DISCRIMINANT ANALYSIS AND STUDY OF POLYMORPHISM IN MYOSTATIN (GDF-8) GENE IN NATIVE POULTRY

Thesis

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

MASTER OF VETERINARY SCIENCE
in
ANIMAL GENETICS AND BREEDING
(Minor Subject: Animal Biotechnology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled **"Discriminant analysis and study of polymorphism in myostatin (GDF-8) gene in native poultry"** submitted for the degree of **M.V.Sc.** in the subject of **Animal Genetics and Breeding** (Minor subject: **Animal Biotechnology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Sumeet Patil** (**L-2019-V-04-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

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

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CERTIFICATE - II

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ABSTRACT

The present investigation was done for discriminant analysis of Punjab Brown native chicken, PB1 and PB2 broiler parent on the basis of body weight and morphometric variables viz. body weight (BW), body length (BL), wing length (WL), shank length (SL), thigh length (TL), breast girth (BG) and keel length (KL), recorded in 50 males and 50 females from each group from day of hatch to 25 weeks, at 5-week intervals. BW was strongly correlated with BL and WL and most other variables had medium to low correlation. Age-wise and gender-wise discriminant functions, with three levels of outcome variable had greater classification accuracy (92.2% to 100%), in comparison to functions with six levels of outcome variable (90.2% to 93.6%). PCR-RFLP analysis in Punjab Brown birds, done to study the polymorphisms in myostatin (MSTN) promoter region revealed three genotypes viz. AA, AC, CC with frequencies 0.46, 0.50, 0.04 in females and 0.45, 0.47, 0.07 in males. A and C alleles frequency were estimated to be 0.71 and 0.29 in females and 0.69 and 0.31 in males. In females, genotype had significant effect ($p \leq 0.05$) on body weight. No such association was found in males. CC genotype had highest BW followed by AC and AA genotypes. Sequencing results indicated A>C transversion at 1196 bp position and a probable breed specific T>C transition at 1280 bp position. Results of the study indicated that discriminant function was effective for segregating Punjab brown birds from coloured broiler population. Observed mutations could be used in developing MAS strategies for improvement of Punjab brown population.

Key words: Punjab Brown, discriminant, PCR- RFLP, myostatin, mutation.

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Signature of the Major Advisor	**Signature of the Student**

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ABBREVIATIONS USED

%	:	Percent
@	:	At the rate of
°C	:	Degree celsius
µg	:	Microgram
µl	:	Microliter
5'UTR	:	5'Untranslated Region
AICRP	:	All India Coordinated Research Project
ANOVA	:	Analysis of Variance
BLAST	:	Basic Local Alignment Search Tool
Bp	:	Base pairs
BW	:	Body weight
DDBQ	:	DNA Data Bank of Japan
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy nucleotide triphosphate
dsDNA	:	Double stranded Deoxy ribonucleic acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
EMBL	:	European Molecular Biological Laboratory
et al	:	Et alia (Latin other workers)
g	:	Grams
GDF-8	:	Growth Differentiation Factor-8
GLM	:	Generalized linear model
i.e.	:	That is
IBL-80	:	Indian Broiler Ludhiana-80
ICAR	:	Indian Council of Agricultural Research
Kb	:	Kilo basepairs
kDa	:	Kilodalton
Kg	:	Kilogram
M	:	Molar
mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
min	:	Minute(s)
ml	:	Milliliter
mM	:	Millimole
mRNA	:	Messenger Ribonucleic Acid
MSTN	:	Myostatin
N	:	Number of observations

NCBI	:	National Center for Biotechnology Information
NEB	:	New England Biolabs
NF-1	:	Nuclear Factor 1
ng	:	Nanogram
nm	:	Nanometer
NTC	:	No Template Control
OD	:	Optical density
PB1	:	Punjab Broiler 1
PB2	:	Punjab Broiler 2
PCI	:	Phenol-Chloroform-Isoamyl Alcohol
PCR	:	Polymerase Chain Reaction
pH	:	Negative log of hydrogen ion concentration
pmoles	:	Pico moles
RE	:	Restriction Enzyme
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic Acid
rpm	:	Rotations per minute
S.E.	:	Standard Error
Sca I	:	*Streptomyces caespitosus*
sec	:	Second(s)
SNPs	:	Single Nucleotide Polymorphisms
SRF	:	Serum Response Factor
SSCP	:	Single-strand conformation polymorphism
TAE	:	Tris Acetate EDTA
Taq	:	*Thermus aquaticus*
TE	:	Tris EDTA
U	:	Unit
UK	:	United Kingdom
USA	:	United States of America
UV	:	Ultra Violet
V	:	Volts
vis-à-vis	:	In relation to
viz.	:	Namely
w/v	:	Weight/Volume
σ^2	:	Variance

CHAPTER I

INTRODUCTION

Presently, India is one of the dominant producer of poultry eggs and meat in the world. Over the years, the poultry industry in the country has endured a major structural shift, from being a backyard activity to an organized sector. This growth can be attributed to many factors like growing incomes, along with the rise of vertically integrated poultry farming systems that have reduced consumer prices by lowering production and marketing costs. The Indian poultry industry, which includes broilers and eggs, was valued at 1,750 billion rupees in 2018, is expected to increase to 4,340 billion rupees by 2024, with a compound annual growth rate (CAGR) of 16.20 percent between 2019 and 2024 (Department of Animal Husbandry and Dairying, 2017a).

Population of India is expected to increase by 34% by the year 2050 and to meet the dietary recommendations of Indian Council for Medical Research (ICMR) for animal products for a population of more than 1.7 billion people, the livestock industry would need to upgrade its production to 186.2 million tonnes of milk, 18.7 million tonnes of meat, and 306 billion eggs every year. Milk, meat and eggs production will have to accelerate beyond their present levels and increase by 1.5, 3.7 and 4.7 times, respectively. Fulfilling the nutritional needs for this enormous livestock population with the same limited resource base of land and water will be a massive challenge (National Institute of Animal Nutrition and Physiology, 2013). The whole poultry sector of India can be sub-categorized under two main categories. One major part is the organized commercial sector which contributes around 77% of poultry production. While the other unorganized backyard sector contributes approximately 23% of poultry production (TANUVAS, 2012).

However, it is to be noted that the major growth in poultry production has been through exotic germplasm and development of their crosses or varieties, whereas the native poultry breeds are still found to be lacking in performance with respect to the meat and egg production. Despite their poor productivity, local chicken in the tropics can thrive and perform with erratic feed and water supplies with minimal healthcare. They are part of a well-balanced farming system that provides high-quality

animal protein and emergency cash income to rural people and play an important role in the rural community's sociocultural life. Local chickens are excellent mothers and good sitters, despite being modest growers and poor layers of comparatively smaller sized eggs (Padhi, 2016). Because of their vigilance, light body weight, greater shank length, camouflagic characteristics, and aggression, native fowl are capable of self defence against predators. Meat from native fowl has substantially more amino acids (arginine and lysine) than meat from foreign birds, and is prized for its pigmentation, flavour, leanness, and appropriateness for specific recipes. It also commands higher premium. Native fowl's brown-shelled eggs are higher in threonine and valine, have a better flavour (TANUVAS, 2012). Therefore, in a resource constrained rural Indian setup, indigenous chicken genetic resources are backbone of backyard poultry production as well as a potential resource for economic upliftment of the poorer sections of the society (Department of Animal Husbandry Dairying and Fisheries, 2017b).

Punjab Brown is one such native poultry breed found in the Gurdaspur district of Punjab, with average weight of cocks and hens as 2.15±0.94 kg and 1.57±0.04 kg, respectively. The age at sexual maturity is 20 to 24 weeks and they have been reported to annually produce 60 to 80 brown shelled eggs, having average egg weight of 46.0±1.91 g (Vij et al., 2006). Due to its unique morphometry, it has the ability to perform in extreme climatic conditions with minimal input. Therefore, it is imperative to identify and study important discriminatory variables, which define the uniqueness of Punjab Brown as well as investigate the unexplored genetic variability in candidate genes, which can help in breed improvement by developing improved selection strategies.

Characterisation of native chicken in comparison to synthetic poultry populations can be made using various quantitative and qualitative morphological variables. There is a necessity to recognize such variables in Punjab Brown native poultry breed. Multivariate statistical approach can be used to identify the discriminant variables which would help in developing the best discriminant function. Various morphometric variables such as breast girth, tibial length, comb length and height, wattle height and length have been used in developing discriminant function for indigenous chicken (Musa et al., 2018). Therefore, there is a need to evolve a

discriminant function that shall help in segregating out the purebred from heterogenous poultry flocks. Discriminant analysis is a powerful classificatory technique to describe characteristics that are specific to distinct groups and classify cases into pre-existing groups based on similarities between that case and the other cases belonging to the groups.

Myostatin (MSTN) is a well-known muscle growth-limiting factor (GDF-8). It belongs to the superfamily of secreted growth and differentiation proteins known as transforming growth factors-β (TGF-β) (McPherron & Lee, 1997). MSTN gene consists of two introns and three exons (Bellinge et al., 2005). It is secreted as the latent protein of 38 kDa comprising of 376 amino acids whereas, biologically active, processed and matured MSTN protein is 15 kDa (Wolfman et al., 2003). It is known to be expressed during embryonic development and in adult muscle (Baron et al., 2002). MSTN is chiefly expressed in skeletal muscles which control the growth of the muscles by suppressing proliferation and differentiation of myoblast cells in mammals (Ríos et al., 2002). Polymorphisms in different regions of MSTN gene have been associated with performance variables in different studies (Bhattacharya & Chatterjee, 2013; Mendias et al., 2008; Paswan et al., 2014; Tripathi et al., 2012). Therefore, exploring polymorphism in this vital candidate gene will help to develop selection strategies favouring certain genotypes over others for achieving higher body weight at an early age and augmenting the performance in the desired direction. Keeping these in view, the present investigation was conducted with the following objectives:

1. Morphological characterization and discriminant function analysis for Punjab Brown native poultry vis-à-vis parent lines of IBL-80 broiler variety.
2. Study on polymorphisms in promoter region of myostatin gene in Punjab Brown native poultry.

CHAPTER II

REVIEW OF LITERATURE

The review of literature cited for the present study of Discriminant Analysis along with Myostatin gene polymorphism in Punjab Brown of chicken is presented under the following headings:

2.1 Discriminant analysis of Morphometric traits in Poultry

2.2 GDF-8 gene and the association of its polymorphism with economic traits in sire line of broiler variety

2.1 Discriminant Analysis of Morphometric traits in Poultry

Rosário et al. (2008) recorded feed intake (g), live weight (g), feed conversion (g/g), carcass weight (g), breast weight (g), leg weight (g), average live weight and carcass weight for multivariate canonical discriminant analysis in 1920 chicks housed in 48 pens, with eight treatments, defined as the combination of three commercial broiler chicken strains (Arbor Acres, AgRoss 308, Cobb 500) and one experimental strain (RX). The most significant traits differentiating treatments were average live and carcass weight, while the disparity between average feed intake and the average live weight plus feed conversion was utilised to categorize them. Within strain, there was a notable difference between sexes, with Cobb 500 and AgRoss 308 having the best multivariate performance mean. They suggested analysing performance data sets using canonical discriminant analysis because of the reduction from six original traits to only two canonical variables.

Ajayi et al. (2012) used data from 273 randomly selected 12-weeks-old indigenous chickens of normal feathered, frizzle-feathered, naked-neck and Anak Titan genotypes to morphologically classify the chicken genotypes using multivariate discriminant analysis. The best discriminating factors to distinguish the chicken genotypes were breast girth (BG), keel length (KL), thigh length (TL), shank length (SL), and wing length (WL). The AT and NF genotypes had the largest distance (72.54), while the NN and FF genotypes had the smallest distance (4.27). Classification results revealed that 85.2% of AT genotype was found to be accurately categorized into their source population. Yet, 22.7 percent of NF birds were

misclassified to be in NN, while 33.3 percent of NN birds were misclassified as NF. These findings suggest that these two indigenous chicken genotypes have a high rate of gene transfer highlighting the need for conservation of this breed.

Yakubu et al. (2012) used multivariate discriminant analysis in Nigerian indigenous, exotic and crossbred turkeys to differentiate their morphological and heat-tolerant traits. Body weight (BW), Keel Length (KL), Body Length (BL), Breast Girth (BG), Shank Length (SL), Thigh Length (TL), rectal temperature (RT), pulse rate (PR), respiratory rate (RR) and heat stress index (HI) were measured from 228 turkeys. The best discriminating variables to distinguish the three genetic groups were BW, TL, HI. The indigenous and exotic turkeys had the largest Mahalanobis distance (36.68) whereas the indigenous turkeys and their crossbred counterparts had the lowest distance 7.97. Such findings might help with the adoption of indigenous turkey conservation and enhancement plan for the long-term development of animal genetic resources.

