# A STUDY ON GENETIC POLYMORPHISM OF MYOSTATIN (*GDF8*) GENE IN NELLORE AND MACHERLA BROWN SHEEP

*By*

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Mr.**D.VENKATA PRANEETH**, **GVM/14-001** has satisfactorily prosecuted the course of research and that the thesis entitled **A STUDY ON GENETIC POLYMORPHISM OF MYOSTATIN GENE (*GDF8*) IN NELLORE AND MACHERLA BROWN SHEEP** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

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# CERTIFICATE

This is to certify that the thesis entitled **A STUDY ON GENETIC POLYMORPHISM OF MYOSTATIN(*GDF8*) GENE IN NELLORE AND MACHERLA BROWN SHEEP** submitted in partial fulfilment of the requirements for the degree of **"Master of Veterinary Science"** of Sri Venkateswara Veterinary University, Tirupati is a record of the bonafide research work carried out by Mr.D.Venkata Praneeth under my guidance and supervision. The subject of the thesis has been approved by the Student's advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of Investigations have been duly acknowledged by the author of the thesis.

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***D.VENKATA PRANEETH. . .***

# DECLARATION

I. D. VENKATA PRANEETH hereby declare that the thesis entitled **A STUDY ON GENETIC POLYMORPHISM OF MYOSTATIN GENE (*GDF8*) IN NELLORE AND MACHERLA BROWN SHEEP** submitted to Sri Venkateswara Veterinary University, Tirupati for the degree of Master of Veterinary Science is the result of original research work done by me. I also declare that the materials contained in this thesis have not been published earlier.

**Date:**

**Place :** Gannavaram **(D.Venkata Praneeth)**

## LIST OF SYMBOLS AND ABBREVATIONS

| S.No | Symbol | Full form |
|---|---|---|
| 1 | % | Percent |
| 2 | °C | Degree Celsius |
| 3 | Cm | Centimeter |
| 4 | Ng | Nanogram |
| 5 | µg | Microgram |
| 6 | Mg | Milligram |
| 7 | G | Gram |
| 8 | Kg | Kilogram |
| 9 | i.e | that is |
| 10 | *et al.* | and others |
| 11 | *viz.* | namely, that is to say, as follows |
| 12 | Df | degrees of freedom |
| 13 | Min | Minute |
| 14 | Ml | Millilitre |
| 15 | µl | Microlitre |
| 16 | M | Molarity |
| 17 | Mm | Millimoles |
| 18 | µM | micromoles |
| 19 | N | Normality |
| 20 | pH | Negative logarithm of hydrogen ion concentration |
| 21 | Rpm | revolutions per minute |
| 22 | EDTA | Ethylene Diamine Tetra Acetic acid |
| 23 | NaCl | Sodium Chloride |
| 24 | $MgCl_2$ | Magnesium chloride |
| 25 | SDS | Sodium Dodecyl Sulphate |
| 26 | TE | Tris EDTA |
| 27 | UV | Ultra Violet |

| | | |
|---|---|---|
| 28 | V | Volts |
| 29 | @ | At the rate of |
| 30 | TBE | Tris Borate EDTA |
| 31 | DNA | Deoxyribo Nucleic acid |
| 32 | RNA | Ribo Nucleic acid |
| 33 | Kb | Kilo base pair |
| 34 | bp | Base pair |
| 35 | OD | Optical density |
| 36 | FAO | Food and Agricultural Organization |
| 37 | PCR | Polymerase Chain Reaction |
| 38 | $N_a$ | Observed number of alleles |
| 39 | $N_e$ | Effective number of alleles |
| 40 | $H_o$ | Observed Heterozygosity |
| 41 | $H_e$ | Expected Heterozygosity |
| 42 | $F_{IS}$ | Inbreeding coefficient |
| 43 | PIC | Polymorphism Information Content |
| 44 | HWE | Hardy-Weinberg Equilibrium |
| 45 | PCR-RFLP | Polymerase chain reaction restriction fragment Length polymorphism |
| 46 | GDF8 | Growth differentiation factor 8 |
| 47 | FAOSTAT | Food and Agriculture Organization Statistical Data base |
| 48 | UTR | Untranslated region |
| 49 | SNP | Single Nucleotide Polymorphism |

| | | |
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## ABSTRACT

The primary objective in small animal production is to have higher meat production. The present study is aimed at understanding natural variation of *GDF8* locus in Nellore and Macherla Brown sheep genetic groups by using PCR-RFLP. DNA was isolated from 100 sheep blood samples using a modified high salt method. Two sets of primers were designed to amplify regions of exon 3 and intron 1 of *GDF8* gene. A total of 100 blood samples from the three sheep populations were used for amplification of exon 3 and 65 samples were used for amplification of intron 1. The PCR products were subjected to RFLP with *Hae*III restriction enzyme for exon 3 and *Hpy*CH4V restriction enzyme for intron 1. Based on different genotypes obtained the genotypic and allelic frequencies were determined. In the present study exon 3 is monomorphic in all the samples and hence, further analysis could not be performed on this locus. The intron 1 region was polymorphic representing *HH, Hh, hh* genotypes. The heterozygosity values for intron 1 region were 0.48, 0.49 and 0.47 for Nellore Jodipi, Nellore Brown and Macherla Brown genetic groups respectively. The PIC values for

Nellore Jodipi, Nellore Brown and Macherla Brown are 0.37, 0.37 and 0.36 respectively, suggesting considerable amount of variation exist in these populations. The allelic frequencies of Nellore Jodipi, Nellore Brown and Macherla Brown were 0.59 and 0.41, 0.56 and 0.44, 0.62 and 0.38 for H and h alleles, respectively. Diversity estimates ($F_{IS}$) were negative for the three populations indicating that there is no differentiation among the three populations. The test for Hardy Weinberg Equilibrium indicated that the three populations are departing from the equilibrium assumptions. No significant ($P>0.05$) association of a genotype with body weights at different ages was observed in the present study in both the genetic groups of Nellore Jodipi and Nellore Brown. A new PCR-RFLP marker is designed for intron 1 in this study which is found to be polymorphic and useful in population studies.

# CHAPTER I

# INTRODUCTION

The primary objective in small animal production is to have higher meat production. In India, unlike western countries the main source of meat is sheep and chicken. Sheep in India make valuable contribution to the livelihood of economically weaker sections as they are mainly raised by nomads. Sheep population in India was 63 million (FAOSTAT, 2014) accounting to 4.46 % of the world population. India stands third in the world in terms of population . In the current scenario, there is great demand for export of quality sheep meat across the world. Hence it is worth concentrating in improvement of sheep production.

One of the constraints in achieving this objective is the productivity of Indian sheep breeds which is relatively less compared to exotic breeds. Average meat yield of Indian sheep was 12kg/animal while that from China was 15.5kg/animal and 22.4kg/animal in Australia (FAOSTAT, 2011). Careful breeding practices and marker-assisted selection of individuals can bring drastic changes in this scenario.

Underlying assumption in quantitative genetic theory is that heritability of agronomically important traits like meat production is regulated by multiple genes with small individual effects. Genomic tools required to detect such genes have been developed during last two decades. Myostatin (also known as *growth differentiation factor 8*, abbreviated as *GDF*-8) has been discovered and proved to have major effect on meat production (Georges, 2010). *GDF8* is a myokine, a protein that is produced and released by myocytes that acts on muscle cells autocrine function to inhibit myogenesis *i.e*. muscle cell growth and differentiation. It has three exons and two introns located on OAR2 of the sheep genome. Natural myostatin mutations are discovered in farm and domestic animals like cattle, sheep, dog and horse. In sheep a hypomorphic mutation has been detected at the *GDF 8* locus (*c.2360G>A*) which is associated with increased

muscle mass (Clop *et al.*, 2006; Boman *et al.*, 2010). This mutation was initially identified in heavily muscled Belgian Texel sheep. Subsequently it has been discovered that this mutation causes illegitimate target site for microRNAs (miRNA) that are heavily expressed in skeletal muscle, thus reducing *GDF 8* expression levels, causing muscular hypertrophy. The mutation was subsequently shown to segregate in other sheep breeds and cause increased muscle mass in several sheep breeds including Charollais, New Zealand Texel, Australian White Suffolk, Polled Dorset etc. ( Kijas *et al*., 2007; Hadjipavlou *et al*., 2008; Takeda *et al*., 2010). Indeed, haplotype analysis in an Australian sheep resource population indicated that there is a possibility of existence of other *GDF8* hypomorphs. Apart from the well characterized hypomorph *c.2360G>A*, other mutations also shown to increase muscle mass (Johnson *et al.,* 2009). The hypomorphs of *GDF8* are used in marker-assisted selection in many countries to achieve success in improving mutton production (Boman *et al.,* 2009; Lee 2007). Understanding natural variants of such genes would help in developing marker assisted breeding strategies in sheep.

Different molecular tools are available to detect natural variation. PCR-RFLP is relatively simple and economical tool to identify natural variation at specific loci. Though the information obtained is limited, co-dominant nature of the tool makes it as marker of choice for identifying natural variation at a given locus. The principle involves PCR amplification of the given region and digesting the fragment with specific restriction enzyme that can cleave at the specific location if there is a mutation.

India is rich in sheep genetic diversity with 42 recognized sheep breeds and several unclassified locally adopted sheep genetic groups. Andhra Pradesh is native to Nellore breed of sheep, which is the tallest breed in India and mutton type breed. It is predominant in Nellore and Prakasham districts and in their adjoining areas. There are three variants in Nellore breed mainly Jodipi, Brown and Palla. Macherla brown is a

locally adopted genetic group present in and around Macherla region of Guntur district. Macherla Brown sheep is distributed along the banks of river Krishna flowing through Guntur, Krishna, Prakasam and Nalgonda districts of Andhra Pradesh and Telangana states. The home tract of this breed is mainly Nagarjunasagar dam areas of Guntur and Nalgonda districts. Macherla Brown is known for heat tolerance, disease resistance and thrives well in harsh climatic conditions and scarce feeding conditions.

Studies on the polymorphism of Myostatin gene in Nellore are scanty and the perusal of literature reveals no such studies in Macherla Brown sheep.

With this background, the present study is aimed at understanding natural variation of *GDF8* locus in the two meat type sheep *viz.* Nellore and Macherla brown with the following objectives.

**Objectives:**

1. To determine the genetic polymorphism of *GDF8* locus using *PCR-RFLP* in Nellore and Macherla brown sheep.
2. To determine the population genetic parameters *viz*. heterozygosity and polymorphism information content.
3. To study the association of *GDF8* gene variants if any, with growth traits in Nellore sheep.

