EFFECTS OF PROTEIN SUPPLEMENTS ON FERTILITY AND PHYLOGENIC INFERENCE OF FERTILITY GENES IN INDIGENOUS SHEEP OF BANGLADESH

A thesis

By

MD. IQBAL HOSSAIN

Roll No.: 0116/03

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DEPARTMENT OF GENETICS AND ANIMAL BREEDING

FACULTY OF VETERINARY MEDICINE

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in the partial fulfillment of the requirements for the degree of

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Supervisor	**Head**
	Chairman of the Examination committee

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CHITTAGONG-4225, BANGLADESH

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Authorization

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The Author
June, 2018

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List of Abbreviations

A	Arginine
bp	Base pair
BCS	Body condition score
BER	Bangladesh economic review
BMP-15	Bone morphogenetic protein-15
C	Cytosine
CM	Centimeter
CP	Crude protein
CRD	Completely randomized design
dNTPs	Deoxynucleotide triphosphates
ddNTPs	Dideoxynucleotide triphosphates
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylene demine tetra acetic acid
e.g.	Example
FAO	Food and Agricultural Organization
Fec	Fecundity
FSH	Follicle stimulating hormone
G	Guanine
GDF-9	Growth differentiation factor-9
GDP	Gross domestic product
Kcal	Kilo-calorie
Kg	Kilogram
LSD	Least Significant Difference
ME	Metabolizable energy
MJ	Mega jule
ML	Milliliter
MP	Microbial protein

NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
RDP	Rumen degradable protein
RFLP	Restriction fragment length polymorphism
SC	Scrotal circumference
SNP	Single Nucleotide Markers
T	Tyrosine
UDP	Rumen un-degradable protein
%	Percentage
µl	Microliter

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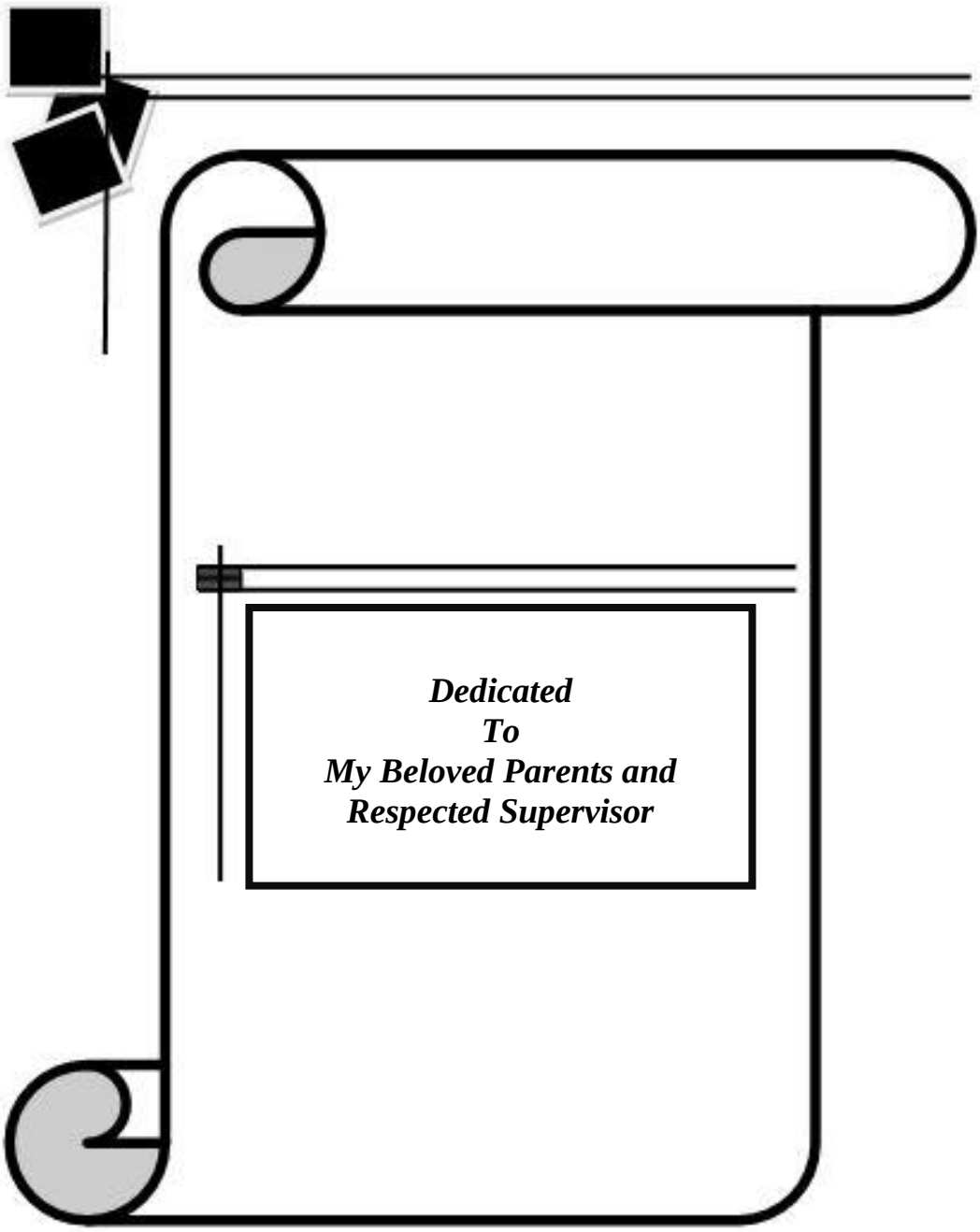
Dedicated
To
My Beloved Parents and
Respected Supervisor

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Abstract

The study was carried out to know the effects of protein supplementations on fertility and inference of fertility genes. Three iso-caloric but different graded levels of protein containing rations were feed to three different groups of sheep. For molecular study, DNA was extracted from the whole blood sample for PCR of GDF9 and BMP15 fertility genes and purified PCR products were sequenced with the help of Sanger sequencer. Sequence alignment, pair and multi-alignment comparison of the BMP15 and GDF9 gene were performed with MEGA6 software. The semen volume, sperm counts, percentages of normal and viable sperm were higher in Treatment 2 than the other two treatments. Ewes treated with supplemented, protein concentrate reached conception at earlier age (Treatment 1, 7.4±0.09 months and Treatment 2, 6.67±0.18 months) than control (8.37± 0.15 months). Conception rate was significantly higher in treated groups than control group. The gestation length and lambing interval were significantly higher in control (155.25±4.1 days, 188.25±2.3 days, respectively) than treated groups. Lamb's birth weights of three groups were ranged from 1.11 to 1.55 kg. The designated sequences of BMP15 gene and GDF9 gene were revealed 100% homology with the sequence of Pelibuey sheep. In addition, the sequences of BMP15 gene showed 99% similarity with the Balochi and Lohi breed of sheep and GDF9 gene asserted that sequences were 95% harmonious with the Awassi breed. The evolutionary divergences in indigenous sheep ranged from 0.00 to 3.35 for BMP15 gene and 0.00 to 0.77 for GDF9 gene with clear differences within indigenous sheep. In the phylogenetic tree nucleotide sequence of BMP15 was closely related with Pelibuey sheep and Rahmani sheep breed had strong association with GDF9. Jukes-Cantor (JC) model designed with the 3866.36 BIC score and Kimura 2-parameter model carried 6222.80 BIC score were the best fitted models for BMP15 gene and GDF9 gene, respectively. The overall nucleotide frequencies of BMP15 were 27.87% (A), 26.34% (T/U), 21.70% (C), and 24.10% (G) and GDF9 were 20.22% (A), 26.32% (T/U), 27.91% (C) and 25.54% (G), respectively. The study indicated that the influence of nutrition on reproductive performance and these genomic inceptions will be open a new era in the indigenous sheep production of Bangladesh.

Key words: Indigenous sheep, Characters, Protein supplements, Fertility gene inference.

Chapter-1: Introduction

In Bangladesh, the gap between the population and animal protein availability is more severe in nature. Peoples are facing nutritional deficiency that is from protein shortage. Mutton (sheep meat) can plays an important role in supplying animal protein with comparatively low prices for the people of Bangladesh.

Among the livestock species, sheep may contribute a significant amount of protein and create opportunities for income generation to the peoples of Bangladesh (Alam et al., 2006). About 3.35 million sheep are distributed all over the country (BER, 2017). Almost all of them are non-descriptive indigenous in nature, having 15 to 25 kg average body weight and probably originated from south-eastern to sub-tropical regions with adaptive capacity to hot humid climate (Mukherjee, 2000). The characteristics of indigenous sheep of Bangladesh are: grey in color, with black or white patches, and the face, ear and feet are mostly light black with coarse wool. In Bangladesh, sheep is generally managed under rural condition and they are sparsely distributed in some areas such as Rajshahi, Dinajpur, Bogra, Rangpur, Tangail districts and in the delta region of Noakhali area (Bindon, 1984). However, farmer, who keeps a significant number of sheep that is not ideally productive due to poor genetic merit (Alam et al., 2001; Rahman et al., 2014), indiscriminate breeding, poor nutrition and management (Alam et al., 2006), seasonal fluctuations in feed resources and diseases.

Reproductive perfection and traits of farm animal is mainly dependent on nutritional conditions (Kochapakdee et al., 1994) where under nutrition results in the loss of body weight and body condition, delays the onset of puberty, increases the post-partum onset of estrus, interferes with normal ovarian cyclicity by decreasing gonadotropin secretion and increases infertility (Legesse, 2008). For the improvement of the production and reproduction status of animals it may require improved rations specially protein as a source of essential amino acids and (in the case of ruminants) as a nitrogen source for rumen micro-flora. The quality of the protein in a feed is dependent on both amino acid profile and the digestibility. Proteins are complex organic compounds of high molecular weight. The monomer of proteins is made up of amino acids which are indispensable and dispensable. In the ruminant, dietary protein can be classified as either rumen degradable protein (RDP) or as rumen un-degradable

protein (UDP). The protein requirement of an animal is dependent on its physiological status and level of production. Amino acids are the most important regulators of pre-implantation development and inclusion of specific amino acids in embryo culture media was shown to overcome the so-called "developmental blocks" observed in many species. Steeves and Gardner (1999) demonstrated that the embryo has a switch in its requirements for amino acids as it develops the zygote to the blastocyst.

Semen is the combined secretion of male reproductive glands containing the male gametes (spermatozoa) and seminal fluids produced in the testis and male reproductive tract (Hafez and Hafez, 2013). The quality of semen plays a major role in determining the fertility and reproductive efficiency of any livestock production. There are a number of studies such as Brown (1994); Rekik et al., (2007) concerning about the effects of special nutrients, particularly proteins on reproductive parameters in the ram. In ruminants low planes of nutrition during the pre-pubertal period delay testicular growth and the onset of puberty by inhibiting the development of a mature reproductive endocrine system reported by Brown (1994). Severe underfeeding can even permanently impair sperm output (Brown, 1994; Fernández et al., 2005). Previous studies Zohara et al. (2014) have reflected the importance of protein supplementations on growth and productivity of goats and sheep. On the other hand, it has long been established that rams reared on higher levels of feeding grow faster and attain puberty at younger ages than rams on low planes of nutrition (Fernández et al., 2005). It is proved that, appropriate nutritional management is essential for successful mating in sheep flocks (Fortune, 1993; Fernández et al., 2005).

Nutritional influences on reproduction in farm animals were probably recognized soon after domestication where Clark (1934) provided the first experimental evidence for gonadal effects when he related the effect of "flushing" on lambing rate in sheep to an increase in ovulation rate. In case of female nutrition during gestation not only affects maternal body weight gain, body condition, and reproductive performance (Hess et al., 2005) but also affects prenatal and postnatal offspring growth and development (Wu et al., 2006; Zohara et al., 2014). Improved diet in the ewe increases ovulation rate and modifies the morphological and functional quality of the embryo recovered (Ruiz-Lozano, 2003). Several genes (such as BMP15, GDF9 etc.) which are related with ovulation rate and litter size have been shown to affect female fecundity in sheep (Barzegari et al., 2010; Javanmard et al., 2011). Among them,

BMP15 genes exclusively expressed in the oocyte, which are responsible for a huge range of cellular behaviours including the development and maturation of the oocytes (Hanrahan et al., 2004; Javanmard et al., 2011). On the other hand, autosomal GDF9 has an essential role in controlling the follicular growth through its influence on granulosa cell function (Barzegari et al., 2010; Javanmard et al., 2011).

The advances of modern technologies (PCR-RFLP, Sanger sequencer, RNA sequencing analyzer, Next generation sequencer etc.) are concerning about the animal fertility traits and reproductive performance in animal and uses of promoted technologies for identification of gene responsible for fertility traits. Gene isolation technique is the most influential technique that helps to recognize the gene related with reproductive efficiency specially fecundity traits. Recently, DNA isolation and gene (GDF9 and BMP15) identification has been widely used in domestic animals, including sheep in order to identify the litter size (Barzegari et al., 2010) and ovulation (Hanrahan et al., 2004; Barzegari et al., 2010). However, no depth study has yet been done on the semen quality, fertility and gene related with fertility traits in sheep using available feed resources (protein supplements) in our country. Undeniable, protein supplements using concept in sheep ration will help to open a new horizon in countable improvement of the semen quality, fertility traits and reproductive efficiency in its totality. This exploration will be subsidiary for realize the effects of feed supplements (protein) on semen quality as well as gene related with fertility and efficient result will be accomplished in breeding and selection programme. For this purpose the study was carried out with the following objectives.

Objectives:

- To know the effects of protein supplements on fertility traits in Sheep.
- To know the genes, those responsible for fertility traits and their phylogenic inference in Sheep.