Ogah (2013) collected data on body weight (BW), shank length (SL), body length (BL), thigh length (TL), wing length (WL), keel length (KL), chest circumference (CC) and body width (BD) from three Nigerian indigenous chicken genotypes viz. naked neck (NN), frizzled feather (FF) and normal feather (NF) chickens and conducted canonical discriminant analysis. Two discriminant functions were extracted and it was observed that BW, BD and TL had the highest discriminatory power of all the variables. The frizzled feathered chickens excelled in terms of performance and distance between genotypes, allowing for the development of an indigenous chicken improvement programme.

Daikwo et al. (2015) measured ten morphometric traits on 436 frizzle feathered and 610 normal feathered chickens in north-central Nigeria. Stepwise canonical discriminant analysis identified eight morphological variables (BG, BL, SL, WL, KL, neck length, bird height, head circumference) with the greatest discriminating power between the best two genotypes. 100% of the individuals in the chicken population were correctly classified using the extracted discriminant function. The classification accuracy of the function was cross-validated using the split-sample method and indicated a 100% success rate. The findings might be supplemented by

DNA-based approaches for better chicken genetic resource protection and management.

Adeyemi & Oseni (2018) used Body Weight (BW), Shank Length (SL), Thigh Length (TL), beak length, Abdomen circumference (AC), tail feather (TF), snood length (SN), spur length (SP) and caruncle (CR) eight morphometric variables to discriminate three colour variants (white, black and lavender) of Nigerian indigenous turkey populations and evaluated phenotypic resemblances and differences. They found black and lavender variants to be closest in all the examined traits. Between black and lavender, the pairwise squared Mahalanobis distance was 6.745, whereas the distances between white and each of black and lavender were 9.242 and 57.595, respectively ($P \leq 0.05$). BW, SL, and AC were the most discriminating morphometric variables in distinguishing the three colour variations. These findings might point to within population intrinsic diversity in morphometric characteristics among indigenous turkeys.

Musa et al. (2018) used variables viz. Breast girth (BG), WL, tibial length, comb length (CL), comb height (CH), wattle length and wattle height (WH) for discriminant analysis in Nigerian chicken genotypes and observed intermingling between normal feathered and necked neck genotypes while the necked feathered and frizzled feather were almost distinct. They reported that the morphometric traits BG and tibial length as being most correlated with body weight. Some morphometric characteristics were shown to be genotype specific and it was suggested that these may be used as markers of body weight.

Venkatas et al. (2019) used discriminant analysis for different genotypes of broiler breed Anak Titan. Based on morphometric characteristics, the analysis proved useful in distinguishing purebred and crossbred chicken. For analysis, they used data on Body Length (BL), Body Girth (BG), Thigh Length (TL), Shank length (SL), Keel Length (KL), Wing Length (WL) and wingspan (WS) variables, collected at weekly intervals. The purebred Anak Titan had a considerably higher mean weight than the purebred and crossbred indigenous chickens, indicating that BW was the main discriminating criterion. The outcomes of this study might aid in making breeding decisions in smallholder poultry production, especially in developing nations with limited resources.

Melesse et al. (2021) did a study to differentiate native chicken of Metekel, Kaffa, Bale and Sheka areas of Ethiopia based on morphometric measurements using multivariate analysis on data of Live weight (LW), breast circumference (BC), body length (BL), wingspan (WS), shank circumference (SC), shank length (SL), keel length (KL), neck length (NL) and back length (BkL) recorded from 3069 adult native chickens. Two clear groups in which chickens of Bale and Sheka were in one group and Metekel and Kaffa in other, were generated by cluster analysis. Three significant canonical variables (CAN) were found. 73.2 and 14.6 percent of the overall variations were accounted for by CAN1 and CAN2, respectively. The scatter plot obtained by canonical discriminant analysis revealed that CAN1 effectively differentiated between Metekel and Kaffa chickens, whereas CAN2 effectively discriminated against Bale and Sheka chicks. 95.3, 94.9, 92.3, and 82.2 percent of Metekel, Bale, Kaffa, and Sheka chickens were accurately classified into their respective populations by discriminant analysis. Multivariate analysis of morphometric characteristics was shown to provide a suitable basis for classifying indigenous chicken populations into distinct groups.

2.2 GDF-8 gene Polymorphism and Association Studies

McPherron & Lee, (1997) observed that myostatin null mice exhibited 2 to 3 fold increase in the skeletal muscle mass due to hyperplasia and hypertrophy. Mutations in the myostatin gene were also associated with the double-muscling phenotype in cattle (Grobet et al., 1998; McPherron & Lee, 1997).

Gu et al. (2002) identified total 5 SNPs in the chicken myostatin gene by PCR-SSCP. Three SNPs localized in the 5'-regulatory region was due to 3 single point mutations which were G304A, A322G, C334T, respectively. The other two SNPs were G167A and T177C, respectively. Two SNPs in the 3'-regulatory region of the myostatin gene were A7263T and A6935G. Frequency of the BB genotype was very high (0.70) while the AA genotype was only 0.033 in 5'-regulatory region (P60/P61) in Beijing youji population. The genotype frequencies (P93/P94) were significantly different among the lines ($P \leq 0.01$). Frequencies of the EE genotype in Beijing Youji were lower than those in the other lines, while frequencies of the EE genotype in Baierji and Hyline Layer were higher than the rest. Frequency of allele C (P80/P81) in

9 breeds was higher than that of allele D. Frequency of genotype MM (P76/P77) was low, and that of MN was high in 7 breeds.

Bhattacharya and Chatterjee, (2013) concluded that myostatin gene was polymorphic and had a significant effect on growth traits in broiler chickens. They studied the polymorphism in myostatin gene in exons 1, 2 and 3 using single-stranded conformation polymorphism followed by sequencing and revealed polymorphism of the gene in PB-1 and CB broiler lines and IWI layer line. A total of 13 haplotypes were observed across 3 chicken lines. Myostatin haplogroups had a significant effect on BW at 28, 42, and 49 d of age in the PB-1 line. At 14th day and 49th day significant association of haplogroups was observed with BW in the CB line. Though myostatin gene was polymorphic but had no significant association with growth traits in IWI layer line.

Paswan et al. (2014) reported single nucleotide polymorphisms (SNPs) attributable to transition mutations in the promoter region of myostatin (GDF-8) gene in broiler chicken. Three genotypes AA, AB and BB with frequencies 0.63, 0.28 and 0.09, respectively, were identified. BB genotype significantly affected body weight at hatching, implicating the role of the identified SNPs in the chicks' muscle development.

Khaerunnisa et al. (2016) did a study to detect the polymorphism of T4842G mutation in the myostatin gene in Indonesian chickens and assess their effects on carcass characteristics. The gene polymorphism was identified with the PCR-RFLP method using the *BsrI* restriction enzyme. Genotyping was performed on 332 chickens from 7 chicken populations of Indonesia (Kampung, Sentul, Merawang, Cobb broiler, F1 cross breed of Kampung x layer, F1 cross breed of Kampung x Cobb and F2 cross breed of Kampung x Cobb broiler). The myostatin locus was polymorphic in all populations. Results from the analysis of the allele and genotype frequency showed that the T allele had a higher frequency than the G allele in all populations, except for the F1 crossbreed of the Kampung x Cobb broiler chicken population. A significant effect was found between genotype and carcass characteristics in the F2 crossbred Kampung x Cobb broiler chickens. A SNP in exon 2 was associated with live weight, carcass weight, breast weight, thighs weight, drum

sticks weight, wings weight, breast muscle weight, thighs muscle weight, drum sticks muscle weight and free water.

Lassiter et al. (2019) studied Gene expression targeting breast muscle samples obtained from 8–10-week-old Pedigree Male (PedM) broilers. They individually phenotyped the broilers for Feed Efficiency (FE) between 6 & 7 weeks of age. High FE phenotype's breast muscle analysis revealed that muscle development may be promoted by downregulation of numerous parts of the myostatin pathways in amalgamation with up-regulation of genes that boost muscle production and growth implying its role in inhibiting muscle growth.

Ye et al. (2007) assessed effects of myostatin polymorphisms in 3 commercial broiler chicken lines on feed conversion efficiency, mortality, growth, ultrasound breast depth, eviscerated carcass weight, breast %, leg defects, blood oxygen level, and hen antibody titre to infectious bursal disease (IBD) vaccine. 5 SNPs segregating these lines w.r.t. myostatin gene were identified forming basis for 8 haplotypes. Varying frequencies of SNP allele and haplotypes were found between lines. Significant association of GDF-8 SNPs, found with mortality, growth, blood oxygen levels and hen antibody titer to IBD vaccine, often inconsistent across lines. These results suggest pleiotropic effects of myostatin gene on broiler performance.

Tanjung et al. (2019) studied association of GDF-8 gene mutations with the BW of hybrid chicken crossbreed resulting from BC1 Broiler and F1 Kamper. The discovered SNP was analyzed by the Pearson correlation test with 49 days old chicken BW. The BW of the hybrid chicken is more compared to Pelung chicken but lower than the Broiler. Two Adenine insertions, one Guanine deletion, and four substitutions (C2244G, G2283A, T4842G, G7378T) in 3 exons yield nine haplotypes. Protein sequence of 6 haplotypes differed while 3 had protein sequences identical to myostatin protein. Strong positive correlation (r=0.736) between MSTN protein and mutant to 49-days-old chicken body weight especially the Adenine insertion to 2099-2100bp of GDF-8 gene. T4842G substitution had strong negative correlation (r = -0.773) with 49 days old chicken BW. Adenine insertion at 2099-2100 bp of GDF-8 gene could be a potential genetic marker of heavier body weights for hybrid chicken.

Zhang et al. (2019) did a study to correlate GDF-8 polymorphisms with chicken production performance. Correlation between the genotypes and 1 to10

weeks BW and 73rd day Carcass traits was investigated in 180 Dehang broilers by direct PCR product sequencing. Four haplotypes on the basis of 5 identified SNPs were reconstructed. Four of these SNPs were significantly associated with few growth variables($p \leq 0.05$), but no significant association with carcass traits was found. Association study revealed no significant correlation for rs314431084. The AA, GG, CC, and TT genotypes of rs313622770, rs313744840, rs316247861, rs317126751 were good for the growth. Significant association of diplotypes with weight of chest and leg muscle ($p < 0.05$) was found. The SNPs in GDF-8 gene could be exploited as potential markers for MAS during chicken breeding.

Dementeva et al. (2017) used PCR-RFLP to analyze allele and genotype in the Smena-8 cross of G5 line of Cornish chicken breed. Two single nucleotide substitutions i.e., G/A at MST2109 and G/C at MST2244 were considered for exon 1. In MST2244, deoxynucleotide G predominates over C, while deoxynucleotide A predominates over G in MST2109 In the case of MST2109 and there were no variations in the production traits across the genotypes. There was a statistically significant difference in live weight at seven days between the CC and G2G2 genotypes ($p \leq 0.01$), as well as between the CG2 and G2G2-genotypes ($p \leq 0.05$). At seven days of age, G2G2 chicks weighed substantially more than CC (179.5 g) and CG2 (193.95 g) birds. It was found that the CC and G2G2 genotypes had statistically significant variations in weight at 33 days ($p \leq 0.05$). Myostatin allele frequencies in Cornish breed line G5 may be estimated as a result of this research. Myostatin gene genotypes in chickens could be taken into consideration in the breeding process in the broiler poultry business based on the results of the study.

Mohammed (2018) genotyped 300 Ross 308 chicks for myostatin gene and its relation with some qualitative carcass and morphometrics traits in broiler chicken. RFLP was done using *AciI, BbvI* and *BbsI* restriction enzymes. For the (GG, GA, and AA), the genotypic frequency was 32 percent, 54 percent, and 14 percent for the restriction enzyme *AciI*. They were extremely significant, and they had a substantial ($p \leq 0.05$) influence on carcass yield % as well. Breast width and body length were significantly affected ($p \leq 0.05$) by the myostatin genotypes throughout the third and sixth weeks. There was a 6.78 percent, 86 percent, and 31.36 percent distribution of

GG, GA, and AA genotypes respectively for the myostatin gene by restriction enzyme *BbsI*. In the third week, the variations between these percentages were extremely significant, and in the sixth week, the genotypes of myostatin gene had a significant ($p \leq 0.05$) influence on leg length, body size and comb height. Ninety eight and two percent of the myostatin gene genotypes (AA and GA) were found in the study samples, respectively, and the differences between these numbers were highly significant. The genotypes of the myostatin gene had a statistically significant ($p \leq 0.01$) effect on leg length and thigh thickness.

Ewuola et al. (2018) classified mutations in the myostatin gene of chosen chicken species at the investigated locus as part of a computational genetics study. Functional analysis of non-synonymous single nucleotide polymorphism (nsSNP) using PROVEAN with myostatin sequences from 12 chickens, 4 quails and 4 turkeys obtained from the GenBank, revealed three amino acid substitutions (Y11F, P20Q and G3R) in chicken, three in turkey (F155W, N65P and K95A) one in quail (Y100R) and were all returned neutral, indicating their beneficial impacts. Information from nucleotide sequences indicated that members of the phasianidae family were clustered together and closely related (chicken, quail and turkey).