# CHAPTER – II

# REVIEW OF LITERATURE

The literature published on the significance of myostatin gene, polymorphism and association studies of myostatin gene with body weights and population parameters is reviewed in the following sections.

## 2.1 Significance of Myostatin gene

The genetic cause of muscular hypertrophy was unknown for a long time. The biological function of *GDF8* was first determined in mice, where the *GDF8* null animals are significantly larger than wild type animals and show a large and widespread increase in skeletal muscle mass (McPherron *et al*., 1997 a). The physical location of bovine myostatin gene was revealed by FISH technique of YAC clones, indicated that bovine myostatin gene lies close to centromere of bovine chromosome (BTA2) at 2q11, (Smith *et al*., 1997). Gene sequencing of myostatin has been done in different species like cattle, sheep, chicken, goat, pig and in all of these species the gene has three exons and two introns (McPherron *et al*., 1997 b).

The *GDF8* is the major regulator of myogenesis and it functions as a negative regulator of muscle growth in mammals. The *GDF8* gene is associated with increased skeletal muscle mass (double muscling) in cattle (Marchitelli *et al.,* 2003), dogs (Mosher *et al.,* 2007), mice (McPherron *et al*., 1997; Mendias *et al.,* 2008). Studies conducted in breeds of chickens, have shown different mutations in the myostatin gene and the significant association of it with fat metabolism and body weight traits (Zhang *et al*., 2011).

In sheep, the myostatin gene is located on chromosome two. For the first time muscular hypertrophy was found in Belgian Texel breed. Twenty single-nucleotide polymorphisms (SNPs) were identified in the region of *GDF8* in Texel sheep. Among

these are, SNP g+6223G>A in the 3' untranslated region (3'UTR) which cause the muscular hypertrophy (Clop *et al*., 2006). Later, this SNP has been found in various sheep breeds. Thus, *MSTN* g+6223G>A (previously designated as g+6723G>A) has been suggested as a useful gene marker for breeding to improve carcass traits in sheep (Kijas *et al*., 2007; Hadjipavlou *et al*., 2008; Johnson *et al*., 2009).

**2.2 Polymorphic studies of Myostatin gene in sheep**

Single nucleotide polymorphism (SNP; g.6223G>A) has been detected in the 3' UTR of *GDF8* gene in Belgian Texel sheep. This SNP has been found to affect muscular hypertrophy in Belgian Texel sheep (Clop *et al*., 2006).

The SNP, g+6723G>A, which is known to increase muscularity with in the Belgian Texel sheep was also tested in Australian breeds *viz.* White Suffolk, Poll Dorset and Lincoln. The loss of function allele (g+6723A) had significant effects on slaughter measurements of muscling. Further, it was observed that fatness had largest impact within the white Suffolk breed (Kijas *et al*., 2007).

Sequencing of the coding region of the myostatin gene in Norwegian Spælsau ram lamb revealed a one base-pair insertion mutation (*c.120insA*) producing a premature stop codon in amino acid position 49 which resulted in production of non functional myostatin protein (Boman *et al*., 2009b).

PCR-RFLP studies of exon 3 portion of myostatin gene in Sanjabi sheep revealed three genotypes MM, Mm, mm indicating the polymorphic nature (Soufy *et al*., 2009).

The analysis of intron1 of myostatin gene using PCR-SSCP technique revealed five variants designated as A,B,C,D,E in case of New Zealand Romney sheep (Hickford *et al*., 2010). Similarly, attempt was made to study the polymorphism of myostatin gene

locus c.960deIG in Indonesian local sheep breeds by using PCR-SSCP studies but the result showed no polymorphism in this gene (Sumantri *et al*., 2011).

Polymorphism studies using PCR-RFLP in native Dalagh sheep breed of Iran revealed that exon 3 of myostatin gene was monomorphic (Azari *et al*., 2012).

A study of myostatin gene polymorphism in Zel sheep breed of Iran using PCR-SSCP techniques indicated intron 1 was monomorphic and intron 2 was polymorph whereas, PCR-RFLP studies of exon 3, showed that all were monomorphic (Dehnavi *et al.*, 2012).

Studies on myostatin gene in Makoei sheep of Iran was investigated by PCR SSCP. Four SSCP patterns, representing four different genotypes were identified (Farhadian *et al*., 2012).

SNP analysis of *GDF8* gene in a panel of 45 DNA samples that includes 9 sheep breeds (Muzaffarnagri, Malpura, Magra, Nali, Chokla, Deccani, Madgyal, Ganjam and Garole) revealed three SNPs, two in promoter and one in intron1 region of myostatin gene (Arora *et al*., 2013).

A study was conducted regarding genetic polymorphism of exon 3 fragment of myostatin gene in Egyptian and Saudi sheep breeds namely Barki, Ossimi, Rahmani , Najdi and Harri The results indicated all these breeds were monomorphic (Elkorshy *et al*., 2013).

Sequencing of promoter region of myostatin gene in Arabic and Kordi sheep breeds revealed three SNP at positions of 430,450 and 530 (Kamanagar *et al*., 2014)

In Meharbans sheep,.PCR-RFLP studies of exon 3 region of myostatin gene revealed two genotypes (Jamshidi *et al.*, 2014)

Molecular analysis of exon 3 fragment of myostatin genes in synthetic Bulgarian milk sheep population using PCR-RFLP revealed that myostatin gene was monomorphic (Georgieva *et al*.,2015).

Variation of intron 1 of ovine *GDF8* gene was observed with PCR-SSCP analysis and a total of six genotypes were observed in case of New Zealand Romney sheep (Ibrahim *et al*., 2015).

PCR-RFLP studies on amplification of 497 bp fragment of myostatin gene revealed two genotypes in Egyptian sheep breeds such as Barki, Rahmani and Osseimi (Mahrous *et al*., 2015).

Similarly in an attempt to identify polymorphism in coding sequence of *GDF8* in indigenous meat type sheep breeds, there are three polymorphic sites in the 5'UTR, one in exon 1 and one in the exon 2 regions. The mutations are located at c.539T > G and c.821T > A in the exon 1 and exon 2, respectively, (Pothuraju *et al*., 2015)**.**

Polymorphic studies of myostatin gene in Russian sheep breed of Dzhalginsky merino revealed 20 SNPs (Trukhachev *et al*., 2015).

The PCR-RFLP studies of exon 3 region of myostatin in Northeast Bulgarian Merino sheep breed revealed monomorphic pattern (Dimitrova *et al*., 2016).

Polymorphism of myostatin gene was determined by PCR-RFLP in Teleorman Black head lambs which revealed two genotypes of Mm, mm (Lazar *et al*., 2016).

Othman *et al*. (2016) conducted experiments on exon 3 fragment of myostatin gene in Egyptian sheep breeds of Barki, Rahmani and Ossimi and found them as monomorphic.

Similarly, genetic polymorphism and PCR-RFLP studies in sheep breeds such as Madras red, Mecheri and Nilagiri breeds of Tamilnadu indicate that these breeds were found to be monomorphic indicating myostatin gene is highly conserved and could be of use in evolutionary studies (Sahu *et al*., 2016).

Quirino *et al* . (2016) reported in the Santa Ines and crossbred (Santa Ines x Dorper) sheep breed of Brazil and found that there was no mutation for the myostatin

gene whereas the crossbred (Santa Ines x Dorper) sheep were carriers of this mutation in 3'UTR region.

A summary of PCR-RFLP studies on myostatin gene of different authors is presented in Table 1.

### 2.3 Association Studies of Myostatin gene in sheep

Two SNPs which were located in myostatin region had a significant association with muscle depth in case of commercial Charollais sheep (Hadjipavlou *et al*., 2008).

The impact of two known myostatin mutations was examined in field experiments with Norwegian white sheep and found that there was significant impact of both mutations on body conformation and fat (Boman *et al*., 2010).

Five variants (designated A-E) of the myostatin gene (*MSTN*) were obseved on analysis of randomly selected sheep from a variety of breeds and composite breeds in New Zealand (Hickford *et al*., 2010) and the study found that the presence of allele A in a lambs genotype was associated with decreased leg, loin and total yield of lean meat, whereas the presence of allele B was associated with increased loin yield and proportion loin yield. The effect of the number of allele copies present was investigated, and it was found that the absence of A, or the presence of two copies of B, was associated with increased mean leg yield, loin yield and total yield but associated with decreased shoulder yield, similarly two copies of A were associated with decrease in proportion of loin yield. However, no association with allele C was found.

PCR-SSCP polymorphism of intron 1 of myostatin gene in Baluchi sheep revealed three variants namely p1,p2 and p3, of which the p1 was known to be associated with breeding values of growth traits in Baluchi sheep (Ansary *et al*., 2011).

**Table 1. Summary of PCR-RFLP studies of *GDF8* gene in India and abroad**

| Amplified region | Restriction enzyme | Population | Monomorphic/Polymorphic (Genotypes) | References |
|---|---|---|---|---|
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Dalagh sheep of Iran | Monomorphic (mm) | Azari *et al*. (2012) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Zel sheep of Iran | Monomorphic (mm) | Dehnavi *et al*. (2012) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Egyptian and Saudi sheep breeds (Barki, Ossimi, Rahmani, Najdi and Harri) | Monomorphic (mm) | Elkorshy *et al*.(2013) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Mehraban sheep of Iran | Polymorphic (mm,Mm) | Jamshidi *et al*. (2014) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Synthetic Population Bulgarian Milk sheep | Monomorphic (mm) | Georgieva *et al*.(2015) |
| Myostatin fragment 497 bp fragment | *Dra*I | Barki, Rahmani and Osseimi (Egyptian sheep breeds) | Polymorphic (AB, BB) | Mahrous *et al*. (2015) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | North East Bulgarian Merino sheep | Monomorphic (mm) | Dimitrova *et al*. (2016) |
| Exon 3 of *GDF8* 337 bp fragment | *Hae*III | Teleorman Black Head lambs | Polymorphic (mm, Mm) | Lazar *et al*. (2016) |
| Part of 5'UTR, exon 1 and part of intron 1 | *Msp*I *and Hae*III | Madras Red, Mecheri and Nilagiri sheep breeds | Monomorphic for both | Sahu *et al*. (2016) |

Different polymorphisms of myostatin gene were identified in Zel sheep of Iran but no significant effect of genotypes of myostatin gene was observed on yearling weights (Dehnavi *et al*., 2012).

Polymorphism in ovine myostatin gene is associated with birth weight but not with weight gain in Iranian Makoei sheep (Farhadian *et al*., 2012).

Arora *et al*. (2013) observed three SNP in Indian sheep breeds and the presence of favourable alleles is known to be associated with increased fore quarter weight and mutton tenderness.