Chapter 2: Review of Literature

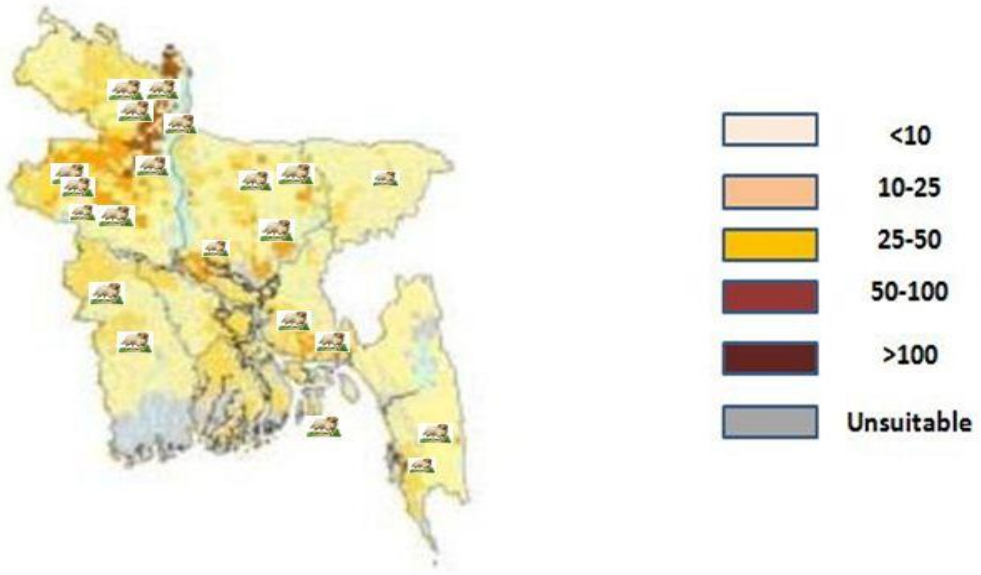
2.1. Current status of sheep rearing in Bangladesh

Bangladesh is endowed with livestock where it acts as an integral component of agriculture and makes multifaceted contributions to the growth and development in the agricultural sectors which is nearly about to 14.74% of total GDP (BER, 2017). The contribution of livestock production is almost 3.31% which is the second highest among all other sub-sector of agriculture in Bangladesh (BER, 2017). The livestock resources of Bangladesh are mainly based on cattle, goat, sheep, buffalo, and poultry.

Table 2.1: Livestock population of Bangladesh (BER, 2017-2018)

Livestock species	Livestock populations (In millions)
Cattle	24.09
Sheep	3.46
Goat	26.1
Buffalo	1.49
Poultry	338
Total livestock	393.14

Sheep is a species among them which may contribute a significant amount in the protein content and income generation to the people of Bangladesh (Alam et al., 2001). There are about 1000 distinct sheep breeds in the world, of which 233 are found in Asia (FAO, 2003). Native sheep (*Ovis aries*) might be originated from the wild Urial (*Ovis orientalis vignei*) of Asia (Hassan and Talukder, 2012). Sheep are distributed all over the country, but greater population of sheep is in the districts of Rajshahi, Dinajpur, Bogra, Rangpur, Tangail and in the delta region of Noakhali where many char lands are available (Hossain et al., 1997). Almost all of the sheep are indigenous in nature and some of them are crossbred like (indigenous × garole) in Bangladesh (Khan et al., 2009; Hassan and Talukder, 2012). They are slow in body growth and small in body size. They are inferior to produce hides and wool (Mukherjee, 2000) which are coarse in nature with high modulation.



Picture 2.1: Sheep distribution area in Bangladesh (Distance: Square/Km)

The weather of Bangladesh is favorable for sheep production, as they can be maintained under rural conditions because of their ability to adapt to harsh environment, poor management and feeding practices. Under traditional feeding system, the sheep are raised by grazing on harvested or fellow lands, roads, and canal sides (Sultana et al., 2010) for plane land and char area, almost without concentrate supplementation. However, farmer who keeps a significant number of sheep that is not ideally productive due to nutrition and management (Aalm et al., 2006) and seasonal fluctuations in feed resources (Mukasa-Mugerwa et al., 2002). Nutrient source especially protein source will make a revolutionary changes on the reproductive and productive status of sheep population and might overcome this misery conditions.

2.2. Protein sources, mechanism of absorption and its function

Protein feeds" contain high levels of protein (over 15%) includes grass and forage crops, soybean meal, cottonseed meal, protein concentrate, rapeseed meal, palm kernel, field beans, sunflower seed meal, distillers dark grains and various other products e.g. wheat feed, maize gluten, malt culms, lucerne, linseed meal etc (Genever et al., 2014). Proteins are complex organic compounds of high molecular weight. The monomer of proteins is made up of amino acids which are indispensable and dispensable. Amino acids are produced when proteins are hydrolyzed by enzymes, acids or alkalis. Certain amino acids can be produced from others by a process known as transamination, but the carbon skeletons of a number of amino

acids cannot be synthesized in the animal body and these are referred to as indispensable or essential amino acids. However, in the case of the ruminant, all the indispensable amino acids can be synthesized by the rumen microorganisms. In the ruminant, dietary protein can be classified as either rumen degradable protein (RDP) or as rumen un-degradable protein (UDP). Rumen un-degradable protein passes through the rumen and is digested in the small intestine into amino acids that is absorbed by the animal. On the other hand rumen degradable protein is degraded in the rumen and is transformed to either protozoal or microbial protein (MP) which was reported by Knight (1980) where he narrated that the soybean feeds may be beneficial due to their lower levels of ruminal degradation. This protozoal and microbial protein enters the small intestine and is digested to amino acids that are absorbed by the animal. However, maximum rates of growth can be achieved with a balanced mixture of microbial Protein and complementary dietary amino acids in a suitable form. Most of the nitrogen required by the animal is used for protein synthesis. This CP content is a measure of the nitrogen present in the foodstuff, but gives little indication of its value to the animal. Before the food becomes available to the animal it must undergo digestion, during which it is broken down to simpler substances which are absorbed into the body.

The quality of the protein in a feed is dependent on both amino acid profile and the digestibility. The protein requirement of an animal is dependent on its physiological status and level of production. Animals require protein as a source of essential amino acids and (in the case of ruminants) as a nitrogen source for rumen microflora. In ruminants, the protein flowing to the duodenum is the sum of the amount of dietary protein that escapes ruminal degradation and the quantity of microbial protein synthesized in the rumen, which depends on the fermentable energy as well as the degradable protein content of the diet (AFRC, 1993). Therefore, from the digestible crude protein of the diet the amount and quality of protein arriving at the small intestine cannot be adequately predicted. Amino acids are among the most important regulators of pre-implantation development and inclusion of specific amino acids in embryo culture media was shown to overcome the so-called “developmental blocks” observed in many species. Steeves and Gardner (1999) demonstrated that the embryo has a switch in its requirements for amino acids as it develops the zygote to the

blastocyst. Work by Davis et al. (2002) has indicated significant effects of protein on ovulation rate of ewes.

2.3. Problems with low protein in diet

Dietary nutrients usually protein are the major important factors affecting production performance in sheep (Shahjalal et al., 1992). Lassoued et al. (2004) reported that, ewe lambs reach a live weight of approximately 28 kg, less 6 kg than their supplemented counterparts and only 44% attained puberty in comparison to 84% of their supplemented counterparts, if un-supplemented between 6 (weaning time) and 12 months of age. Chronic low protein intake by animal is a basic problem (Attah et al., 2006) which may be attributed to low productivity. It has been reported that in ruminants low planes of nutrition during the pre-pubertal period delay testicular growth and the onset of puberty by inhibiting the development of a mature reproductive endocrine system (Pruitt et al., 1986). It is well documented that semen quality and sexual activity in rams are reduced due to protein deficient feeds (Brown, 1994; Jibril et al., 2011). Protein metabolism and their deficiency may impair spermatogenesis and libido in males and fertility, embryonic development and survival, post-partum recovery activities, milk production, offspring development and their survival in females (Mitchell et al., 2003; Kheradmand et al., 2006). Due to shortage of nutrition low-fecundity occurs in the majority of domestic sheep is one of hindrance for the improvement of sheep production (Chen et al., 2015). Enjalbert (2006) reported that, many reproductive health disorders in animals due to diet inadequacy. The loss of body weight and body condition, low conception, low lambing rates, delays the onset of puberty, increases the post-partum onset of estrus, interferes with normal ovarian cyclicity by decreasing gonadotropin secretion and increases infertility in under nutrition (Boland et al., 2001).

Nutrition during gestation affects both maternal body weight gain, body condition, and reproductive performance (Wettemann et al., 2003), prenatal and postnatal offspring growth and development (Godfrey and Barker, 2000; Wu et al., 2006). Nutrition during the early post-natal period can also have a permanent effect on adult litter size in sheep and by inference probably so too in other multiple-ovulating ruminant species such as the sheep. For example, pre-weaning growth restriction of 12% in hill ewe lambs caused a significant permanent reduction in their subsequent prolificacy as adult ewes (Rhind and Russell, 1998). It is reported that in the case of

female offspring a low feeding level during the first 3 months of the 5-month pregnancy in ewes resulted in their female offspring, at 20 months of age, having a lower mean ovulation rate than those from adequately nourished. This reduction in ovulation rate due to early in utero nutrition occurred in the absence of any shift, either during foetal or adult life, in pituitary gonadotrophins (Rae et al., 2001).

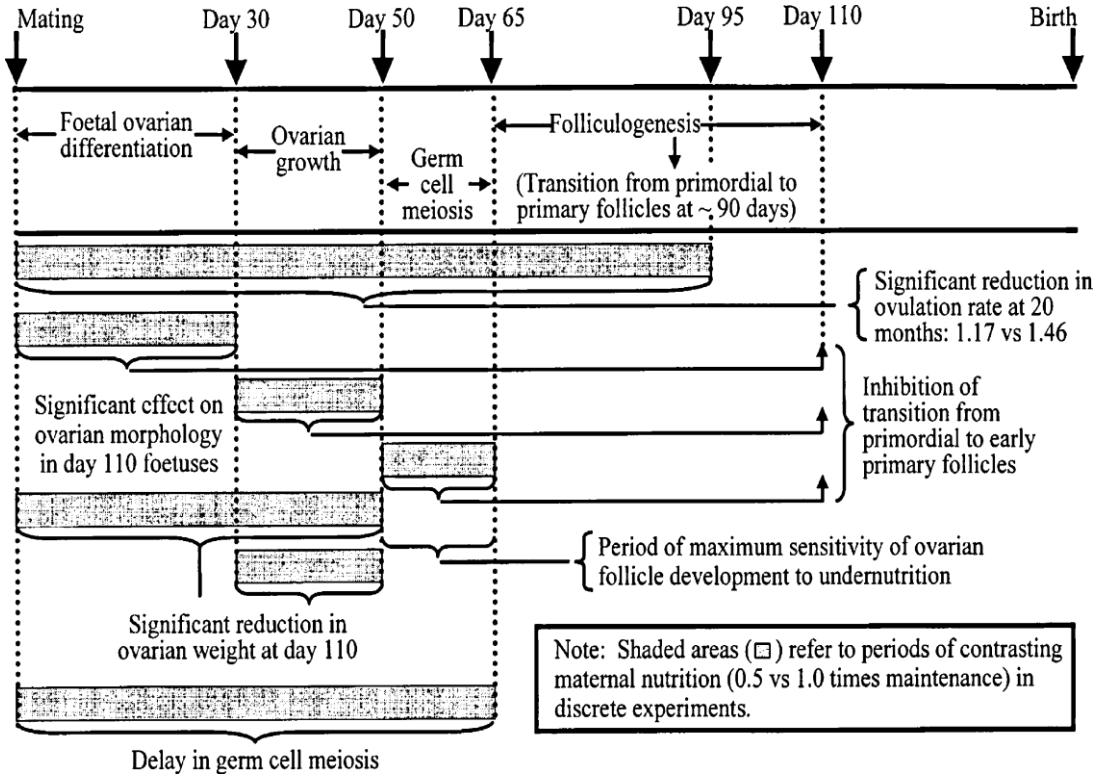


Figure 2.1: Summary of foetal ovarian effects (Mcevoy and Robinson, 2002; Rae et al., 2002)**.**

2.4. Relation of protein with reproduction

The effect of nutrition on the reproductive processes may depend upon the "net nutritional status", a term which encompasses endogenous and exogenous sources of nutrients available to the ewe (Downing and Scaramuzzi, 1991). Several studies (Kheradmand et al., 2006; Zohara et al., 2014) have documented the interrelationship between feed intake and reproductive performance in adult rams. There are a number of studies concerning the effects of special nutrients, particularly proteins (with or without day length) on reproductive parameters in the ram (Fernández et al., 2005). Protein supply above maintenance requirements effects in reproductive parameters, such as testicular size, semen quality, testosterone secretion (Fernández et al., 2005).

There were fewer differences in the viability of sperm cells of male ruminants placed on different protein diets (Kheradmand et al., 2006; Jibril et al., 2011).

In this respect the effect of strategic supplementary feeding with protein on ewe productivity under range conditions. El-Hag et al. (1998) recorded a significant improvement in lambing rates and a marked reduction in abortion rates of supplemented ewes in comparison to those managed on range land. It appears that the reproductive response to improved planes of nutrition is breed dependent and according to, the higher is the inherent prolificacy level of the breed, the higher is its reproductive response in terms of ovulation rate and litter size to improved levels of nutrition prior to and during mating. In addition to the foetal and early post-natal nutritional effects on adult ovulation rate referred to earlier in this review there are times during adult life when ovulation rate is also particularly sensitive to nutrient supply. In ewes one of these times is 6 months prior to mating when ovarian follicles emerge from the primordial pool and become committed to growth. There is now evidence that the reduction in ovulation rate can be prevented by improved nutrition (flushing) in the 10-day period prior to mating. Indeed the critical window for the stimulatory effect of improved nutrition may be even shorter than 10 days. Thus in a recent review of the scientific literature on short-term nutritional flushing Viñoles Gil (2003) concluded that its beneficial effect could be imparted over as short a period from Day 8 to Day 4 before ovulation. Research into methods of improving the efficiency of ruminant multiple ovulation production from oocytes when using these reproductive technologies is obtained by aspirating ovarian follicles which provides new information on the impact of oocyte donor nutrition on oocyte quality. Critical windows during ovulation rate in ewes are particularly sensitive to nutrition are narrated in Table 2.2.

Table 2.2: Critical windows during ovulation rate in ewes (Robinson et al., 2002 ; Viñoles Gil, 2003).

Nutritionally sensitive window	**Target organ**	**Mechanism**
Foetus (Days 50–65)	Foetal ovary	Alteration in germ cell meiosis
Adults		
6 months prior to ovulation	Ovary	Alteration in the number of follicles leaving the primordial pool
The 10 days preceding ovulation	Hypothalamus, pituitary	Changes in ovarian follicular growth and atresia, oocyte quality, ovulation rate
Days 8–4 preceding ovulation	Ovary	Changes in ovarian follicular growth and atresia, oocyte quality, ovulation rate

2.5. Effects of protein on semen quality

Semen volume is one of the important factors in semen evaluation and reproduction performance in the males. It has long been established that rams reared on higher levels of feeding grow faster and attain puberty at younger ages than rams on low planes of nutrition (Sutama and Edey, 1985). A number of studies have demonstrated that the spermatogenesis in rams is responsive to increases in protein intake. This effect has been related to an increase in testicular size because it is due to an increase in the volume of seminiferous epithelium and in the diameter of seminiferous tubules (Hötzel et al., 1998; Al-kawmani et al., 2014). The total number of spermatozoa per ejaculate can be affected by improved diet (Fernández et al., 2005).