CHAPTER III

MATERIALS AND METHODS

3.1 Location of Study &Data Collection

The current study was carried out in three genetic groups i.e., Punjab Brown native chicken (PBN), and broiler parent lines viz. PB1 (sire line) and PB2 (dam line), maintained under the ICAR-AICRP Poultry Breeding Project, at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Primary data pertaining to body weight and morphometric variables was recorded from 50 males and 50 females from each genetic group (PBN, PB1 and PB2) at 5 weeks interval till 25 weeks of age.

3.1.1 Selection Procedure

For the present study, chicks of each group were regenerated by randomly mating 50 males with 250 females of each group. Artificial insemination of the hens was done with semen from cocks of respective strain at Poultry Breeding farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Wing tags were attached on right wing of birds, for individual identification and facilitation of data recording.

3.1.2 Management and feeding

For the chick, grower, and laying stages of the birds, standard management procedures were followed. The feeding of birds was done conforming to the ICAR-AICRP feeding schedule and initially upto chick stage (upto 5 weeks age), birds were fed *ad libitum*, followed by restricted feeding commencing at 5 weeks and continuing through the grower and laying stages of the birds. *Ad libitum* clean and cool water was offered to birds throughout. The feeding records were maintained on door cards of every flock. Feed efficiency of each group was calculated from these records. All the birds were raised under uniform environmental, managemental as well as feeding conditions so as to minimize the effect of environment.

3.1.3 Vaccination and deworming

The entire flock was vaccinated covering diseases such as Mareks disease, New Castle disease (F1, R2b, and Lasota strains), Infectious Bursal disease, and Fowl

pox. Routine deworming was followed at every three months, to avert losses accounted to parasitic infestations.

3.2 Description of experiments and measurements

3.2.1 Experiment No. 1: Morphological characterization and discriminant function analysis for Punjab Brown native poultry vis-à-vis parent lines broiler variety.

Phenotypic data pertaining to traits body weight and morphometric variables were collected. Variables were recorded as mentioned in table 1 in agreement with FAO (2012) guidelines for measuring the variables. The following variables were documented for the investigation:

1. Sex/Gender
2. Sire number
3. Dam number
4. Day old body weight (BWT0) (in g)
5. 5 weeks body weight (BWT5) (in g)
6. 10 weeks body weight (BWT10) (in g)
7. 15 weeks body weight (BWT15) (in g)
8. 20 weeks body weight (BWT20) (in g)
9. 25 weeks body weight (BWT25) (in g)
10. Body length at 5 weeks (BL5) (in cm)
11. Body length at 10 weeks (BL10) (in cm)
12. Body length at 15 weeks (BL15) (in cm)
13. Body length at 20 weeks (BL20) (in cm)
14. Body length at 25 weeks (BL25) (in cm)
15. Wing Length at 5 weeks (WL5) (in cm)
16. Wing Length at 10 weeks (WL10) (in cm)
17. Wing Length at 15 weeks (WL15) (in cm)
18. Wing Length at 20 weeks (WL20) (in cm)

19. Wing Length at 25 weeks (WL25) (in cm)
20. Shank length at 5 weeks (SL5) (in cm)
21. Shank length at 10 weeks (SL10) (in cm)
22. Shank length at 15 weeks (SL15) (in cm)
23. Shank length at 20 weeks (SL20) (in cm)
24. Shank length at 25 weeks (SL25) (in cm)
25. Thigh length at 5 weeks (TL5) (in cm)
26. Thigh length at 10 weeks (TL10) (in cm)
27. Thigh length at 15 weeks (TL15) (in cm)
28. Thigh length at 20 weeks (TL20) (in cm)
29. Thigh length at 25 weeks (TL25) (in cm)
30. Breast girth at 5 weeks (BG5) (in cm)
31. Breast girth at 10 weeks (BG10) (in cm)
32. Breast girth at 15 weeks (BG15) (in cm)
33. Breast girth at 20 weeks (BG20) (in cm)
34. Breast girth at 25 weeks (BG25) (in cm)
35. Keel length at 5 weeks (KL5) (in cm)
36. Keel length at 10 weeks (KL10) (in cm)
37. Keel length at 15 weeks (KL15) (in cm)
38. Keel length at 20 weeks (KL20) (in cm)
39. Keel length at 25 weeks (KL25) (in cm)

Figure 1: Measurement of Body length (FAO, 2012)

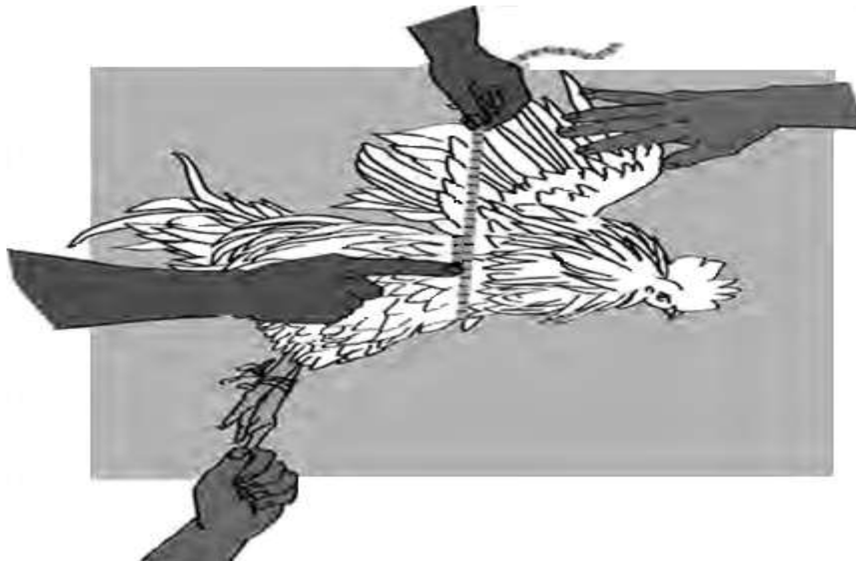


Figure 2: Measurement of Body Girth (FAO, 2012)

Figure 3: Measurement of Shank length (FAO, 2012)

Figure 4: Punjab Brown Female at Poultry Breeding Farm, GADVASU, Ludhiana

Figure 5: Punjab Brown Male at Poultry Breeding Farm, GADVASU, Ludhiana

Table 1: Measurement of various traits and instruments used as per guidelines of FAO (2012)

S No.	Trait	Measurement	Instrument
1	Body Weight (BW)	Recording the weight displayed on display of weighing balance	Digital Weighing Balance
2	Body Length (BL)	Bird was restrained in lying position with fully stretched sigmoid neck and Body length was measured from the tip of the beak to the tip of the tail (excluding feathers).	Graduated flexible measuring tape
3	Wing Length (WL)	Measured as the distance between the tip of the phalanges and the coracoids-humerus joint.	Graduated flexible measuring tape
4	Shank Length (SL)	shank length was measured from the hock joint to the spur of either leg.	Graduated flexible measuring tape
5	Thigh Length (TL)	Thigh length was measured as the distance between the hock joint and the pelvic joint	Vernier Callipers
6	Breast girth (BG)	Breast Girth was measured as the circumference of the breast around the deepest region of the breast	Graduated flexible measuring tape
7	Keel Length (KL)	Keel length was taken as the distance between the anterior and posterior ends of the keel.	Vernier Callipers

3.2.1.1 Multivariate analysis of variance (MANOVA) of variables

To study the effect of genetic group or strain, all variables were subjected to multivariate analysis of variance (MANOVA). Age and sex wise analysis of data pertaining to each variable was done. As the sex of the birds was assessed at 6 to 7 weeks of age, therefore for variables recorded at day of hatch and 5 weeks age, were analyzed with pooled sexes. At day old stage only body weight (BW0) was recorded to avoid any stress to the newly hatched chicks. MANOVA was carried out to study the effect of genetic group or strain. General linear model MANOVA option in IBM SPSS Statistic (24.0) software package was used for this study.

3.2.1.2 Discriminant Analysis

Discriminant function was given by Fisher (1936) with a purpose to discriminate the individuals belonging to two different populations showing some degree of overlapping. A function Z was defined as;

$$z = b_1 x_1 + b_2 x_2 \ldots\ldots + b_n x_n$$

Where, $x_1, x_2 \ldots\ldots, x_n$,. are the variables measured, and $b_1, b_2, \ldots\ldots b_n$, are the weighing coefficients. The b_i values are estimated such based on z values, the ratio of variance between populations to that of within the populations would be maximized. The maximization of this ratio leads to a set of simultaneous equations which after solution provide the desired b_i values.

Assuming 3 variables, the simultaneous equations will take the following form:

$$b_1' + b_2' r_{12} + b_3' r_{13} = d_1'$$

$$b_1' r_{12} + b_2' + b_3' r_{23} = d_2'$$

$$b_1' r_{13} + b_2' r_{23} + b_3' = d_3'$$

……. equation I

A solution of these equations in terms of matrix is as follows;

$$\boldsymbol{b} = \boldsymbol{R}^{-1} \boldsymbol{d}$$

Where**,** b is the column vector of coefficients, R^{-1} is the inverse of the correlation matrix and D is the column vector for d'_i values.

In equation (I) the various components are:

$$r_{12} = \frac{\sum(x_1 x_2)}{\sqrt{\sum(x_1)^2 \sum(x_2)^2}}$$

$$r_{13} = \frac{\sum(x_1 x_3)}{\sqrt{\sum(x_1)^2 \sum(x_3)^2}}$$

$$r_{23} = \frac{\sum(x_2 x_3)}{\sqrt{\sum(x_2)^2 \sum(x_3)^2}}$$

Similarly

$$d_1' = \frac{d_1}{\sqrt{\sum(x_1)^2}} \qquad \text{with} \qquad d_1 = x_1^1 - x_1^2$$

$$d_2' = \frac{d_3}{\sqrt{\sum(x_3)^2}} \qquad \text{with} \qquad d_1 = x_3^1 - x_2^2$$

$$d_3' = \frac{d_2}{\sqrt{\sum(x_2)^2}} \qquad \text{with} \qquad d_1 = x_2^1 - x_3^2$$

$$b_1' = \frac{b_1}{\sqrt{\sum(x_1)^2}}, \quad b_2' = \frac{b_2}{\sqrt{\sum(x_2)^2}}, \quad b_1' = \frac{b_3}{\sqrt{\sum(x_3)^2}}$$

Where, x_i^1 and x_i^2 indicate mean of ith character in population 1 and 2 respectively. (Singh & Chaudhary, 1977)

For the present study discriminant analysis was carried out by using IBM SPSS Statistic (24.0) software package. Seven morphometric variables (i.e., BL, WL, WS, SL, TL, BG and KL) along with body weight (BW) variable were used in the discriminant analysis. In order to assess the covariance between the variables used, the correlation among all the body weight and morphometric variables was estimated. The discriminant function combined several quantitative variables, each of which made an independent contribution to the overall discriminant analysis. The discriminant analysis extracted discriminant functions, based on the number of independent variables used or one less than that number of levels (n) of dependent variable (n – 1). Eigen value indicating the percent variance explained by each function was estimated, this indicated the efficacy of discriminant function. The relative importance of the morphometric variables in discriminating the three groups was assessed through the significance of the discriminant function for which Wilk's

lambda and Chi-square estimates were obtained and their significance was checked at $p<0.05$. Both standardised and unstandardized coefficients of each variable were estimated and these coefficients indicated the most important explanatory variables in the discriminant function.

Accuracy of the classification was evaluated using cross-validation statistics. The ability of the most discriminating function to correctly categorise the chicken genotypes was studied by classification (or misclassification) analysis.

3.2.2 Experiment No. 2: Effect of GDF-8 gene polymorphism on economic traits in the Punjab Brown

3.2.2.1 Chemicals / equipment and laboratory wares / buffers and solutions / enzymes

All the chemicals, equipments, laboratory wares and miscellaneous items used for the study have been enlisted in Annexure-I, while the details of buffers, solutions and enzymes have been given in Annexure-II.

3.2.2.2 Experimental Animals

One eighty blood samples (90 male and 90 females) were collected from Punjab Brown breed birds maintained at Poultry Breeding Farm, GADVASU, Ludhiana. Males and females were classified into three groups viz. stunted, medium and high growth on the basis of BWT5 of the individuals.

3.2.2.3 Collection of blood samples:

With help of 24-gauge needle and 2 ml syringe, 1 ml of venous blood was collected from wing vein of birds under sterile conditions in 2 ml microcentrifuge tubes containing 50 microlitres of 2.7% EDTA solution as anticoagulant. The tube was tightly capped and shaken gently to facilitate through mixing of blood with the anticoagulant. Blood samples were transported to the laboratory in an ice box containing ice packs and stored in the refrigerator at -20°C till the isolation of genomic DNA.