Ibrahim *et al*. (2015) reported that *GDF8* polymorphism is mainly associated with loin yield and percentage loin yield but has no effect on birth weight, weaning weight and growth rate in New Zealand Romney sheep.

Polymorphism of myostatin gene in Kurdi sheep of Northern Khorasan revealed the presence of three genotypes mm, Mm and MM. It was shown m allele was significantly associated with body weight (Akbari *et al*., 2015).

Trukhachev *et al.* (2015) conducted polymorphism studies in Dzhalginsky Merino sheep and found twenty SNPs of which three SNPs have negative effect on body parameters and three SNPs have no significant influence on this parameters.

Quirino *et al*. (2016) indicated that the heterozygote animals for the mutation had higher cold carcass weights and the animals carrying the mutation had also greater fat thickness in Santa Ines and cross bred sheep of Brazil.

## 2.4 Population parameters

Genotypic frequencies of MM, Mm and mm genotypes of myostatin gene were reported as 2%, 1.33% and 96.7% respectively and allele frequencies as 3% and 97% for M and m alleles in case of Sanjabi sheep breed of Iran, but the population was not in HWE (Soufy *et al*., 2009).

Polymorphic study in New Zealand Romney sheep identified six genotypes, which were AA (46.6%), AB (30.2%), AC (13.3%), BB (5.8%), BC (3.5%) and CC (0.6%) with allele frequencies of 68.3%, 22.7% and 9% for A, B and C respectively (Hickford *et al*., 2010).

Polymorphism of myostatin gene in Zel sheep revealed that intron 2 was polymorph and three genotypes were found which were AA(73,5%), AB(4%), BB (22.5%) and the allelic frequencies of A and B were found to be 0.04 and 0.37, respectively and the population at this locus was not in accordance to HWE (Dehnavi *et al*., 2012).

The PCR-SSCP analysis in Iranian Makoei sheep revealed the presence of five alleles and allele frequencies were found to be 0.4185, 0.0815, 0.2283, 0.2065, and 0.0652 for A, B, C, D and E, respectively. Four genotypes were observed with genotypic frequencies of 0.413, 0.293, 0.130 and 0.163 for AD, AC, AE, and BC respectively (Farhadian *et al*., 2012).

Jamshidi *et al.* (2014) conducted polymorphic studies in Mehraban sheep of Iran and observed two genotypes Mm (0.05), and mm (0.947). The allelic frequencies were 0.97 and 0.03, respectively for m and M alleles, The HWE in the population under study was not established.

PCR-SSCP analysis of intron1 of *GDF8* in New Zealand Romney lambs reported the presence of six genotypes AA (0.111), AB (0.367), AC (0.1), BB (0.288), BC (0.128) and CC (0.006) representing three alleles A, B, and C with frequencies of 0.34, 0.54 and 0.12, respectively (Ibrahim *et al* ., 2015)

PCR-RFLP studies of myostatin gene in Egyptian sheep breeds observed the presence of two genotypes AB and BB and the highest heterozygosity observed (0.45) was in Barki sheep. The lowest values, 0.15 were in Osseimi, but Rahmani has

moderate value of 0.35 for ($H_o$). The frequency of the B allele was significantly higher than that of A allele, especially in Osseimi breed (Mahrous *et al*., 2015).

Polymorphic study of myostatin gene by PCR-RFLP in Teleorman Black Head lambs revealed two genotypes of mm (16.67%) and Mm (83.33%). The M allele frequency was 42% and m allele frequency was 58%. Observed values of myostatin genotypes were found in Hardy Weinberg Disequilibrium (Lazar *et al*., 2016).

On perusal of literature it was observed that no polymorphism or association studies were available on sheep genetic groups of Andhra Pradesh. Hence, an effort was made to understand the nature (monomorphic/polymorphic) of the myostatin gene in some of the genetic groups of Andhra Pradesh.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Experimental Animals

Blood samples were collected from 25 Nellore Brown sheep (Figure 1) breed from the Livestock Research Station (LRS), Siddarampuram, 28 Nellore Jodipi sheep (Figure 2) breed from Livestock Research Station, Palamaner. Blood samples from 47 Macherla Brown sheep (Figure 3) were collected from their home tract from different farmer flocks. Care was taken to collect blood samples from unrelated individuals.

### 3.1.2 Chemicals

Following are the chemicals used in the present study-

- Ammonium chloride (Himedia)
- Boric acid (Himedia)
- Chloroform (Fisher Scientific)
- Ethidium bromide (Himedia)
- Ethyl alcohol (Jiangsu Huaxi)
- Ethylene Diamino Tetra Acetic acid (EDTA) (Himedia)
- *Hae*III Restriction enzyme (Thermo scientific)
- *Hpy*CH4V Restriction enzyme (New England Biolabs)
- Iso amyl alcohol (Fisher Scientific)
- Magnesium chloride (Himedia)
- Nuclease free water (Himedia)
- Potassium chloride (Merck)
- Primers (Bioserve)
- Proteinase-k (Himedia)
- Sodium acetate (Himedia)

**Figure 1. Nellore Brown Ewe and Ram from LRS, Siddarampuram**

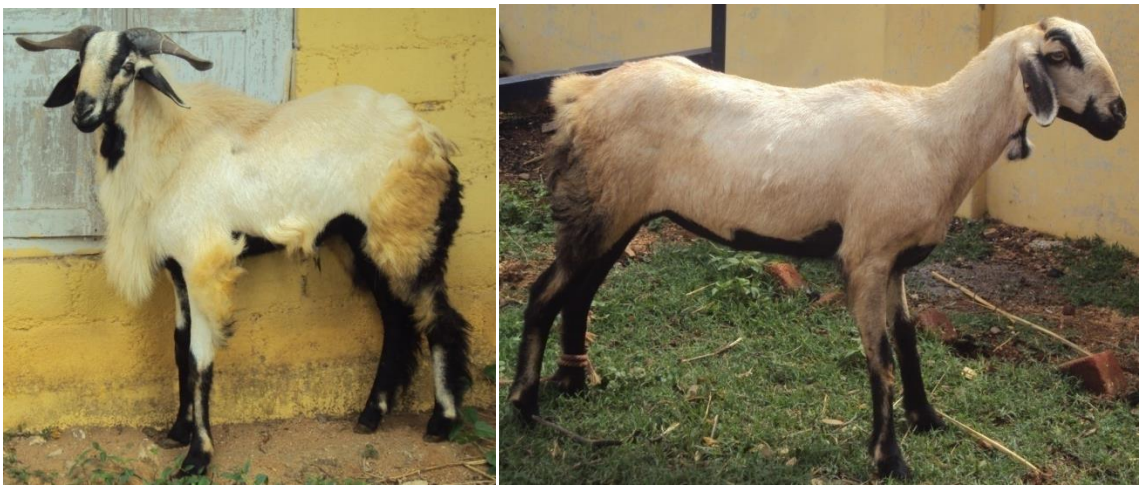

**Figure 2. Nellore Jodipi Ram and Ewe from LRS, Palamaner**

**Figure 3. Macherla Brown Ram and Ewe from its home tract**

- Sodium chloride (Himedia)
- Sodium Dodecyl Sulphate (Himedia)
- Tris hydroxyl methyl amino methane hydrochloride (Himedia)
- Ultra Pure Agarose (Invitrogen)
- 100 bp ladder (Promega)
- 50 bp ladder (Thermo scientific, promega)
- 25 bp ladder (Bio basic inc)

## 3.2 Methods

### 3.2.1 Sample collection, transport and storage

Approximately 4 ml of venous blood was collected from Jugular vein of the animal in a 5 ml vacutainer. Immediately after collection, samples are stored on ice. The samples were then transported to the lab and stored at –20$^{o}$ C.

### 3.2.2 Preparation of Solutions / Reagents

The compositions of different solutions / reagents used in DNA isolation were as follows:

**EDTA (10 per cent ) solution**

| | | |
|---|---|---|
| Ethylene Diamine Tetra Acetic Acid (EDTA) | : | 1g |
| Triple glass distilled water to make up to | : | 10 ml |

**RBC Lysis Buffer**

| | | |
|---|---|---|
| Ammonium chloride | : | 8.0235 g |
| Potassium chloride (10 mM) | : | 0.7455 g |
| EDTA | : | 0.0372 g |

Triple glass distilled water to make up to 1000 ml and autoclaved and stored at 4$^{0}$C.

**Tris Buffer Saline**

| | | |
|---|---|---|
| Potassium chloride | : | 0.0373 g |
| Tris (hydroxymethyl) aminomethane HCl | : | 0.0303 g |
| Magnesium chloride (10 mM) | : | 1 ml |
| Sodium chloride (0.4 M) | : | 8.1800 g |
| Triple glass distilled water to make up | : | 1000 ml |

pH adjusted to 7.4 and autoclaved and stored at $4^0$C.

**Sodium Dodecyl Sulphate (10 per cent SDS)**

| | | |
|---|---|---|
| Sodium dodecyl sulphate | : | 10 g |
| Triple glass distilled water to make up to | : | 100 ml |

**Proteinase – k**

| | | |
|---|---|---|
| Proteinase – k | : | 20 mg |
| Triple glass distilled water to make up to | : | 1 ml |

Stored at - 20°C

**Saturated Sodium Chloride**

| | | |
|---|---|---|
| Sodium chloride | : | 29.22 g |
| Triple glass distilled water to make up to | : | 100 ml |

**TE buffer (DNA Storage buffer)**

| | | |
|---|---|---|
| Tris (1M, pH 8.0) | : | 1 ml |
| EDTA (0.5M, pH 8.0) | : | 200 µl |

Triple glass distilled water to make up to 100ml and autoclaved and stored at room temperature.

**Tris-EDTA buffer (TE buffer pH 8.0)**

| | | | |
|---|---|---|---|
| Tris (hydroxymethyl) aminomethane HCl (10 mM) | pH 7.6 | : | 1.2114 g |
| EDTA(0.1 mM) | | : | 0.3722 g |

Triple glass distilled water to make up to 1000 ml. pH adjusted to 8.0

Autoclaved and stored at $4^0$C.

**Ethanol (95 per cent)**

| | | |
|---|---|---|
| Ethanol 99.9 per cent | : | 95 ml |
| Distilled water | : | 5 ml |

**Ethanol (70 per cent)**

| | | |
|---|---|---|
| Ethanol 99.9 per cent | : | 70 ml |
| Distilled water | : | 30 ml |

**0.5 M EDTA**

| | | |
|---|---|---|
| EDTA | : | 18.612g |
| Triple glass distilled water to make up to | : | 100 ml |

**Chloroform and Isoamyl Alcohol Solution**

| | | |
|---|---|---|
| Chloroform | : | 24 ml |
| Isoamyl alcohol | : | 1 ml |

**Preparation of 10X TBE buffer (stock) 500 ml**

| | | | |
|---|---|---|---|
| Tris base | | : | 54 g |
| EDTA | - | : | 4.65 g |
| Boric acid | - | : | 27.5 g |

Make up to 500 ml with triple distilled water.