In Merino rams in particular, the production of spermatozoa has been shown to be very responsive to changing nutrition in a number of studies using a variety of techniques (Murray et al., 1990). Despite there were some negative findings in sheep (Tilton et al., 1964), the weight of evidence has led to wide acceptance of the strong,

direct relationships between plane of nutrition, testicular mass and the number of spermatozoa available for ejaculation, for the small ruminants at least testicular growth and function on nutritional responses in Merino rams.

2.6. Effects of protein on ewe fertility

Fertility traits have a major impact on efficiency and profitability in sheep production (Jansson, 2014). Developing fertility traits may therefore be of big interest for sheep producers (Kumm, 2009). Nutrition influences on ruminant fertility directly by the supply of specific nutrients required for the processes of oocyte development, ovulation, fertilization, embryo survival and the establishment of pregnancy. Recent studies on nutrition and ruminant fertility extends from whole animal responses to the intricate cellular and molecular events that control gamete production, embryo development, conceptus growth and implantation. It also deals with effects during embryonic and foetal life on the timing of puberty and subsequent adult fertility. The functional unit of female gonad is ovarian follicle which includes oocyte, surrounding granulosa cells and external theca cells (Knight and Glister, 2003; Orisaka et al., 2009). It has been proved that improved diet in the ewe increases ovulation rate and modifies the morphological and functional quality of the oocytes and embryo recovered (Kheradmand et al., 2006). Whereas, ovulation rate is the primary source of variation in prolificacy, both within and between breeds (Webb et al., 1999) but unfortunately, it is poorly understood in female mammals (Shimasaki et al., 2004; Vireque et al., 2008).

High protein supplements fed to ewes for 32 days increased ovulation rates above those of ewes receiving less protein, without increasing live weight above that of control ewes (Davis et al., 1982). When the interaction between age and level of protein feeding is considered, it would appear that maiden ewes are more sensitive to inadequate protein feeding and embryonic mortality is greater than for mature ewes. This finding agreed with the Bennett (1964) that under nutrition significantly reduced the lambing percentage of 2-year-old primiparous ewes. Increased levels of protein in the diet have, in relation with increasing ovulation rates, increased the circulating levels of FSH during the latter half of the estrus cycle (Knight, 1980). Haresign (1981) suggested that, flushing for one cycle may extend its effects by preventing the late atresia of follicles and Louw et al. (1974) reported a greater ovarian follicular response to gonadotrophin in ewes that were on a high plane of nutrition. Molle et al.

(1995) showed that flushing Sarda ewes with soybean meal, while mated on mature grassland, was found to be effective in improving reproductive performance. In particular, ovulation rate increased by 0.40 per ewe ovulating and prolificacy tended to be higher by 0.30 lambs per ewe lambing in flushed ewes, compared with the controls.

Table 2.3: Effects of protein on ram semen quality and ewe fertility

Protein Sources	**Semen quality**				**Ewe fertility**	
	Breed name	*Volume*	*Total sperm. (%)*	*Live sperm. (%)*	*Breed name*	*Fertility (%)*
Low protein (Control)	Bakhtiary rams[(1)]	+	+	+	Indigenous ewes [(4)]	83
	Kivircik rams[(2)]	+	+		Sudanese Desert Ewes[(5)]	75
	Yankasa rams[(3)]	+	+	+	Awassi ewes[(6)]	81
High protein (Treatment)	Bakhtiary rams[(1)]	+++	+++	++	Indigenous ewes[(4)]	92
	Kivircik ram[(2)]	+++	+++		Sudanese Desert ewes [(5)]	82
	Yankasa rams[(3)]	+++	+++	+++	Awassi ewes[(6)]	96

[[(1)](Kheradmand et al., 2006); [(2)](Elmaz et al., 2007); [(3)](Jibril et al., 2011); [(4)](Zohara et al., 2014); [(5)](Idris et al., 2010); [(6)](Al-Haboby et al., 1999)]

Among the fertility traits in sheep population ovulation rate and litter size are paramount fertility traits and where those can be genetically regulated by many genes with small effects and sometimes additionally by single genes with major effects, called fecundity (Fec) genes The entire study about fecundity genes have a paramountcy effects on farming sector and livestock economy.

2.7. Genes responsible for fertility traits (litter size and ovulation)

Identifying the genes is a common starting point in the understanding the molecular mechanisms underlying biological processes including differentiation and development. Nowadays world are moves faster which provides various tools to scientist for sharpening the research activities. The genetic materials are found in the form of deoxyribonucleic acid (DNA) in the domesticated sheep (*Ovis aries*) in chromosomes. Ovulation rate and litter size are important fertility traits in sheep and are of high economic value (Notter, 2008), where those can be genetically regulated by many genes with small effects and sometimes also by single genes with major effects, called fecundity (Fec) genes (Drouilhet et al., 2009). The study of fecundity genes is of great importance in the farming industry (Nicol et al., 2009). Characteristics for the presence of a major gene affecting fertility in a population include high variation in ovulation rate and litter size combined with high repeatability.

It has been discussed that ovulation rate and the subsequent litter size are the main factors for improving reproductive rate in sheep (Bradford, 1972). Traditional selection for improving litter size in sheep is difficult due to the sex-limited nature and low heritability of the trait (5%–10%). In addition, the lack of knowledge on the number of the genes controlling this trait and the possible gene interactions are the other limitations for this trait. Molecular genetics can overcome these limitation offering new opportunities to the improvement of reproductive traits, as it supplies tools to analyse genetic variability directly at the DNA level with the possibility of detecting the individual genes influencing the reproductive characteristics.

Several genes (such as GDF9, BMP15) have recently been shown to affect female fecundity in domesticated sheep (Galloway et al., 2000; Hanrahan et al., 2004; Davis, 2005). In recent years, a number of natural genetic mutations associated with ovulation rates in sheep breeds have been identified including point mutations in the Growth differentiation factor 9 (GDF9), Bone morphogenetic protein 15 (BMP15) (Hanrahan et al., 2004) and bone morphogenetic protein (BMPR1B) (Souza et al., 2001) genes. BMP- 15 is a growth factor and a member of the TGFß super family that is specifically expressed in oocytes. Bone morphogenetic protein acts through a cascade of other proteins (the SMAD pathway) that are responsible for a huge range of cellular behaviours including the development and maturation of oocytes

(Galloway et al., 2000; Shimasaki et al., 2004). This gene regulates granulosa cell proliferation and differentiation by promoting granulosa cell mitosis and suppressing follicle stimulating hormone receptor expression, all of which play a pivotal role in female fertility in mammals (Otsuka et al., 2000). Without BMP15, oocytes continue to grow in the absence of granulosa cell proliferation until they can no longer be supported by the residual granulosa cells, where upon they degenerate (Galloway et al., 2000). The BMP15 gene has been mapped to sheep chromosome X and contains 2 exons. They constitute either non-conservative amino acid substitutions (FecXI, FecXB and FecXL) or premature stop codons (FecXG, FecXH and FecXR) and have a dosage-dependent effect. All mutations in this gene show the same phenotype like as ovulation rates are markedly increased in the heterozygotes, while the homozygotes demonstrate primary ovarian failure resulting in complete sterility (Galloway et al., 2000; Hanrahan et al., 2004; Monteagudo et al., 2009).

Like BMP15, the autosomal GDF9 has an essential role in controlling the follicular growth through its influence on granulosa cell function (Galloway et al., 2000; Hanrahan et al., 2004). GDF9 is a growth factor and a member of the transforming growth factor β super-family that is secreted by oocytes in growing ovarian follicles (Juengel et al., 2002), which is essential for growth and differentiation of early ovarian follicles. The changing concentrations of GDF9 in vivo leads to incremental changes in ovulation rate in sheep (Hanrahan et al., 2004). Absence of GDF9 blocks the follicular growth at the primary stage in homozygotes, resulting in sterility, while inactivation of only one copy of GDF9 increases the ovulation rate (Hanrahan et al., 2004; Monteagudo et al., 2009). This may confer a fecundity advantage on the heterozygotes (Gemmell and Slate, 2006). Ovine GDF9 spans approximately 2.5 kb and contains two exons and one intron. Exon 1 spans 397 bp and encodes for amino acids 1–134, while exon 2 spans 968 bp and encodes for amino acids 135–456. The single intron spans 1,126 bp. The mutations in this gene cause increased ovulation rate and twin and triplet births in heterozygotes, and complete primary ovarian failure in homozygotes resulting in total infertility in some prolific breeds of sheep (Hanrahan et al., 2004).

Table 2.4: Genes related with major effect on fertility and ovulation rate

Authors	**Breed (Country)**	**Gene (Chromosome)**
Zarazaga et al. (1998)	Lacaune (France)	BMPR-1BC
Drouilhet et al. (2013)	Lacaune (France)	FecL
Galloway et al. (2000)	Romney (New Zealand).	BMP15 (X)
Hanrahan et al. (2004)	Belclare, Cambridge (Ireland)	
Barzegari et al. (2010)	Moghani, Ghezel (Iran)	
Bodin et al. (2002)	Lacaune (France)	
Monteagudo et al. (2009)	Rasa Aragonesa (Spain)	
Drouilhet et al. (2009)	Lecaune (France)	
Hanrahan et al. (2004)	Belclare, Cambridge (Ireland)	GDF9 (5)
Polley et al. (2010)	Garole (India)	GDF9 (5)

2.8. Gene isolation techniques

2.8.1. PCR-RFLP

The development of the polymerase chain reaction (PCR) technique has revolutionary effects on modern molecular genetics which explosively increases the systematic and population research (Schmid et al., 2000). This method has high resolution power. It also permits exceedingly detailed description of genetic variation in DNA and molecular component. This technique uses two short single strand DNA primers (typically 20 bp to 25 bp long) to initiate DNA replication at a specific point on the DNA molecule. A thermo stable DNA polymerase is then used to copy the DNA by extending the primers and synthesizing complementary strands of DNA. Repeatedly denaturing the DNA, re-annealing the primers, and copying the DNA will exponentially amplify the target sequence between the primers. Following the amplification process, sufficient DNA is available for analysis directly by electrophoresis. The PCR-amplified DNA can be digested with a restriction enzyme

and visualized by gel electrophoresis to determine if the PCR fragments have been cleaved known as restriction fragment length polymorphism (RFLP) which was the earliest form of deoxyribonucleic acid (DNA) marker used to construct the first true genomic maps. Polymerase chain reaction and PCR-RFLP are frequently used in diagnostic testing to determine the genotype at a known genetic mutation. There are a large number of restriction enzymes, each of which has a different specific recognition site.

2.8.2. RNA-seq analyzer

Due to development of molecular, sequencing and bioinformatics analysis technologies, high throughput RNA deep sequencing (RNA-seq) provides a platform for measuring large-scale gene expression patterns (Chen et al., 2015). Recently, in order to identify the differentially expressed genes (DEGs) and novel transcript units, the RNA-seq has been widely used in domestic animals, including goats (Xu et al., 2013), sheep (Chen et al., 2015) and others. Additionally, the efficacy of RNA-seq has also been demonstrated in mammalian reproductive tissues, for example in sheep oocyte, granulosa cell and ovary (Miao and Luo, 2013).

2.8.3. Microsatellite DNA

Microsatellites are simple, tandemly repeated di- to tetra-nucleotide sequence motifs flanked by unique sequences. They are valuable as genetic markers because they are co-dominant, detect high levels of allelic diversity, and are easily and economically assayed by the polymerase chain reaction (PCR) (Mccouch et al., 1997). Microsatellites have been the most widely used markers for genetic diversity estimation in recent years because they are highly polymorphic and evenly distributed over the genome (Sharma et al., 2013). Single Nucleotide Markers (SNPs) are now the markers of choice in QTL analysis and genomic selection. For situations with poor or absent pedigree information, use of SNP markers for genetic diversity estimation can be helpful. Silió et al. (2010) reported that, used of SNP data for genetic diversity estimation in livestock breeds. This new technique permit molecular biologists and geneticists to systematically evaluated and compare the large area of the genome. Most of this analysis based on the PCR amplification of microsatellite DNA which has been particularly useful for genomic mapping and to study the genetic variation comparing between domestic sheep and big horn sheep (Forbes et al., 1995).

Table 2.5: Various techniques used for gene isolation

Breed(Country)	Techniques name	Sample
Lacaune Sheep (France)	Real-time quantitative PCR	Granulosa cells, Follicular fluid[(1)]
Qira Black Sheep and Hetian Sheep (China)	Real-time quantitative PCR	Ovaries[(2)]
Afshari, Baluchi, Makui, and Mehraban (Iran)	PCR-RFLP	Blood[(3)]
Moghani and Ghezel (Iran)	PCR-RFLP	Blood[(4)]
Han and Surabaya (China)	Next-generation sequencing	Blood[(5)]

[(1)(Drouilhet et al., 2013); (2)(Chen et al., 2015); (3)(Javanmard et al., 2011); (4)(Barzegari et al., 2010); (5)(Miao and Luo, 2013); (6)(Dai et al., 2012)]

2.8.4. Sanger sequencing

Sanger sequencing is a targeted sequencing technique that uses oligonucleotide primers to seek out specific DNA regions. Sanger sequencing begins with denaturation of the double-stranded DNA (Jamuar et al., 2016). The single-stranded DNA is then annealed to oligonucleotide primers and elongated using a mixture of deoxynucleotide triphosphates (dNTPs), which provide the needed arginine (A), cytosine (C), tyrosine (T), and guanine (G) nucleotides to build the new double-stranded structure. In addition, a small quantity of chain-terminating dideoxynucleotide triphosphates (ddNTPs) for each nucleotide is included. The sequence will continue to extend with dNTPs until a ddNTP attaches. As the dNTPs and ddNTPs have an equal chance of attaching to the sequence, each sequence will terminate at varying lengths. Each ddNTP (ddATP, ddGTP, ddCTP, ddTTP) also includes a fluorescent marker. When a ddNTP is attached to the elongating sequence, the base will fluoresce based on the associated nucleotide. By convention, A is indicated by green fluorescence, T by red, G by black, and C by blue. A laser within the automated machine used to read the sequence detects a fluorescent intensity that is translated into a “peak.” When a heterozygous variant occurs within a sequence, loci will be captured by two fluorescent dyes of equal intensity. When a homozygous

variant is present, the expected fluorescent color is replaced completely by the new base pair's color.