3.2.2.4 Extraction of Genomic DNA from blood

Genomic DNA was isolated from whole blood by phenol-chloroform-isoamyl alcohol method (Sambrook & Russell, 2001) with minor changes as described below:

1. The blood samples, which were stored at -20°C were taken out and thawed properly to make a uniform solution.
2. 250µl of Blood was transferred to 2 ml microcentrifuge tubes and 1000µl of poultry lysis buffer and 2 µl of Proteinase K (20mg/ml) were added to it and kept for overnight incubation at 56°C in water bath.
3. Next morning, the tubes were centrifuged at 15000 rpm for 15 minutes at 20°C.
4. The supernatant was transferred into autoclaved 2 ml microcentrifuge tubes, and equal volume of equilibrated phenol (Tris saturated phenol of pH 8.0) was added and mixed thoroughly by continuous inversion with inverting machine (Hulamixer™ Sample Mixer, Invitrogen™) for 15 min till a light coffee coloured uniform solution without any balls of phenol is formed.
5. The mixture was centrifuged at 15000 rpm for 15min at 20°C.
6. The upper aqueous phase containing DNA was transferred into fresh autoclaved 2 ml Eppendorf tubes by means of wide bore tips. Care was taken during transfer of viscous aqueous phase so that the lower organic phase (containing phenol, cell lysate, proteins etc.) and white interface (containing proteins) is not disturbed.
7. The extraction step was repeated (as in steps 4 to 6) once with equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and then with Chloroform:Isoamyl alcohol (24:1).
8. Supernantant was transferred to 1.5 ml autoclaved eppendorf tubes after Extraction with Chloroform:Isoamyl alcohol (24:1).
9. To this extracted phase 0.6 volume of chilled isopropanol and 0.1 volume of 3M sodium acetate (pH 5.5) was added and mixed gently by inversion and kept at room temperature for 15 minutes to allow the precipitation of DNA.
10. Then the contents were centrifuged at12000 rpm for 15 min at 4°C.
11. The supernatant was discarded by gentle inversion without disturbing DNA pellet.
12. The DNA pellet then washed twice with 0.5 ml of 70% ethanol and Eppendorf tubes were centrifuged at 12000 rpm for 15 min at room temperature.
13. Finally, DNA pellet was air dried after inverting in blotting paper to remove the traces of ethanol. The pellet was dissolved in 60µl of nuclease free water (NFW).

14. DNA samples were incubated in water bath at 65°C for 1 hour to inhibit DNase activity and to dissolve DNA pellet properly into NFW.
15. Samples were stored at 4°C for a week so that DNA is dissolved completely and subsequently at -20°C till use.

3.2.2.5 Evaluation of quality, purity and concentration

The genomic DNA isolated from the blood was checked for its quality, purity and concentration. DNA samples of good quality, purity and concentration were used for further analysis.

3.2.2.5.1 Quality of DNA

The quality of the genomic DNA was checked by agarose gel electrophoresis. For this purpose, 0.8 % agarose w/v suspension in 1X TAE buffer was prepared and heated in microwave until the agarose was completely melted and dissolved to give a clear transparent solution. Then, it was cooled to about 50-60°C and ethidium bromide (10mg/ml) @ 10µl per 100ml of agarose solution was added and mixed gently. Meanwhile, the agarose casting tray was prepared and the comb was put in proper position leaving 0.1 cm gap between the tips of the comb teeth and floor of casting tray so that wells were completely sealed by the agarose. The agarose solution was poured into the gel casting tray and its level was adjusted. The agarose was allowed to cool and comb was removed from the gel casting tray and it was submerged in the gel tank containing 1X TAE buffer. For loading the samples 1µl of DNA was diluted with 20µl autoclaved distilled water, 5µl of diluted DNA along with 1.0 µl of gel loading dye (6X) were mixed and loaded into the wells. Electrophoresis was performed at 80V for 30min. Then the gel was visualised and photographed under gel documentation system. Intact DNA samples devoid of smearing were inferred to be of good quality and were used in the present investigation.

3.2.2.5.2 Purity of DNA

The purity of genomic DNA was assessed by spectrophotometry (Nanodrop). The optical density (OD) value at 260 nm indicated the amount of nucleic acid present while that of 280 nm gave the amount of protein. The ratio of optical density (OD) values at 260 nm to that of 280 nm was used as criterion for checking the purity of DNA. For this purpose, 2 µl of stock DNA sample was used for

taking the reading in spectrophotometer. The reading was taken against NFW as a blank. Samples showing OD ratio (260/280) between 1.8 to 1.9 were used for further analysis.

3.2.2.5.3 Concentration of DNA

The concentration of stock DNA was calculated by taking OD value at 260nm. The following formula was employed for the purpose; DNA concentration (µg/ml) = OD_{260} × dilution factor × 50 (1 OD value at 260nm is equivalent to 50µg dsDNA/ml). From the stock solution of DNA working solution was prepared so that each sample has a concentration of 80 to 100 ng/µl of DNA.

3.2.2.6 PCR amplification

3.2.2.6.1 PCR amplification of 605bp fragment of GDF-8 gene of poultry (*Gallus gallus domesticus*)

The promoter region of GDF-8 gene was amplified from isolated genomic DNA of poultry birds. A 605bp fragment was amplified by using the corresponding set of reported forward and reverse primers (Kumar et al., 2007) as indicated in the following table (Table 2).

Table 2: Forward and reverse primer sequences used for amplification of promoter region of GDF-8 gene

Primer	Primer sequence	Size of Amplicon
GDF-8 Forward	5′CAAAATGTTTATTCCTGCTCA3′	605bp
GDF-8 Reverse	5′CTAAACAGATCCGGGACAGC3′	

The primers were stored at -20°C. The primers were diluted with nuclease free water to make the stock solution with concentration of 100 pmoles/µl. The working solution of primer was prepared by 10 fold dilution of stock primer solution in nuclease free water, so that each has a concentration of 10 pmoles/µl.

3.2.2.6.2 Optimization of PCR reaction mixture

The reaction mixture was optimised using variable concentrations of different components for amplification of specific region of GDF-8 gene in a final volume of 25µl. The optimized PCR reaction mix that was finally used for amplification is as follows (Table 3);

Table 3: Optimized PCR reaction mixture

S. No.	Reaction components	Volume	Final concentration
1.	5X Colourless GoTaq® Flexi Buffer	5 µl	1X
2.	dNTPs	0.5 µl	10mM
3.	$MgCl_2$	1.5 µl	1.5 mM
4.	Forward primer	0.8 µl	10pmol/ µl
5.	Reverse primer	0.8 µl	10pmol/ µl
6.	GoTaq® Flexi DNA Polymerase	0.25 µl	5U/ µl
7.	Nuclease free water	15.15 µl	-
8.	Genomic DNA (template)	1.0 µl	(80-100ng/ µl)
	Total	**25 µl**	

3.2.2.6.3 PCR reaction conditions for amplification

The components of optimized PCR reaction were taken in an ice box. A master mix was prepared for the required number of reactions by adding the reaction components in the following order, nuclease free water, 5X Colorless GoTaq® Flexi Buffer, dNTP mix, $MgCl_2$, forward and reverse primers and finally GoTaq® Flexi DNA Polymerase (Promega, USA) enzyme. The 24 µl of master mix was distributed into 0.2ml of each PCR tubes duly labelled and marked. Finally, 1 µl of genomic DNA was added to the PCR tubes followed by gentle mixing and reaction mix was spun at 3000 rpm for 10 seconds. The PCR tubes with the reaction mixture were put in a thermo cycler (Applied Biosystems). The PCR amplification was optimised by the trial of various annealing temperature and was used in further study. (Table 4)

Table 4: PCR programme for amplification of target sequence

S. No.	Steps	Temperatures	Time	Remarks
1.	Initial denaturation	94°C	5min	
2.	Cyclic denaturation	95°C	45sec	35 cycles
3.	Cyclic annealing	57°C	30sec	
4.	Cyclic extension	72°C	45sec	
5.	final extension	72°C	8 min	
6.	Storage	4°C	∞	

3.2.2.6.4 Checking of amplicons

After completion of PCR program, the PCR products were stored at 4°C. Horizontal submarine agarose gel electrophoresis was carried out to check different PCR amplified products, 5 µl of PCR product was mixed with 1 µl of 6X loading dye and then loaded in the wells on gel. The amplicons were analysed by running on 2% agarose gel at 85 V for 25-30 min in 1X TAE buffer along with marker (100 kb DNA ladder, Promega, USA). The gel stained with ethidium bromide was viewed under UV transilluminator and documented by gel documentation system (Syngene, UK) and image was captured and saved for further analysis.

3.2.2.7 Restriction digestion of PCR products

The amplicons of high quality were digested by restriction enzyme. To identify the restriction fragment length polymorphism (RFLP). *Sca I* restriction enzymes was used for the different fragments of GDF-8 gene (promoter region).

3.2.2.7.1 Restriction digestion of 605 bp fragment of GDF-8 gene of chicken by *Sca I*

The 605 bp fragment comprising of promoter region of GDF-8 gene of chicken was digested by *Sca I* enzyme. The recognition sequence of *Sca I* restriction enzymes is AGT↓ACT.

3.2.2.7.2 Optimization of Restriction enzyme (RE) digestion

The various combinations of digestion mixtures were tried. The optimized RE digestion mixture is as follows (Table 5).

Table 5: Optimized RE digestion mixture (GDF-8 gene)

S. No.	Reaction components	Amount
1.	Nuclease free water	8 µl
2.	10x buffer R for RE	1 µl
3.	Restriction enzyme (10 U/µl)	0.5 µl
4.	PCR product	0.5 µl
	Total	**10 µl**

3.2.2.7.3 Setting up of Restriction enzyme digestion

All the components required for RE digestion were put on ice. Nuclease free water, buffer and enzymes were added in the order for required number of reactions in a 0.2 ml PCR tubes to make a master mix. Then, the master mix was dispensed into labelled 0.6 ml eppendorf tubes and PCR products were added to the corresponding tubes. All the above steps were carried out on ice. The tubes for RE digestion were spun shortly and incubated as per the conditions recommended for RE. After digestion the RE digests were stored at 4 °C.

3.2.2.7.4 Digestion condition

The restriction enzyme digestions were carried out as per the RE manufacture's guidelines in the following condition;

Restriction enzyme	Temp	Time Incubation
1. *Sca I*	37°C	4 hours

3.2.2.8 Gel electrophoresis of RE digested PCR product

3.2.2.8.1 Agarose gel electrophoresis

Four µl of 6x Purple loading dye (Bromophenol blue) was added in the digested products and mixed properly. The mixture was then loaded in the wells. The digested products fragments were electrophoresed in 2.5% w/v agarose gel which was stained with ethidium bromide, at 60 V 1 hr in 1x TAE buffer. The gel was then viewed under gel documentation system and images were captured at different zoom levels. For comparison, an undigested sample was also loaded in one well and ran along with the RE digests.

3.2.2.8.2Visualization of RE digests and documentation

The gels containing RE digests were viewed and photographed under gel documentation system to determine the genotype/patterns of GDF-8 gene (promoter region) of chicken. Photographs of all the gels were stored in the computer system of the gel documentation system and used for future analysis of genotype pattern of birds.

3.2.2.8.3 Estimation of the size of the restricted fragments

To determine the size of the restriction fragments 100bp DNA ladder (Promega, USA) was used. Five µl of the ladder was loaded in one well and run parallel to the digested products in agarose gel electrophoresis.

3.2.2.8. Association of genotypes with body weight variables

Statistical analysis to study the significant association of genotypes with body weight variables was done by general linear model, multivariate analysis of variance (MANOVA) option in IBM SPSS Statistic (24.0) software package.

3.2.2.9 Nucleotide sequencing of the DNA samples

3.2.2.9.1 Elution of DNA from low melting agarose

The amplicons of GDF-8 genes were eluted and sent for sequencing. For elution, amplicons were electrophoresed in 2% low melting agarose gel stained with ethidium bromide, at 100 V for 30 minutes in 1X TAE buffer. The gel was then viewed under UV transilluminator in very low intensity in order to avoid any UV induced mutation in the amplified DNA. DNA was purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega). The manufacture's protocol was followed which is described here.

3.2.2.9.2 Wizard® SV Gel and PCR Clean-Up System Kit Protocol (using a micro centrifuge)

The Wizard® SV Gel and PCR Clean-Up System was designed to extract and purify DNA fragments of 100bp to 10kb from standard or low-melt agarose gels in either Tris acetate (TAE) or Tris borate (TBE), or to purify PCR products directly from a PCR amplification. The Wizard® SV Gel and PCR Clean-Up System is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. After electrophoresis to separate the DNA fragments, the band(s) of interest is excised and dissolved in the presence of guanidine isothiocyanate (Membrane Binding Solution). Alternatively, after amplification, an aliquot of the PCR is added to the Membrane Binding Solution and directly purified. The system allows a choice of methods for isolation of DNA from the dissolved agarose gel slice or PCR amplification. DNA can be isolated using microcentrifugation to force the dissolved gel slice or PCR product through the membrane while simultaneously binding the DNA on the surface of the silica. After washing the isolated DNA fragment or PCR product, the DNA is eluted in water.