### 3.2.3 DNA Extraction

#### 3.2.3.1 Isolation of genomic DNA from whole blood

DNA was isolated from blood samples using a modified high salt method (Miller *et al*., 1988; Aravindakshan *et al.*, 1997).

The steps followed for isolation of DNA from blood samples are given below,

i. Three ml of blood and nine ml of RBC lysis buffer were taken in a 15 ml of centrifuge tube. This tube was inverted several times and incubated in ice with shaking for 10 minutes for complete lysis of red blood cells. Nuclear material was pelleted by centrifugation at 4000 rpm at room temperature for 10 minutes and discarded the supernatant.

ii. Nuclear pellet was washed with 10 ml of RBC lysis buffer and centrifuged once again. This step was repeated for three to four times till the clear nuclear pellet was obtained.

iii. Nuclear pellet was resuspended in 10 ml Tris buffer saline (PH 7.4) and centrifuged at 3000 rpm for 10 minutes. The pellet was collected and the procedure was repeated 3 times.

iv. 250 µl of TE buffer (PH 8.0) was added to the pellet and vortexing was performed. To this tube 5 µl of proteinase-k and 12 µl of 0.5M EDTA were added and mixed

thoroughly. To this solution 500 µl of SDS was added with gentle mixing and incubated overnight at 55 $^0$C. After incubation 150 µl of saturated NaCl was added and shaken vigorously. To this mixture equal volume of Chloroform: Isoamylalcohol (24:1) was added, mixed and centrifuged at 13,000 rpm for 15 minutes at 24 $^0$C.

v. Aqueous upper phase was transferred to fresh tube without disturbing the interphase.

vi. Finally DNA was precipitated by adding double the volume of 95 per cent ethanol and 1/3 th volume of 3M Sodium Acetate to the supernatant collected and inverted several times until the DNA was precipitated and kept at 4 $^0$C for 2 hours and centrifuged at 13,000 rpm for 30 minutes at 4 $^0$C.

vii. The supernatant was discarded and 300 µl of 70 per cent ethanol was added and centrifuged at 13,000 rpm for 5 minutes at room temperature.

viii. The supernatant was discarded and the pellet was air dried.

ix. The dried DNA pellet was resuspended in 300 µl of TE buffer (pre warmed) and kept at 37 $^0$C for 10 minutes for dissolution.

x. The resuspended pellet was stored at -20 $^0$C until further use.

**3.2.3.2 Checking the presence and purity of DNA**

On extraction of DNA, existence and concentration was checked by agarose gel electrophoresis. Agarosoe gel of 0.8 per cent was prepared by boiling agarose in 1X TBE buffer. The prepared gel was poured into an electrophoresis plate and left at room temperature for about 30 minutes for polymerization. One µl of 6x loading buffer (bromophenol blue dye) and 6 µl of distilled water were mixed and then loaded into the wells of the gel. The DNAs were run on agarose gel at 90 V for about 40 minutes in 1x TBE buffer, stained in 0.5 µl/ml ethidium bromide solution and was visualized under UV light. DNA was quantified by using NanoDrop™ 2000/2000c (Thermo Fisher Scientific) using the convention that 1 absorbance unit at 260 nm equals 50µg per ml. Purity of

DNA was judged on the basis of OD ratio at 260:280 and samples having the acceptable purity i.e. 1.7 to 2.0 were used for further analysis.

### 3.2.4 PCR amplification of Myostatin

#### 3.2.4.1 Primer design and genotypic profiles

Two sets of primers to amplify regions of exon 3 and intron 1 of myostatin gene were used in the present study. Published primers from previous studies (Azari *et al*.,2012; Dehnavi *et al*., 2012) were used for primer BLAST to confirm the primer complimentarity for exon 3 region of myostatin gene. Based on the primer BLAST results the published primer sequences were modified so as to amplify the ovine genomic DNA unambiguously. The primer sequences were given in Table 2. The targeted region with the RFLP site is given in Figure 4 and Figure 5.The expected allelic profile is as follows, if there is a mutation that generate restriction site for *Hae*III enzyme then the allelic profile will be three fragments with sizes 125bp 118bp and 94bp, which is referred as *mm* genotype. In the absence of mutation *Hae*III enzyme cannot cleave the fragment and then give rise to only 337bp fragment, which is referred as *MM* genotype. Heterozygote will include a combination of allelic profiles referred as *Mm* genotype.

To amplify intron 1 region, the ENSEMBL database was screened for SNPs and the mutations were analyzed for possible RFLP pattern. In intron 1 a mutation g.118144833G>T (rs119102825) is forming restriction site for *Hpy*CH4V enzyme. Hence a PCR-RFLP assay was designed using *Hpy*CH4V restriction enzyme for the mutation in intron 1 of *GDF8*. Primers were designed to amplify the region using Primer 3 program (Untergasser *et al*., 2012). The targeted region with the RFLP site is given in Figure 5. The primer details are given in Table 2. The primers were designed in such a way that there is an internal restriction site control for the fragment. If there is a mutation in intron 1 that generate cleavage site for *Hpy*CH4V, then it gives three fragments 269bp, 115bp and 74bp. The genotype is represented as *hh* in this study. If there is no mutation,

**Table 2. Details of Primers to amplify region of *GDF8* gene**

| Primer | Sequence | Tm | GC % | Length | Amplicon length | Accession No. |
|---|---|---|---|---|---|---|
| Exon3_F | **5'**TAGGAGAGATTTTGGGCTTGA**3'** | 50 | 42.9 | 21 | 337 | NC_019459 |
| Exon3_R | **5'**TCATGAGCACCCACAGCGATC**3'** | 56 | 57.1 | 21 | | |
| Intron1_F | **5'**GCTTTTTAGCTCTCTAAGGAAACA**3'** | 52 | 37.5 | 24 | 458 | NC_019459 |
| Intron1_R | **5'**TGCTTGTGGAGACAAAACAAT**3'** | 49 | 38.1 | 21 | | |

TAGGAGAGATTTTGGGCTTGATTGTGATGAGCACTCCACAGAATCTCGATGC
TGTCGTTACCCTCTAACTGTGGATTTTGAAGCTTTTGGATGGGATTGGATTAT
TGCACCTAAAAGATATAA**GGCC**AATTACTGCTCTGGAGAATGTGAATTTTT
ATTTTTGCAAAAGTATCCTCATACCCATCTTGTGCACCAAGCAAACCCCAAA
GGTTCAGCC**GGCC**CTTGCTGTACTCCTACAAAGATGTCTCCAATTAATATGC
TATATTTTAATGGCAAAGAACAAATAATATATGGGAAGATTCCAGGCATGG
TAGTAGATCGCTGTGGGTGCTCATGA

**Figure 4. Target Sequence of a part of exon 3 of *GDF8* locus in sheep** (Highlighted regions are the HaeIII restriction enzyme cleavage sites)

TGCTTGTGGAGACAAAACAATAAATCCTCAAGACTAGAAGCCATAAAAATC
CAAATCCTCAGTAAGCTTCGCCTGGAAACAGCTCCTAACATCAGCAAAGAT
GCTATAAGACAACTTTTGCCCAAGGCTCCTCCACTCCGGGAACTGATTGATC
AGTACGATGTCCAGAGAGATGACAGCAGCGACGGCTCCTTGGAAGACGATG
ACTACCACGTTACGACGGAAACGGTCATTACCATGCCCACGGAGTGTGAGT
AGTTCTGCTAG**G**G**GCA**GAGCAACGACTCTGCTGACTGCTGTTCTAGTGTTCAT
GAGAAACCGATCTATTTTCAGGCTCTTTTAACAAGCTGCTGGCTTGTACGTA
AGGAGGAGGGCAAAGAGCTTTT**TGCA**AGACTTCATGAGAAATATGCTAATG
AGACTGAAAGCTGCTACATTATCTGTTTCCTTAGAGAGCTAAAAAGC

**Figure 5. Target region to amplify mutation in Intron I of *GDF8* locus in sheep** (Highlighted regions are the *Hpy*CH4V restriction enzyme cleavage sites; The site in red colour indicates putative SNP)

then the profile will be 384bp and 74bp, referred as *HH*. If the individual is heterozygous for the mutation then the allelic profile will be a combination of the two profiles and is referred as *Hh*.

The custom designed desalted primers were obtained from Bioserve India Pvt. Ltd., Hyderabad.

**3.2.4.2 Primer dilution**

The primer stock solutions were prepared as per manufacturer's instruction and a working primer solution of 10µM is prepared.

**3.2.4.3 PCR protocol and reaction conditions**

DNA samples isolated from 100 sheep were amplified for exon 3 region and, out of which 65 samples were amplified for intron 1 region in the present study. PCR was set up in a total reaction volume of 15 µl. Fourteen µl of reaction volume comprising 2x master mix, nuclease free water and forward and reverse primers which was aliquoted in each PCR tube and one µl of template was added to each tube to make the final volume. Each reaction volume contained.

| **PCR Components** | | **Volume** |
|---|---|---|
| DNA Template | - | 1.0 µl |
| Primer Forward (10 pmoles/µl) | - | 0.5 µl |
| Primer Reverse (10 pmoles/µl) | - | 0.5 µl |
| 2 x PCR Master Mix | - | 7.5 µl |
| Nuclease free water | - | 5.5 µl |
| Total | - | 15.0 µl |

PCR mix was prepared for one additional sample to cover pipetting error. The PCR conditions were optimized by setting different time-temperature combinations for annealing and extension process. PCR was carried out in total volume of 15µl in a 200 µl PCR tube. The combination that gave best result, in terms of yield and specificity of the product was further used for amplification. PCR was carried out in a thermal cycler (Eppendorf, Master cycler TM, Germany).

The conditions that were employed were initial denaturation at 94 $^0$C / 2 min, denaturation at 94 $^0$C / 30 sec, annealing at 54$^0$C / 30 sec, extension at 72 $^0$C / 1 min for 34 cycles and final extension at 72 $^0$C / 10 min for amplification of exon 3 and initial denaturation at 94$^0$C / 2 min, denaturation at 94$^0$C / 45 seconds, annealing at 52$^0$C/ 45 seconds, extension at 72$^0$C/ 2 min for 34 cycles and final extension at 72$^0$C/ 10 min for intron 1

### 3.2.5 Agarose gel electrophoresis

The PCR products were verified for amplification by electrophoresing them on 1.5 % agarose gel. The agarose gel was prepared by boiling 0.6 gm of agarose in 40 ml of 1X TBE. Electrophoresis was carried out at 110V for approximately 45 minutes.