2.9. Summary of literature review

- Sheep plays an important role for income generation to the landless people and alleviates protein shortage by providing mutton (sheep meat) with low prices. Developing fertility traits may be a big interest for sheep producers and nutritional influences on fertility traits directly by the supply of specific nutrients required for the processes of spermatogenesis in rams, oocyte development, ovulation, fertilization, embryo survival and the establishment of pregnancy in ewes.
- The ovulation rate and the litter size are the main factors for improving reproductive rate in sheep where GDF9, BMP15 genes directly responsible for ovulation rate and litter size and mutations in GDF9, BMP15 genes cause increased ovulation rate and twin and triplet births in heterozygotes sheep.
- There several molecular techniques such as, Polymerase chain reaction (PCR), Microsatellites marker, RNA sequencer (RNA-seq) and Sanger sequencer those has revolutionary effects on modern molecular biology which explosively increases the systematic and population research by performing gene sequencing and measuring large-scale gene expression patterns within concise time frame.
- Even though in Bangladesh, most of the research in the case of sheep are mainly biological study, incase interrelate study of the effects of nutrients (protein supplements) fertility traits of sheep tells sorry tale. In Bangladesh molecular study of fertility genes of GDF9 and BMP15 with litter size and ovulation rate in indigenous sheep still untouched. So it has become subject of concern for conducting this study. These results that will be generated helps to further explore the genomic complexities of indigenous sheep and effects of nutrients supplementation on sheep that may alter reproductive performance of ewes to increase and improve the sheep production in Bangladesh.

Chapter 3: Materials and Methods

3.1. Study area

The study was carried out at the Hathazari Thana and Bakolia Thana, situated in the Chittagong District close to the Bay of Bengal and lying between latitudes 22º20'0" N to 22º32'30"N and between longitude 91º 47' 30" E to 91º 52'30" E which was taken by the using of widely used Garmin etrex GPS machine (Dutta et al., 2011) during the month of January 2017 to December 2017.

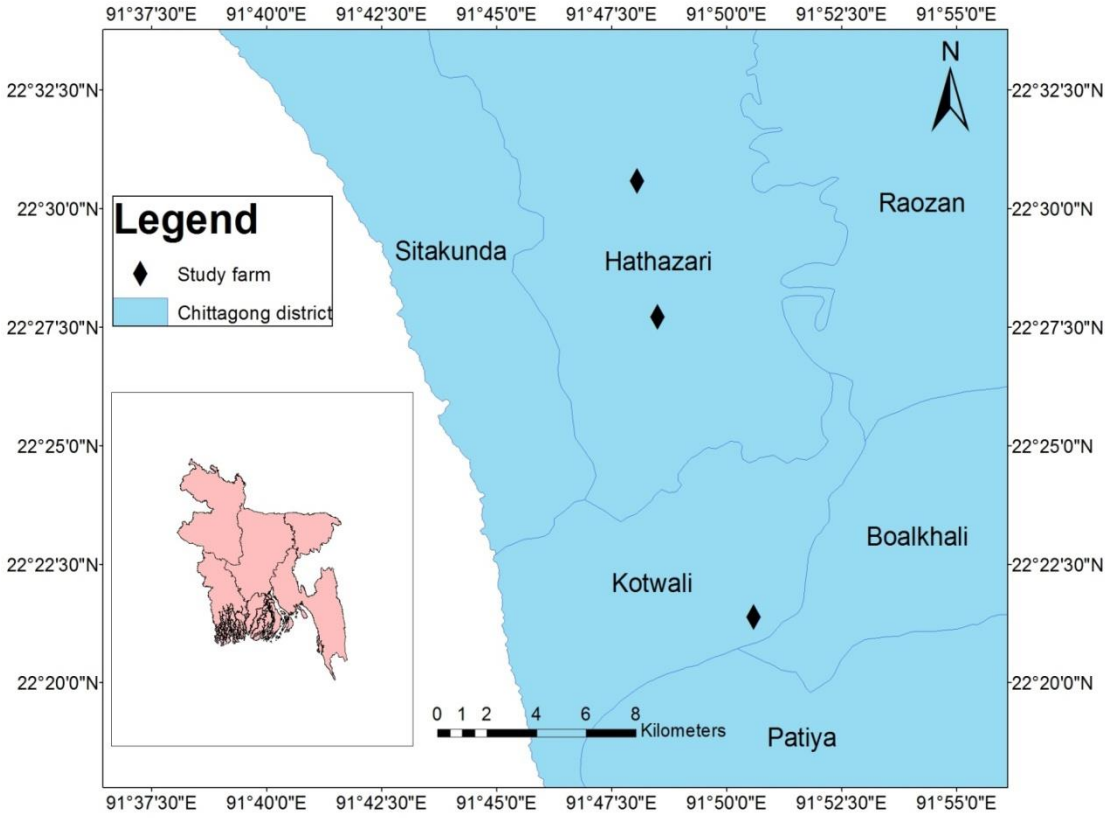


Figure 3.1: Study area map (By using of ArcGIS 10.2.1)

3.2. Experimental animal's selection

Thirty six indigenous sheep were selected from three different areas [Modonhat/T2 (11), Hathazari sadar/T1 (16) and Bakolia /T0 (9)] on the basis of direct visit, observation their body condition score (BCS), health status and normal clinical conditions. Then the sheep were randomly divided into three treatment groups according to location which was heterogeneous in nature and ages were ranged from

one to five years. The study design with treatment and replication are narrated in bellow Table 3.1**.**

Table 3.1: Study design with treatment and replications

	T0 (Animals number)	T1 (Animals number)	T2 (Animals number)
Replication pattern	T0.A(3)	T1.A(4)	T2.A(4)
	T0.B(3)	T1.B(4)	T2.B(4)
	T0.C(3)	T1.C(4)	T2.C(3)
		T1.D(4)	

The three groups were managed under semi-intensive system, kept in separate pens and fed individually. They were acclimatized and observed regularly for two weeks with a view to screening haemoparasites and helminthes by the farmers and researcher.

3.3. Experimental ration formulation and design

Three iso-caloric rations (12 MJ/kg DM ME) containing graded level of protein were formulated (11.68% CP for control/To, 12.95% CP for T1 and 13.96% CP for T2) by using of conventional feed stuffs (Table 3.1) and concentrate mixtures were supplied (0.5 kg/day/sheep) to all groups of sheep. Protein-concentrate (PRO-PAK) was provided in both T1 and T2 groups, but there no protein concentrate was added to the formulated ration of control group. All of the groups grazed for 8 to 9 hours per day in pasture land.

Picture 3.1: Feed mixing

Table 3.2: Animals number, ingredients and chemical composition of experimental ration

Treatment groups	Ingredients												Calculated chemicals composition			
	Animals number	Maize	Rice Polish	Wheat bran	Soybean oil	Soybean meal	Protein concentrate	Dicalcium phosphate	DL-Methionine	Vitamin B premix	Common salt	Total amount	Total Crude Protein (%)	Total Crude Fiber (%)	Ether Extract (%)	Total Energy, ME (MJ)
Treatment 1(T1)	16	56.5	22	10	0.75	5	2.5	1.15	0.6	1	0.5	100	12.94	4.46	6.19	12.00
Treatment 2(T2)	11	54.5	22	10	0.75	5	4.5	1.15	0.6	1	0.5	100	13.96	4.48	6.32	12.00
Control(T0)	09	59	22	10	0.75	5	0	1.15	0.6	1	0.5	100	11.68	4.44	6.03	12.00

3.4. Semen collection and evaluation

At the mid of the trial, all rams were subjected to a fertility test in all groups. Scrotal diameter was recorded from the first week to until the end of experiment in cm for find out the correlation with semen volume. The rams were trained for semen collection by artificial vagina (AV) method using receptive restrained ewes. During the trials period semen collection was done several times in order to assess the semen quality. The ejaculates were assessed for physical, biochemical and microscopic test. Collected semen samples were evaluated as following the procedure described by Zemjanis (1970).

- The volume of semen was obtained directly from the calibrated tube and recorded. Microscopic examination for wave pattern (gross sperm motility) was determined by placing a drop of raw undiluted semen on a pre-warmed slide with cover-slip and viewed using a field microscope at 40X magnification. Sperm concentration was measured using Neubauer-haemocytometer according to the method of the Organisation, (1999). Live and dead ratio of the sperm cells was determined as described by Esteso et al., (2006). A thin smear of the semen sample was made on clean grease free glass slide and stained with eosin-nigrosin stain for enumeration of live dead ratio. Sperm abnormalities were determined by making a thin smear of the semen sample on clean grease free glass slide and fixed with buffered normal saline. In both cases three hundred thirty three sperm cells were counted per slide using light microscopy at 40X magnification.

Picture 3.2: Semen collection and evaluation

3.5. Record keeping for the fertility traits (female) in the experimental group

Data regarding fertility traits (for example, age at first lambing, gestation period, lambing interval, lamb weight) were recorded during the experimental period from the farm areas (Modonhat, Hathazari sadar and Bakolia) in a consecutive manner.

3.5.1. Lamb birth weight, weaning weight, pre-weaning average daily weight gain of lamb and puberty of sheep

Within 24 hours of the new born, date of birth, birth weight, type of birth, sex of lamb were recorded. Body weight was recorded weekly by a top loading weighing machine. Age at puberty was recorded by the onset of first behavioral estrus. Rams puberty was measured by the copulatory behavioral evaluation called a "serving capacity test" (Perkins et al., 2007). Weaning weight was recorded on 60^{th} day by the following formula:

$$\textbf{Pre-weaning average daily gain (wt/kg)} = \frac{\text{Weaning weight} - \text{Birth weig ht}}{\text{Weaning weight}} \times 100$$

3.5.2. Pregnancy period (gestation length) of ewes

Pregnancy was determined by trans-abdominal palpation and history of mating. Gestation length was counted from the day of service to the day of onset of labor. Conception rate, lambing and fertility rate, litter size was calculated by the following formula (Landais and Cissoko, 1986).

Conception rate (%) = $\frac{\text{Number of ewes pregnant}}{\text{Ewes present to rams}} \times 100$

Lambing rate (%) = $\frac{\text{Number of ewes lambing}}{\text{Number of ewes mated}} \times 100$

Lamb survival rate (%) = $\frac{\text{Number of offspring weaned}}{\text{Number of offspring produced}} \times 100$

Litter size (prolificacy) = $\frac{\text{Number of lambs}}{\text{Number of ewes lambing}} \times 100$

3.6. Molecular study of fertility genes of sheep

For molecular mechanisms of above fertility traits (especially litter size and ovulation) mainly blood was used as raw material, for understanding the underlying biological processes. In this consequences blood sample was collected from ewes and the detail procedure blood collection, DNA extraction, PCR, gene sequencing of PCR product were described as below:

3.6.1. Blood collection

Twenty blood samples from the all three treatments (T0/Control=6; T1=10; T2=4) were collected from the jugular vein of mature ewes with vacutainer tube containing 0.5 M EDTA (pH=8). All samples were delivered back to the laboratory in an ice box and stored at -20°C to the freezer by the use of aseptic means.

3.6.2. DNA extraction

DNA was extracted from the whole blood samples using FavorPrep^{TM} blood genomic DNA extraction mini kit. At first, 200 µl whole blood samples was transferred to a micro-centrifuge tube with 20 µl proteinase-k and 200 µl FABG buffer and mixed thoroughly by pulse-vortexing. Then the sample was incubated for 15 minutes at 60^0C temperature and kept vortexing the sample for every 3-5 minutes interval for lysating the sample. Added 200 µl of 100 % ethanol and mixed through by pulse vortexing for 10 second and briefly spined the tube for removing the drops from the inside of the tube. After that, the mixture transferred to FABG column with collection tube and centrifuged at 6000×g for a minute. Spined the FABG mini column after adding 400 µl W1 buffer at 18000×g (full speed) for 30 seconds and 750 µl wash buffer at 18000×g for 30 seconds, respectively and discarded the flow through in a consecutive manner. Centrifugation was continuing for additional 3 minutes to dry the column and finally transferred FABG mini column to the elution tube where 120 µl elution buffers added to center of the column. The total DNA was collected and stored at -20^0C after completing the final centrifugation for 1 minute in full speed.

Picture 3.3: DNA extraction

3.6.3. Primer designing

Table 3.3: Summary of general characteristics of investigated genes (BMP15 and GDF9) in this study based on (Hanrahan et al., 2004; Maitra et al., 2016).

	BMP15	GDF9
Primer Sequence	Forward Primer-5'-TCCCTAAAGGCCTGAAAGAGT-3'	Forward Primer-5'-GAAGACTGGTATGGGGAAATG -3'
	Reverse Prime-5'-GCTGAAGGCAAGGAATAGAATC-3'	Reverse Prime-5'- CCAATCTGCTCCTACACACCT-3'
Amplified region	Exon 2	Exon 1
PCR Product(bp)	575	462
Annealing temperature°C	56	60

3.6.4. Polymerase chain reaction (PCR)

Primers for PCR of GDF9 and BMP15 genes were cited from Hanrahan et al., (2004) and Maitra et al., (2016) (Above Table 3.2). Polymerase chain reaction (PCR) was carried out in a final reaction volume of 25 µl on i-cycler (BIO-RAD, USA). PCR cocktail consisted of 50 to 100ng of genomic DNA, 200µM of each dNTPs, 50pM of each primer, 0.5 units of *Taq* DNA polymerase and *Taq* buffer having 1.5 mM MgCl2 for each reaction. The PCR cycle was accomplished by denaturation for 1 min at 94°C; 35 cycles of denaturation at 94°C for 45sec, 45sec annealing time with 56°C temperature for BMP15 and 60°C temperature for GDF9, respectively, extension step at 72°C for 45sec with a final extension at 72°C for 5 min. The PCR products were visualized following electrophoresis through 1.8% ethilium bromide stained agarose gel and the fragments were photographed under gel documentation unit and their sizes were estimated using a 1k bp DNA ladder (Fermentas International, Inc.).

3.7. Gene sequencing of PCR product

3.7.1. PCR product purification

Mixed 5 µl of a post-PCR reaction product with 2 µl of ExoSAP-IT (enzyme: ExoASP-IT) a combined 7 µl reaction volume. Then, it incubated at 37°C for 15 min for degrading the remaining primers and nucleotides. Finally, for inactivate the ExoSAP-IT enzymatic reaction mixed sample was incubated at 80°C for 15 min.

3.7.2. Sequencing

The purified PCR products were Sanger-sequenced with big dye terminator v3.1 sequencing kit and a 3730xl automated sequencer (Applied Biosystems, Foster City, CA). After-that**,** nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea) by using of ABI PRISM 3730XL Analyzer (96 capillary type).

3.8. Statistical analysis

Data recorded for the semen motility, live death ratio, number of ewes conceived, conception rate, weight at first conception, gestation period and lamb number per sub-

group were analyzed by using completely randomized design (CRD). The collected data were corrected and analyzed by using the statistical package SAS (SAS, 2008). The mean differences were compared using least significant difference (lsd) (Steel et al., 1997) at 5% level of significance. Descriptive statistics were used to determine the mean and standard error of mean.

The nucleotides sequences were analyzed to determine the nucleotide sequences comparison, evolutionary divergence, evolutionary relationships and maximum composite likelihood estimation by using NCBI BLAST (Johnson et al., 2008; Madden, 2013) and MEGA6 software (Tamura et al., 2013).