1. First the PCR products were loaded and run on agarose gel in 1X TAE Buffer at 85V for 35 minutes using standard gel electrophoresis protocol.

2. The weight of 1.5 ml microcentrifuge tube is recorded on weighing balance.
3. PCR Product was visualized on a UV transilluminator.
4. The PCR amplicon of interest was expunged from gel along with least possible amount of agarose gel with the help of a sharp razor blade.
5. Care was taken to keep the gel under UV light for minimum possible time to reduce nicking and degradation of DNA.
6. Excised gel slice was transferred to the weighed microcentrifuge tube and new weight was recorded. The weight of empty microcentrifuge tube was subtracted from the total weight to get the weight of the excised gel slice.
7. To this microcentrifuge tubes Membrane Binding Solution was added at a ratio of 10µl of solution per 10mg of agarose gel slice and incubated at 55 °C for 10 minutes or until the gel slice was completely dissolved.
5. The mixture was vortexed and centrifuged at 10000 rpm for 30 s at room temperature to ensure that the contents were at the bottom of the tube.
6. One SV Minicolumn is placed in a collection tube for each dissolved gel slice.
7. The dissolved gel mixture is transferred to the SV Minicolumn assembly, incubated for 1 minute at room temperature and SV Minicolumn Assembly is centrifuged @ 14,000 rpm for 1 minute.
8. SV Minicolumn is removed from the Spin Column assembly and the liquid in the Collection Tube is discarded.
9. SV Minicolumn is placed back into the collection tube and washed with700µl of Membrane Wash Solution, previously diluted with 95% ethanol.
10. SV Spin Column Assembly is centrifuged at 14000 rpm for 1 minute
11. The collection tube is again emptied and washing step was repeated with 500µl of Membrane Wash Solution and the SV Minicolumn assembly was centrifuged @ 14000 rpm for 5 minutes at room temperature.
12. The collection tube is emptied and the column assembly is recentrifuged for 1 minute to evaporate any residual ethanol.
13. The SV Minicolumn is transferred to a clean 1.5ml microcentrifuge tube.

14. 50µl of Nuclease-Free Water was added directly to the centre of the column without touching the membrane with the pipette tip. After Incubation at room temperature for 1 minute, Centrifugation for 1 minute at 14,000rpm was done.
15. SV Minicolumn was discarded, and the microcentrifuge tube containing the eluted DNA was stored at –20°C.

The eluted DNA was quantified using Nanodrop and then sent for the DNA sequencing to Bioserve Technologies Private Limited, Hyderabad. The sequencing of DNA was done using ABI 3730 (48 capillary) DNA Analyzer (Thermo Scientific). The sequences were then analysed using online tools i.e., BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Alibaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/) for gene regulation study. The output result files of Clustal Omega were saved in Jalview (.jvl) format for SNP detection and viewed in JalView2 software.

CHAPTER IV

RESULTS AND DISCUSSION

Under the experiment data were recorded on individuals of three treatment groups viz. Punjab Brown (PBN), PB1 and PB2, at a regular interval of 5 weeks starting from day of hatch (day 0) till the age of 25 weeks, all the body weight and morphometric variables were measured. On the hatch day, only body weight of chicks was recorded to avoid any stress to the chicks. Accurate gender detection, in all the three groups, was done at 6 weeks of age based on vent sexing, plumage as well as growth pattern of comb and wattles. Therefore, the analysis of data was done for pooled sexes till 5 weeks of age and gender-wise analysis was carried out for variables recorded 10 weeks onwards.

4.1 Multivariate analysis of variance (ANOVA) and Post hoc test results

The results of the multivariate ANOVA performed using GLM procedure in IBM SPSS Statistic (24.0), revealed that there was significant difference ($p \leq 0.05$) in body weight and morphometric variables between the three treatment groups. All variables showed an increasing trend with increasing age of the birds (Tables 6 & 7).

Post hoc test revealed that body weight of pooled sexes, measured at day 0 (BW0) and 5 weeks age (BW5) of PB1 birds was significantly ($p \leq 0.05$) higher followed by PB2 and Punjab Brown birds. It was observed that in both genders the BW variables recorded from 10th week to 25th week age had highest mean estimates for PB1 followed by PB2 and Punjab Brown birds. Though PB1 birds had higher mean body weight but there was no significant difference between the weight variables (BW10, BW15, BW20 and BW25) of PB1 and PB2 birds in both sexes.

The body length variables (BL10, BL15, BL20 and BL25) were highest for PB2 group of birds followed by PB1 and Punjab Brown birds. In both sexes, the Punjab brown birds had significantly ($p \leq 0.05$) lower BL10, BL15, BL20, in comparison to PB1 and PB2 birds. In case of BL25 there was no significant difference between Punjab Brown and PB1 birds.

Punjab Brown females had significantly higher ($p \leq 0.05$) wing length (WL) and shank length (SL) from PB1 and PB2 females across age groups. In adult females, the PB1 females had higher WL25 in comparison to PB2 birds. In case of male birds,

the estimates of WL20 and WL25 of mature PB1 birds were significantly higher ($p \leq 0.05$). Between the three genetic groups, there was no significant difference in the male birds with respect to SL10, SL15, SL20 and SL25 variables. SL25 was highest in PB1 males (9.29 centimetres).

In both genders the thigh length variables (TL10, TL15, TL20 and TL25) were highest ($p \leq 0.05$) in PB1 followed by Punjab Brown and PB2. The thigh length was least in PB2 birds among the three groups in either sex.

The keel lengths measured at different ages in either sex were least for Punjab brown birds. PB2 male and females had significantly ($p \leq 0.05$) higher KL10, KL15 and KL20 estimates among the treatment groups. In mature birds the mean estimates of KL25 was higher ($p \leq 0.05$) in PB1 among the three treatment groups.

The body girth variables of female broiler lines PB1 and PB2 were higher than Punjab brown females. BG10, BG15 and BG20 estimates of PB2 females were highest ($p \leq 0.05$) among all the groups. PB1 females had higher ($p \leq 0.05$) estimate for BG25 in comparison to PB2 and Punjab Brown birds. The observations of body girth were different in male birds i.e., in case of male birds, the body girth measurements seemed to have increased for native and PB1 birds, post 15 weeks of age. The Punjab Brown males had significantly higher ($p \leq 0.05$) estimates for BG15, BG20 and BG25 followed by PB1 and PB2 birds.

Table 6: Mean estimates body weight variables of pooled sexes of three genetic groups measured at 5 weeks age

Trait	PB1 (N = 100)	PB2 (N = 100)	Native (N = 107)
BW0	$45.57^{a} \pm 0.41$	$39.94^{b} \pm 0.33$	$37.66^{c} \pm 0.33$
BW5	$1191.56^{a} \pm 10.98$	$1008.19^{b} \pm 12.98$	$715.04^{c} \pm 20.09$
BL5	$29.7^{a} \pm 0.13$	$25.56^{b} \pm 0.12$	$21.78^{c} \pm 0.17$
WL5	$14.87^{a} \pm 0.12$	$12.41^{b} \pm 0.12$	$9.79^{c} \pm 0.12$
SL5	$6.86^{a} \pm 0.06$	$5.85^{b} \pm 0.05$	$3.15^{c} \pm 0.08$
TL5	$7.53^{a} \pm 0.06$	$4.35^{b} \pm 0.06$	$3.97^{c} \pm 0.09$
KL5	$7.67^{a} \pm 0.08$	$5.73^{b} \pm 0.04$	$3.79^{c} \pm 0.19$
BG5	$19.51^{a} \pm 0.16$	$20.85^{b} \pm 0.11$	$14.27^{c} \pm 0.17$

Means with different superscripts have significant difference between columns

Table 7: Mean estimates for body weight variables males and females of all genetic groups measured at different age groups

Traits	PB1 F (N = 64)	PB2 F (N = 59)	Native F (N = 50)	PB1 M (N=50)	PB2 M (N=51)	Native M (N=57)
BW: Body Weight (grams)						
BW10	1517.64^{a} ± 33.17	1478.98^{a} ± 22.55	987.24^{b} ± 7.58	1784.32^{a} ± 40.7	1728.90^{a} ±36.22	1274.07^{b} ±26.51
BW15	1674.2^{a} ± 30.17	1597.29^{ab} ± 22.31	1543.08^{b} ± 29.02	2177.87^{a} ± 48.98	2093.76^{a} ±36.71	1808.77^{b} ±27.69
BW20	2097.19^{a}±42.1	2078.49^{a}±33.35	1915.5^{b}±31.67	2623.94^{a}±68.76	2607.96^{a}±51.63	2080.00^{b}±39.39
BW25	2472.06^{a}±49.27	2453.42^{a} ± 34.81	2096.76^{b} ± 36.32	2996.47^{a} ± 61.96	3002.06^{a} ±52.43	2619.05^{b}±39.11
BL: Body Length (centimetres)						
BL10	39.94^{a}±0.18	40.16^{a}±0.15	35.35^{b}±0.24	41.30^{a}±0.22	41.47^{a}±0.17	36.17^{b}±0.23
BL15	41.11^{a}±0.26	41.38^{a}±0.14	38.03^{b}±0.25	43.14^{a}±0.21	42.73^{a}±0.17	38.80^{b}±0.23
BL20	43.26^{b}±0.27	45.71^{a}±0.54	40.88^{c}±0.28	44.40^{b}±0.26	46.72^{a}±0.61	41.74^{c}±0.27
BL25	45.85^{ab}±0.32	46.28^{a}±0.53	44.66^{b}±0.24	46.30^{ab}±0.31	47.30^{a}±0.60	45.44^{b}±0.22
WL: Wing Length (centimetres)						
WL10	21.50^{b} ± 0.15	22.19^{a} ± 0.16	21.80^{ab} ± 0.16	22.81^{a} ± 0.14	23.12^{a} ± 0.19	21.77^{b} ± 0.17
WL15	22.70^{b} ± 0.20	22.96^{b} ± 0.17	23.64^{a} ± 0.15	24.20 ± 0.19	23.88 ± 0.2	23.59 ± 0.16
WL20	23.92^{b} ± 0.20	23.63^{b} ± 0.18	24.93^{a} ± 0.14	25.48^{a} ± 0.21	24.54^{b} ± 0.21	24.90^{ab} ± 0.16
WL25	25.65^{a} ± 0.21	24.39^{b} ± 0.19	26.00^{a} ± 0.13	26.88^{a} ± 0.26	25.32^{b} ± 0.22	25.99^{b} ± 0.15

