPR1B* gene was constructed using MEGA 7. The maximum likelihood (ML) method was used as a statistical method K2+G model and 1000 bootstrap replications were used to assess the reliability of the tree. The gene *BMPR1B* in this study is denoted by a triangle

4.6 Pairwise distance matrix (PWD)

A distance matrix is a square matrix (two-dimensional array) containing the distances, taken pairwise, between the sets. The Distance Matrix Explorer is used to display results from the pairwise distance calculations.

4.6.1 *GDF9*

Where Flying Fox and DH Camel are the most distant from the goat; with a phylogenetic distance of 0.145 and 0.143 (Fig. 4.18).

4.6.2 *BMP15*

Wolf and Bat are the most distant from *BMP15* gene with a phylogenetic distance of 0.185 and 0.180 (Fig. 4.19)

4.6.3 *BMPR1B*

Red-Deer and Elk are the most distant from *BMPR1B* gene with a phylogenetic distance of 0.027 (Fig. 4.20)

GDF9_Current_Work		0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.004	0.007
JN100109|Chi|Lezhi_black	0.001		0.000	0.000	0.001	0.000	0.001	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.003	0.007
NM_001285708|Chi	0.001	0.000		0.000	0.001	0.000	0.001	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.003	0.007
JN100108|Tibetan|mRNA	0.001	0.000	0.000		0.001	0.000	0.001	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.003	0.007
FJ712709|Black_Begal	0.002	0.001	0.001	0.001		0.001	0.002	0.002	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.002	0.004	0.008
KY780296|Tibet_cashmere	0.001	0.000	0.000	0.000	0.001		0.001	0.001	0.002	0.000	0.001	0.000	0.000	0.000	0.001	0.001	0.003	0.007
EU883989|GDF9	0.002	0.001	0.001	0.001	0.002	0.001		0.002	0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.002	0.004	0.007
EF446168|GDF9	0.002	0.001	0.001	0.001	0.002	0.001	0.002		0.002	0.001	0.002	0.001	0.001	0.001	0.002	0.002	0.004	0.007
KF758766|GDF9	0.004	0.002	0.002	0.002	0.004	0.002	0.004	0.004		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.007
JN587295|Chi_Jhakrana	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.002		0.001	0.000	0.000	0.000	0.001	0.001	0.003	0.007
FJ472652|Chi_Markhoz	0.002	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.004	0.001		0.001	0.001	0.001	0.002	0.002	0.004	0.008
MK421573|Chi_Nandidurga	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.002	0.000	0.001		0.000	0.000	0.001	0.001	0.003	0.007
MK421572|Chi_Nandidurga3	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.002	0.000	0.001	0.000		0.000	0.001	0.001	0.003	0.007
MK421567|Chi|Bidri1	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.002	0.000	0.001	0.000	0.000		0.001	0.001	0.003	0.007
MK421570|Chi_Nandidurga1	0.002	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.004	0.001	0.002	0.001	0.001	0.001		0.002	0.004	0.008
LC586428|Chi	0.002	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.004	0.001	0.002	0.001	0.001	0.001	0.002		0.004	0.008
KR063137|Goat	0.011	0.009	0.009	0.009	0.011	0.009	0.011	0.011	0.007	0.009	0.011	0.009	0.009	0.009	0.011	0.011		0.007
AB058416|Bta	0.031	0.030	0.030	0.030	0.031	0.030	0.031	0.029	0.028	0.030	0.031	0.030	0.030	0.030	0.031	0.031	0.028	
EU267798|Yak	0.032	0.031	0.031	0.031	0.032	0.031	0.032	0.030	0.029	0.031	0.032	0.031	0.031	0.031	0.032	0.032	0.029	0.001
FJ529501|Buffalo	0.028	0.026	0.026	0.026	0.028	0.026	0.028	0.025	0.024	0.026	0.028	0.026	0.026	0.026	0.028	0.028	0.026	0.008
GQ227503|Moschus_Deer	0.036	0.035	0.035	0.035	0.036	0.035	0.036	0.034	0.032	0.035	0.036	0.035	0.035	0.035	0.036	0.036	0.032	0.024
XM_040240121|_Oryx	0.019	0.018	0.018	0.018	0.019	0.018	0.019	0.019	0.018	0.018	0.019	0.018	0.018	0.018	0.019	0.019	0.023	0.034
XM_005890431|_Wild_Yak	0.034	0.032	0.032	0.032	0.034	0.032	0.034	0.031	0.030	0.032	0.034	0.032	0.032	0.032	0.034	0.034	0.030	0.002
XM_022589005|_Beluga_Whale	0.081	0.080	0.080	0.080	0.081	0.080	0.081	0.079	0.080	0.080	0.081	0.080	0.080	0.080	0.081	0.081	0.082	0.078
XM_007461344|_Baiji	0.082	0.081	0.081	0.081	0.082	0.081	0.082	0.080	0.081	0.081	0.082	0.081	0.081	0.081	0.082	0.082	0.084	0.080
XM_029217693|_Nar_Whale	0.082	0.081	0.081	0.081	0.082	0.081	0.082	0.080	0.081	0.081	0.082	0.081	0.081	0.081	0.082	0.082	0.084	0.081
XM_036847515|_Whale	0.086	0.085	0.085	0.085	0.086	0.085	0.086	0.084	0.085	0.085	0.086	0.085	0.085	0.085	0.086	0.086	0.088	0.086
XM_007188566|Sca_Whale	0.094	0.093	0.093	0.093	0.094	0.093	0.094	0.092	0.093	0.093	0.095	0.093	0.093	0.093	0.094	0.095	0.096	0.093
XM_004420206|_White_Rhino	0.129	0.127	0.127	0.127	0.129	0.127	0.129	0.127	0.127	0.127	0.129	0.127	0.127	0.127	0.129	0.129	0.126	0.119
XM_001504427|_Horse	0.114	0.112	0.112	0.112	0.114	0.112	0.114	0.112	0.112	0.112	0.114	0.112	0.112	0.112	0.114	0.114	0.117	0.108
XM_006212821|_Alpaca	0.132	0.130	0.130	0.130	0.132	0.130	0.132	0.129	0.127	0.130	0.132	0.130	0.130	0.130	0.132	0.132	0.132	0.123
XM_014856804|_Donkey	0.118	0.117	0.117	0.117	0.118	0.117	0.118	0.117	0.117	0.117	0.118	0.117	0.117	0.117	0.118	0.118	0.121	0.112
XM_010981399|_Camel	0.143	0.142	0.142	0.142	0.143	0.142	0.143	0.140	0.139	0.142	0.143	0.142	0.142	0.142	0.143	0.143	0.143	0.137
XM_010971602|_DH_Camel	0.143	0.142	0.142	0.142	0.143	0.142	0.143	0.140	0.139	0.142	0.143	0.142	0.142	0.142	0.143	0.143	0.143	0.134
XM_008571635|_Flying_Lemur	0.139	0.138	0.138	0.138	0.139	0.138	0.139	0.137	0.135	0.138	0.140	0.138	0.138	0.138	0.139	0.140	0.138	0.130
XM_039879918|_Flying_Fox	0.145	0.143	0.143	0.143	0.145	0.143	0.142	0.142	0.140	0.143	0.145	0.143	0.143	0.143	0.145	0.145	0.143	0.140