Chapter 4: Results

4.1. Fertility traits of male (seminal traits)

The mean and standard error of mean (SEM) values of different seminal traits are shown in Table 4.1. As shown in Table 4.1, semen volume was statistically significant (P<0.05) among the three groups. The semen volume was higher in Treatment 2 which was 0.94 ml than the other two groups and it might be due to the variation in ration, farm management system, age differences and body size of the sheep. In the case of sperm count Treatment 2 contains 4.25×10^6 sperm, which was higher than the other two groups and SEM value was 0.018 that's console those groups were significantly different. Seminal pH was statistically significant among the three groups, which was slightly alkali in nature ranged from 6.95 to 7.43. Among the three groups scrotal diameter was more sizably voluminous in Treatment 2 than the other two groups, which were not statistically significant (P>0.05).

Table 4.1: Various seminal traits of ram

Traits	Treatment 1	Treatment 2	T0/Control	SEM	P-value
Semen volume(ml)	0.88^{ab}	0.94^{a}	0.81^{b}	0.013	0.03
Sperm count (10^6)	4.15^{ab}	4.25^{a}	4.085^{b}	0.018	0.04
pH	7.07^{b}	6.95^{b}	7.43^{a}	0.028	0.002
Scrotal diameter (cm)	8.47	20.82	13.10	3.28	0.34

[Means with different superscripts in the same row differ significantly among the treatment groups (P<0.05)]

4.2. Sperm morphology and viability

Percentage of sperm morphology and picture of sperm viability are shown in Figure 4.1 and Picture 4.1, 4.2 respectively. In Figure 4.1, percentage of normal sperm was highly assiduous in case of treatment groups (91.16% in Treatment 1and 94.6% in Treatment 2) than the control group. Here 14.50% sperm was abnormal in nature in case of control, which was higher than the other two groups. Among the three groups these values have no significant difference (P>0.05). Sperm viability was not statistically significant among the three groups where Treatment 2 contain 87.33% viable sperm, which was higher than the other two groups.

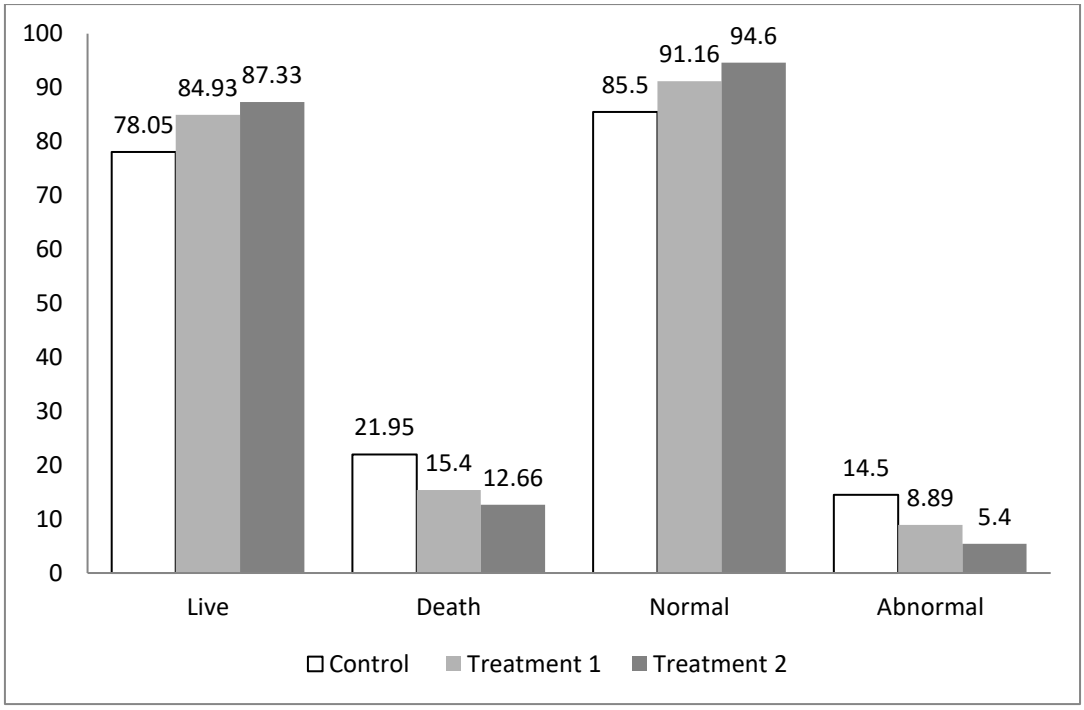
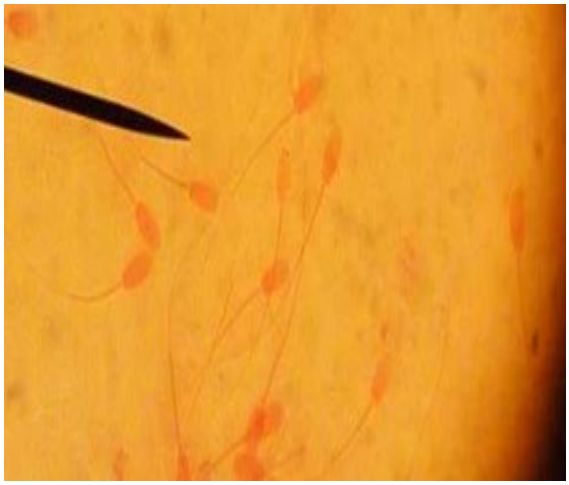


Figure 4.1: Percentage of sperm morphology



Picture 4.1 : Normal and abnormal sperm

Picture 4.2: Live and death sperm

4.3. Reproductive parameters of female (fertility traits)

The various reproductive parameters (fertility traits) of indigenous ewes are depicted in the Table 4.2. The age at first conception varied with the differences of treatments. Ewe treated with supplemented group (protein concentrate) reached conception at earlier age (Treatment 1, 6.75±0.09 months and Treatment 2, 6.67±0.18 months) than control group (8.37±0.15 months). Weight at first conception was significantly

different (P<0.05) among the three groups where weight at conception was 11±0.22 kg in case of Treatment 2 group which was higher than the other two groups.

Table 4.2: Mean ± standard error of reproductive parameter (fertility traits) of indigenous ewes

Parameters	**Control**	**Treatment 1**	**Treatment 2**	**P value**
Age at first conception (month)	8.37±0.15	6.75±0.09	6.67±0.18	0.10
Weight at first conception (kg)	8.82^{b} ±0.23	9.57^{ab}± 0.16	11^{a}±0.22	0.04
Conception rate (%)	66.66^{a}±21.16	71.42^{ab}±18.41	100	0.02
Gestation period (days)	155.25^{a}±4.1	153.7^{ab}±4.0	148^{b} ± 4.2	0.05
Lambing interval (days)	188.25^{a}±2.3	186.57^{ab}± 5.9	176^{b} ±4.6	0.01
Lamb number (parturition time)	1.5±0.6	1.57 ±0.5	1.75 ±0.5	0.79
Lamb birth weight at parturition (kg)	1.11^{b}±0.03	1.24^{b}±0.02	1.56^{a} ±0.03	0.002

[Means with different superscripts in the same row differ significantly among the treatment groups (P<0.05)]

Conception rate was 100% in Treatment group 2 which was higher in percentage than the other two groups. The gestation length in indigenous ewes varied from 148 to 155 days. The gestation length was significantly higher in control group (155.25±4.1 days) compared with treated groups. In control group lambing interval was 188.25±2.3 days, which was significantly different and longer than the other two groups (P<0.05). Among the three groups number of lamb has no significant difference which was more in number in case of Treatment 2 (1.75±0.5). Among the three groups of lambs mean birth weights were significantly difference and ranged from 1.11kg to 1.55 kg. The birth weight of lambs born in Treatment 2 groups was 1.56±0.3 kg which was higher when compared with lambs born in other two groups.

4.4. Molecular study of fertility genes (BMP15 and GDF9)

For genetic assessment 20 samples were tested for the presence of BMP15 and GDF9 genes randomly from each group whose amplicon sizes were 575 bp and 462 bp, respectively narrated in the Table 4.3. Among the all samples 18 (90%) samples were positive for both BMP15 and GDF9 genes where typical amplicon sizes of the gene products measured by PCR, which are shown in Figures 4.2, 4.3 respectively.

Table 4.3: Percentage fertility genes with amplicon sizes

Fertility genes	**Amplicon size**	**No. of sample tested**			**Positive tested**			**Total Positive (%)**
		T1	**T2**	**C/T0**	**T1**	**T2**	**C/T0**	
BMP15	575bp	8	5	7	7	5	6	90%
GDF9	462bp	8	5	7	7	5	6	90%

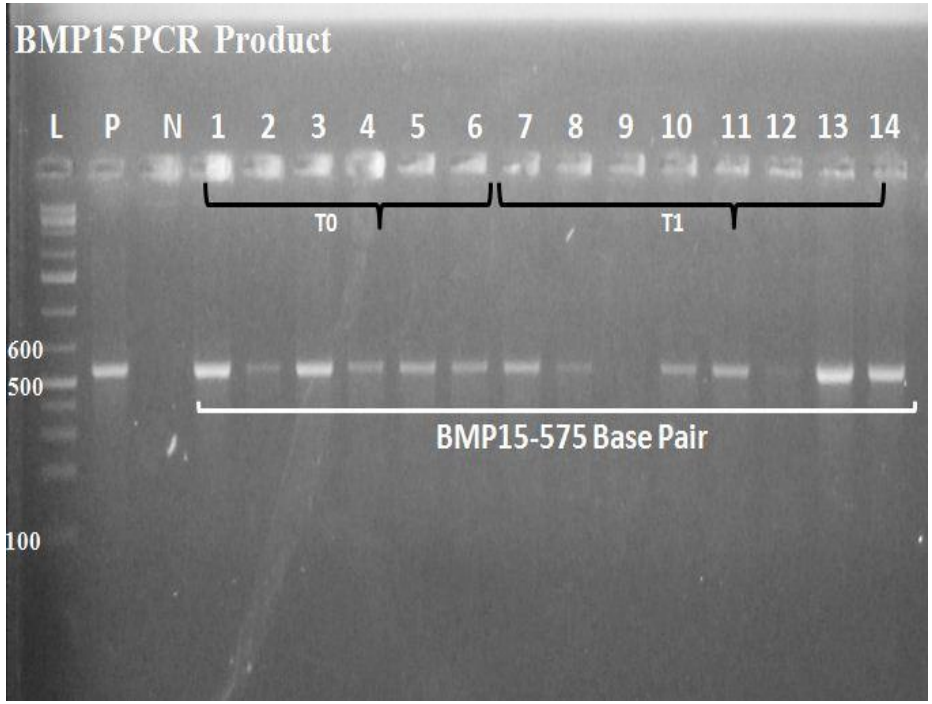


Figure 4.2.a: PCR products of BMP15 gene 575bp amplicon size

L=ladder (ladder size >1kb), P=positive control, N=negative control, 1..14=sample

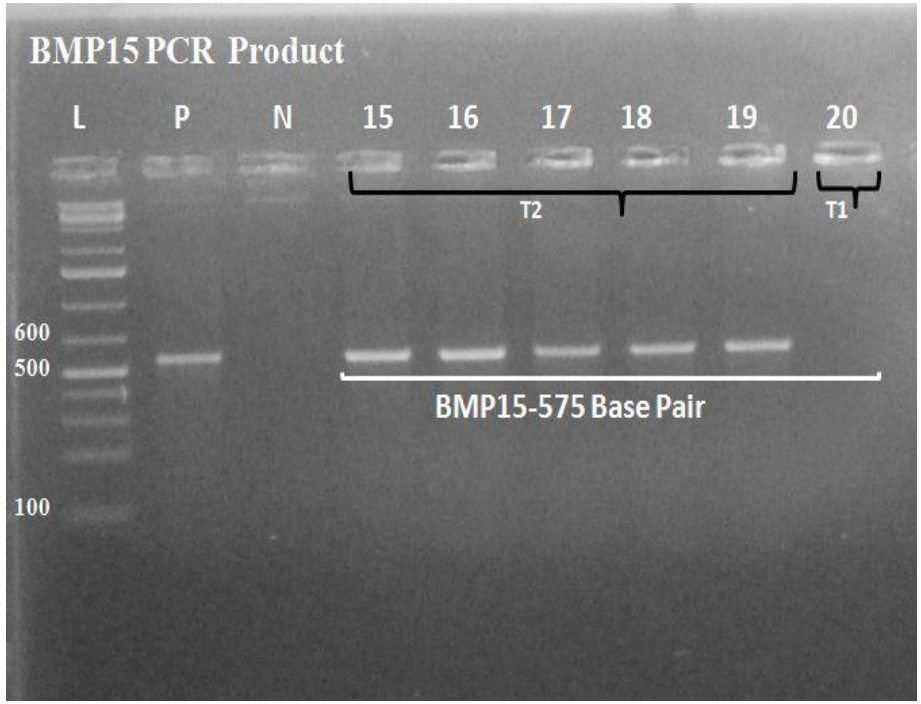


Figure 4.2.b: PCR products of BMP15 gene 575bp amplicon size

L=ladder (ladder size >1kb), P=positive control, N=negative control, 15..20=sample