### 3.2.6 RFLP genotyping

The PCR products were subjected to RFLP with *Hae*III enzyme for exon 3 and *Hpy*CH4V for intron 1 fragment of *GDF8*. *Hae*III enzyme cleaves after GG in the recognition site GGCC while *Hpy*CH4V cleaves after TG in TGCA recognition site.

#### 3.2.6.1 Standardised protocol used for digestion of PCR products

The *Hae*III restriction enzyme digestion was set up in 15µl reaction volume as follows

| Components | Amount |
|---|---|
| 1) Restriction enzyme (10 units/µl) | 0.5µl |
| 2) 10x red buffer | 1.5µl |
| 3) PCR product | 8µl |
| 4) Nuclease free water | 5µl |

The reaction mix was incubated at 37 $^{0}$C in water bath for overnight. The digested product was then electrophoresed on 2.5% agarose gel for genotyping.

The *Hpy*CH4V restriction enzyme digestion reaction was also setup in 10µl reaction volume as follows-

| Components | Amount |
|---|---|
| 1) Restriction enzyme (10 units/µl) | 0.2µl |
| 2) 10x red buffer | 1.0µl |
| 3) PCR product | 3µl |
| 4) Nuclease free water | 5.8µl |

Incubation time/temperature maintained was 37 ˚C for 6 hours in water bath. The digested product was then electrophoresed on 2 % agarose gel for genotyping.

### 3.2.7 Population genetic analysis

#### 3.2.7.1 Allele and genotype frequencies

PCR-RFLP is a bi allelic system and hence, the maximum possible genotypes are three. From the gels the genotypes were scored directly as homozygous or heterozygous. The genotype frequencies are calculated as follows-

$$\text{Genotype frequency} = \frac{\text{Proportion of animals with a particular genotype}}{\text{Total number of animals}}$$

Based on the genotypes, the allele frequencies were estimated as

$$\text{Frequency of an allele(p)} = \frac{2(\text{Number of homozygotes}) + (\text{Number of heterozygote})}{2(\text{Total Number of individuals})}$$

The expected and observed number of genotypes for individual populations was

calculated using Genepop (v4.2) on the web software package (Raymond and Rousset 1995).

### 3.2.7.2 Testing for Hardy-Weinberg equilibrium and population differentiation

The polymorphic locus was used to test for equilibrium in each of the population using Genepop (v4.2). The null hypothesis is that the gametes unite at random. The population differentiation is tested using $F_{IS}$ values Weir and Cockerham (1984) and Robertson and Hill (1984).

### 3.2.8 Association studies

An attempt was made to study the genotype association with body weights at birth, 3 months, 6 months and 9 months of age in Nellore sheep. ANOVA was performed to observe the significant differences of body weights between the genotypes of Nellore sheep breed.

# CHAPTER IV

# RESULTS

## 4.1 Blood Samples and Phenotypic Data

To study the polymorphism of *GDF8* gene of sheep in exon 3 and intron 1 a total of 100 blood samples from unrelated individuals were collected. Twenty five sheep belong to Nellore Brown located in Livestock Research Station (LRS), Siddarampuram, 28 sheep belong to Nellore Jodipi sheep located in Livestock Research Station (LRS), Palamaner and 47 Macherla Brown genetic group of sheep were used in the present study. Phenotypic information on body weights at birth, 3 months, 6 months, 9 months and 12 months age were collected for the samples collected from both the Livestock Research Station, Palamaner and Siddarampuram and is presented in Tables 3 and 4. The Macherla Brown sheep samples were collected from the home tract of the genetic group from farmers at field level from different flocks by enquiring farmers regarding unrelated individuals from flock. There was no systemic data on body weights at different ages available for this genetic group.

## 4.2 Isolation of Genomic DNA

Genomic DNA was isolated from the blood by high salt method (Miller *et al*., 1988) (Figure 6). The quality and concentration of genomic DNA isolated was shown in Table 5. The DNA concentration was in the range of 20 ng/µl to 765 ng/µl. Purity of the DNA was assessed by absorbance at 260/280 nm was in the range of 1.24 to 2.2 and majority of them were in the range of 1.4 to 2 (Table 5).

**Table 3. Body Weights (Kg) of Nellore Jodipi sheep collected at different ages from LRS, Palamaner**

| S. No. | Animal No. | D.O.B | Sex | Birth wt. | Wt. at 3 months | Wt. at 6 months | Wt. at 9 months | Wt. at 12 months |
|---|---|---|---|---|---|---|---|---|
| 1 | F-528 | 23-06-15 | F | 3.0 | 16.0 | 18.5 | 21.0 | 24.0 |
| 2 | F-626 | 19-09-15 | F | 4.0 | 14.0 | 18.5 | 22.5 | 24.5 |
| 3 | N-908 | 18-01-10 | F | 3.2 | 13.0 | 18.5 | 21.5 | 23.0 |
| 4 | F-376 | 15-11-14 | M | 3.2 | 13.0 | 18.0 | 22.0 | 24.5 |
| 5 | F-117 | 01-10-14 | M | 3.9 | 12.5 | 17.1 | 20.0 | 25.0 |
| 6 | E-664 | 17-10-12 | F | 3.5 | 15.2 | 20.0 | 25.0 | 27.5 |
| 7 | F-540 | 25-07-15 | F | 3.5 | 14.5 | 16.0 | 22.5 | 26.8 |
| 8 | F-221 | 26-09-14 | M | 3.0 | 16.8 | 23.0 | 26.5 | 29.8 |
| 9 | F-304 | 16-10-14 | M | 3.5 | 15.2 | 22.8 | 25.0 | 29.0 |
| 10 | F-566 | 31-07-15 | F | 3.5 | 13.8 | 16.2 | 21.0 | 26.6 |
| 11 | F-532 | 24-06-15 | F | 2.8 | 15.8 | 19.5 | 22.0 | 25.5 |
| 12 | F-288 | 10-09-14 | M | 3.0 | 16.5 | 25.0 | 28.0 | 31.0 |
| 13 | F-87 | 25-12-13 | M | 2.8 | 15.2 | 21.5 | 22.5 | 29.0 |
| 14 | E-946 | 21-10-13 | F | 3.8 | 19.2 | 23.4 | 25.1 | 26.5 |
| 15 | E-163 | 26-12-10 | F | 3.0 | 14.5 | 19.5 | 23.0 | 25.0 |
| 16 | RA-91 | 23-09-14 | F | 2.8 | 15.0 | 19.0 | 24.0 | 27.0 |
| 17 | F-602 | 17-08-15 | F | 3.2 | 14.0 | 18.7 | 21.5 | 25.0 |
| 18 | N-909 | 19-01-10 | F | 3.2 | 11.5 | 17.0 | 20.0 | 22.0 |
| 19 | F-672 | 10-01-13 | M | 3.2 | 18.5 | 26.5 | 29.0 | 31.0 |
| 20 | E-695 | 16-05-15 | F | 2.9 | 15.0 | 18.5 | 23.5 | 26.0 |
| 21 | F-27 | 19-05-14 | F | 3.0 | 15.0 | 20.5 | 24.0 | 28.0 |
| 22 | F-282 | 10-09-14 | M | 3.0 | 15.4 | 24.2 | 27.0 | 29.0 |
| 23 | E-78 | 13-10-10 | F | 2.5 | 12.0 | 15.0 | 23.0 | 25.0 |
| 24 | F-216 | 25-09-14 | F | 3.2 | 21.0 | 24.4 | 26.5 | 29.6 |
| 25 | F-260 | 10-05-14 | M | 3.3 | 19.0 | 27.8 | 30.0 | 31.0 |
| 26 | E-350 | 26-09-11 | F | 2.8 | 10.8 | 14.0 | 19.0 | 23.5 |
| 27 | F-193 | 20-09-14 | M | 3.3 | 19.0 | 25.8 | 27.0 | 28.0 |
| 28 | F-204 | 24-09-14 | M | 3.5 | 19.4 | 25.4 | 27.5 | 29.0 |

**Table 4. Body Weights (Kg) of the Nellore Brown sheep collected at different ages from LRS, Siddarampuram**

| S. No. | Animal No. | D.O.B | Sex | Birth wt. | Wt. at 3 months | Wt. at 6 months | Wt. at 9 months |
|---|---|---|---|---|---|---|---|
| 1 | 201 | 12-12-10 | F | 3.0 | 13.5 | 15.5 | 18.5 |
| 2 | 250 | 11-02-11 | F | 3.5 | 16.5 | 18.5 | 19.5 |
| 3 | 113 | 16-07-10 | F | 3.0 | 8.5 | 10.0 | 19.0 |
| 4 | 204 | 16-12-10 | F | 3.0 | 14.5 | 15.5 | 17.0 |
| 5 | 112 | 13-07-10 | F | 2.7 | 9.0 | 18.0 | 19.0 |
| 6 | 96 | 02-05-10 | F | 3.0 | 9.6 | 10.0 | 16.5 |
| 7 | 110 | 07-07-10 | F | 2.6 | 9.3 | 13.5 | 17.5 |
| 8 | 108 | 02-11-10 | F | 3.5 | 8.2 | 10.0 | 14.0 |
| 9 | 233 | 10-04-11 | F | 2.8 | 13.5 | 14.5 | 17.0 |
| 10 | 121 | 26-10-10 | F | 3.0 | 10.5 | 14.5 | 18.3 |
| 11 | 89 | 25-01-10 | F | 3.5 | 10.5 | 12.5 | 17.5 |
| 12 | 84 | 19-01-10 | F | 2.5 | 10.0 | 12.0 | 18.5 |
| 13 | 221 | 10-11-11 | F | 3.5 | 15.5 | 16.5 | 17.5 |
| 14 | 174 | 21-07-10 | F | 2.8 | 13.5 | 15.5 | 17.5 |
| 15 | 91 | 27-01-10 | F | 2.6 | 9.0 | 13.5 | 16.5 |
| 16 | 99 | 02-07-10 | F | 3.5 | 10.5 | 14.5 | 18.5 |
| 17 | 244 | 13-10-11 | F | 3.5 | 14.5 | 16.5 | 17.5 |
| 18 | 122 | 27-10-10 | F | 2.9 | 9.5 | 11.0 | 20.2 |
| 19 | 246 | 14-10-11 | F | 2.8 | 14.5 | 16.5 | 17.0 |
| 20 | 114 | 20-07-10 | F | 3.5 | 8.8 | 9.5 | 18.0 |
| 21 | 207 | 25-12-10 | F | 3.0 | 15.5 | 16.5 | 18.5 |
| 22 | 203 | 12-08-10 | F | 2.8 | 13.5 | 15.5 | 16.5 |
| 23 | 98 | 02-06-10 | F | 3.0 | 8.2 | 13.5 | 18.2 |
| 24 | 194 | 24-11-10 | F | 3.5 | 12.5 | 15.5 | 17.5 |
| 25 | 150 | 30-10-10 | F | 3.5 | 7.5 | 16.2 | 18.5 |