GDF9_Current_Work	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.024	0.030	0.030	0.030	0.030
JN100109|Chi|Lezhi_black	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
NM_001285708|Chi	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
JN100108|Tibetan|mRNA	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
FJ712709|Black_Begal	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.024	0.030	0.030	0.030	0.030
KY780296|Tibet_cashmere	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
EU883989|GDF9	0.008	0.007	0.009	0.006	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.024	0.030	0.030	0.030	0.029
EF446168|GDF9	0.007	0.006	0.008	0.005	0.008	0.017	0.017	0.017	0.018	0.020	0.026	0.024	0.027	0.024	0.029	0.029	0.029	0.029
KF758766|GDF9	0.007	0.006	0.008	0.005	0.007	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.029	0.029	0.029	0.029
JN587295|Chi_Jhakrana	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
FJ472652|Chi_Markhoz	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.025	0.030	0.030	0.030	0.030
MK421573|Chi_Nandidurga	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
MK421572|Chi_Nandidurga3	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
MK421567|Chi|Bidri1	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.026	0.024	0.027	0.024	0.030	0.030	0.029	0.030
MK421570|Chi_Nandidurga1	0.008	0.007	0.009	0.005	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.025	0.030	0.030	0.030	0.030
LC586428|Chi	0.008	0.007	0.009	0.006	0.008	0.017	0.018	0.018	0.019	0.020	0.027	0.024	0.027	0.025	0.030	0.030	0.030	0.030
KR063137|Goat	0.007	0.007	0.008	0.006	0.008	0.018	0.018	0.018	0.019	0.021	0.026	0.024	0.027	0.025	0.030	0.030	0.029	0.030
AB058416|Bta	0.001	0.003	0.006	0.008	0.002	0.017	0.017	0.018	0.019	0.020	0.025	0.023	0.026	0.023	0.029	0.028	0.028	0.029
EU267798|Yak		0.003	0.007	0.009	0.002	0.017	0.018	0.018	0.019	0.020	0.025	0.023	0.026	0.024	0.029	0.028	0.028	0.029
FJ529501|Buffalo	0.009		0.006	0.008	0.004	0.016	0.017	0.017	0.018	0.020	0.025	0.023	0.025	0.023	0.028	0.028	0.028	0.029
GQ227503|Moschus_Deer	0.025	0.023		0.010	0.007	0.017	0.017	0.018	0.019	0.020	0.026	0.024	0.026	0.024	0.029	0.028	0.029	0.029
XM_040240121|_Oryx	0.035	0.030	0.041		0.009	0.018	0.018	0.019	0.020	0.022	0.027	0.023	0.027	0.024	0.030	0.030	0.029	0.030
XM_005890431|_Wild_Yak	0.004	0.011	0.026	0.035		0.017	0.017	0.018	0.019	0.020	0.025	0.023	0.026	0.023	0.029	0.028	0.028	0.029
XM_022589005|_Beluga_Whale	0.080	0.074	0.079	0.085	0.078		0.004	0.002	0.006	0.007	0.020	0.018	0.020	0.018	0.022	0.022	0.023	0.025
XM_007461344|_Baiji	0.081	0.076	0.078	0.086	0.080	0.012		0.004	0.006	0.008	0.020	0.018	0.020	0.018	0.022	0.022	0.024	0.025
XM_029217693|_Nar_Whale	0.082	0.077	0.080	0.088	0.081	0.004	0.012		0.006	0.008	0.020	0.018	0.020	0.018	0.022	0.022	0.024	0.025
XM_036847515|_Whale	0.088	0.082	0.084	0.094	0.089	0.020	0.020	0.021		0.004	0.023	0.019	0.021	0.019	0.024	0.024	0.026	0.026
XM_007188566|Sca_Whale	0.094	0.090	0.092	0.101	0.093	0.029	0.029	0.030	0.011		0.023	0.020	0.023	0.020	0.025	0.025	0.027	0.028
XM_004420206|_White_Rhino	0.120	0.119	0.123	0.129	0.119	0.091	0.095	0.094	0.104	0.110		0.014	0.030	0.015	0.033	0.033	0.026	0.027
XM_001504427|_Horse	0.110	0.108	0.114	0.110	0.108	0.080	0.081	0.083	0.088	0.093	0.064		0.026	0.002	0.029	0.029	0.024	0.025
XM_006212821|_Alpaca	0.125	0.120	0.124	0.132	0.123	0.088	0.091	0.090	0.098	0.103	0.142	0.126		0.026	0.005	0.005	0.031	0.033
XM_014856804|_Donkey	0.114	0.112	0.118	0.114	0.112	0.081	0.082	0.084	0.089	0.095	0.065	0.004	0.127		0.029	0.029	0.024	0.026
XM_010981399|_Camel	0.139	0.134	0.137	0.146	0.137	0.102	0.103	0.102	0.110	0.116	0.157	0.140	0.018	0.141		0.002	0.033	0.035
XM_010971602|_DH_Camel	0.136	0.131	0.134	0.146	0.134	0.102	0.103	0.102	0.110	0.116	0.157	0.140	0.018	0.141	0.005		0.033	0.035
XM_008571635|_Flying_Lemur	0.131	0.126	0.135	0.134	0.136	0.104	0.108	0.107	0.118	0.124	0.118	0.108	0.145	0.110	0.158	0.158		0.029
XM_039879918|_Flying_Fox	0.142	0.140	0.137	0.142	0.142	0.117	0.121	0.120	0.131	0.134	0.129	0.119	0.157	0.123	0.169	0.171	0.138	

Figure 4.18: PWD matrix of *GDF9* gene created using MEGA7 software

my_seq|BMP15|		0.000	0.000	0.001	0.002	0.002	0.001	0.001	0.004	0.004	0.004	0.006	0.006	0.005	0.005	0.005	0.006	0.006	0.007	0.010	0.010	0.011	0.011	0.011	0.019	0.021
JF824148_Chi_Tibetan_goat	0.000		0.000	0.001	0.002	0.002	0.001	0.001	0.004	0.004	0.004	0.006	0.006	0.005	0.005	0.005	0.006	0.006	0.007	0.010	0.010	0.011	0.011	0.011	0.019	0.021
JF824149_Chi_Lezhi_black	0.000	0.000		0.001	0.002	0.002	0.001	0.001	0.004	0.004	0.004	0.006	0.006	0.005	0.005	0.005	0.006	0.006	0.007	0.010	0.010	0.011	0.011	0.011	0.019	0.021
EU847289_Chi_Boer	0.001	0.001	0.001		0.002	0.002	0.000	0.000	0.003	0.003	0.003	0.005	0.006	0.005	0.005	0.005	0.006	0.006	0.006	0.010	0.010	0.011	0.011	0.011	0.019	0.021
EU847284_Chi_Yunling_Black	0.004	0.004	0.004	0.003		0.003	0.002	0.002	0.004	0.004	0.004	0.006	0.006	0.005	0.006	0.006	0.006	0.006	0.007	0.011	0.011	0.011	0.011	0.011	0.019	0.021
EU888137_Chi_Black_Bengal	0.004	0.004	0.004	0.003	0.005		0.002	0.002	0.004	0.004	0.004	0.006	0.006	0.005	0.005	0.006	0.006	0.006	0.007	0.011	0.011	0.011	0.011	0.011	0.019	0.021
MK421566_Chi_isolate_Nandidurga4	0.001	0.001	0.001	0.000	0.003	0.003		0.000	0.003	0.003	0.003	0.005	0.006	0.005	0.005	0.005	0.006	0.006	0.006	0.010	0.010	0.011	0.011	0.011	0.019	0.021
MK421564_Chi_isolate_Nandidurga2	0.001	0.001	0.001	0.000	0.003	0.003	0.000		0.003	0.003	0.003	0.005	0.006	0.005	0.005	0.005	0.006	0.006	0.006	0.010	0.010	0.011	0.011	0.011	0.019	0.021
NM_001114767.2_Ovis_aries	0.010	0.010	0.010	0.009	0.012	0.012	0.009	0.009		0.000	0.000	0.005	0.006	0.004	0.005	0.004	0.005	0.005	0.006	0.010	0.010	0.010	0.010	0.011	0.018	0.020
KT853038_Ovis_aries	0.010	0.010	0.010	0.009	0.012	0.012	0.009	0.009	0.000		0.000	0.005	0.006	0.004	0.005	0.004	0.005	0.005	0.006	0.010	0.010	0.010	0.010	0.011	0.018	0.020
KT013295_Ovis_aries_Afshari	0.010	0.010	0.010	0.009	0.012	0.012	0.009	0.009	0.000	0.000		0.005	0.006	0.004	0.005	0.004	0.005	0.005	0.006	0.010	0.010	0.010	0.010	0.011	0.018	0.020
JQ326273_Bubalus_bubalis	0.023	0.023	0.023	0.021	0.024	0.024	0.021	0.021	0.018	0.018	0.018		0.004	0.004	0.006	0.004	0.006	0.006	0.006	0.011	0.011	0.011	0.011	0.011	0.019	0.021
EF375880_Bubalus_bubalis	0.027	0.027	0.027	0.025	0.028	0.028	0.025	0.025	0.023	0.023	0.023	0.012		0.004	0.006	0.005	0.006	0.006	0.007	0.011	0.011	0.011	0.011	0.011	0.019	0.021
XM_005898488_Pred_Bos_mutus	0.018	0.018	0.018	0.017	0.020	0.020	0.017	0.017	0.013	0.013	0.013	0.010	0.014		0.005	0.002	0.005	0.005	0.006	0.010	0.010	0.010	0.010	0.011	0.018	0.020
XM_040265988_Pred_Oryx_dammah	0.021	0.021	0.021	0.020	0.023	0.023	0.020	0.020	0.016	0.016	0.016	0.024	0.028	0.018		0.005	0.006	0.006	0.006	0.010	0.010	0.010	0.010	0.010	0.019	0.020
DQ463368_Bos_taurus	0.021	0.021	0.021	0.020	0.023	0.023	0.020	0.020	0.016	0.016	0.016	0.013	0.017	0.003	0.021		0.006	0.006	0.006	0.010	0.010	0.011	0.011	0.011	0.018	0.021
XM_043897208_Pred_Cervus_elaphus	0.027	0.027	0.027	0.025	0.028	0.028	0.025	0.025	0.021	0.021	0.021	0.027	0.031	0.021	0.024	0.024		0.000	0.003	0.010	0.010	0.010	0.010	0.010	0.017	0.019
XM_043459547_Pred_Cervus_canadensis	0.027	0.027	0.027	0.025	0.028	0.028	0.025	0.025	0.021	0.021	0.021	0.027	0.031	0.021	0.024	0.024	0.000		0.003	0.010	0.010	0.010	0.010	0.010	0.017	0.019
XM_020901080_Pred_Odocoileus_virginianus	0.032	0.032	0.032	0.031	0.034	0.034	0.031	0.031	0.027	0.027	0.027	0.028	0.032	0.024	0.030	0.027	0.010	0.010		0.010	0.010	0.011	0.011	0.011	0.018	0.020
XM_030846710_Pred_Globicephala_melas	0.073	0.073	0.073	0.071	0.075	0.075	0.071	0.071	0.067	0.067	0.067	0.073	0.073	0.067	0.068	0.070	0.067	0.067	0.075		0.001	0.002	0.003	0.003	0.016	0.019
XM_004325009.3_Pred_Tursiops_truncatus	0.075	0.075	0.075	0.073	0.076	0.076	0.073	0.073	0.068	0.068	0.068	0.075	0.074	0.068	0.070	0.071	0.068	0.068	0.076	0.001		0.003	0.003	0.003	0.016	0.019
XM_029240827_Pred_Monodon_monoceros	0.076	0.076	0.076	0.075	0.078	0.078	0.075	0.075	0.070	0.070	0.070	0.076	0.076	0.070	0.071	0.073	0.070	0.070	0.078	0.005	0.006		0.001	0.003	0.016	0.019
XM_022563769_Pred_Delphinapterus_leucas	0.078	0.078	0.078	0.076	0.079	0.079	0.076	0.076	0.071	0.071	0.071	0.078	0.078	0.071	0.073	0.074	0.071	0.071	0.079	0.006	0.008	0.001		0.003	0.016	0.019
XM_007450928_Pred_Lipotes_vexillifer	0.076	0.076	0.076	0.075	0.078	0.078	0.075	0.075	0.070	0.070	0.070	0.076	0.076	0.070	0.071	0.073	0.070	0.070	0.078	0.008	0.009	0.008	0.009		0.017	0.019
XM_006754222.2_Pred_Myotis_davidii	0.180	0.180	0.180	0.177	0.181	0.181	0.177	0.177	0.171	0.171	0.171	0.173	0.175	0.169	0.175	0.169	0.161	0.161	0.169	0.138	0.140	0.138	0.140	0.146		0.020
XM_038448952_Pred_Canis_lupus_familiaris	0.185	0.185	0.185	0.183	0.186	0.187	0.183	0.183	0.176	0.176	0.176	0.185	0.181	0.176	0.178	0.180	0.167	0.167	0.175	0.162	0.165	0.162	0.160	0.166	0.174	