Traits	PB1 F (N = 64)	PB2 F (N = 59)	Native F (N = 50)	PB1 M (N=50)	PB2 M (N=51)	Native M (N=57)
SL: Shank Length (centimetres)						
SL10	$7.59^{c} \pm 0.06$	$8.26^{b} \pm 0.07$	$8.63^{a} \pm 0.12$	8.52 ± 0.08	8.67 ± 0.07	8.66 ± 0.1
SL15	$8.12^{c} \pm 0.05$	$8.45^{b} \pm 0.07$	$8.90^{a} \pm 0.12$	8.78 ± 0.09	8.87 ± 0.07	8.93 ± 0.11
SL20	$8.61^{b} \pm 0.05$	$8.63^{b} \pm 0.06$	$9.08^{a} \pm 0.12$	9.04 ± 0.09	9.06 ± 0.07	9.10 ± 0.11
SL25	$9.01^{ab} \pm 0.06$	$8.83^{b} \pm 0.06$	$9.18^{a} \pm 0.13$	9.29 ± 0.09	9.26 ± 0.07	9.20 ± 0.11
TL: Thigh Length (centimetres)						
TL10	$10.35^{a} \pm 0.07$	$8.75^{b} \pm 0.07$	$8.72^{b} \pm 0.12$	$10.99^{a} \pm 0.08$	$9.16^{b} \pm 0.08$	$9.20^{b} \pm 0.11$
TL15	$10.97^{a} \pm 0.07$	$9.19^{c} \pm 0.08$	$9.57^{b} \pm 0.12$	$11.60^{a} \pm 0.08$	$9.57^{c} \pm 0.08$	$10.06^{b} \pm 0.11$
TL20	$11.68^{a} \pm 0.08$	$9.44^{c} \pm 0.07$	$10.00^{b} \pm 0.12$	$12.10^{a} \pm 0.08$	$9.83^{c} \pm 0.08$	$10.48^{b} \pm 0.11$
TL25	$12.28^{a} \pm 0.09$	$9.70^{c} \pm 0.08$	$10.33^{b} \pm 0.13$	$12.55^{a} \pm 0.10$	$10.07^{c} \pm 0.08$	$10.77^{b} \pm 0.11$
KL: Keel Length (centimetres)						
KL10	$9.68^{b} \pm 0.1$	$10.92^{a} \pm 0.04$	$6.83^{c} \pm 0.06$	$9.8^{b} \pm 0.12$	$10.51^{a} \pm 0.04$	$10.52^{a} \pm 0.08$
KL15	$12.93^{b} \pm 0.15$	$14.12^{a} \pm 0.03$	$7.48^{c} \pm 0.07$	$12.9^{b} \pm 0.17$	$13.70^{a} \pm 0.03$	$11.14^{c} \pm 0.08$
KL20	$15.35^{a} \pm 0.15$	$15.44^{a} \pm 0.04$	$8.27^{b} \pm 0.09$	$15.27^{a} \pm 0.18$	$15.01^{a} \pm 0.05$	$11.88^{b} \pm 0.09$
KL25	$17.59^{a} \pm 0.27$	$16.18^{b} \pm 0.05$	$8.61^{c} \pm 0.11$	$17.82^{a} \pm 0.32$	$15.73^{b} \pm 0.05$	$12.17^{c} \pm 0.11$
BG: Body Girth (centimetres)						
BG10	$27.92^{b}\pm0.20$	$28.55^{a}\pm0.10$	$23.01^{c} \pm 0.17$	$27.35^{a} \pm 0.24$	$27.04^{a} \pm 0.12$	$25.37^{b} \pm 0.17$
BG15	$28.35^{b}\pm0.20$	$29.44^{a}\pm0.11$	$26.06^{c} \pm 0.16$	27.93 ± 0.24	27.94 ± 0.12	28.45 ± 0.16
BG20	$29.83^{a}\pm0.21$	$29.86^{a}\pm0.12$	$28.09^{b} \pm 0.16$	$29.55^{b} \pm 0.25$	$28.35^{c} \pm 0.13$	$30.51^{a} \pm 0.16$
BG25	$32.13^{a}\pm0.21$	$30.27^{b}\pm0.12$	$29.95^{b} \pm 0.19$	$31.77^{a} \pm 0.26$	$28.74^{b} \pm 0.13$	$32.38^{a} \pm 0.19$

Means with different superscripts have significant difference between columns for males and females

The mean estimates reflected the effect of selection programme that has been followed in these three groups of birds. The PB1 and PB2 parent birds have been maintained under the AICRP poultry breeding project for more than 45 generations. Selection in case of PB1 birds has been done solely on the basis of 5 week body weight whereas in PB2, which is the dam line, in addition to 5 weeks body weight, the performance with respect to 36 weeks egg production is also given weightage in selection of these birds. The base population of Punjab Brown, which is native breed and has lower growth performance, has been established more recently since 2015 and these birds have been selected for 8 week body weight and 36 weeks egg production.

4.2 Correlation estimates between body weight and morphometric variables.

The correlation estimates between the variables was obtained from the data recorded in pooled sexes. BW5 had a strong correlation with BL5 (0.82) and WL5 (0.72) variables at 5 weeks age. The estimates of BW5 were moderate with TL5 (0.48), KL5 (0.66) and BG5 (0.56). The lowest correlation of BW5 was observed with SL5 (0.37).

Among morphometric variables strong correlation was observed between WL5 and BL5 (0.88) followed by the correlation estimate of KL5 and BG5 (0.68). Correlation estimates were lower between WL5, SL5 and TL5 variables (Table 8).

Table 8: Correlation between body weight and morphometric variables at 5 weeks of age in pooled sexes

	BW5	BL5	WL5	SL5	TL5	KL5	BG5
BW5	1*						
BL5	0.82*	1*					
WL5	0.72*	0.88*	1*				
SL5	0.37*	0.40*	0.46*	1*			
TL5	0.48*	0.46*	0.47*	0.28*	1*		
KL5	0.66*	0.36*	0.20*	0.08*	0.31*	1*	
BG5	0.56*	0.45*	0.35*	0.04*	0.26*	0.68*	1*

* indicates significant at $p<0.05$

Similar observations were recorded with respect to correlation estimates of body weight and morphometric variables recorded at 10, 15, 20 and 25 weeks of age in either sex (Table 9 to 12).

Table 9: Correlation between body weight and morphometric variables at 10 weeks of age in separate sexes

Gender		BW10	BL10	WL10	SL10	TL10	KL10	BG10
Female	BW10	1*						
	BL10	0.80*	1*					
	WL10	0.82*	0.89*	1*				
	SL10	0.56*	0.44*	0.47*	1*			
	TL10	0.36*	0.36*	0.39*	0.25*	1*		
	KL10	0.28*	0.37*	0.34*	0.04*	0.25*	1*	
	BG10	0.20*	0.39*	0.37*	0.06*	0.21*	0.54*	1*
Male	BW10	1*						
	BL10	0.91*	1*					
	WL10	0.90*	0.89*	1*				
	SL10	0.53*	0.44*	0.47*	1*			
	TL10	0.58*	0.55*	0.59*	0.34*	1*		
	KL10	0.76*	0.77*	0.72*	0.42*	0.44*	1*	
	BG10	0.87*	0.91*	0.81*	0.44*	0.49*	0.81*	1*

* indicates significant at p<0.05

Table 10: Correlation between body weight and morphometric variables at 15 weeks of age in separate sexes

Gender		BW15	BL15	WL15	SL15	TL15	KL15	BG15
Female	BW15	1*						
	BL15	0.92*	1*					
	WL15	0.65*	0.61*	1*				
	SL15	0.49*	0.43*	0.33*	1*			
	TL15	0.36*	0.34*	0.34*	0.25*	1*		
	KL15	0.25*	0.21*	0.15*	-0.04*	0.20*	1*	
	BG15	0.39*	0.32*	0.35*	0.08*	0.21*	0.47*	1*
Male	BW15	1*						
	BL15	0.89*	1*					
	WL15	0.85*	0.84*	1*				
	SL15	0.52*	0.39*	0.47*	1*			
	TL15	0.55*	0.53*	0.54*	0.31*	1*		
	KL15	0.62*	0.62*	0.55*	0.31*	0.37*	1*	
	BG15	0.88*	0.88*	0.78*	0.44	0.47*	0.68*	1*

* indicates significant at p<0.05;

Table 11: Correlation between body weight and morphometric variables at 20 weeks of age in separate sexes

Gender		BW20	BL20	WL20	SL20	TL20	KL20	BG20
Female	BW20	1*						
	BL20	0.70*	1*					
	WL20	0.64*	0.46*	1*				
	SL20	0.49*	0.38*	0.31*	1*			
	TL20	0.32*	0.28*	0.27*	0.25*	1*		
	KL20	0.18*	0.14*	0.14*	-0.06*	0.13*	1*	
	BG20	0.31*	0.30*	0.31*	0.04*	0.14*	0.40*	1*
Male	BW20	1*						
	BL20	0.66*	1*					
	WL20	0.84*	0.59*	1*				
	SL20	0.50*	0.34*	0.44*	1*			
	TL20	0.54*	0.41*	0.52*	0.35*	1*		
	KL20	0.49*	0.33*	0.50*	0.18*	0.27*	1*	
	BG20	0.89*	0.62*	0.73*	0.44*	0.46*	0.51*	1*

* indicates significant at $p<0.05$

Table 12: Correlation between body weight and morphometric variables at 25 weeks of age in separate sexes

Gender		BW25	BL25	WL25	SL25	TL25	KL25	BG25
Female	BW25	1*						
	BL25	0.71*	1*					
	WL25	0.67*	0.50*	1*				
	SL25	0.52*	0.42*	0.36*	1*			
	TL25	0.29*	0.24*	0.28*	0.23*	1*		
	KL25	0.19*	0.15*	0.07*	0.03*	0.10*	1*	
	BG25	0.31*	0.26*	0.28*	0.04*	0.11*	0.31*	1*
Male	BW25	1*						
	BL25	0.67*	1*					
	WL25	0.83*	0.58*	1*				
	SL25	0.51*	0.36*	0.48*	1*			
	TL25	0.54*	0.39*	0.50*	0.37*	1*		
	KL25	0.43*	0.27*	0.43*	0.20*	0.21*	1*	
	BG25	0.84*	0.62*	0.74*	0.44*	0.42*	0.46*	1*

* indicates significant at $p<0.05$

4.3 Discriminant analysis

4.3.1 Discriminant Functions with three levels of outcome variable

Age wise canonical discriminant analysis was done for male and female variables separately. For different age group (10, 15, 20 and 25 weeks) of birds two discriminant functions were extracted for each gender (Table 13). Such discriminant functions had 3 levels (PB1, PB2 and Punjab Brown native) of outcome variable. The discriminant analysis for 5 week body weight was done with data of pooled sexes.

4.3.1.1 Discriminant analysis with three levels of outcome variable with data of 5 weeks age group birds

All the variables used for discriminant analysis were significant ($p<0.01$) for the discriminant function. The first discriminant function had the highest Eigen value (14.71) and explained higher variance (71.86%). The second discriminant function explained lower variance (28.14%). Cumulatively, both discriminant functions explained 100% of the variance and were highly significant ($p<0.01$) on the basis of estimates of Wilk's Lambda (0.01 and 0.15) and chi-square (1404.22 and 575.24) for the two functions. The structure matrix results reported the pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions, the results indicated that SL5, BL5 and TL5 had largest absolute correlation with the first discriminant function, whereas SL5 and BG5 had higher correlation with the second discriminant function (Table 14). In compliance with the structure matrix results the first discriminant function coefficients were loaded highly for SL5 (0.936), BL5 (Table 14) and TL5 (0.554) and the second function had higher coefficient estimates for SL5 (0.847) and BG5 (0.774) (Table 15). Group wise mean discriminant score indicated the distance between the groups (table 16). Cross validation results of classification analysis indicated a 100% success rate (Table 17).

4.3.1.2 Discriminant analysis with three levels of outcome variable with data of 10, 15, 20 and 25 weeks age group birds.

Gender wise discriminant analysis was done for the body weight and morphometric variables recorded at 10, 15, 20 and 25 weeks age in the PB1, PB2 and Punjab Brown native chicken. Two discriminant functions were extracted from the

analysis, and the first discriminant function explained higher variance in case of both females and males. In case of females the first discriminant function explained 83.72%, 88.18%, 86.85%, 75.97% variance and for males the first discriminant function explained 84.07%, 87.54%, 85.37%, 71.94% variance at 10, 15, 20 and 25 weeks age, respectively. In all age groups and in both genders the functions derived had significant ($p \leq 0.01$) Wilk's lamda and Chi-square estimates (Table 13).

In females with respect to the first discriminant function, structure matrix analysis revealed the most explanatory variable in the discriminant functions of 10, 15, 20 and 25 weeks age groups in females, were KL10, KL15, KL20 and KL25, respectively. This keel length (KL) variable also had the highest canonical discriminant function coefficient in all the discriminant functions. Whereas, in case of males the structure matrix analysis revealed that BL10, KL15, KL20 and BG25 were the most explanatory variables in the discriminant functions of 10, 15, 20 and 25 weeks age groups. These variables also had higher canonical discriminant function coefficient in the discriminant functions (Tables 14 and 15). Classification results revealed that all functions had very high discriminating power and correctly classified the cases (Table 17).