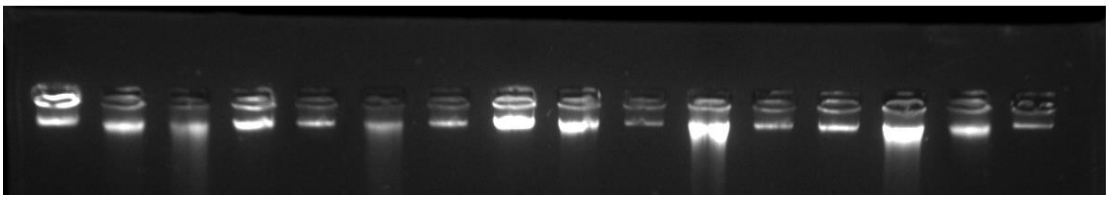

**Figure 6. Genomic DNA isolated from a subset of sheep used in the study on 1% agarose gel**

**Table 5. Quality and quantity of genomic DNA isolated from sheep blood samples**

| S. No. | Animal no | λ26o | λ 260/280 | Concentration of DNA (ng/µl) |
|---|---|---|---|---|
| ***Nellore Jodipi*** | | | | |
| 1 | F-566 | 2.128 | 1.87 | 106.4 |
| 2 | F-532 | 1.149 | 1.92 | 057.5 |
| 3 | N-909 | 1.319 | 1.9 | 065.9 |
| 4 | F-540 | 2.577 | 1.84 | 128.8 |
| 5 | E-664 | 0.54 | 2.2 | 027.0 |
| 6 | F-528 | 0.48 | 1.62 | 024.0 |
| 7 | F-626 | 2.445 | 1.87 | 122.3 |
| 8 | N-908 | 0.9 | 1.42 | 045.0 |
| 9 | F-376 | 0.42 | 2.12 | 021.0 |
| 10 | F-117 | 2.067 | 1.86 | 103.3 |
| 11 | F-221 | 0.72 | 1.5 | 036.0 |
| 12 | F-304 | 1.321 | 1.97 | 066.1 |
| 13 | F-288 | 1.22 | 1.32 | 061.0 |
| 14 | F-87 | 0.46 | 1.27 | 023.0 |
| 15 | E-946 | 1.361 | 1.52 | 068.0 |
| 16 | E-163 | 3.713 | 1.88 | 185.7 |
| 17 | RA-91 | 1.075 | 1.97 | 053.7 |
| 18 | F-602 | 1.24 | 1.95 | 062.0 |
| 19 | F-672 | 4.778 | 1.77 | 238.9 |
| 20 | E-695 | 2.687 | 1.91 | 134.3 |
| 21 | F-27 | 0.44 | 2.16 | 022.0 |
| 22 | E-78 | 0.833 | 2.06 | 041.6 |
| 23 | F-216 | 2.696 | 1.32 | 134.8 |
| 24 | F-260 | 1.791 | 1.52 | 089.5 |
| 25 | E-350 | 2.828 | 1.65 | 141.4 |
| 26 | F-193 | 0.885 | 2.04 | 044.3 |
| 27 | F-204 | 1.73 | 1.68 | 086.5 |
| 28 | F-282 | 1.92 | 1.64 | 096.0 |
| ***Nellore brown sheep*** | | | | |
| 29 | 204 | 14.5 | 1.51 | 725.0 |
| 30 | 112 | 5.43 | 1.3 | 271.5 |
| 31 | 96 | 0.408 | 1.75 | 020.4 |
| 32 | 110 | 0.48 | 2.2 | 024.0 |

Contd...,

| | | | | |
|---|---|---|---|---|
| 33 | 108 | 6.77 | 1.68 | 338.9 |
| 34 | 233 | 0.4 | 1.36 | 020.0 |
| 35 | 89 | 0.476 | 1.49 | 023.8 |
| 36 | 84 | 0.54 | 1.24 | 027.0 |
| 37 | 221 | 3.676 | 1.55 | 183.8 |
| 38 | 174 | 5.091 | 1.5 | 382.0 |
| 39 | 91 | 0.522 | 2.2 | 026.1 |
| 40 | 99 | 0.42 | 1.35 | 021.0 |
| 41 | 244 | 15 | 1.55 | 750.0 |
| 42 | 246 | 14.5 | 1.62 | 725.0 |
| 43 | 114 | 0.52 | 2.1 | 026.0 |
| 44 | 207 | 7.803 | 1.48 | 390.1 |
| 45 | 203 | 0.48 | 1.75 | 024.0 |
| 46 | 98 | 5.5 | 1.43 | 275.0 |
| 47 | 194 | 0.506 | 2.15 | 025.3 |
| 48 | 150 | 3.084 | 1.6 | 154.2 |
| 49 | 121 | 0.512 | 2.1 | 025.6 |
| 50 | 201 | 6.2 | 1.56 | 310.0 |
| 51 | 250 | 7.3 | 1.45 | 365.0 |
| 52 | 122 | 0.414 | 1.39 | 050.7 |
| 53 | 113 | 1.935 | 1.26 | 096.8 |
| ***Macherla brown sheep*** | | | | |
| 54 | m5 | 10.9 | 1.73 | 545.0 |
| 55 | m38 | 1.719 | 1.5 | 086.0 |
| 56 | m47 | 14.38 | 1.7 | 719.0 |
| 57 | a pos | 15.3 | 1.51 | 765.0 |
| 58 | m51 | 12.84 | 1.6 | 642.0 |
| 59 | m19 | 2.181 | 1.29 | 109.1 |
| 60 | m9 | 14.46 | 1.49 | 723.0 |
| 61 | m37 | 5.267 | 1.5 | 263.4 |
| 62 | m45 | 0.502 | 1.65 | 025.1 |
| 63 | m58 | 12.86 | 1.93 | 643.2 |
| 64 | m28 | 1.78 | 1.76 | 089.0 |
| 65 | m41 | 3.871 | 1.52 | 193.6 |
| 66 | m55 | 6.169 | 1.53 | 308.4 |
| 67 | m26 | 1.53 | 1.36 | 076.5 |
| 68 | m16 | 15.04 | 1.83 | 752.0 |
| 69 | m23 | 1.48 | 1.42 | 074.0 |
| 70 | m40 | 2.749 | 1.6 | 137.4 |
| 71 | m48 | 1.583 | 1.62 | 079.1 |

Contd...,

| | | | | |
|---|---|---|---|---|
| 72 | m10 | 3.631 | 1.43 | 181.6 |
| 73 | m75 | 1.132 | 1.6 | 056.6 |
| 74 | m42 | 3.752 | 1.45 | 187.6 |
| 75 | m54 | 1.238 | 1.53 | 061.9 |
| 76 | m44 | 0.49 | 1.47 | 024.5 |
| 77 | m76 | 11.88 | 1.54 | 594.0 |
| 78 | m60 | 1.384 | 1.69 | 069.2 |
| 79 | m63 | 2.2 | 1.81 | 110.0 |
| 80 | m8 | 2.52 | 1.94 | 126.0 |
| 81 | m82 | 9.09 | 1.42 | 454.5 |
| 82 | m32 | 5.448 | 1.57 | 272.4 |
| 83 | m15 | 4.369 | 1.52 | 218.5 |
| 84 | b pos | 4.586 | 1.35 | 229.3 |
| 85 | m4 | 3.115 | 1.55 | 155.8 |
| 86 | m24 | 14.2 | 1.51 | 710.0 |
| 87 | m72 | 7.305 | 1.57 | 365.2 |
| 88 | m22 | 0.856 | 1.69 | 042.8 |
| 89 | m41 | 3.871 | 1.36 | 193.6 |
| 90 | m39 | 0.483 | 1.63 | 024.2 |
| 91 | m74 | 1.94 | 1.76 | 097.0 |
| 92 | m59 | 14.58 | 1.48 | 729.0 |
| 93 | d pos | 7.673 | 1.56 | 383.7 |
| 94 | m81 | 10.4 | 1.69 | 520.0 |
| 95 | m69 | 1.59 | 1.65 | 080.0 |
| 96 | m73 | 0.559 | 1.34 | 028.0 |
| 97 | c pos | 3,702 | 1.5 | 185.1 |
| 98 | m66 | 11.68 | 1.7 | 584.0 |
| 99 | m17 | 4.39 | 1.43 | 219.5 |
| 100 | m53 | 15.2 | 1.62 | 760.0 |

### 4.3 PCR amplification of *GDF8* loci

Primers designed were targeted to amplify two SNPs of *GDF8*, one located in exon 3 and the other located in intron 1 of the gene. The region targeting exon 3 was 337 bp and the region targeting intron 1 was 458 bp in length. Both the fragments were amplified unambiguously. A single clean product without primer dimer was obtained for both the fragments (Figures 7 and 8). A total of 100 samples were used for exon 3 amplification and 65 samples were used for intron 1 amplification. The 100 samples for exon 3 include 28 samples belonging to Nellore Brown, 25 samples to Nellore Jodipi and 47 samples to Macherla Brown. Sixty five samples that were used to amplify intron 1 region include 22 samples of Nellore Brown, 18 samples of Nellore Jodipi and 25 samples of Macherla Brown sheep. The Annealing temperatures maintained for exon 3 and intron 1 were $54^0$C and $52^0$C respectively.