Figure 4.19: PWD matrix of *BMP15* gene created using MEGA7 software

FINAL_SEQ_BMPR1B		0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
NM_001285575|Chi|BMPR1B	0.003		0.000	0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.004	0.004	0.004	0.004
DQ060443|Chi|BMPR1B_Cds	0.003	0.000		0.001	0.002	0.001	0.001	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.004	0.004	0.004	0.004
FJ712710|Chi|Blackbengal_Cds	0.002	0.003	0.003		0.001	0.000	0.000	0.001	0.001	0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
JN100106|Chi|Tibetan_Cds	0.003	0.003	0.003	0.001		0.001	0.001	0.002	0.000	0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005	0.005
EU847285|Chi|Yunlingblack_Cds	0.002	0.003	0.003	0.000	0.001		0.000	0.001	0.001	0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
EU847290|Chi|Boerbreed_Cds	0.002	0.003	0.003	0.000	0.001	0.000		0.001	0.001	0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
JN100107|Chi|Lezhi_Black_Cds	0.005	0.006	0.005	0.003	0.003	0.003	0.003		0.002	0.002	0.002	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005	0.005	0.004	0.005	0.005	0.005	0.005	0.005	0.005
JN049449|Chi|BMPR1B_Cds	0.003	0.003	0.003	0.001	0.000	0.001	0.001	0.003		0.001	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005	0.005
GU359045|Chi|BMPR1B_Cds	0.004	0.006	0.005	0.002	0.003	0.002	0.002	0.005	0.003		0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
DQ666418|Chi|BMPR1B_cds	0.006	0.007	0.007	0.004	0.005	0.004	0.004	0.007	0.005	0.006		0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
KF758765|Chi|BMPR1B_Cds	0.012	0.012	0.012	0.010	0.010	0.010	0.010	0.012	0.010	0.012	0.014		0.001	0.000	0.001	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
AF298885|Sheep|BMPR1B_Cds	0.012	0.012	0.012	0.010	0.010	0.010	0.010	0.012	0.010	0.012	0.014	0.001		0.001	0.001	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004
AF357007|Sheep|BMPR1B_mRNAcds	0.012	0.012	0.012	0.010	0.010	0.010	0.010	0.012	0.010	0.012	0.014	0.000	0.001		0.001	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
EU183347|Sheep|mRNA_Cds	0.014	0.015	0.015	0.012	0.013	0.012	0.012	0.015	0.013	0.014	0.017	0.003	0.003	0.003		0.003	0.003	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
XM_040237828|Prdt|oryx_X6_mRNA	0.019	0.019	0.019	0.018	0.019	0.018	0.018	0.021	0.019	0.020	0.022	0.014	0.014	0.014	0.017		0.000	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
XM_040237823|Prdt|oryx_X1_mRNA	0.019	0.019	0.019	0.018	0.019	0.018	0.018	0.021	0.019	0.020	0.022	0.014	0.014	0.014	0.017	0.000		0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
XM_019962427|Prdt|zebu_X1_mRNA	0.024	0.025	0.025	0.024	0.024	0.024	0.024	0.027	0.024	0.026	0.028	0.017	0.016	0.017	0.019	0.020	0.020		0.000	0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
XM_019962436|Prdt|zebu_X9_mRNA	0.024	0.025	0.025	0.024	0.024	0.024	0.024	0.027	0.024	0.026	0.028	0.017	0.016	0.017	0.019	0.020	0.020	0.000		0.000	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
XM_019962431|Prdt|zebu_X5_mRNA	0.024	0.025	0.025	0.024	0.024	0.024	0.024	0.027	0.024	0.026	0.028	0.017	0.016	0.017	0.019	0.020	0.020	0.000	0.000		0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
XM_027544025|Prdt|zebu_hybrid_X4_mRNA	0.025	0.026	0.026	0.024	0.025	0.024	0.024	0.027	0.025	0.027	0.029	0.018	0.017	0.018	0.019	0.021	0.021	0.001	0.001	0.001		0.000	0.000	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
XM_027544029|Prdt|zebu_hybrid_X8_mRNA	0.025	0.026	0.026	0.024	0.025	0.024	0.024	0.027	0.025	0.027	0.029	0.018	0.017	0.018	0.019	0.021	0.021	0.001	0.001	0.001	0.000		0.000	0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
XM_027544034|Prdt|zebu_hybrid_X13_mRNA	0.025	0.026	0.026	0.024	0.025	0.024	0.024	0.027	0.025	0.027	0.029	0.018	0.017	0.018	0.019	0.021	0.021	0.001	0.001	0.001	0.000	0.000		0.001	0.001	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.004	0.004
NM_001105328|Prdt|cattle_mRNA	0.026	0.026	0.026	0.025	0.026	0.025	0.025	0.028	0.026	0.027	0.029	0.019	0.017	0.019	0.020	0.022	0.022	0.001	0.001	0.001	0.002	0.002	0.002		0.000	0.002	0.002	0.002	0.003	0.003	0.004	0.004	0.004	0.004
BC134547|cattle|clone_mRNA_cds	0.026	0.026	0.026	0.025	0.026	0.025	0.025	0.028	0.026	0.027	0.029	0.019	0.017	0.019	0.020	0.022	0.022	0.001	0.001	0.001	0.002	0.002	0.002	0.000		0.002	0.002	0.002	0.003	0.003	0.004	0.004	0.004	0.004
XM_005907860|Prdt|wildyalk_X2_mRNA	0.026	0.026	0.026	0.025	0.026	0.025	0.025	0.028	0.026	0.027	0.029	0.019	0.017	0.019	0.020	0.020	0.020	0.005	0.005	0.005	0.006	0.006	0.006	0.007	0.007		0.000	0.002	0.003	0.003	0.004	0.004	0.004	0.004
XM_005907859|Prdt|wildyalk_X1_mRNA	0.026	0.026	0.026	0.025	0.026	0.025	0.025	0.028	0.026	0.027	0.029	0.019	0.017	0.019	0.020	0.020	0.020	0.005	0.005	0.005	0.006	0.006	0.006	0.007	0.007	0.000		0.002	0.003	0.003	0.004	0.004	0.004	0.004
XM_010848331|Prdt|bison_X3_mRNA	0.026	0.027	0.027	0.025	0.026	0.025	0.025	0.028	0.026	0.027	0.030	0.019	0.017	0.019	0.020	0.020	0.020	0.004	0.004	0.004	0.005	0.005	0.005	0.005	0.005	0.007	0.007		0.003	0.003	0.004	0.004	0.004	0.004
XM_044946207|Prdt|Waterbuffalo_X4_mRNA	0.025	0.026	0.026	0.024	0.025	0.024	0.024	0.027	0.025	0.027	0.029	0.019	0.017	0.019	0.020	0.019	0.019	0.009	0.009	0.009	0.010	0.010	0.010	0.010	0.010	0.012	0.012	0.012		0.000	0.004	0.004	0.004	0.004
XM_044946214|Prdt|waterbuffalo_X13_mRNA	0.025	0.026	0.026	0.024	0.025	0.024	0.024	0.027	0.025	0.027	0.029	0.019	0.017	0.019	0.020	0.019	0.019	0.009	0.009	0.009	0.010	0.010	0.010	0.010	0.010	0.012	0.012	0.012	0.000		0.004	0.004	0.004	0.004
XM_043871281|Prdt|reddeer_X1_mRNA	0.027	0.028	0.028	0.027	0.027	0.027	0.027	0.029	0.027	0.029	0.031	0.022	0.020	0.022	0.023	0.021	0.021	0.019	0.019	0.019	0.019	0.019	0.019	0.020	0.020	0.019	0.019	0.019	0.022	0.022		0.000	0.000	0.000
XM_043871289|Prdt|reddeer_X9_mRNA	0.027	0.028	0.028	0.027	0.027	0.027	0.027	0.029	0.027	0.029	0.031	0.022	0.020	0.022	0.023	0.021	0.021	0.019	0.019	0.019	0.019	0.019	0.019	0.020	0.020	0.019	0.019	0.019	0.022	0.022	0.000		0.000	0.000
XM_043438645|Prdt|Elk_X6_mRNA	0.027	0.028	0.028	0.027	0.027	0.027	0.027	0.029	0.027	0.029	0.031	0.022	0.020	0.022	0.023	0.021	0.021	0.019	0.019	0.019	0.019	0.019	0.019	0.020	0.020	0.019	0.019	0.019	0.022	0.022	0.000	0.000		0.000
XM_043438647|Prdt|Elk_X8_mRNA	0.027	0.028	0.028	0.027	0.027	0.027	0.027	0.029	0.027	0.029	0.031	0.022	0.020	0.022	0.023	0.021	0.021	0.019	0.019	0.019	0.019	0.019	0.019	0.020	0.020	0.019	0.019	0.019	0.022	0.022	0.000	0.000	0.000	