Table 13: Summary of Canonical Discriminant Functions with discriminant analysis having three levels of outcome variable

Age (Weeks)	Gender	Function	Eigen value	% of Variance	Cumulative %	Canonical Correlation	Wilk's lamda	Chi-square	df	Sig. (p value)
5	**M+F**	**1**	14.71	71.86	71.86	0.97	0.01	1404.22	14	0.00
		2	5.76	28.14	100	0.92	0.15	575.24	6	0.00
10	**Female**	**1**	20.38	83.72	83.72	0.98	0.01	778.97	14	0.00
		2	3.96	16.28	100	0.89	0.20	267.53	6	0.00
	Male	**1**	14.81	84.07	84.07	0.97	0.02	610.49	14	0.00
		2	2.81	15.93	100	0.86	0.26	199.18	6	0.00
15	**Female**	**1**	17.17	88.18	88.18	0.97	0.02	683.68	14	0.00
		2	2.30	11.82	100	0.83	0.30	199.43	6	0.00
	Male	**1**	16.70	87.54	87.54	0.97	0.02	609.41	14	0.00
		2	2.38	12.46	100	0.84	0.30	181.28	6	0.00
20	**Female**	**1**	17.29	86.85	86.85	0.97	0.02	700.12	14	0.00
		2	2.62	13.15	100	0.85	0.28	214.77	6	0.00
	Male	**1**	17.35	85.37	85.37	0.97	0.01	639.09	14	0.00
		2	2.97	14.63	100	0.87	0.25	205.56	6	0.00
25	**Female**	**1**	8.49	75.97	75.97	0.95	0.03	593.66	14	0.00
		2	2.69	24.03	100	0.85	0.27	217.83	6	0.00
	Male	**1**	12.19	71.94	71.94	0.96	0.01	645.02	14	0.00
		2	4.75	28.06	100	0.91	0.17	260.73	6	0.00

Table 14: Structure Matrix ordering the most explanatory to least explanatory variables in the with discriminant analysis having three levels of outcome variable

Variable	Function	5 weeks	10 weeks		15 weeks		20 weeks		25 weeks	
			Female	Male	Female	Male	Female	Male	Female	Male
SL	1	.620*	-0.123	-0.016	-0.096	-0.018	-.085*	-0.009	-0.056	-0.009
	2	0.303	.234*	-.051*	-.222*	-.038*	-0.015	-.013*	.093*	.022*
BL	1	.580*	.333*	.433*	.207*	.326*	0.135	.167*	.068*	-.077*
	2	0.017	0.041	-0.147	0.095	0.117	-.209*	-0.083	-0.062	0.011
TL	1	.551*	0.135	0.186	0.021	0.071	0.088	-0.007	0.245	0.158
	2	-0.354	-.509*	.593*	.786*	.790*	.866*	.794*	.839*	.608*
WL	1	.451*	0.002	.119*	-0.059	0.039	-.092*	-0.009	0.245	0.064
	2	0.046	.125*	-0.093	-.094*	.069*	0.050	.159*	.839*	.161*
KL	1	.337*	.635*	-0.089	.845*	.344*	.942*	.443*	.947*	-0.278
	2	0.024	0.503	-.266*	0.152	-0.225	0.032	0.355	-0.058	.681*
BW	1	.326*	.269*	.249*	0.039	.142*	0.038	.158*	.179*	-.122*
	2	0.092	-0.036	-0.014	.133*	0.101	-.120*	0.110	-0.046	0.083
BG	1	0.360	.417*	.178*	.261*	-.048*	.149*	-.160*	0.181	.324*
	2	.534*	0.117	0.008	-0.071	-0.009	0.004	0.121	.294*	0.116

*Largest absolute correlation between each variable and any discriminant function

Table 15: Canonical discriminant function coefficients (unstandardized) with discriminant analysis having three levels of outcome variable

Variable	Function	5 weeks	10 weeks		15 weeks		20 weeks		25 weeks	
			Female	Male	Female	Male	Female	Male	Female	Male
BG	1	0.081	0.159	-0.621	-0.024	-1.571	-0.183	-1.422	-0.088	1.206
	2	0.774	-0.193	0.907	-0.215	-0.122	0.021	0.125	0.261	0.019
BL	1	0.942	0.775	1.722	0.727	1.356	0.071	0.099	-0.041	-0.070
	2	-0.411	-0.209	-0.794	-0.208	0.178	-0.123	-0.133	-0.062	-0.032
BW	1	-0.009	0.004	0.001	-0.006	0.002	0.000	0.005	0.001	-0.005
	2	0.004	-0.003	0.002	0.003	0.000	-0.001	-0.001	-0.002	-0.002
KL	1	0.483	0.918	-1.292	1.195	0.845	1.267	0.903	0.677	-0.381
	2	-0.659	1.513	-1.228	0.031	-0.649	-0.041	0.455	-0.108	0.597
SL	1	0.936	-0.402	-0.126	0.163	0.082	0.038	-0.053	-0.224	-0.073
	2	0.847	0.853	-0.209	-0.841	-0.187	-0.084	-0.245	0.184	-0.183
TL	1	0.554	0.072	0.150	-0.170	-0.181	0.016	-0.204	0.289	0.528
	2	-0.742	-1.156	1.452	1.447	1.624	1.502	1.608	1.211	1.171
WL	1	-0.313	-1.450	-1.095	-0.096	-0.577	-0.152	-0.575	-0.186	0.289
	2	-0.072	0.624	-0.262	-0.306	-0.275	0.073	-0.046	0.235	0.001
constant	1	-23.562	-13.619	-16.258	-30.617	-10.660	-10.724	29.236	-3.963	-27.722
	2	-2.974	-6.406	11.038	8.190	-4.965	-9.743	-15.400	-20.409	-14.700

Table 16: Group wise mean discriminant score of different age groups with discriminant analysis having three levels of outcome variable

Strain	Function	5 weeks	10 weeks		15 weeks		20 weeks		25 weeks	
			Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
PB1	**1**	4.872	2.913	3.477	1.401	2.808	2.508	1.678	2.432	0.537
	2	-1.584	-2.232	2.008	1.894	2.057	1.852	2.494	1.620	3.256
PB2	**1**	-0.137	2.788	2.34	3.776	3.334	2.757	4.302	1.132	-4.589
	2	3.435	2.452	-2.139	-1.568	-1.780	-1.955	-1.665	-2.166	-1.137
NATIVE	**1**	-4.425	-7.019	-4.961	-6.248	-5.299	-6.463	-5.233	-4.449	3.663
	2	-1.730	-0.036	0.258	-0.574	-0.103	-0.064	-0.566	0.483	-1.668

Table 17: Cross validation results of classification analysis for discriminant analysis having three levels of outcome variable

Age (Weeks)	Sex	PB1 (%)	PB2 (%)	Native (%)
5	M+F	100	100	100
10	F	100	100	100
	M	100	98	100
15	F	100	100	100
	M	100	100	100
20	F	98.4	98.3	100
	M	100	96.1	100
25	F	92.2	100	100
	M	97.9	100	100

4.3.2 Discriminant analysis with six levels of outcome variable with data of 10, 15, 20 and 25 weeks age group birds.

Discriminant analysis was also done by considering 6 levels (3 strains and 2 genders) of outcome variable viz. PB1M (PB1 male), PB1F (PB1 female), PB2M (PB2 male), PB2F (PB2 female), NativeM (native male) and NativeF (native female). Data on all the independent variables were used for developing age specific discriminant functions. For this discriminant analysis the data of birds belonging to 10, 15, 20 and 25 weeks age group was used.

For different age groups of birds the discriminant analysis extracted 5 canonical discriminant functions. Wilk's lamda and Chi-square statistics indicated that all discriminant functions were significant ($p<0.01$). Only the 5th discriminant functions of age group 20 and 25 weeks were non significant. In each age group the first discriminant function explained the maximum variance, ranging from 56.6 to 72.2%. On cumulative basis all 5 discriminant functions, of each age group of birds, explained 100% of the variance. Structure matrix analysis revealed that the most explanatory variable, for the first discriminant function of 10,15, 20 and 25 weeks age

groups, were BL10, KL15, KL20 and KL25, respectively. Accordingly, also these variables also had highest Canonical Discriminant Function Coefficients in their respective discriminant functions. Classification analysis revealed that the discriminant functions (with 6 levels of outcome variable) of 10, 15, 20 and 25 weeks age groups of birds had 93.9%, 95.1%, 93.6% and 90.2% accuracy in correctly classifying the original grouped cases, respectively.

Rosário et al. (2008) recommended the use of performance data for canonical discriminant analysis, in which they used 6 independent variables and extracted 5 discriminant functions, with the first function explaining 89.35% of the total variance. They reported that in the first discriminant function of the 6 variables feed intake and live weight had the most explanatory power. Their discriminant analysis had 8 levels of the outcome variable. Ajayi et al. (2012) used body weight (BW), wing length (WL), wing span (WS), shank length (SL), tibial length (TL), body length (BL), body girth (BG), keel length (KL) for discriminant analysis in indigenous chicken genotypes (normal feathered, frizzled and naked neck) and broiler breed Anak Titan (AT). Their analysis revealed that the best discriminant function classified 85.2% of AT correctly. Twenty-two point seven of frizzled feathered were misclassified as naked neck and 33.3% of naked neck was misclassified as normal feathered. (Ogah, 2013) used discriminant analysis for studying the divergence between three Nigerian indigenous chicken genotypes (normal feathered, frizzled and naked neck) using morphometric variables viz. body weight, body length, chest circumference, shank length, thigh length, keel length, wing length and body width. They extracted 2 significant ($p<0.01$) discriminant functions with the first function explaining 59.7% of the total variance, and in the first function body weight and body length were the most explanatory variables. Daikwo et al. (2015) used discriminant analysis for discriminating between normal feathered and frizzle feathered chickens of Nigeria. They used breast girth, body length, shank length, bird height, head circumference, wing length, neck length, and keel length for extracting a single discriminant function. The single discriminant function explained 100% of variance and had 100% classification accuracy. Musa et al. (2018) derived discriminant function with 100% classification power for normal feathered (NF), frizzled feathered (FF) and naked

neck (NN) Nigerian chicken genotypes. They used explanatory variables such as breast girth (BG), wing length (WL), tibial length (TL), comb length (CL), comb height (CH), wattle length (WL) and wattle height (WH). Among the morphometric traits BG and TL were most correlated with body weight variable. The discriminant analysis revealed lesser divergence between NF and NN genotypes. The discriminant functions derived in their experiment explained 75 to 84% of the total variance. Venkatas et al. (2019) used the discriminant analysis for studying the divergence between indigenous chicken genotypes (normal feathered, frizzled and naked neck) and broiler breed Anak Titan in South African chicken. Analysis was helpful for differentiation of purebred and crossbred chicken based on morphometric traits. For analysis they used data on body length (BL), breast girth (BG), thigh length (TL), shank length (SL), keel length (KL), wing length (WL) and wing span (WS), collected at weekly intervals upto 20 weeks of age. All variables were significant ($p<0.01$) for discriminant function analysis and their discriminant functions had 100% classification power. Shoyombo et al. (2019) used 10 variables viz. body weight (BW), body length (BL), breast girth (BG), shank length (SL), thigh length (TL), comb length (CL), comb height (CH), wing length (WL), wattle length (WAL) and wattle thickness (WT) for stepwise discriminant analysis and found that seven morphometric traits, TL, SL, WAL, WL, CL, CH and BL, were significant ($p<0.001$) for discriminating three dual-purpose chicken breeds (Shika, Kuroiler, and Sasso) of Nigerian chicken.

Table 18: Summary of Canonical Discriminant Functions with six levels of outcome variable

Age (Weeks)	Function	Eigen value	% of Variance	Cumulative %	Canonical Correlation	Wilk's lamda	Chi-square	df	Sig. (p value)
10	1	11.29	56.6	56.6	0.958	0.002	1956.60	35	0.00
	2	6.159	30.9	87.4	0.928	0.027	1152.54	24	0.00
	3	1.608	8.1	95.5	0.785	0.196	521.69	15	0.00
	4	0.842	4.2	99.7	0.676	0.512	214.49	8	0.00
	5	0.06	0.3	100.0	0.238	0.943	18.69	3	0.00
15	1	11.986	67.9	67.9	0.961	0.004	1798.77	35	0.00
	2	3.026	17.1	85.0	0.867	0.047	977.05	24	0.00
	3	1.796	10.2	95.2	0.801	0.191	530.65	15	0.00
	4	0.811	4.6	99.8	0.669	0.534	201.08	8	0.00
	5	0.034	0.2	100.0	0.182	0.967	10.74	3	0.01
20	1	13.163	72.2	72.2	0.964	0.005	1701.10	35	0.00
	2	2.99	16.4	88.6	0.866	0.070	851.56	24	0.00
	3	1.802	9.9	98.5	0.802	0.280	408.03	15	0.00
	4	0.265	1.5	100.0	0.458	0.784	77.81	8	0.00
	5	0.008	0.0	100.0	0.088	0.992	2.47	3	0.48
25	**1**	7.261	58.0	58.0	0.938	0.009	1513.22	35	0.00
	2	3.928	31.4	89.4	0.893	0.074	836.46	24	0.00
	3	0.818	6.5	95.9	0.671	0.362	325.30	15	0.00
	4	0.485	3.9	99.8	0.571	0.659	133.79	8	0.00
	5	0.022	0.2	100.0	0.148	0.978	7.092	3	0.07