### 4.4 PCR-RFLP

#### 4.4.1 Polymorphism of *GDF8* at exon 3

The exon 3 amplicons were digested with *Hae*III restriction enzyme to detect SNP. The digested products showed a pattern of three fragments, 125 bp, 118 bp and 94 bp (Figure 9). All the tested animals showed same pattern of allelic profile in the three populations indicating absence of polymorphism at exon 3 of *GDF8.*

#### 4.4.2 Polymorphism of *GDF8* at intron 1

The primers for the fragment were designed in such a way that it includes a control restriction enzyme site. Irrespective of presence or absence of the putative SNP, there will be a fragement of 74 bp, (Figure 10) the amplified intron 1 fragment were digested with *Hpy*CH4V restriction enzyme. The resultant profile showed all the three possible genotypes viz HH (269 bp, 115 bp, and 74 bp) in presence of mutation at the putative SNP in the intron 1 of *GDF-8*, the second profile showed 384 bp and 74 bp

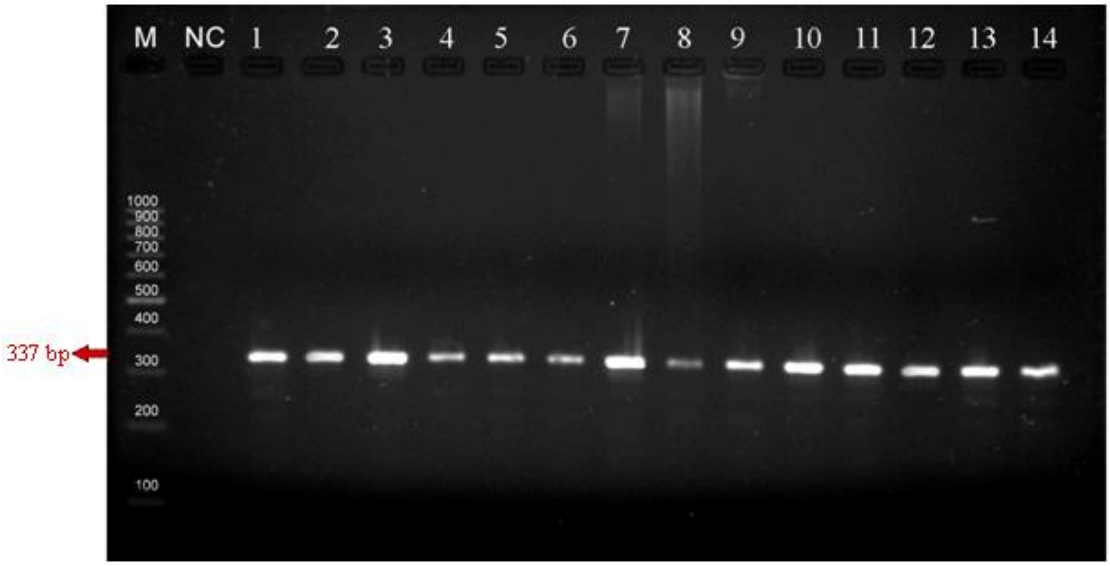


**Figure 7. Amplicons of exon 3, *GDF8*** (M: 100bp marker; NC: Negative Control; Lane 1-4: Nellore Jodipi Samples; Lane 5-8: Nellore Brown Samples; Lane 9-14: Macherla Brown samples)

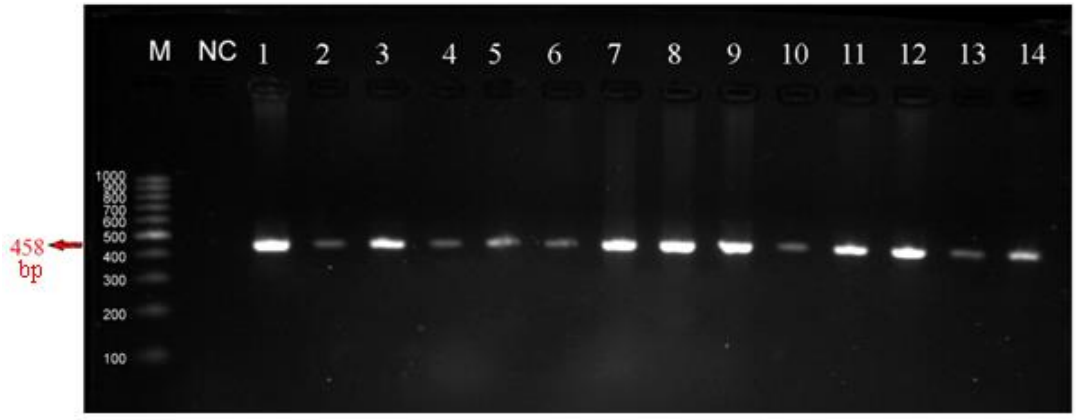


**Figure 8. Amplicons of intron 1, *GDF8*** (M: 100bp marker; NC: Negative Control; Lane 1-4: Nellore Jodipi Samples; Lane 5-8: Nellore Brown Samples, Lane 9-14: Macherla Brown samples)

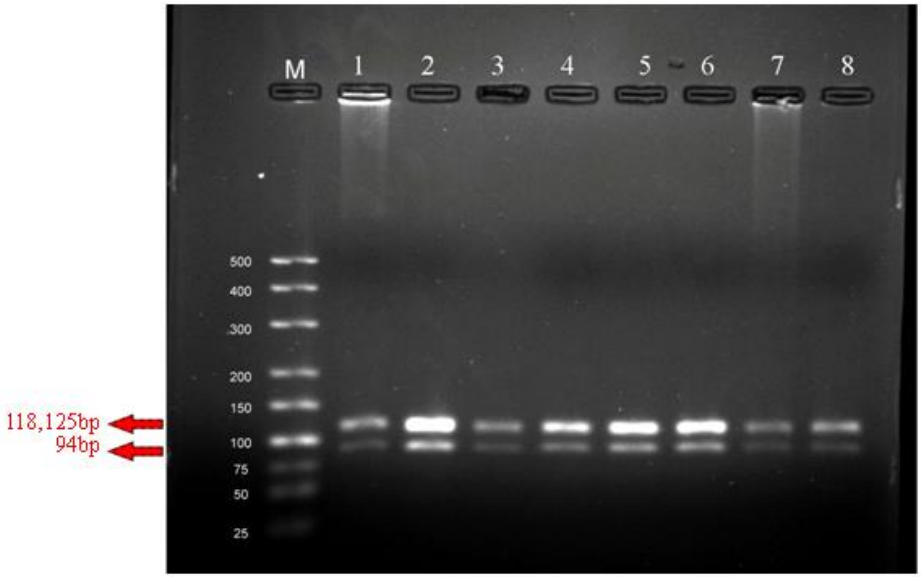


**Figure 9 : Photograph of PCR-RFLP product of exon 3** (M: 25bp marker, Lane (1-2): Nellore Jodipi; Lane (2-4): Nellore Brown; Lane (5-8): Macherla Brown)

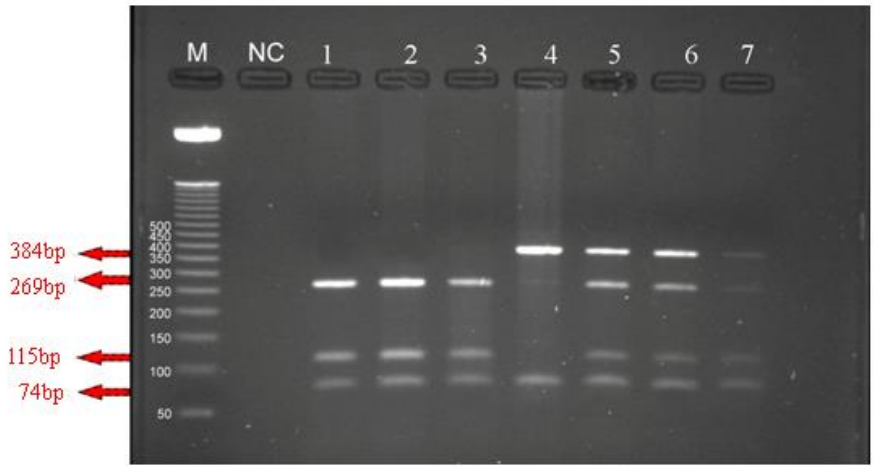


**Figure 10: Photograph of PCR-RFLP product of intron 1** ( M: 50bp Marker; NC: Negative control; Lanes 1, 2, 3: HH genotype; Lane 4: hh genotype; Lane 5, 6, 7: Hh genotype)

fragment referring to hh genotype in the absence of mutation. A third pattern was a combination of the two (384 bp, 269 bp, 115 bp and 74 bp) patterns (Figure 10) referred as Hh genotype. Population wise observed and expected number of genotypes was presented in Table 6.

### 4.5. Population analysis

In the present study exon 3 is monomorphic in all the samples analyzed, hence, further analysis could not be performed for this locus.

The intron 1 is polymorphic in all the three populations with hh genotype at very low frequency (Table 6). The heterozygosity of the locus in each population is presented in Table 7. The heterozygosity values suggest that there is considerable amount of variation exist at this locus. The usefulness of the marker as indicated by polymorphic information content (PIC) also indicates moderate usefulness of the marker (Table 7) for population studies in all the three populations. Two diversity estimates ($F_{IS}$) were obtained (Weir and Cockerham, 1984: Robertson and Hill, 1984). Both the estimates were negative for the three populations indicating that there is no differentiation among the three populations (Table 8). The test for Hardy Weinberg Equilibrium indicated that the Nellore brown (P=0.0499), Nellore Jodipi (P=0.0341) and Macherla Brown (P=0.0032) are departing from the equilibrium assumptions.

### 4.6 Association of *GDF8* polymorphism with growth traits in sheep

In the present study exon 3 is monomorphic. Hence, no association analysis could be performed. The sample size was small to interpret conclusively using association studies. However, an attempt was made to verify the association of genotypic variants of intron 1 with body weights at birth, 3, 6, 9 and 12 months age. The phenotypic information of the two populations under study was presented in Tables 3

**Table 6. Heterozygosity and genetic diversity at intron 1 of *GDF8* locus in different genetic groups of sheep**

| Population | No. | Observed Genotypic frequency | | | Expected Genotypic frequency | | | Expected homozygotes | Expected heterozygotes |
|---|---|---|---|---|---|---|---|---|---|
| | | HH | Hh | hh | HH | Hh | hh | | |
| Nellore Jodipi | 22 | 5 | 16 | 1 | 7.56 | 10.88 | 3.56 | 11.12 | 10.88 |
| Nellore Brown | 18 | 3 | 14 | 1 | 5.43 | 9.14 | 3.43 | 8.86 | 9.14 |
| Macherla Brown | 25 | 6 | 19 | 0 | 9.49 | 12.02 | 3.49 | 12.98 | 12.02 |

**Table 7. Allele frequencies of the SNP located at intron 1 of *GDF8*gene**

| Population | H | h | Heterozygosity | PIC |
|---|---|---|---|---|
| Nellore Jodipi | 0.59 | 0.41 | 0.48 | 0.37 |
| Nellore Brown | 0.56 | 0.44 | 0.49 | 0.37 |
| Macherla Brown | 0.62 | 0.38 | 0.47 | 0.36 |

**Table 8. Population differentiation based on intron 1 polymorphism in *GDF8* gene**

| Population | Weir and Cockerham's $F_{IS}$ | Robertson and Hill's $F_{IS}$ |
|---|---|---|
| Nellore Jodipi | -0.4867 | -0.4925 |
| Nellore Brown | -0.5556 | -0.5625 |
| Macherla Brown | -0.6000 | -0.6048 |

and 4, respectively. The mean (±SEM) body weights at different ages are presented in tables 9 and 10. ANOVA was performed to observe the significant differences with respect to body weights that correspond to different genotypes in Nellore brown and Nellore Jodipi populations. No significant ($P>0.05$) association of a genotype with body weights at different ages was observed in the present study in both the genetic groups.