Figure 4.20: PWD matrix of *BMPR1B* gene created using MEGA7 software

4.7 Evolutionary convergence/divergence and selection pressure analysis

The type of selection pressure operating on the target sequences was determined using Z-test. Z-test of selection based upon the average number of synonymous substitutions and an average number of non-synonymous substitutions. Z-test considered a non-synonymous rate elevated above the synonymous rate as evidence for Darwinian selection. The variance of the difference between the non-synonymous and synonymous was computed with bootstrapping value of 1000. The specific sequence whether it is positive/negative selection is determined by its number of Non-synonymous (DN) and a number of synonymous (DS) values. If the DN>DS then it is assumed that the target sequence is under positive selection and if DN<DS then the target sequence is under negative selection pressure (Nei & Gojobori, 1986). The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) in favor of the alternative hypothesis (dN > dS) (below diagonal) is shown. Where dS are synonymous substitution per site and non-synonymous substitution per site represented by dN. Values of P less than 0.05 are considered significant at the 5% level

4.7.1 *GDF9, BMP15,* and *BMPR1B*

If DN<DS then the target sequence is under negative selection pressure. Values of P less than 0.05 are considered significant at the 5% level. These results signify that the sequence of *GDF9* within species has undergoes negative selection and has been selected by the nature (Fig. 4.21, 4.22 and 4.23)

GDF9_Current_Work		-1.001	-1.001	-1.001	0.553	-1.001	0.553	-1.417	-1.736	-1.001	-1.417	-1.001	-1.001	-1.001	-1.417	-1.417	0.906	2.044	1.927	2.399	2.771	2.668	1.966	3.118	3.037	3.037	2.677	2.534	4.056	3.845	2.855	4.211	2.719	2.819	4.827	4.558
JN100109|Chi|Lezhi_black	1.000		0.000	0.000	1.002	0.000	1.002	-1.001	-1.417	0.000	-1.001	0.000	0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
NM_001285708|Chi	1.000	1.000		0.000	1.002	0.000	1.002	-1.001	-1.417	0.000	-1.001	0.000	0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
JN100108|Tibetan|mRNA	1.000	1.000	1.000		1.002	0.000	1.002	-1.001	-1.417	0.000	-1.001	0.000	0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
FJ712709|Black_Bagal	0.291	0.159	0.159	0.159		1.002	1.420	0.553	0.165	1.002	0.552	1.002	1.002	1.002	0.553	0.553	1.435	2.356	2.240	2.706	3.045	2.989	2.275	3.336	3.255	3.255	2.898	2.752	4.243	4.039	3.052	4.400	2.913	3.012	5.007	4.738
KY780296|Tibet_cashmere	1.000	1.000	1.000	1.000	0.159		1.002	-1.001	-1.417	0.000	-1.001	0.000	0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
EU883989|GDF9	0.291	0.159	0.159	0.159	0.079	0.159		0.553	0.165	1.002	0.552	1.002	1.002	1.002	0.553	0.553	1.435	2.356	2.240	2.706	3.045	2.989	2.275	3.336	3.255	3.255	2.898	2.752	4.243	4.039	3.052	4.400	2.913	3.012	5.007	4.510
EF446168|GDF9	1.000	1.000	1.000	1.000	0.291	1.000	0.291		-1.736	-1.001	-1.417	-1.001	-1.001	-1.001	-1.417	-1.417	0.907	2.283	2.163	2.647	2.990	2.668	2.199	3.277	3.196	3.196	2.834	2.687	4.123	3.916	2.990	4.281	2.851	2.951	4.958	4.689
KF758766|GDF9	1.000	1.000	1.000	1.000	0.434	1.000	0.434	1.000		-1.417	-1.736	-1.417	-1.417	-1.417	-1.736	-1.736	1.545	2.403	2.282	2.772	3.100	2.808	2.316	3.197	3.116	3.116	2.754	2.609	4.214	4.010	3.055	4.373	2.914	3.014	5.080	4.841
JN587295|Chi_Jhakrana	1.000	1.000	1.000	1.000	0.159	1.000	0.159	1.000	1.000		-1.001	0.000	0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
FJ472652|Chi_Markhoz	1.000	1.000	1.000	1.000	0.291	1.000	0.291	1.000	1.000	1.000		-1.001	-1.001	-1.001	-1.417	-1.417	0.906	2.043	1.926	2.398	2.770	2.668	1.966	3.117	3.036	3.036	2.675	2.533	4.054	3.844	2.853	4.210	2.717	2.818	4.826	4.556
MK421573|Chi_Nandidurga	1.000	1.000	1.000	1.000	0.159	1.000	0.159	1.000	1.000	1.000	1.000		0.000	0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
MK421572|Chi_Nandidurga3	1.000	1.000	1.000	1.000	0.159	1.000	0.159	1.000	1.000	1.000	1.000	1.000		0.000	-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
MK421567|Chi|Bidri1	1.000	1.000	1.000	1.000	0.159	1.000	0.159	1.000	1.000	1.000	1.000	1.000	1.000		-1.001	-1.001	1.112	2.163	2.045	2.522	2.880	2.809	2.082	3.199	3.117	3.117	2.756	2.611	4.123	3.916	2.924	4.281	2.786	2.886	4.894	4.624
MK421570|Chi_Nandidurga1	1.000	1.000	1.000	1.000	0.291	1.000	0.291	1.000	1.000	1.000	1.000	1.000	1.000	1.000		-1.417	0.907	2.046	1.929	2.400	2.773	2.669	1.968	3.122	3.041	3.041	2.680	2.538	4.060	3.849	2.859	4.215	2.724	2.824	4.831	4.562
LC586428|Chi	1.000	1.000	1.000	1.000	0.291	1.000	0.291	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.907	2.045	1.928	2.400	2.772	2.668	1.968	3.120	3.039	3.039	2.679	2.536	4.058	3.848	2.857	4.213	2.722	2.822	4.829	4.560
KR063137|Goat	0.183	0.134	0.134	0.134	0.077	0.134	0.077	0.183	0.062	0.134	0.183	0.134	0.134	0.134	0.183	0.183		1.746	1.622	2.522	2.532	2.900	1.667	3.041	2.960	2.960	2.600	2.460	4.280	3.996	3.050	4.357	2.911	3.010	4.951	4.712
AB058416|Bta	0.022	0.016	0.016	0.016	0.010	0.016	0.010	0.012	0.009	0.016	0.022	0.016	0.016	0.016	0.021	0.022	0.042		-1.001	-0.010	1.781	2.717	-0.121	3.067	2.986	3.126	2.907	2.837	4.065	3.747	2.878	4.118	2.801	2.644	4.271	4.765
EU267798|Yak	0.028	0.022	0.022	0.022	0.013	0.022	0.013	0.016	0.012	0.022	0.028	0.022	0.022	0.022	0.028	0.028	0.054	1.000		-0.245	1.648	2.607	-0.534	2.989	2.907	3.048	2.830	2.762	3.998	3.678	2.811	4.050	2.736	2.579	4.206	4.701
FJ529501|Buffalo	0.009	0.006	0.006	0.006	0.004	0.006	0.004	0.005	0.003	0.006	0.009	0.006	0.006	0.006	0.009	0.009	0.006	1.000	1.000		2.587	3.081	-0.068	3.299	3.216	3.354	3.131	2.978	4.242	3.531	3.003	4.297	2.920	2.764	4.692	5.109
GQ227503|Moschus_Deer	0.003	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.001	0.002	0.003	0.002	0.002	0.002	0.003	0.003	0.006	0.039	0.051	0.005		3.657	1.694	3.623	3.271	3.407	3.263	3.110	4.036	3.842	3.186	4.208	2.975	2.820	5.254	5.058
XM_040240121|_Orya	0.004	0.003	0.003	0.003	0.002	0.003	0.002	0.004	0.003	0.003	0.004	0.003	0.003	0.003	0.004	0.004	0.002	0.004	0.005	0.001	0.000		2.460	3.319	3.239	3.374	3.190	2.808	3.967	3.668	3.053	4.040	2.976	3.075	4.612	4.601
XM_005890431|_Wild_Yak	0.026	0.020	0.020	0.020	0.012	0.020	0.012	0.015	0.011	0.020	0.026	0.020	0.020	0.020	0.026	0.026	0.049	1.000	1.000	1.000	0.046	0.008		2.951	2.869	3.011	2.928	2.723	3.963	3.642	2.771	4.016	2.696	2.537	4.172	4.663
XM_022589005|_Beluga_Whale	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.000	0.001	0.002		1.231	1.083	0.986	0.631	3.334	3.288	1.876	3.424	1.800	1.915	4.373	4.707
XM_007461344|_Baiji	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.002	0.110		0.690	1.407	1.211	3.108	3.206	1.948	3.343	1.722	1.838	4.157	4.497
XM_029217693|_Nar_Whale	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.000	0.000	0.002	0.140	0.246		0.834	0.700	3.389	3.343	1.795	3.477	1.570	1.688	4.419	4.750
XM_036847515|_Whale	0.004	0.003	0.003	0.003	0.002	0.003	0.002	0.003	0.003	0.003	0.004	0.003	0.003	0.003	0.004	0.004	0.005	0.002	0.003	0.001	0.001	0.001	0.002	0.163	0.081	0.203		0.873	3.299	3.016	2.221	3.153	1.996	2.106	4.220	4.735
XM_007188566|Sco_Whale	0.006	0.005	0.005	0.005	0.003	0.005	0.003	0.004	0.005	0.005	0.006	0.005	0.005	0.005	0.006	0.006	0.008	0.003	0.003	0.002	0.001	0.003	0.004	0.204	0.114	0.243	0.192		2.999	2.697	1.906	2.836	1.691	1.803	3.930	4.494
XM_004420206|_White_Rhino	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.001	0.002		2.221	2.792	2.382	2.727	2.825	2.888	3.154
XM_001504427|_Horse	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001	0.002	0.004	0.014		3.097	1.743	3.203	3.301	3.346	2.912
XM_006212821|_Alpaca	0.003	0.002	0.002	0.002	0.001	0.002	0.001	0.002	0.001	0.002	0.003	0.002	0.002	0.002	0.003	0.003	0.001	0.002	0.003	0.002	0.001	0.001	0.003	0.032	0.027	0.038	0.014	0.030	0.003	0.001		3.224	0.812	1.270	3.801	3.754
XM_014856804|_Donkey	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.003	0.009	0.042	0.001		3.326	3.424	3.474	3.296
XM_010981399|_Camel	0.004	0.003	0.003	0.003	0.002	0.003	0.002	0.003	0.002	0.003	0.004	0.003	0.003	0.003	0.004	0.004	0.002	0.003	0.004	0.002	0.002	0.002	0.004	0.037	0.044	0.059	0.024	0.047	0.004	0.001	0.209	0.001		-0.212	3.592	4.143
XM_010971602|_DH_Camel	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.002	0.003	0.003	0.002	0.005	0.006	0.003	0.003	0.001	0.006	0.029	0.034	0.047	0.019	0.037	0.003	0.001	0.103	0.000	1.000		3.687	4.173
XM_008571635|_Flying_Lemur	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.001	0.000	0.000	0.000	0.000		4.994
XM_039879918|_Flying_Fox	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.000	0.001	0.000	0.000	0.000	