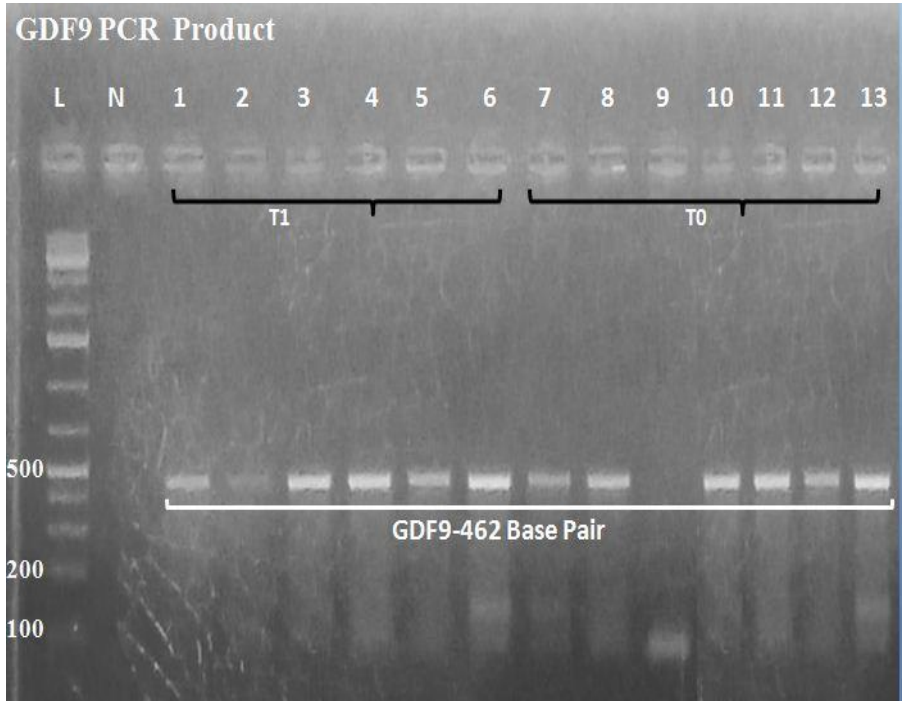


Figure 4.3.a: PCR products of GDF9 gene 462bp amplicon sizes

L=ladder (ladder size>1kb), N=negative control, 1..13=sample

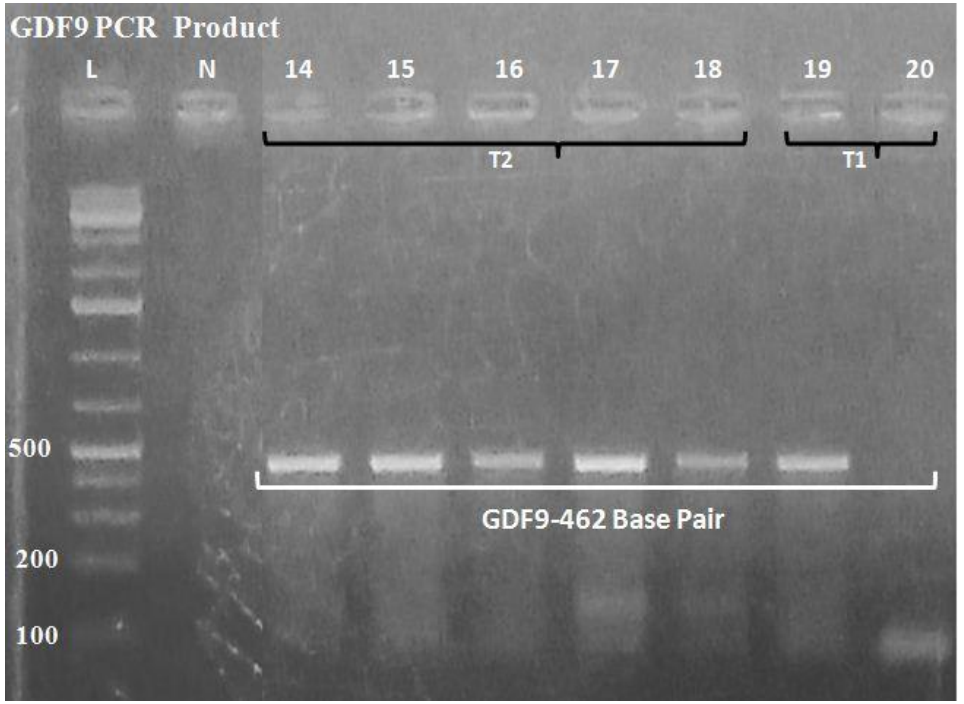


Figure 4.3.b: PCR products of GDF9 gene 462bp amplicon sizes

L=ladder (ladder size>1kb), N=negative control, 14..20=sample

4.5. Nucleotide sequences comparison of BMP15 and GDF9 gene

The sequence of same region was subjected to alignment analysis and it was observed that sequence of this fragment was closely similar with the other sheep sequences (Pelibuey sheep, Norwegian white sheep etc.). The designated sequences of BMP15 gene are shown in (Figure 4.4a and 4.4b, respectively) and GDF9 genes are shown in (Figure 4.5a and 4.5b, respectively). Those figures were revealed 100% homology with the sequence of Pelibuey sheep breed, similarly the nominal sequences of GDF9 gene showed 100% identity with the sequence of Norwegian white sheep. On the other hand the color key comparison of nucleotides sequences by NCBI blast are shown in Appendix II (Figure 4.7 and Figure 4.8, respectively). The designated sequences of BMP15 gene are shown in 99% similar with NCBI accession no: JN655672.1 (Appendix II-Figure 4.9) and GDF9 genes are shown 95% similar with NCBI accession no: KT357485.1 (Appendix II-Figure 4.10).

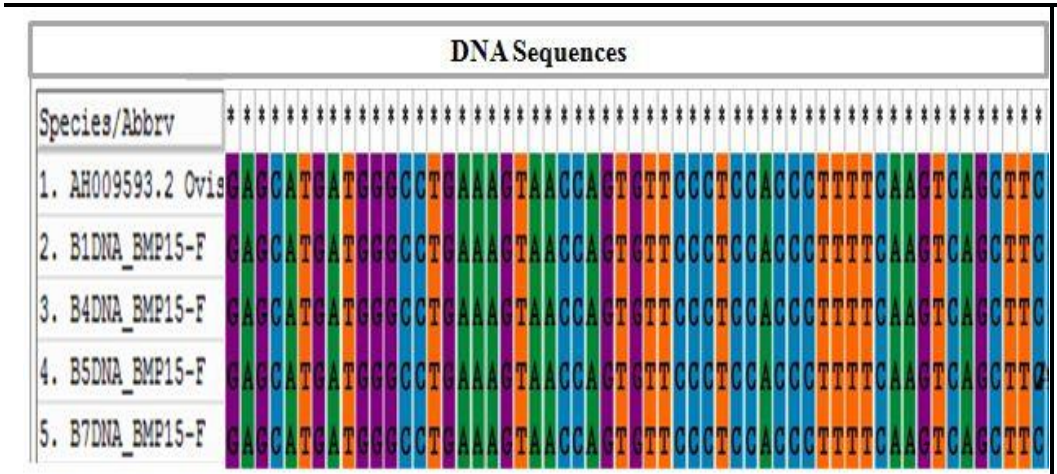


Figure 4.4.a: Nucleotide sequences comparison of BMP15 gene

Score	Expect	Identities	Gaps	Strand
883 bits(978)	0.0	489/489(100)%	0/489(0%)	Plus/Plus

```
Query  1     GCAGGCAGTATTGCATCGGAAGTTCCTGGCCCCTCCAGGGAGCATGATGGGCCTGAAAGT  60
Sbjct  833   GCAGGCAGTATTGCATCGGAAGTTCCTGGCCCCTCCAGGGAGCATGATGGGCCTGAAAGT  892
Query  61    AACCAGTGTTCCCTCCACCCTTTTCAAGTCAGCTTCCAGCAGCTGGGCTGGGATCACTGG  120
Sbjct  893   AACCAGTGTTCCCTCCACCCTTTTCAAGTCAGCTTCCAGCAGCTGGGCTGGGATCACTGG  952
Query  121   ATCATTGCTCCCCATCTCTATACCCCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTA  180
Sbjct  953   ATCATTGCTCCCCATCTCTATACCCCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTA  1012
Query  181   CACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAGTGAGCTGGTG  240
Sbjct  1013  CACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAGTGAGCTGGTG  1072
Query  241   GATCAGAATGTCCCTCAGCCTTCCTGTGTCCCTTATAAGTATGTTCCCATTAGCATCCTT  300
Sbjct  1073  GATCAGAATGTCCCTCAGCCTTCCTGTGTCCCTTATAAGTATGTTCCCATTAGCATCCTT  1132
Query  301   CTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGATTGCCCAGTCC  360
Sbjct  1133  CTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGATTGCCCAGTCC  1192
Query  361   TGCACATGCAGGTGACGGCAAAGGTGCAGCTAGCTCAGGTTTGCCCAAGAAATTCGAAAA  420
Sbjct  1193  TGCACATGCAGGTGACGGCAAAGGTGCAGCTAGCTCAGGTTTGCCCAAGAAATTCGAAAA  1252
Query  421   GGATTTATAATAAATACTGTTAAATCTGAGAGTGCTCAACCCAAGTGCTCTACCCAATCT  480
Sbjct  1253  GGATTTATAATAAATACTGTTAAATCTGAGAGTGCTCAACCCAAGTGCTCTACCCAATCT  1312
Query  481   GTAGATTCT  489
Sbjct  1313  GTAGATTCT  1321
```

Figure 4.4.b: The 100% identity sequence of *Ovis aries* BMP15 gene (sequence ID: KT853038.1).

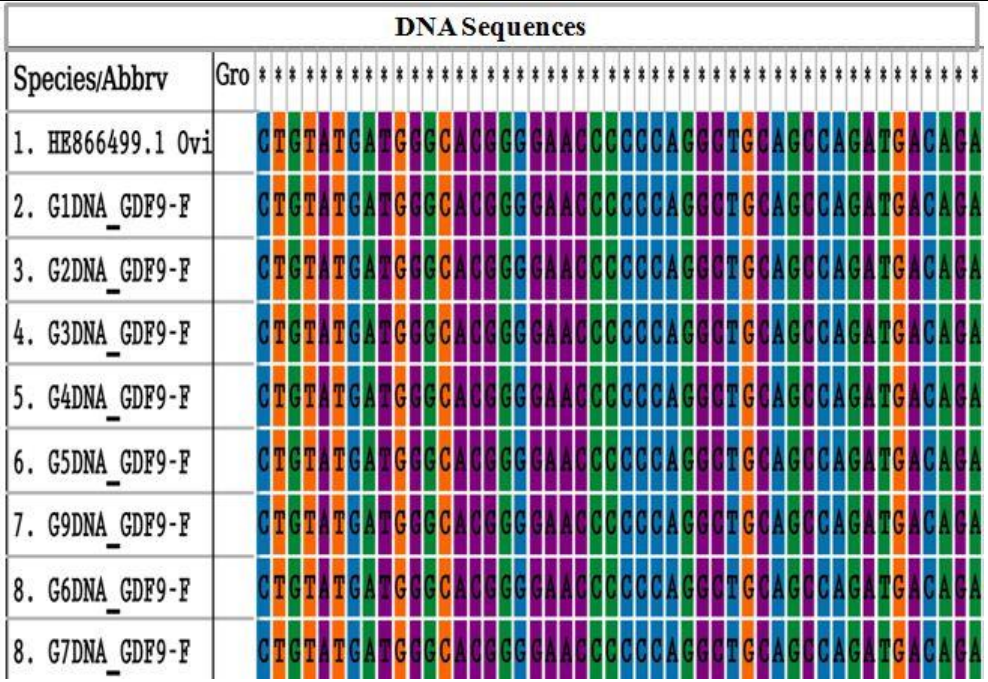


Figure 4.5.a: Nucleotide sequences comparison of GDF9 gene

Score	Expect	Identities	Gaps	Strand
984 bits(758)	0.0	379/379(100)%	0/379(0%)	Plus/Plus

```
Query  1    CTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCCTATTAGCCTTGATTCTCTGCCTTCT  60
Sbjct  41   CTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCCTATTAGCCTTGATTCTCTGCCTTCT  100
Query  61   AGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCC  120
Sbjct  101  AGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCC  160
Query  121  TTGCTGAACCATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGTT  180
Sbjct  161  TTGCTGAACCATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGTT  220
Query  181  CTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAGCTTTGCGCTAC  240
Sbjct  221  CTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAGCTTTGCGCTAC  280
Query  241  ATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGGACCCCTAAATCCAACAGACGC  300
Sbjct  281  ATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGGACCCCTAAATCCAACAGACGC  340
Query  301  CACCTCTACAACACTGTTCGGCTCTTCACCCCCTGTGCTCAGCACAAGCAGGCTCCTGGG  360
Sbjct  341  CACCTCTACAACACTGTTCGGCTCTTCACCCCCTGTGCTCAGCACAAGCAGGCTCCTGGG  400
Query  361  GACCTGGCGGCAGGTGTGT  379
Sbjct  401  GACCTGGCGGCAGGTGTGT  419
```

Figure 4.5.b: The 100% identity sequence of *Ovis aries* GDF9 genes (sequence ID: HE866499.1).

4.6. Estimates of evolutionary divergence within sequences of BMP15 and GDF9 gene

Estimates of evolutionary divergence of both BMP15 and GDF9 gene among the 8 nucleotide sequences obtained from the indigenous sheep species were shown in Table 4.4. Divergence is the average number of base substitutions at each site, between all the base pairs within the groups. The arrangement positions of codon were 1st+2nd+3rd+Noncoding chronologically. There were a total of 318 positions for BMP15 gene and 369 positions for GDF9 gene in the final dataset. The evolutionary divergences of neucleotide sequences in indigenous sheep ranged from 0.00 to 3.35 for BMP15 gene and 0.00 to 0.77 for GDF9 gene with clear differences within in indigenous sheep.

Table 4.4: Estimates of evolutionary divergence between sequences

Nucleotide sequences	1	2	3	4	5	6	7	8
1	0							
2	n/c							
3	n/c	2.91						
4	0.06	2.06	3.35					
5	n/c	n/c	2.17					
6	n/c	0.02	3.01	1.94	n/c			
7	n/c	0.00	2.90	2.07	n/c	0.02		
8	n/c	0.00	2.91	2.07	n/c	0.02	0.0	0

[n/c= not possible to estimate evolutionary distances and column numbers are same as row numbers, 1.B2DNA BMP 15-R, 2.B6DNA BMP 15-R, 3.B1DNA BMP 15-R, 4.B4DNA BMP 15-R, 5.B5DNA BMP 15-R, 6.B7DNA BMP 15-R, 7. B9DNA BMP 15-R]

Nucleotide sequences	1	2	3	4	5	6	7	8
1	0							
2	0.73							
3	0.72	0.72						
4	0.72	0.76	0.73					
5	0.72	0.72	0.00	0.73				
6	0.72	0.72	0.00	0.73	0.00			
7	0.74	0.74	0.73	0.72	0.73	0.73		
8	0.77	0.72	0.73	0.71	0.73	0.73	0.72	0

[Column numbers are same as row numbers, 1.G1 DNA GDF9-F,2.G2 DNA GDF9-F, 3.G3 DNA GDF9-F, 4.G4 DNA GDF9-F,5.G5 DNA GDF9-F, 6.G9 DNA GDF9-F, 7.G6 DNA GDF9-F, 8.G7 DNA GDF9-F]

4.7. Evolutionary relationships taxa of BMP15 gene and GDF9 gene

The evolutionary history was inferred using the Neighbor-Joining method. The branching pattern of the tree was used to determine the most closely related pair of the sequences. The tree were drawn to scale, with the sum of branch length = 2.54 for BMP15 gene and 2.21 for GDF9 gene (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree which are shown in Figure 4.6a and Figure 4.6b, respectively. A final alignment was obtained by repeated this procedure until it reaches the root of a tree. The evolutionary distances were computed using the p-distance method. In the eve of BMP15 genes nucleotide sequence of (NCBI accession no: KT853038.1, AH003593.2, KTO13294.1) were closely related with B1DNA BMP15-R than the sequence (NCBI accession no: HM583333.1). Among them nucleotide sequence of sheep breed (NCBI accession no: KT357481.1) was closely related with G1DNA GDF9F than the (sequences (NCBI accession no: KT357485.1, KT853039.1 respectively) for GDF9.