**Table 9. Mean Body weights (Kg) in different genotypes of intron 1 of *GDF8* in Nellore Jodipi sheep**

| Phenotype | Genotype | | |
|---|---|---|---|
| | HH (n=5) | Hh(n=16) | hh(n=1) |
| Birth weight | 03.28±0.20 | 03.21±0.85 | 03.20 |
| Weight at 3 months | 15.26±0.50 | 14.68±0.45 | 18.50 |
| Weight at 6 months | 18.90±1.13 | 20.06±0.66 | 26.50 |
| Weight at 9 months | 23.20±0.91 | 23.23±0.58 | 29.00 |
| Weight at 12 months | 26.22±1.02 | 26.47±0.60 | 31.00 |

**Table 10. Mean Body weights (Kg) in different genotypes of intron 1 of *GDF8* in Nellore Brown sheep**

| Phenotype | Genotype | | |
|---|---|---|---|
| | HH(n=3) | Hh(n=14) | hh(n=1) |
| Birth weight | 03.03±0.26 | 03.06±0.98 | 03.50 |
| Weight at 3 months | 10.77±1.88 | 11.74±0.71 | 07.50 |
| Weight at 6 months | 13.33±1.87 | 14.36±0.69 | 16.20 |
| Weight at 9 months | 18.00±0.50 | 17.56±0.36 | 18.50 |

# CHAPTER V

# DISCUSSION

As per the report of working group of animal husbandry and dairying in India for the 12$^{th}$ five year plan (2012-17), though India ranks 3$^{rd}$ in world sheep population, ranks 7$^{th}$ with respect to meat and wool production. The plausible reasons include unorganized sheep breeding practices and no practice of selective breeding etc. Identification of elite germplasm is a constraint for selective breeding. Markers play a key role in identification of elite animals (Dodgson *et al*., 1997). Single nucleotide polymorphisms (SNP) markers are useful tools in genetic association studies (De Bakkar *et al*., 2005). PCR-RFLP is a simple and relatively economical tool when few SNP loci need to be studied (Ota *et al*., 2007). Perusal of literature revealed that there were no studies involved with respect to Myostatin (*GDF8*) polymorphism in Nellore sheep breed and local sheep genetic groups in Andhra Pradesh. Hence the present study was undertaken to study the polymorphism of *GDF8* using PCR-RFLP technique in Nellore and Macherla brown sheep.

## 5.1 DNA isolation

Good quality genomic DNA from sheep whole blood collected in $K_2$-EDTA vacutainers was isolated using high salt method (Miller *et al*., 1988; Aravindakshan., 1997). The method is proved to be quick, reliable and known to produce yield of DNA that is almost equivalent to phenol extraction method in sheep (Montgomery and Sise, 1990). In the present study majority of the DNA isolations were having OD λ260/280 ratios in the range of 1.4 to 2.0 indicating relatively good quality genomic DNA.

**5.2 PCR amplification**

The unambiguous PCR amplification of exon 3 and intron 1 regions of *GDF-8* is obtained at annealing temperature of $54^0$C and $52^0$C, respectively. The exon 3 primer that is published in literature showed three mutations in the forward primer region and one mutation in the reverse primer binding region. These mutations were corrected accordingly in the primers used in the present study to amplify the SNP in intron 1 (rs119102825).

**5.3 PCR-RFLP of exon 3 and intron 1 of *GDF8* gene**

The exon 3 polymorphism is extensively studied in sheep (Azari *et al*., 2012; Dehnavi *et al*., 2012). Majority of the studies indicated that the SNP in exon3 is showing less variation (Soufy *et al*.,2009; Lazar et al.,2016) and in most cases it is monomorphic (Dehnavi *et al*., 2012; Georgieva *et al*., 2015 ; Dimitrova *et al*., 2016). Further it should be noted that there is no SNP reported in the SNP database for the putative RFLP site in exon 3. In the present study the marker is monomorphic in all the populations studied. Absence of reported SNP in exon 3 and monomorphism reported in majority of the studies raises concern on usefulness of this marker for population studies. In addition, the fragment includes two restriction sites for the *Hae*III enzyme. Previous studies have not mentioned which site actually shows polymorphism. Owing to the ambiguity future studies on *GDF-8* exon 3 polymorphism should be designed carefully.

In the present study the SNP database was searched for additional reported SNPs to identify possible PCR-RFLP markers. One such SNP was observed at (g.118144833G>T) in intron 1. The polymorphism associated with the SNP is confirmed in Iranian and Moracan sheep included in Nextgen project

(http://projects.ensembl.org/nextgen/). Hence a set of primer pairs were designed to amplify this region of *GDF8* in the present study. The SNP at this position leads to a variant for a restriction enzyme named *Hpy*CH4V. Another restriction site for the same enzyme in the vicinity was included which can be used as positive control for the restriction digestion. Sixty five samples comprising 22 Nellore Jodipi sheep, 18 Nellore brown sheep and 25 Macherla brown sheep were used for intron 1 study. The genotypes are indicated as HH, Hh and hh . This newly designed PCR-RFLP variant is found to be polymorphic in all the three populations with heterozygosity values of 0.48, 0.49 and 0.47 in Nellore Jodipi, Nellore Brown and Macherla Brown sheep populations respectively, indicating its usefulness in population studies. Further, the informativeness of the marker as indicated by polymorphic information content (PIC) is 0.37, 0.37 and 0.36 in respective sheep populations, respectively. In a biallelic marker system the maximum possible PIC value for a given locus is 0.375 (Hildebrand *et al*., 1994). Hence the newly designed PCR-RFLP marker in this study is useful in population studies.

**5.4 Genetic differentiation between different sheep populations in Andhra Pradesh**

In the present study, the polymorphism of the SNP located in intron 1 is used to understand the population dynamics. The $F_{IS}$ values (Table 8) indicated that three populations showed no differentiation within populations (Weir and Cockerham, 1984; Robertson and Hill,1984). All the three populations showed deviations from Hardy – Weinberg assumptions . This may be due to the drift generated by smaller sample size used in the study. Higher sample size may unravel actual reason for the deviations of these populations from Hardy Weinberg Equilibrium.

**5.5 Association studies**

Previously, mutations in the non-coding region of *GDF8* locus were reported to be associated with muscular hypertrophy (Clop *et al*., 2006). The polymorphism observed at the Intron 1 of *GDF8* locus is tested for association with body weights at different ages as body weight is an indicator of meat production. However, these associations must be carefully interpreted as the sample size is very small. The present study failed to observe any association between the genotypes and body weights at different ages ($p>0.05$).

In the present study, it was shown that the PCR-RFLP marker at exon 3 may not be a suitable marker. A new PCR-RFLP marker designed for intron 1 in the present study was found to be polymorphic and useful in population studies. Further studies on larger sample sizes may help in understanding if the SNP variant is associated with meat production traits.

# CHAPTER VI

## SUMMARY

The primary objective in small animal production is to have higher meat production. In India, unlike western countries the main source of meat is sheep and chicken. One of the constraints in achieving this objective is the productivity of Indian sheep breeds which is relatively less compared to exotic breeds. *GDF8* is a myokine, a protein that is produced and released by myocytes that acts on muscle cells' autocrine function to inhibit myogenesis *i.e*. muscle cell growth and differentiation. Understanding natural variants of such genes would help in developing marker assisted breeding strategies in sheep.

Andhra Pradesh is native to Nellore breed of sheep. It is predominant in Nellore, Prakasham districts and their adjoining areas. There are three variants in Nellore breed namely Jodipi, Brown and Palla. Macherla brown is a locally adopted genetic group present in and around Macherla region of Guntur district. The present study is aimed at understanding natural variation of *GDF8* locus in the two meat type sheep *viz.* Nellore and Macherla Brown by using PCR-RFLP, to determine the population genetic parameters and to study the association of *GDF8* gene variants if any, with growth traits in Nellore sheep.

DNA was isolated from blood samples using a modified high salt method. Two sets of primers were designed to amplify regions of exon 3 and intron 1 of myostatin gene in the present study. The PCR products were subjected to RFLP with *Hae*III enzyme for exon 3 and *Hpy*CH4V for intron 1 fragment population genetic analysis was conducted using Genepop (v4.2) on the web software package and the polymorphic locus was used to test for equilibrium in each of the population. To know the nature of population, the population differentiation is tested using $F_{IS}$ values as described by Weir and Cockerham (1984) and

Robertson and Hill (1984). Association studies, on the body weights at different ages with genotype tested by performing ANOVA in Nellore Jodipi and Nellore Brown populations

The present study revealed monomorphic patterns at exon 3 in all the samples analyzed. Hence further analysis could not be performed for this locus. The intron 1 region was polymorphic representing *HH, Hh, hh* genotypes, with *hh* genotype at very low frequency. The heterozygosity values for Intron 1 region is 0.48, 0.49 and 0.47 for Nellore Jodipi, Nellore Brown and Macherla Brown indicating there is considerable amount of variation exist in these populations. PIC values for Nellore Jodipi, Nellore Brown and Macherla Brown are 0.37, 0.37 and 0.36 respectively indicating that the marker is informative. The allelic frequencies of Nellore Jodipi, Nellore Brown and Macherla Brown are 0.59 and 0.41; 0.56 and 0.44; 0.62 and 0.38 representing H and h alleles. $F_{IS}$ values were calculated based on Weir and Cockerham (1984) and Robertson and Hill (1984) and both the results indicated negative values. For the three populations indicating that there is no differentiation among them. The test for Hardy Weinberg equilibrium was conducted for the three populations and the results indicated that the three are departing from the equilibrium assumptions. No significant association ($P>0.05$) of a genotype with body weights at different ages was observed in the present study in both the genetic groups of Nellore Jodipi and Nellore Brown which may be due to small sample size of populations. New PCR-RFLP marker designed for intron 1 in this study was found to be polymorphic that could be highly useful in population studies.

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