Figure 4.21: Negative selection of *GDF9* gene created using MEGA7 software

my_seq|BMP15|		0.000	0.000	-0.979	0.445	0.035	-0.979	-0.979	0.696	0.696	0.696	0.853	0.947	0.885	0.392	1.522	1.049	1.049	1.186	2.249	2.165	2.314	2.435	2.307	2.826	3.515
JF824148_Chi_Tibetan_goat	1.000		0.000	-0.979	0.445	0.035	-0.979	-0.979	0.696	0.696	0.696	0.853	0.947	0.885	0.392	1.522	1.049	1.049	1.186	2.249	2.165	2.314	2.435	2.307	2.826	3.515
JF824149_Chi_Lezhi_black	1.000	1.000		-0.979	0.445	0.035	-0.979	-0.979	0.696	0.696	0.696	0.853	0.947	0.885	0.392	1.522	1.049	1.049	1.186	2.249	2.165	2.314	2.435	2.307	2.826	3.515
EU847289_Chi_Boer	1.000	1.000	1.000		0.707	0.527	0.000	0.000	0.867	0.867	0.867	1.014	1.085	1.058	0.556	1.654	1.177	1.177	1.307	2.332	2.248	2.396	2.517	2.389	2.882	3.575
EU847284_Chi_Yunling_Black	0.329	0.329	0.329	0.240		0.869	0.707	0.707	1.113	1.113	1.113	1.232	1.283	1.271	0.847	1.792	1.366	1.366	1.481	2.434	2.354	2.496	2.612	2.488	2.959	3.648
EU888137_Chi_Black_Bengal	0.486	0.486	0.486	0.300	0.193		0.527	0.527	0.987	0.987	0.987	1.130	1.187	1.173	0.702	1.734	1.269	1.269	1.394	2.391	2.308	2.454	2.573	2.447	2.927	3.614
MK421566_Chi_isolate_Nandidurga4	1.000	1.000	1.000	1.000	0.240	0.300		0.000	0.867	0.867	0.867	1.014	1.085	1.058	0.556	1.654	1.177	1.177	1.307	2.332	2.248	2.396	2.517	2.389	2.882	3.575
MK421564_Chi_isolate_Nandidurga2	1.000	1.000	1.000	1.000	0.240	0.300	1.000		0.867	0.867	0.867	1.014	1.085	1.058	0.556	1.654	1.177	1.177	1.307	2.332	2.248	2.396	2.517	2.389	2.882	3.575
NM_001114767.2_Ovis_aries	0.244	0.244	0.244	0.194	0.134	0.163	0.194	0.194		0.000	0.000	0.897	0.982	1.125	0.813	1.707	1.469	1.469	1.581	2.356	2.271	2.421	2.541	2.413	2.962	3.700
KT853038_Ovis_aries	0.244	0.244	0.244	0.194	0.134	0.163	0.194	0.194	1.000		0.000	0.897	0.982	1.125	0.813	1.707	1.469	1.469	1.581	2.356	2.271	2.421	2.541	2.413	2.962	3.700
KT013295_Ovis_aries_Afshari	0.244	0.244	0.244	0.194	0.134	0.163	0.194	0.194	1.000	1.000		0.897	0.982	1.125	0.813	1.707	1.469	1.469	1.581	2.356	2.271	2.421	2.541	2.413	2.962	3.700
JQ326273_Bubalus_bubalis	0.198	0.198	0.198	0.156	0.110	0.130	0.156	0.156	0.186	0.186	0.186		0.370	0.331	0.649	1.258	1.433	1.433	0.963	2.354	2.269	2.420	2.539	2.413	2.822	3.760
EF375880_Bubalus_bubalis	0.173	0.173	0.173	0.140	0.101	0.119	0.140	0.140	0.164	0.164	0.164	0.356		0.537	0.773	1.291	1.467	1.467	1.047	2.607	2.525	2.668	2.783	2.661	2.866	3.922
XM_005898488_Pred_Bos_mutus	0.189	0.189	0.189	0.146	0.103	0.122	0.146	0.146	0.131	0.131	0.131	0.371	0.296		0.849	1.303	1.785	1.785	1.507	2.560	2.473	2.622	2.741	2.615	3.120	3.831
XM_040265988_Pred_Oryx_dammah	0.348	0.348	0.348	0.290	0.199	0.242	0.290	0.290	0.209	0.209	0.209	0.259	0.221	0.199		1.525	1.551	1.551	1.662	2.090	2.004	2.162	2.288	2.155	3.038	3.687
DQ463368_Bos_taurus	0.065	0.065	0.065	0.050	0.038	0.043	0.050	0.050	0.045	0.045	0.045	0.105	0.100	0.098	0.065		2.203	2.203	1.978	2.858	2.778	2.915	3.026	2.907	3.117	4.048
XM_043897208_Pred_Cervus_elaphus	0.148	0.148	0.148	0.121	0.087	0.103	0.121	0.121	0.072	0.072	0.072	0.077	0.072	0.038	0.062	0.015		0.000	0.938	2.217	2.076	2.280	2.403	2.272	2.767	3.698
XM_043459547_Pred_Cervus_canadensis	0.148	0.148	0.148	0.121	0.087	0.103	0.121	0.121	0.072	0.072	0.072	0.077	0.072	0.038	0.062	0.015	1.000		0.938	2.217	2.076	2.280	2.403	2.272	2.767	3.698
XM_020901080_Pred_Odocoileus_virginianus	0.119	0.119	0.119	0.097	0.071	0.083	0.097	0.097	0.058	0.058	0.058	0.169	0.149	0.067	0.050	0.025	0.175	0.175		2.420	2.339	2.480	2.600	2.471	2.567	3.824
XM_030846710_Pred_Globicephala_melas	0.013	0.013	0.013	0.011	0.008	0.009	0.011	0.011	0.010	0.010	0.010	0.010	0.005	0.006	0.019	0.003	0.014	0.014	0.009		-0.978	-0.377	0.294	-1.023	2.432	3.565
XM_004325009.3_Pred_Tursiops_truncatus	0.016	0.016	0.016	0.013	0.010	0.011	0.013	0.013	0.012	0.012	0.012	0.013	0.006	0.007	0.024	0.003	0.020	0.020	0.010	1.000		-0.720	-0.026	-1.290	2.369	3.503
XM_029240827_Pred_Monodon_monoceros	0.011	0.011	0.011	0.009	0.007	0.008	0.009	0.009	0.008	0.008	0.008	0.009	0.004	0.005	0.016	0.002	0.012	0.012	0.007	1.000	1.000		0.996	-0.081	2.526	3.735
XM_022563769_Pred_Delphinapterus_leucas	0.008	0.008	0.008	0.007	0.005	0.006	0.007	0.007	0.006	0.006	0.006	0.006	0.003	0.004	0.012	0.002	0.009	0.009	0.005	0.385	1.000	0.161		0.413	2.626	3.647
XM_007450928_Pred_Lipotes_vexillifer	0.011	0.011	0.011	0.009	0.007	0.008	0.009	0.009	0.009	0.009	0.009	0.009	0.004	0.005	0.017	0.002	0.012	0.012	0.007	1.000	1.000	1.000	0.340		2.354	3.644
XM_006754222.2_Pred_Myotis_davidii	0.003	0.003	0.003	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.001	0.001	0.001	0.003	0.003	0.006	0.008	0.010	0.006	0.005	0.010		3.343
XM_038448952_Pred_Canis_lupus_familiaris	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	