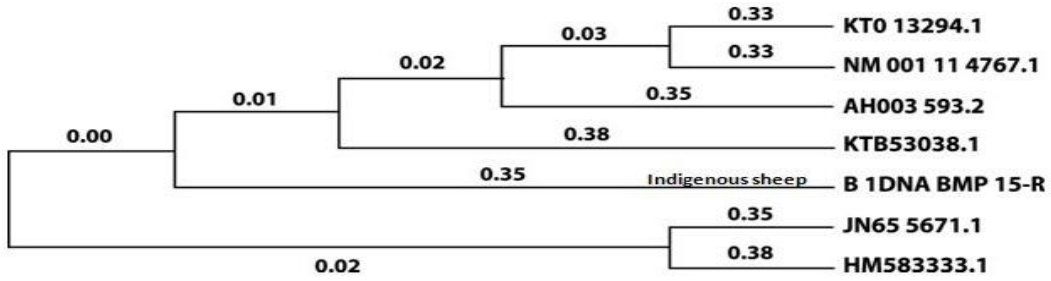


Figure 4.6.a: Phylogenetic tree drawn based on nucleotide sequences of the BMP15 gene

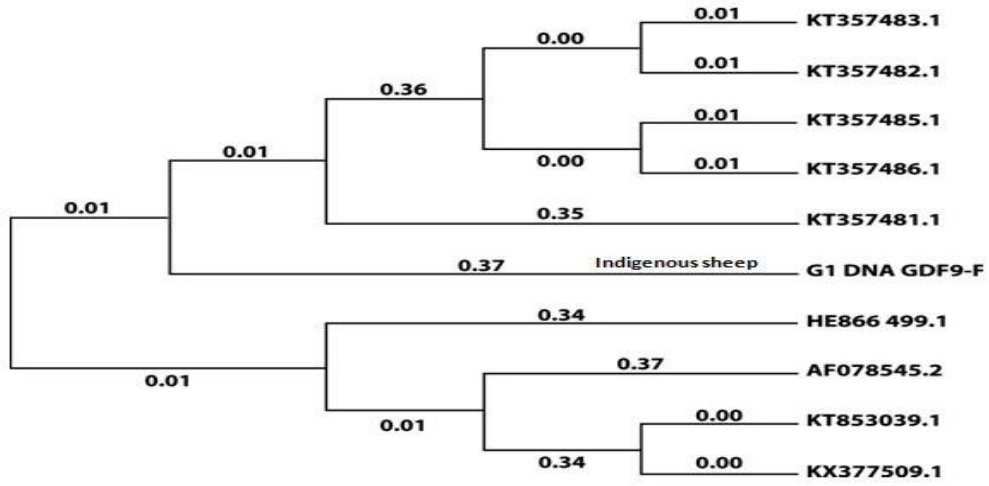


Figure 4.6.b: Phylogenetic tree drawn based on nucleotide sequences of the GDF9 gene

4.8. Maximum likelihood fits of different nucleotide substitution models for BMP15 and GDF9 gene

For all models (Tamura 3-parameter, Kimura 2-parameter and Jukes-Cantor) AIC value (Akaike Information Criterion), BIC (Bayesian information criterion), Maximum Likelihood value (*lnL*), transition bias (*R*) and the number of parameters (including branch lengths) are shown in Table 4.5. Assumed that AIC value and *lnL* were the lowest in case of Tamura 3-parameter model and estimated values of transition bias (*R*) was 0.42 in Kimura 2-parameter model which was lowest among the other models. Jukes-Cantor (JC) model designed with the (3866.36 BIC score) and Kimura 2-parameter model carried 6222.80 score, which were the lowest among the other models for BMP15 gene and GDF9 gene, respectively. For estimating ML values, a tree topology was automatically computed by the using of MEGA6 with the involvement 8 nucleotide sequences. The *lnL* value (-3062.76) and transition bias (*R*) value (0.50) were the lowest in case of Jukes-Cantor model for GDF9 gene.

Table 4.5: Maximum likelihood fits of different nucleotide substitution models

Gene	Model	Parameters	BIC	AIC	*lnL*	*R*
BMP15	JC	13	3866.36	3790.563	-1882.210	0.50
	T92	15	3871.014	3783.582	-1876.696	0.43
	K2	14	3874.065	3792.450	-1882.142	0.42
	JC+I	14	3874.200	3792.586	-1882.210	0.50
GDF9	K2+G	15	6222.80	6133.11	-3051.47	2.76
	JC	13	6229.40	6151.64	-3062.76	0.50
	HKY+G	18	6231.49	6123.89	-3043.83	2.38
	T92+G	16	6234.04	6138.38	-3053.09	2.45

[T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor.]

4.9. Maximum composite likelihood estimate of the pattern of nucleotide substitution of BMP15 and GDF9 gene

The mean distance within species, was estimated according to the sequence data, using the maximum composite likelihood method, which is based on the number of base pairs. Each entry showed that the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values was made equal to 100. Rates of different transitional substitutions are shown in bold and those of

transversionsal substitutions were shown in italics. The overall nucleotide frequencies among 8 nucleotide sequences of BMP15 were 27.87% (A), 26.34% (T/U), 21.70% (C), and 24.10% (G), respectively. There were a total of 318 positions in the final dataset of BMP15 and codon positions were designed as 1st+2nd+3rd+noncoding, respectively. The nucleotide frequencies of GDF9 were 20.22% (A), 26.32% (T/U), 27.91% (C), and 25.54% (G) with total of 369 positions in the final dataset.

Table 4.6: Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	-	*11.7*	*9.64*	**1.64**
T	*12.38*	-	**3.44**	*10.7*
C	*12.38*	**4.17**	-	*10.7*
G	**1.9**	*11.7*	*9.64*	-

Table 4.9: Maximum composite likelihood estimate of the pattern of nucleotide substitution of BMP15

	A	T	C	G
A	-	*1.23*	*1.31*	**24.98**
T	*0.95*	-	**23.61**	*1.2*
C	*0.95*	**22.26**	-	*1.2*
G	**19.78**	*1.23*	*1.31*	-

Table 4.10: Maximum composite likelihood estimate of the pattern of nucleotide substitution of GDF9

Chapter 5: Discussion

5.1. Fertility traits of male (seminal traits)

Reproductive performance of livestock is determined by various factors those are genetic merit, physical environment, nutrition and management. Reproductive well-being and performance of farm animals is largely dependent on their nutritional status. Nutritional factors are perhaps the most crucial in terms of their direct effects on reproductive phenomenon and the potential to moderate the effect of other factors. There was interrelationship between energy intake and reproductive performance in adult rams (Kheradmand et al., 2006; Jibril et al., 2011). Thus, adequate nutrition could encourage mediocre biological types to reach their genetic potential, alleviate the negative effects of a harsh physical environment and minimize the effects of poor management techniques.

Semen volume is one of the important factors in semen evaluation and reproduction performance in the males (Ax et al., 2000). The semen volume was 0.94 ml in Treatment 2 group (protein rich feed) which was higher than the other two groups. This finding were harmonious with the findings of Fernández et al. (2005); Kheradmand et al. (2006) where they denoted that control group generate less volume semen than the treatment groups. On the other hand there was in inconsistent with the query of Jibril et al. (2011) who said that semen volume was not influenced by level of protein. This variation may occur due to changes in protein percentages in ration and farm management system.

Nutrition has effects for the growth and maturation of sertoli cells in newborn lambs. The sertoli cells are strong candidates for foetal programming of future performance, because the number of sertoli cells is highly correlated with the maximum rate of sperm production and sperm concentration. In the present experiment sperm count in Treatment 2 contains 4.25×10^6 sperm which was higher than the other two groups, strongly coincided with the research of Jibril et al. (2011) where they said that increase crude protein intake above the minimum requirements resulted in improved sperm concentration and favours for spermatogenesis.

Scrotal diameter, testicular sizes were affected by nutrition, where testicular growth can be affected when animals are fed above their maintenance requirement. The findings of present studies of scrotal diameter was more sizably voluminous in the

Treatment group 2 than the other two groups which agreement with those obtained by Hötzel et al. (1998); Fernández et al. (2005) where they noticed that the improve protein rich diet was helpful for larger growth of scrotal circumference than the control group, although inconsistent with those obtained by Bielli et al. (1999) who found no significant effect from high dietary protein on testicular dimensions. The voluminous size of scrotal diameter in case of the treatment groups might be attributed to the optimum utilization of dietary protein at about 13.96% CP level as previously reported by Negesse et al. (2001).

5.2. Sperm morphology

Determination of viability and normal sperm percentage is important in assessing semen for semen processing and preservation. Percentage of normal sperm was highly diligent in the case of treatment groups than the control group. Here 94.60% sperm was normal in nature in the case of protein enriched Treatment group 2, which was higher than the other two groups which was consistent with the findings of Fourie et al. (2004); Kheradmand et al. (2006); Jibril et al. (2011) and did not similar with the findings of Barth et al. (2008) who found that the medium or high level of nutrition does not have influence on overall percentage of morphologically normal spermatozoa.

Present study denoted that sperm viability was not statistically significant among the three groups where Treatment group 2 contains 87.33% viable sperm which was higher than the other two groups and strongly coincide with the findings of Jibril et al. (2011). They showed that sperm cells viability in all groups was above 78% where highest viability was recorded in case of treatment groups than the control group and there were no significant differences observed in sperm viability among the treatment groups.

5.3. Female reproductive parameters (fertility traits)

Nutrition during pregnancy had significantly affected the gestation length of the ewes (Holst et al., 1986; Zohara et al., 2014). The gestation length in indigenous ewes varied from 148 to 155 days. The gestation length was significantly higher in control group (155.25±4.1 days) compared with treated groups which was harmonious with the findings of Kabir et al. (2002); Zohara et al. (2014) and inconsistent with research

of Sultana et al. (2011) who observed that gestation length was longer in treatment groups than the control groups.

The lambing interval consists of service period and the gestation period. In control group lambing interval was 188.25±2.3 days which was longer than the other treatment groups but similar with the findings of Hassan and Talukder (2012) and did not supported by the findings of Bhuiyan (2014); Poonia (2008). Therefore, to minimize the lambing interval, post lambing estrus interval and service per conception should be minimized. Through dietary intervention and management which minimizing lambing interval in this study, which will be helpful for improved the sheep production in our country.

A higher litter size may be the result for increase in body weight gain of ewes fed the high energy diet at pre-mating and gestation. The nutrition have ability to alter the liter size of ewes was known, as a rapid improvement in body weight gain is associated with an increase in ovulation rate and lambing rate (Rhind and Mcneilly, 1998; Zohara et al., 2014). The number of lamb born in case of Treatment group 2 was 1.75±0.5, which was higher among the three groups and strongly coincide with the findings of Solomon and Gemeda (2000); Kabir et al. (2002); Sultana et al. (2011) where they showed that liter size was more in number than the control groups. On the other hand this finding was not supported by the work of Banerjee (2008) where they showed there was no variation in case of lamb number among the various groups.

The level of nutrition during the last weeks before parturition acted as modifier for improved the birth weight of lambs. The birth weights of the lambs were also affected by the number of litter size (Duguma et al., 2002). The birth weight affect the lamb's ability to ingest colostrum and receive proper mothering shortly after birth and thus develop an ability to combat infections. The birth weight of lambs born in Treatment 2 groups was 1.56±0.2 kg which was higher when compared with lambs born in other two groups harmonious with the result of Kabir et al. (2004); Zohara et al. (2014) where they cited that birth weight of lambs incase of protein treated groups were higher than the control group, not supported by the findings of Hassan and Talukder (2012) who told that there was no relation of birth weight of lamb with feed groups.

Conception rate in treated groups was higher than the control group which was strongly coincides with the findings of Zohara et al. (2014). Ewe treated with on

supplemented groups (protein concentrate) reached conception at earlier age than the control group. Weight at conception was not significantly different (P>0.05) among the three groups where weight at conception was 11±0.22 kg in the case of Treatment 2 groups which was higher than the other two groups.

5.4. Gene sequencing

5.4.1. Nucleotide sequences comparison of BMP15 and GDF9 gene

The sequence of same region was subjected to close similarity with the other sheep sequences. The designated sequences revealed 100% homology (NCBI accession no: KT853038.1) with the sequence of Pelibuey sheep breed reported by Argüello-Hernández et al. (2014), on the other hand those sequences were 99% similar with designated sequences of Balochi (Nawaz et al., 2013) and Lohi breed of sheep (NCBI accession no: JN655672.1, JN655671.1, respectively). The designated sequences of GDF9 were 100% identical with the sequence of Norwegian white sheep (Våge et al., 2013) and Pelibuey sheep (Argüello-Hernández et al., 2014) (NCBI accession no: HE866499.1, KT853039.1 respectively) where it showed that sequences were 95% harmonious with designated sequences of Suffolk × Awassi crossbred (NCBI accession no: KT357485.1) and Awassi breed (NCBI accession no: KT357483.1).

5.4.2. Estimates of evolutionary divergence between sequences of BMP15 and GDF9 gene

BMP15 and GDF9 genes are known to be specifically expressed in oocytes and to be essential for female fertility in sheep. The evolutionary divergences of nucleotide sequences in indigenous sheep ranged from 0.00 to 3.35, with clear differences within the indigenous sheep in case BMP15 gene. The evolutionary divergences among sequences ranged from 0 to 0.06 that's determined that they are most closely related to each and other, which was supported by the findings of Tajima and Nei (1984). The evolutionary divergences of GDF9 neucleotide sequences in indigenous sheep ranged from 0.00 to 0.77 and divergences among sequences closed 0.00 that's determined that they had close relationship within the nucleotide sequences.

5.4.3. Evolutionary relationships taxa of genes

Phylogenetic trees are important tools for organizing knowledge of biological diversity, and they communicate hypothesized evolutionary relationships among

nested groups of taxa that are supported by shared traits known as synapomorphies Novick et al. (2011). Traditionally, most recent common ancestry is used to interpret taxa relatedness. Taxa that share a more recent common ancestor must be more closely related to each other than to another taxon with a less recent common ancestor (Dees et al., 2014). MEGA6 (Tamura et al., 2013) was a commonly applied program for interpret the taxa relatedness incase of multiple sequence alignment. Using MEGA6, sequences were studied for compared and a tentative measurement of similarity, represented by a distance matrix. This was used to produce a phylogenetic guide tree, using the neighbour-joining (NJ) method (Saitou and Nei, 1987). The branching pattern of the tree was used to determine the most closely related pair of the sequences. Comparative studies of sequences were used in a wide range of taxonomic levels, to evaluate phylogenetic relationships. The phylogeny results of a recent study (Bibinu et al., 2016) based on nucleotide sequences of BMP15 showed a similar clustering of sequences among the various breed those obtained in this study, although there was some intermingling between the species. The designated BMP15 sequences were closely related with the sequence of Pelibuey sheep breed reported by Argüello-Hernández et al. (2014). The GDF9 designated sequences were also almost close-fitting similarity with the taxa of Pelibuey sheep denoted by Argüello-Hernández et al. (2014) on the other hand, it showed close smliar taxa with the Suffolk×Awassi crossbred (NCBI accession no: KT357485.1) and Awassi breed (NCBI accession no: KT357483.1). This close relation in gene sequences might be due to similar environmental conditions and ancestor relatedness.