Figure 4.22: Negative selection of *BMP15* gene created using MEGA7 software

FINAL_SEQ_BMPR1B		-0.635	-0.635	1.068	0.669	1.068	1.068	-0.230	0.669	0.784	-0.026	2.511	2.509	2.511	2.359	3.932	3.932	4.128	4.128	4.128	4.220	4.220	4.220	4.298	4.298	4.160	4.160	4.342	4.267	4.267	4.188	4.188	4.188	4.188
NM_001285575|Chi|BMPR1B	1.000		0.000	-0.357	-0.698	-0.357	-0.357	-1.468	-0.698	-0.293	-1.030	2.155	2.153	2.155	2.029	3.640	3.640	4.041	4.041	4.041	4.133	4.133	4.133	4.216	4.216	4.073	4.073	4.249	4.018	4.018	3.948	3.948	3.948	3.948
DQ060443|Chi|BMPR1B_Cds	1.000	1.000		-0.357	-0.698	-0.357	-0.357	-1.468	-0.698	-0.293	-1.030	2.155	2.153	2.155	2.029	3.640	3.640	4.041	4.041	4.041	4.133	4.133	4.133	4.216	4.216	4.073	4.073	4.249	4.018	4.018	3.948	3.948	3.948	3.948
FJ712710|Chi|Blackbengal_Cds	0.144	1.000	1.000		-0.971	0.000	0.000	-1.927	-0.971	-0.045	-1.047	2.309	2.307	2.309	2.162	4.006	4.006	4.332	4.332	4.332	4.422	4.422	4.422	4.493	4.493	4.361	4.361	4.545	4.330	4.330	4.248	4.248	4.248	4.248
JN100106|Chi|Tibetan_Cds	0.252	1.000	1.000	1.000		-0.971	-0.971	-2.157	0.000	-0.427	-1.307	2.199	2.198	2.199	2.063	3.914	3.914	4.262	4.262	4.262	4.352	4.352	4.352	4.426	4.426	4.291	4.291	4.472	4.253	4.253	4.175	4.175	4.175	4.175
EU847285|Chi|Yunlingblack_Cds	0.144	1.000	1.000	1.000	1.000		0.000	-1.927	-0.971	-0.045	-1.047	2.309	2.307	2.309	2.162	4.006	4.006	4.332	4.332	4.332	4.422	4.422	4.422	4.493	4.493	4.361	4.361	4.545	4.330	4.330	4.248	4.248	4.248	4.248
EU847290|Chi|Boerbreed_Cds	0.144	1.000	1.000	1.000	1.000	1.000		-1.927	-0.971	-0.045	-1.047	2.309	2.307	2.309	2.162	4.006	4.006	4.332	4.332	4.332	4.422	4.422	4.422	4.493	4.493	4.361	4.361	4.545	4.330	4.330	4.248	4.248	4.248	4.248
JN100107|Chi|Lezhi_Black_Cds	1.000	1.000	1.000	1.000	1.000	1.000	1.000		-2.157	-1.262	-1.937	1.898	1.895	1.898	1.792	3.654	3.654	4.057	4.057	4.057	4.149	4.149	4.149	4.231	4.231	4.090	4.090	4.263	4.032	4.032	3.966	3.966	3.966	3.966
JN049449|Chi|BMPR1B_Cds	0.252	1.000	1.000	1.000	1.000	1.000	1.000	1.000		-0.427	-1.307	2.199	2.198	2.199	2.063	3.914	3.914	4.262	4.262	4.262	4.352	4.352	4.352	4.426	4.426	4.291	4.291	4.472	4.253	4.253	4.175	4.175	4.175	4.175
GU359045|Chi|BMPR1B_Cds	0.217	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		-0.850	2.242	2.240	2.242	2.108	3.930	3.930	4.283	4.283	4.283	4.373	4.373	4.373	4.448	4.448	4.313	4.313	4.491	4.272	4.272	4.196	4.196	4.196	4.196
DQ666418|Chi|BMPR1B_cds	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.916	1.913	1.916	1.811	3.652	3.652	4.063	4.063	4.063	4.154	4.154	4.154	4.238	4.238	4.096	4.096	4.266	4.035	4.035	3.970	3.970	3.970	3.970
KF758765|Chi|BMPR1B_Cds	0.007	0.017	0.017	0.011	0.015	0.011	0.011	0.030	0.015	0.013	0.029		1.246	0.000	0.175	4.015	4.015	4.191	4.191	4.191	4.288	4.288	4.288	4.351	4.351	4.219	4.219	4.438	4.319	4.319	4.116	4.116	4.116	4.116
AF298885|Sheep|BMPR1B_Cds	0.007	0.017	0.017	0.011	0.015	0.011	0.011	0.030	0.015	0.013	0.029	0.108		1.246	0.174	4.015	4.015	4.083	4.083	4.083	4.187	4.187	4.187	4.239	4.239	4.109	4.109	4.362	4.254	4.254	3.991	3.991	3.991	3.991
AF357007|Sheep|BMPR1B_mRNAcds	0.007	0.017	0.017	0.011	0.015	0.011	0.011	0.030	0.015	0.013	0.029	1.000	0.108		0.175	4.015	4.015	4.191	4.191	4.191	4.288	4.288	4.288	4.351	4.351	4.219	4.219	4.438	4.319	4.319	4.116	4.116	4.116	4.116
EU183347|Sheep|mRNA_Cds	0.010	0.022	0.022	0.016	0.021	0.016	0.016	0.038	0.021	0.019	0.036	0.431	0.431	0.431		3.734	3.734	3.869	3.869	3.869	3.972	3.972	3.972	4.047	4.047	3.901	3.901	4.121	3.986	3.986	3.779	3.779	3.779	3.779
XM_040237828|Prdt|oryx_X6_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	4.512	4.512	4.512	4.607	4.607	4.607	4.650	4.650	4.340	4.340	4.583	4.375	4.375	4.112	4.112	4.112	4.112
XM_040237823|Prdt|oryx_X1_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000		4.512	4.512	4.512	4.607	4.607	4.607	4.650	4.650	4.340	4.340	4.583	4.375	4.375	4.112	4.112	4.112	4.112
XM_019962427|Prdt|zebu_X1_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.998	0.998	0.998	1.210	1.210	2.174	2.174	2.192	2.995	2.995	3.869	3.869	3.869	3.869
XM_019962436|Prdt|zebu_X9_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000		0.000	0.998	0.998	0.998	1.210	1.210	2.174	2.174	2.192	2.995	2.995	3.869	3.869	3.869	3.869
XM_019962431|Prdt|zebu_X5_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000		0.998	0.998	0.998	1.210	1.210	2.174	2.174	2.192	2.995	2.995	3.869	3.869	3.869	3.869
XM_027544025|Prdt|zebu_hybrid_X4_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.160	0.160	0.160		0.000	0.000	1.494	1.494	2.351	2.351	2.389	3.138	3.138	3.972	3.972	3.972	3.972
XM_027544029|Prdt|zebu_hybrid_X8_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.160	0.160	0.160	1.000		0.000	1.494	1.494	2.351	2.351	2.389	3.138	3.138	3.972	3.972	3.972	3.972
XM_027544034|Prdt|zebu_hybrid_X13_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.160	0.160	0.160	1.000	1.000		1.494	1.494	2.351	2.351	2.389	3.138	3.138	3.972	3.972	3.972	3.972
NM_001105328|Prdt|cattle_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.114	0.114	0.114	0.069	0.069	0.069		0.000	2.482	2.482	2.472	3.209	3.209	4.047	4.047	4.047	4.047
BC134547|cattle|clone_mRNA_cds	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.114	0.114	0.114	0.069	0.069	0.069	1.000		2.482	2.482	2.472	3.209	3.209	4.047	4.047	4.047	4.047
XM_005907860|Prdt|wildyak_X2_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.016	0.016	0.010	0.010	0.010	0.007	0.007		0.000	2.616	3.310	3.310	3.692	3.692	3.692	3.692
XM_005907859|Prdt|wildyak_X1_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.016	0.016	0.010	0.010	0.010	0.007	0.007	1.000		2.616	3.310	3.310	3.692	3.692	3.692	3.692
XM_010848331|Prdt|bison_X3_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.015	0.015	0.009	0.009	0.009	0.007	0.007	0.005	0.005		3.653	3.653	3.912	3.912	3.912	3.912
XM_044946207|Prdt|Waterbuffalo_X4_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		0.000	4.193	4.193	4.193	4.193
XM_044946214|Prdt|waterbuffalo_X13_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	1.000		4.193	4.193	4.193	4.193
XM_043871281|Prdt|reddeer_X1_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
XM_043871289|Prdt|reddeer_X9_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000		0.000	0.000
XM_043438645|Prdt|Elk_X6_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000		0.000
XM_043438647|Prdt|Elk_X8_mRNA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	

Figure 4.23: Negative selection of *BMPR1B* gene created using MEGA7 software

4.8 Protein structure prediction

The pdb file obtained from Raptor (Lovell et al., 2003) for *GDF9*, *BMP15*, and *BMPR1B* (Fig. 4.24, 4.25, and 4.26) was used for the ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php)

4.8.1 *GDF9*

Revealed that highly preferred region includes 95.45% (336) of amino acid residues, all were in favored regions and 3.97% of all regions were in the allowed region. The number of residues in the outlier region was 0.56% (Fig. 4.27).

4.8.2 *BMP15*

Revealed that highly preferred region 91.32% (358) of amino acid residues, all were in favored regions and 6.88% of all regions were in the allowed region. The number of residues in the outlier region was 1.78% (Fig. 4.28).

4.8.3 *BMPR1B*

Revealed that highly preferred region 88.12% (438) of amino acid residues, all were in favored regions and 9.45% of all regions were in the allowed region. The number of residues in the outlier region was 2.41% (Fig. 4.29).

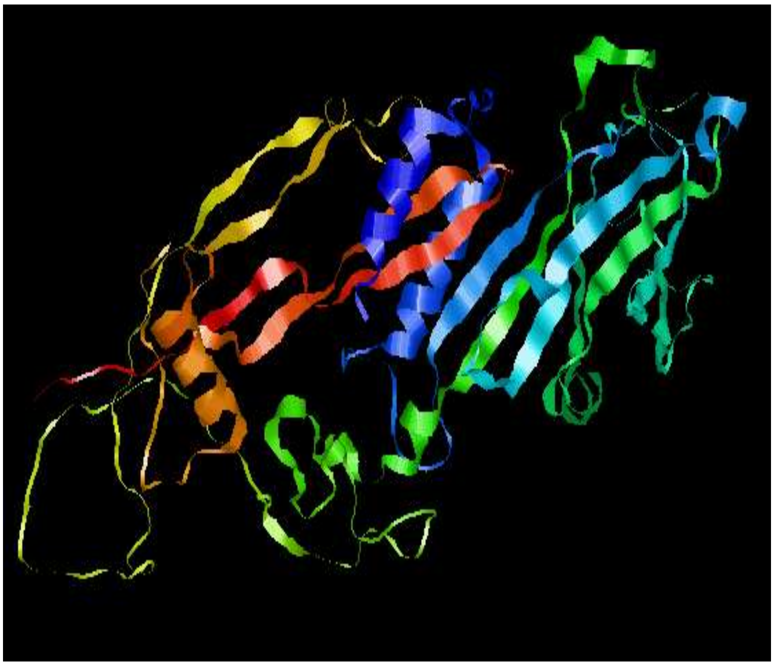


Figure 4.24: Prediction of *GDF9* protein by using RaptorX software

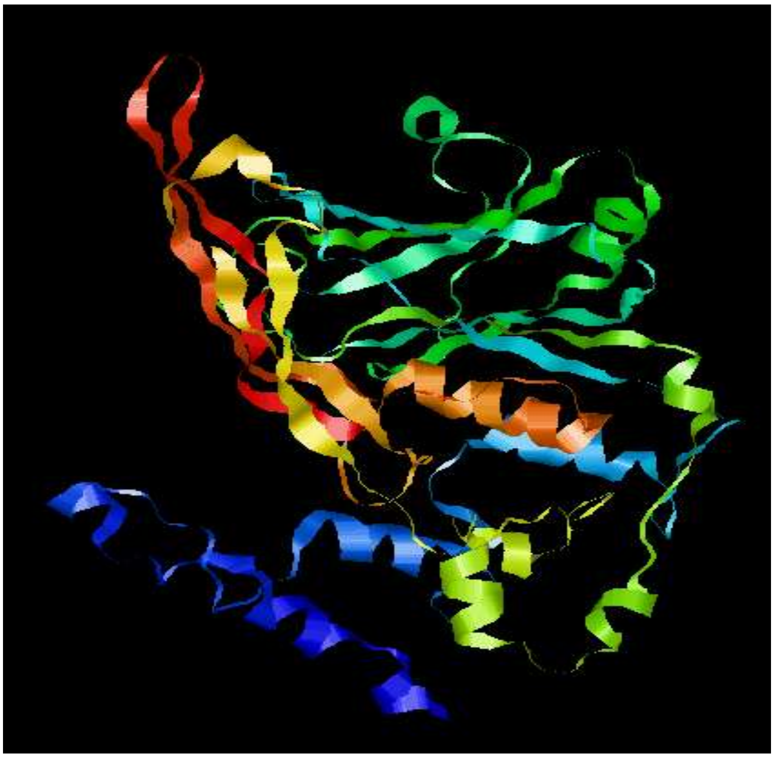


Figure 4.25: Prediction of *BMP15* protein by using RaptorX software

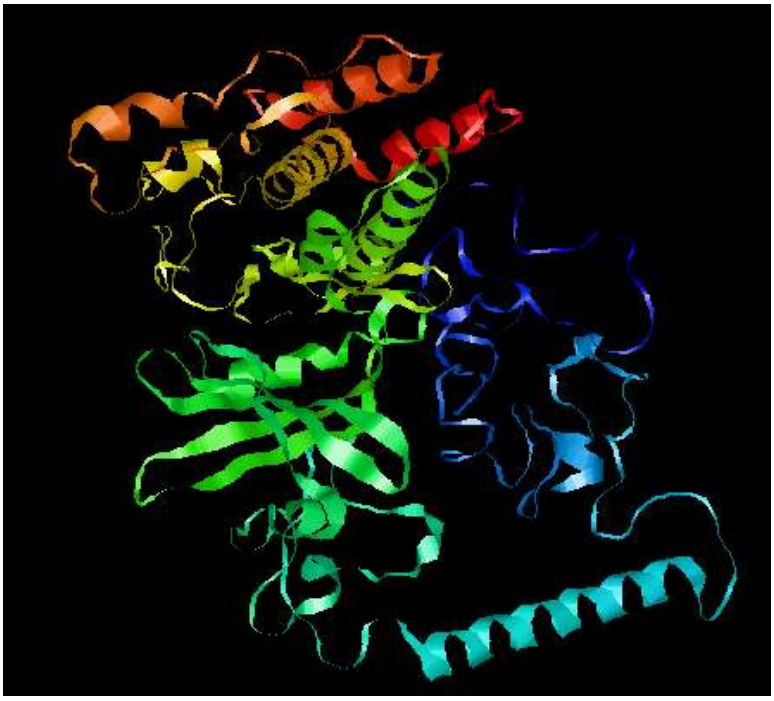


Figure 4.26: Prediction of *BMPR1B* protein by using RaptorX software

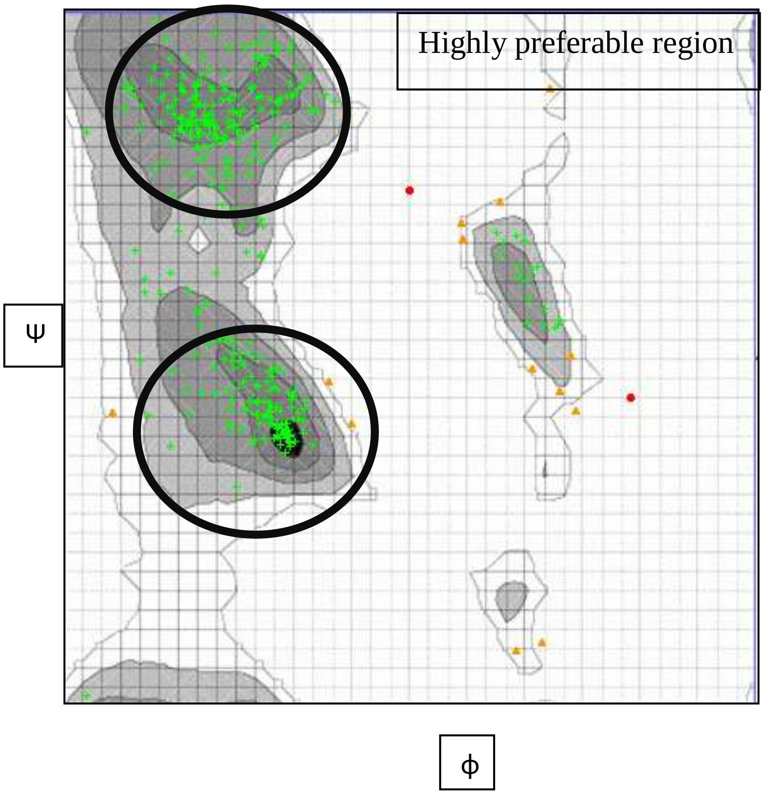


Figure 4.27: Validation of *GDF9* amino-acid residues by using RAMPAGE

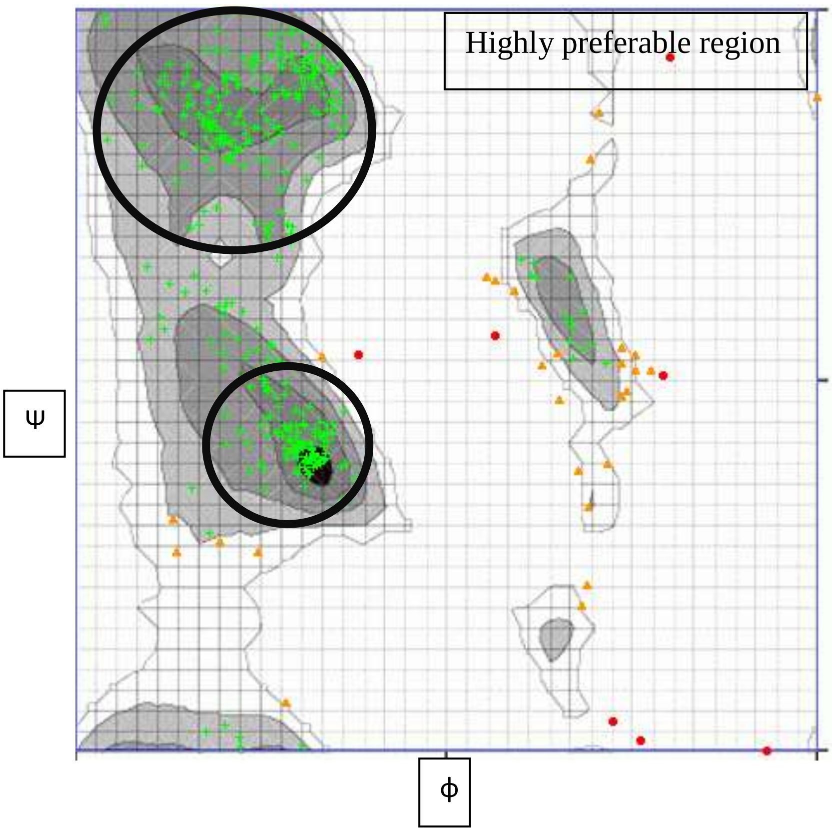


Figure 4.28: Validation of *BMP15* amino-acid residues by using RAMPAGE

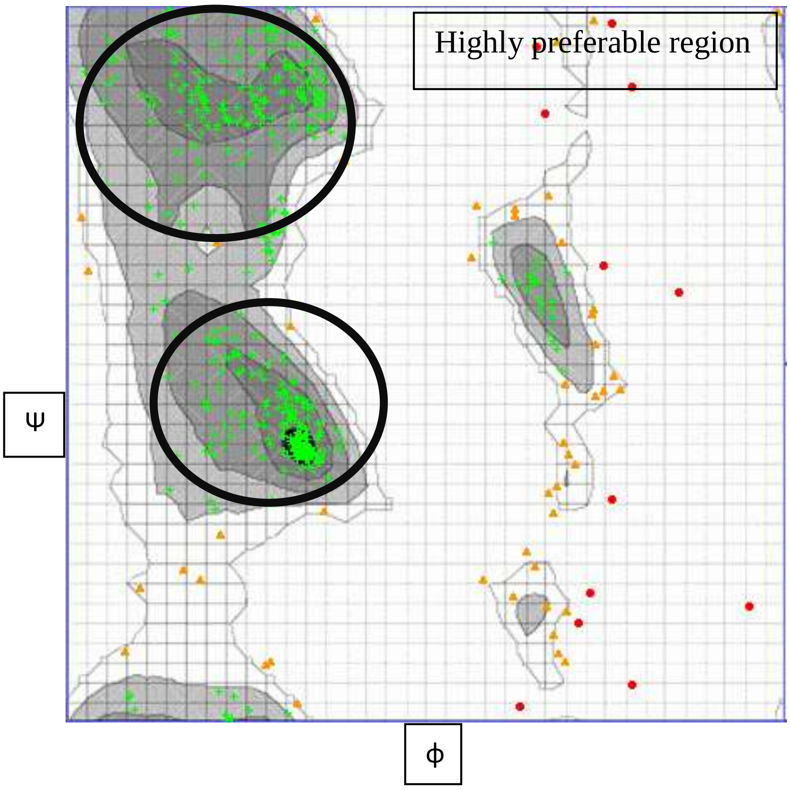


Figure 4.29: Validation of *BMPR1B* amino-acid residues by using RAMPAGE

PROCHECK analysis showed 89.1% residues of *BMP2* protein in favored and allowed region of Ramachandran plot (Bhat et al., 2020). Ramachandran plot for this study had indicated that the *GDF9* protein showed 95.45% residues, *BMP15* protein showed 91.32% residues and *BMPR1B* protein showed 88.12% residues in allowed region all the three protein sequences were found to be present in the favored and allowed region indicating an overall stable protein configuration of the predicted structures. The functional differentiation of four smad4 paralogs in TGF-β signaling pathway of Japanese flounder (*Paralichthys olivaceus*) genes showed all the positively selected amino acid sites with Ramachandran plot method, more than 90% of the area showed efficiency of *GDF9* and *BMP15* (Yu et al., 2020)

CHAPTER – V

SUMMARY AND CONCLUSIONS

The goat is one of the domesticated animals in livestock farming. It has much importance, as it is a multifunctional animal providing meat, milk, and wool for the landless, small, and marginal farmers in our country. It has higher prolificacy and a small generation interval. Goat rearing gives economic support to a large section of the population in rural areas. It is also known as “Poor Man’s Cow”.