5.4. 4. Maximum likelihood fits of different nucleotide substitution models for BMP15 and GDF9 gene

Lowest BIC score model is the best fitted model among the other models reported by, Sung et al. (2013) which was strongly coincide with the present study. Jukes-Cantor (JC) model designed with the (3866.36 BIC score) in BMP-15 genes and Kimura 2-parameter model carried 6222.81 BIC score, which were the lowest among the other models. After considered the observations Jukes-Cantor (JC) and Kimura 2-parameter model were the best fitted for the BMP15 and GDF9 gene, respectively.

5.4.5. Maximum composite likelihood estimate of the pattern of nucleotide substitution of BMP15 and GDF9 gene

In this study, transitional and transversionsal parameters represent a measure of the biochemical similarity of bases and transitional substitutions were occurred more often than the transversionsal substitutions, which was strongly, coincide with the findings of Palero and Crandall (2016). Rates of different transitional substitutions were used to denotes changes in the nucleotide substitutions from (A ↔T, C ↔T), and transversions were used to changes in pyrine to pyrimidine base pair that's denotes the point mutations.

Conclusion

The seminal traits of indigenous sheep were better in Treatment 2 (13.96% CP) than the other two groups that suggest that improved dietary intake above maintenance requirements had positive effects on rams. It may be concluded that ewes treated with on supplemented feed (12.94% CP, 13.96% CP) showed better reproductive performances that presage to rear sheep with on supplements may alter reproductive performance of ewes to increase and improve the sheep production in Bangladesh. The evolutionary divergences and evolutionary history of branching pattern showed the relatedness of the nucleotide sequences. Jukes-Cantor (JC) model and Kimura 2-parameter model were the best fitted models among the other models for BMP15 gene and GDF9 gene, respectively. Although, BMP15 and GDF9 have complex mutagenic reaction may assist to regulate the fertility traits of indigenous sheep. Finally, it can be concluded that the potential role of all the possible mutations in BMP15 and GDF9 on fertility and ovulation rate in indigenous sheep, future investigations including DNA re-sequencing, complete gene sequencing and molecular marker analysis can be done for understanding and perceiving the complexities of the genome. These genomic thoughts will be helpful to conserve the genetic constituents of indigenous sheep and can be applicable tools for the genetic improvement of indigenous sheep of Bangladesh.

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APPENDIX I: Data collection form

Serial No: ------

Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong-4225

Department of Genetics and Animal Breeding

Title: Effects of protein supplements on fertility and phylogenic inference of fertility genes in indigenous sheep of Bangladesh

A questioner on the ram and ewe fertility traits

Owner Name: …….

Area/Village name: …….

Thana/Upazilla: …….

District name: …….

Cell No: …….

Reproductive performances record sheet of the female

Farmer Name	Area	Tag No	Name of the Group	Age at first lambing	Weight at first lambing	Conception rate	Gestation period	Lambing interval	Lamb number	Lamb weight at parturition	Lamb survival rate at birth	Lamb survival rate at Weaning
Abdul Mabud (For example)	Hathazari	E24	Treatment group 2									
	Hathazari	E17	Treatment group 2									
	Hathazari	E2	Treatment group 2									
	Hathazari	E6	Treatment group 2									
Kamal Uddin (For example)	Hathazari	E37	Treatment group 1									
	Hathazari	E9	Treatment group 1									
	Hathazari	E1	Treatment group 1									
	Hathazari	E13	Treatment group 1									
	Hathazari	E18	Treatment group 1									

	Hathazari	E40	Treatment group 1									
	Hathazari	E37	Treatment group 1									
	Hathazari	E20	Treatment group 1									
Naiyem (For example)	Bakolia	E28	Control									
	Bakolia	E10	Control									
	Bakolia	E14	Control									
	Bakolia	E5	Control									
	Bakolia	E9	Control									
	Bakolia	E10	Control									

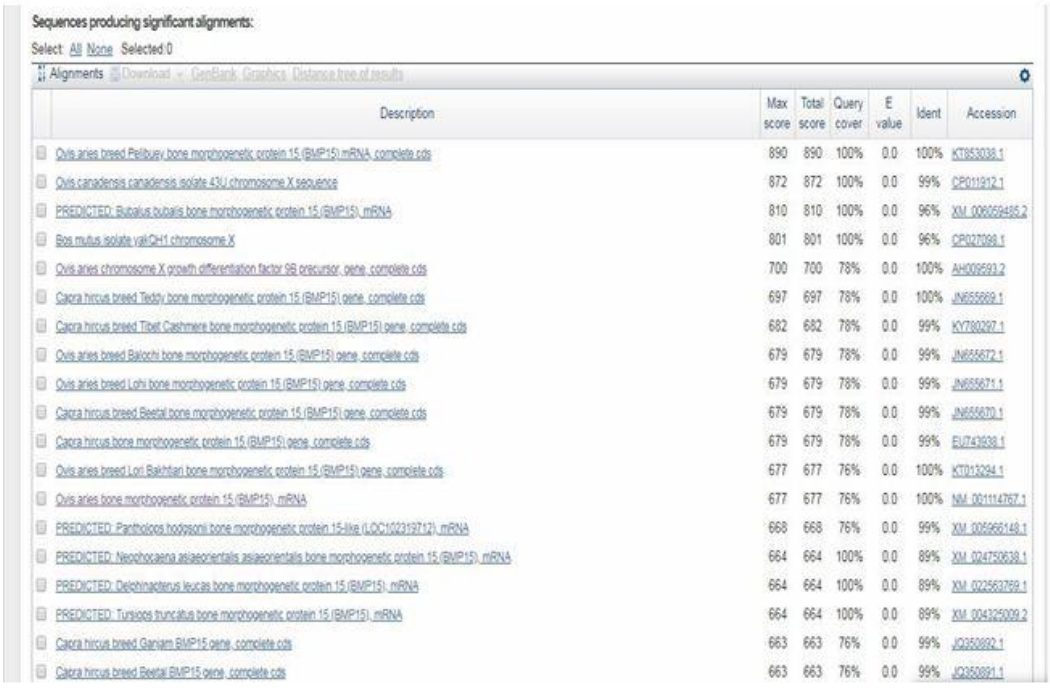
Semen quality record sheet

Farmer Name	Area	Tag No	Name of the Group	pH	Semen volume	Sperm count	Concentration	Sperm viability		Sperm morphology	
								Live	Death	Normal	Abnormal
Kamal Uddin (For example)	Hathazari	R13	Treatment group 2								
	Hathazari	R5	Treatment group 2								
	Hathazari	R18	Treatment group 2								
Abdul Mabud (For example)	Hathazari	R3	Treatment group 1								
	Hathazari	R11	Treatment group 1								
	Hathazari	R17	Treatment group 1								
Naiyem (For example)	Bakolia	R7	Control								
	Bakolia	R9	Control								

Signature of Surveyor and date

APPENDIX II: Nucleotide sequences alignment by NCBI blast

Figure 4.7: Color key for Nucleotide sequences alignment score by NCBI blast


Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Ovis aries breed Pelibuey bone morphogenetic protein 15 (BMP15) mRNA, complete cds	890	890	100%	0.0	100%	KT853038.1
Ovis canadensis canadensis isolate 43U chromosome X sequence	872	872	100%	0.0	99%	CP011912.1
PREDICTED: Bubalus bubalis bone morphogenetic protein 15 (BMP15), mRNA	810	810	100%	0.0	96%	XM_006059486.2
Bos mutus isolate yakCH1 chromosome X	801	801	100%	0.0	96%	CP027098.1
Ovis aries chromosome X growth differentiation factor 9B precursor, gene, complete cds	700	700	78%	0.0	100%	AH009593.2
Capra hircus breed Teddy bone morphogenetic protein 15 (BMP15) gene, complete cds	697	697	78%	0.0	100%	JN655669.1
Capra hircus breed Tibet Cashmere bone morphogenetic protein 15 (BMP15) gene, complete cds	682	682	78%	0.0	99%	KY780297.1
Ovis aries breed Balochi bone morphogenetic protein 15 (BMP15) gene, complete cds	679	679	78%	0.0	99%	JN655672.1
Ovis aries breed Lohi bone morphogenetic protein 15 (BMP15) gene, complete cds	679	679	78%	0.0	99%	JN655671.1
Capra hircus breed Beetal bone morphogenetic protein 15 (BMP15) gene, complete cds	679	679	78%	0.0	99%	JN655670.1
Capra hircus bone morphogenetic protein 15 (BMP15) gene, complete cds	679	679	78%	0.0	99%	EU743938.1
Ovis aries breed Lori Bakhtiari bone morphogenetic protein 15 (BMP15) gene, complete cds	677	677	76%	0.0	100%	KT013294.1
Ovis aries bone morphogenetic protein 15 (BMP15), mRNA	677	677	76%	0.0	100%	NM_001114767.1
PREDICTED: Pantholops hodgsonii bone morphogenetic protein 15-like (LOC102319712), mRNA	668	668	76%	0.0	99%	XM_005966148.1
PREDICTED: Neophocaena asiaeorientalis asiaeorientalis bone morphogenetic protein 15 (BMP15), mRNA	664	664	100%	0.0	89%	XM_024750638.1
PREDICTED: Delphinapterus leucas bone morphogenetic protein 15 (BMP15), mRNA	664	664	100%	0.0	89%	XM_022563769.1
PREDICTED: Tursiops truncatus bone morphogenetic protein 15 (BMP15), mRNA	664	664	100%	0.0	89%	XM_004329009.2
Capra hircus breed Ganjam BMP15 gene, complete cds	663	663	76%	0.0	99%	JQ350892.1
Capra hircus breed Beetal BMP15 gene, complete cds	663	663	76%	0.0	99%	JQ350891.1

Figure 4.8: Nucleotide sequences comparison alignment by NCBI blast

Score	Expect	Identities	Gaps	Strand
679 bits(752)	0.0	382/386(99)%	0/386(0%)	Plus/Plus

```
Query  1     GCAGGCAGTATTGCATCGGAAGTTCCTGGCCCCTCCAGGGAGCATGATGGGCCTGAAAGT  60
Sbjct  6256  GCAGGCAGTATTGCATCTGAAGTTCCTGGCCCCTCCAGGGAGCATGATGGGCCTGAAAGT  6315
Query  61    AACCAGTGTTCCCTCCACCCTTTTCAAGTCAGCTTCCAGCAGCTGGGCTGGGATCACTGG  120
Sbjct  6316  AACCAGTGTTCCCTCCACCCTTTTCAAGTCGGCTTCCAGCAGCTGGGCTGGGATCACTGG  6375
Query  121   ATCATTGCTCCCCATCTCTATACCCCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTA  180
Sbjct  6376  ATCATTGCTCCCCATCTCTATACCCCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTA  6435
Query  181   CACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAGTGAGCTGGTG  240
Sbjct  6436  TACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAATGAGCTGGTG  6495
Query  241   GATCAGAATGTCCCTCAGCCTTCCTGTGTCCCTTATAAGTATGTTCCCATTAGCATCCTT  300
Sbjct  6496  GATCAGAATGTCCCTCAGCCTTCCTGTGTCCCTTATAAGTATGTTCCCATTAGCATCCTT  6555
Query  301   CTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGATTGCCCAGTCC  360
Sbjct  6556  CTGATTGAGGCAAATGGGAGTATCTTGTACAAGGAGTATGAGGGTATGATTGCCCAGTCC  6615
Query  361   TGCACATGCAGGTGACGGCAAAGGTG  386
Sbjct  6616  TGCACATGCAGGTGACGGCAAAGGTG  6641
```

Figure 4.9 : The sequence of *Ovis aries* BMP15 (NCBI accession no: JN655672.1)

Score	Expect	Identities	Gaps	Strand
603 bits(668)	1e-168	361/379(95)%	0/379(0%)	Plus/Plus

```
Query  1     CTTTGGTTTTGCTGCTTTGCCTGGCTCTGTTTTCCTATTAGCCTTGATTCTCTGCCTTCT  60
Sbjct  9     CTTTGGTTTTGCTGCTTTGCCTGGTTCTGTTTTCCTATTACCCTTGATTCTCTGCCTTCT  68
Query  61    AGGGGAGAAGCTCAGATTGTAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCC  120
Sbjct  69    AGGGGAAAAGCTCAAATTGTACCTAGGACTGCGTTGGAATCTGAGGCTGAAACTTGGTCC  128
Query  121   TTGCTGAACCATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGTT  180
Sbjct  129   TTGCTGAACCATTTAGGTGGGAAACACAAACCTGGTCTCCTTTCCCCTCTCTTAAAGGTT  188
Query  181   CTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAGCTTTGCGCTAC  240
Sbjct  189   CTGTATGATGGGCACGGGAAACCCCCCAGGCTGCACCCAAATGACAAAGCTTTGCCCTAC  248
Query  241   ATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGGACCCCTAAATCCAACAGACGC  300
Sbjct  249   ATGAAAAGGCTCTATAAGGCATACGCTACCAGGGAGGGGACCCCTAAATCCAACAAACCC  308
Query  301   CACCTCTACAACACTGTTCGGCTCTTCACCCCCTGTGCTCAGCACAAGCAGGCTCCTGGG  360
Sbjct  309   CACCTCTACAACACTGTTCGGCTCTTCACCCCCTGTGCTCAGCACAAGCAGGCTCCTGGG  368
Query  361   GACCTGGCGGCAGGTGTGT  379
Sbjct  369   GACCTGGCGGCAGGTGTGT  387
```

Figure 4.10: The sequence of *Ovis aries* GDF9 (NCBI accession no: KT357485.1)

Figure 4.11:M6;Alingment Explorer of BMP15 gene

Biography

DR. Md. Iqbal Hossain is a candidate for the degree of MS in Animal Breeding and Genetics under the Department of Genetics and Animal Breeding, Chittagong Veterinary and Animal Sciences University (CVASU). He has passed the secondary School Certificate (SSC) in 2006 and then higher secondary Certificate (HSC) Examination in 2008. He has obtained her Doctor of Veterinary Medicine (DVM) Degree in 2014 (held on 2015) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. He has achieved **National Science and Technology Fellowship** under the Ministry of Science and Technology for his thesis work. He has published several scientific articles in national and international journals. He has great interest on Molecular Genetics and Animal Biotechnology.