Three major fecundity genes have been identified in sheep and goats, namely (1) Bone Morphogenetic Protein Receptor Type 1B (*BMPR1B*; or activin-like kinase 6, ALK6), known as FecB; (2) Growth Differentiation Factor 9 (*GDF9*), known as FecG; and (3) Bone Morphogenetic Protein 15 (*BMP15*) known as FecX. Several mutations have been shown in these genes and these mutations led to increased ovulation rates in heterozygous animals and sterility in homozygous mutant animals.

The present study was undertaken to the sequence of *GDF9*, *BMP15,* and *BMPR1B* coding region of Beetal goat, analyze its sequence to study the evolutionary relationship. The ovaries samples were taken from slaughterhouse which was used for the total RNA isolation, cDNA synthesis was done by kit as per manufacturer’s protocol, and further amplification of target genes coding sequence by using specific primers. PCR showed specific amplicons.

The annotated sequence of *GDF9*, *BMP15,* and *BMPR1B* of the coding region was obtained by custom sequenced through Sanger sequencing. Sequence analysis revealed that these target genes could have evolved due to negative selection over the time. Phylogenetic analysis of these genes by MEGA7 indicated that breeds of goat, ruminants, marine, and non-ruminants form different clusters within the phylogenetic tree, non-ruminants showing that they are evolutionary divergent from the different breeds of the goat. Further, the 3D structure prediction of protein and the validation and stability of protein was done. Drug designing (synthetic analogs) or structural modelling of protein will be beneficial to increase the reproduction performance of the goat.

Conclusions

- Coding sequences of *GDF9*, *BMP15*, and *BMPR1B* genes of Beetal goats of Punjab are being reported for the first time.
- The gene sequences of *GDF9, BMP15,* and *BMPR1B* indicated a high level of conservation among the caprine species, although non-ruminant mammalian species exhibited distance.

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APPENDIX-I

1.) Buffer/ solutions for gel electrophoresis

10X TAE Buffer

Ingredients	Quantity
Distilled water	800 ml
Tris Base	48.4 gm
Glacial Acetic acid	11.42 ml
0.5 M EDTA	20 ml

Makeup to 1000 ml with autoclaved double distilled water.

Working TAE Buffer

10X TAE Buffer 100 ml

Double distilled water (up to) 1000 ml

Stock Ethidium Bromide (50X)

Ethidium Bromide	10mg
Distilled water	1ml

2.) Requirement for Transformation and Cloning

LB Broth

LB Broth (HiMedia)	25g
Distilled water	1000ml

Autoclaved at 15lb/sq inch for 15 min

LB Agar

LB Agar (HiMedia)	40g
Distilled water	1000ml

Autoclaved at 15lb/sq inch for 15 min

LB Agar plates with ampicillin

LB Agar (HiMedia)	40g
Distilled water	1000ml
Ampicillin	100µg/ml

Ampicillin Stock

Ampicillin	100mg
DDW	1ml

Filtered by filter 0.22µm syringe filter under aseptic conditions.

Calcium chloride

100Mm $CaCl_2$ was prepared and chilled at 0°C as required for transformation.

3.) Plasmid Isolation

P1 Solution

50mM Glucose

25Mm Tris-Cl (pH 8.0)

10Mm EDTA (pH 8.0)

Stored at 4°C

P2 Solution

5M NaOH

10%SDS

Freshly prepared

P3 Solution

5M Potassium acetate

VITA

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