Table 19: Structure Matrix with six levels of outcome variable

Age (Weeks)	Function	KL	TL	WL	SL	BW	BL	BG
10	1	0.504	0.203	0.054	-0.100	0.323	.450*	0.412
	2	.649*	-0.243	0.013	0.099	-0.011	-0.090	0.075
	3	0.084	.629*	-0.161	-0.104	0.000	-0.295	-0.081
	4	0.038	0.261	0.416	0.497	.528*	0.399	-0.350
	5	0.363	0.516	.448*	.775*	0.349	0.447	.588*
15	1	.798*	0.027	-0.038	-0.082	0.077	0.271	0.187
	2	0.187	0.533	0.152	0.082	0.501	0.316	-0.106
	3	-0.176	.611*	-0.146	-0.261	-0.251	-0.034	-0.026
	4	0.415	0.223	-0.083	-0.010	-0.027	-0.355	0.447
	5	0.303	0.524	.796*	.766*	.733*	.733*	.669*
20	1	.872*	0.090	-0.056	-0.055	0.117	0.163	0.051
	2	0.151	.700*	0.023	-0.059	-0.182	-0.209	0.215
	3	-0.097	0.515	0.267	0.160	0.463	0.036	-0.251
	4	0.359	0.037	0.381	0.319	.583*	-0.017	.760*
	5	0.186	0.058	.484*	-.592*	0.241	.314*	0.307
25	1	.866*	0.205	-0.033	-0.018	0.218	0.083	0.009
	2	0.199	.649*	0.144	-0.003	-0.130	-0.071	0.408
	3	-0.189	0.614	0.456	.274*	.569*	0.051	-0.004
	4	0.142	-0.116	-0.025	-0.003	0.354	0.015	.726*
	5	0.322	-0.255	.628*	0.001	0.188	-.115*	0.263

Table 20: Canonical Discriminant Function Coefficients (Unstandardized) with six levels of outcome variable

Variable	Function	10 weeks	15 weeks	20 weeks	25 weeks
BW	1	0.002	-0.004	0.001	0.002
	2	-0.001	0.008	-0.003	-0.003
	3	0.004	-0.005	0.004	0.003
	4	0.002	0.003	0.002	0.003
	5	-0.004	-0.002	0.000	-0.001
BL	1	1.043	0.636	0.067	-0.024
	2	-0.530	-0.398	-0.098	-0.018
	3	-0.841	0.518	-0.157	-0.223
	4	0.320	-0.938	-0.257	-0.152
	5	0.113	0.250	0.097	-0.162
WL	1	-1.556	-0.214	-0.296	-0.287
	2	0.407	-0.285	0.120	0.220
	3	-0.169	-0.170	-0.069	0.055
	4	-0.042	-0.104	-0.072	-0.511
	5	0.126	0.300	0.489	0.840
SL	1	-0.484	0.019	-0.057	-0.229
	2	0.348	-0.545	0.003	0.068
	3	-0.347	-0.306	-0.350	-0.171
	4	0.396	0.019	0.270	-0.161
	5	1.374	0.886	-1.526	-0.126
TL	1	0.212	-0.130	0.032	0.239
	2	-0.632	0.717	1.393	1.102
	3	1.322	1.424	0.693	0.685
	4	0.109	0.432	-0.361	-0.322
	5	0.505	0.210	-0.051	-0.682
KL	1	0.628	1.318	1.308	0.709
	2	2.242	0.230	0.064	0.071
	3	0.378	-0.390	-0.132	-0.248
	4	0.349	0.347	0.039	-0.121
	5	-0.110	-0.145	-0.088	0.202
BG	1	0.009	-0.165	-0.336	-0.318
	2	-0.308	-0.695	0.381	0.474
	3	-0.020	0.271	-0.657	-0.290
	4	-0.896	0.521	0.552	0.665
	5	0.444	0.323	0.081	0.036

Variable	Function	10 weeks	15 weeks	20 weeks	25 weeks
Constant	1	-14.035	-24.441	-4.725	3.176
	2	2.078	23.577	-19.854	-24.897
	3	18.258	-22.434	16.782	6.788
	4	0.950	11.940	-7.140	-0.442
	5	-28.724	-30.691	-3.304	-7.557

Table 21: Group wise mean discriminant score at different age groups with six levels of outcome variable

Age (in weeks)	Function	PB1		PB2		Native	
		Male	Female	Male	Female	Male	Female
10	1	2.357	2.592	1.915	1.772	-2.602	-6.612
	2	-2.153	-2.150	0.815	1.883	3.885	-2.707
	3	1.115	0.892	-1.489	-1.436	1.584	-0.784
	4	1.073	-0.947	1.365	-1.038	0.111	-0.091
	5	0.386	-0.262	-0.280	0.252	-0.050	0.017
15	1	0.781	1.722	2.215	3.745	-2.678	-6.564
	2	2.960	0.030	1.256	-2.676	0.126	-1.087
	3	0.774	2.019	-2.169	-0.290	-0.859	0.222
	4	-0.298	0.200	-0.720	-0.102	1.721	-1.083
	5	0.291	-0.225	-0.197	0.173	0.009	0.002
20	1	2.164	2.172	2.581	2.541	-2.969	-7.062
	2	1.194	2.395	-2.729	-1.323	0.682	-0.620
	3	2.257	-0.472	1.145	-1.909	-0.776	0.452
	4	0.351	-0.536	-0.063	-0.020	0.887	-0.567
	5	0.116	-0.066	-0.111	0.103	-0.065	0.041
25	1	2.400	1.792	1.870	1.129	-2.391	-5.064
	2	1.603	2.584	-3.379	-1.484	0.270	0.076
	3	1.156	-0.464	0.876	-1.544	0.345	0.043
	4	-0.182	-0.214	-0.242	0.109	1.337	-0.960
	5	0.247	-0.186	-0.141	0.134	-0.026	0.022

Table 22: Classification analysis with six levels of outcome variable

Age group	Predicted Group Membership					
	PB1		PB2		NATIVE	
	Male	Female	Male	Female	Male	Female
10 weeks	95.7	71.9	98.0	100.0	98.2	98.0
15 weeks	93.6	84.5	96.1	100.0	96.5	100.0
20 weeks	89.4	78.1	90.2	96.6	98.2	100.0
25 weeks	89.4	64.1	94.1	96.6	94.7	98.0

4.4 Genetic Polymorphism in GDF8 promoter region and their association with growth traits

4.4.1 Genomic DNA extraction

The genomic DNA was extracted of from blood samples of 180 Punjab Brown native birds (90 males and 90 females) using phenol chloroform method. The genomic DNA was viewed on 0.8% agarose (Figure 8). The DNA had OD ratio in the range of 1.80 to 1.85 and 2.00 to 2.05 for A260/A280 and A260/230, respectively. The average yield of genomic DNA was 3000 ng/µL (Figure 7 and 8).

4.4.2 PCR-RFLP and estimation of gene and genotype frequency

PCR and restriction enzyme digestion was performed on the DNA samples using the protocol of Kumar et al. (2007). The amplified PCR products of 605 bp, were viewed on 2.5% agarose gel (Figure 9) and were subjected to *Sca I* restriction enzyme digestion. The digested PCR products were of 321 and 284 bp, in case of wild allele and in mutant allele no enzymatic digestion was observed (Figure 10). Therefore, of the total 180 samples screened for genotyping a total three genotypes AA, AC and CC, and two alleles A and C, were observed. The A allele was the wild allele with the restriction site producing 321 and 284 bp digested PCR products. Whereas the C allele, due to the loss of restriction site due to mutation, produced only 605 bp band.

The genotype frequency were estimated in both sexes, in females the AA, AC, CC genotype frequency were 0.46, 0.50, 0.04 and in males they were estimated to be 0.45, 0.47, 0.07, respectively. Similarly, the gene frequency of A and C allele were estimated in females as 0.71 and 0.29; in males they were 0.69 and 0.31, respectively.

The estimates of gene and genotype frequencies indicated that the C allele existed very low in the form of homozygotes and mostly existed in heterozygote. This may be due to artificial and natural selection acting against this allele (tables 23 and 24).

Table 23: Estimates of genotype frequency in Punjab Brown native chicken

Gender	Genotype	N	Genotype Frequency
F	AA	41	0.46
	AC	45	0.50
	CC	4	0.04
M	AA	41	0.46
	AC	43	0.47
	CC	6	0.07

Table 24: Estimates of gene frequency of alleles in Punjab Brown native chicken.

Gender	Allele	Gene frequency
F	A (p)	0.71
	C (q)	0.29
M	A (p)	0.69
	C (q)	0.31

4.4.3 Effect of genotype on growth performance

The effect of genotype on body weight at 0 day, 5, 15, 20 and 25 weeks was studied in Punjab Brown females and males. The effect of genotype was not significant on day old body weight in both genders. In case of females, the CC genotype had significantly ($p<0.05$) higher body weight followed by AC and AA genotypes. Similar observations were also seen in case of males however, the effect of genotype was not statistically significant on body weight variables in males (Table 25).

Table 25: Association of genotype with growth performance in Punjab Brown native chicken

Gender	Variable	AA	AC	CC
F	BW0	36.34 ± 0.59	36.45 ± 0.56	36.37 ± 1.89
	BW5	655.54^{b} ± 13.07	652.86^{b} ± 12.47	767.40^{a} ± 41.84
	BW10	1046.12^{b} ± 27.4	1048.58^{b} ± 26.15	1285.00^{a} ± 87.73
	BW15	1574.17^{b} ± 37.74	1596.29^{ab} ± 36.02	1841.50^{a} ± 120.81
	BW20	1916.83^{b} ± 42.36	1922.2^{ab} ± 40.43	2205.0^{a} ± 135.62
	BW25	2209.02^{b} ± 55.59	2219.60^{b} ± 53.06	2615.50^{a} ± 177.96
M	BW0	37.56 ± 0.46	38.05 ± 0.45	39.49 ± 1.14
	BW5	732.43 ± 42.73	741.85 ± 16.85	761.99 ± 17.24
	BW10	1147.86 ± 87.93	1201.27 ± 34.68	1251.14 ± 35.48
	BW15	1585.57 ± 109.75	1693.22 ± 43.28	1750.00 ± 44.28
	BW20	1890.00 ± 135.98	1988.76 ± 53.63	2022.09 ± 54.86
	BW25	2678.84 ± 62.64	2644.93 ± 61.24	2729.57 ± 155.26

Means with different superscripts have significant difference between columns

4.4.4 Estimation of Average effect of gene

Estimates of the average effect of the gene indicated that A (α_1) allele had an increasing negative effect on body weight variables with increasing age, whereas C (α_2) allele had an increasing positive effect on body weight variables with increasing age.

Further, with increasing age groups, increasing negative estimates were also observed for α (average effect of gene substitution). Indicating that A allele reduced the population mean when substituted the C allele in the population.

Table 26: Estimates average effect of genes in Punjab Brown native chicken

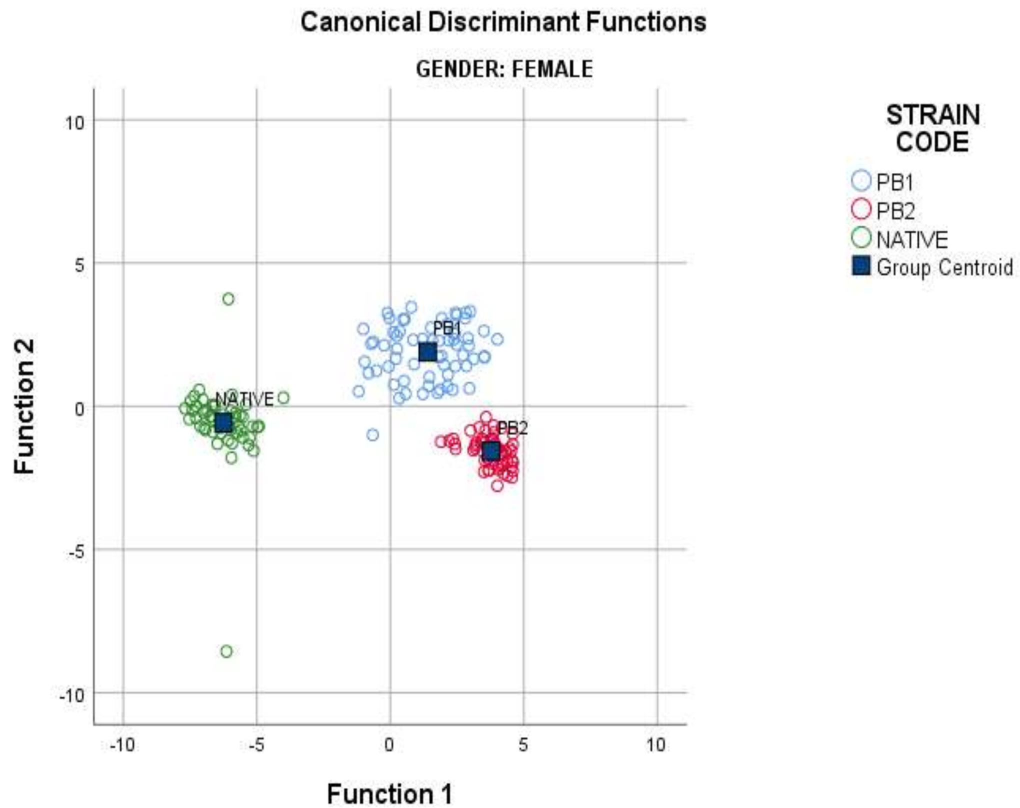
Gender	Trait	Mean	-a (AA)	d (AC)	a (CC)	α (Average effect of gene substitution)	$\alpha_1 = A$	$\alpha_2 = C$
F	**BW0**	36.39	-0.02	0.10	0.02	-0.06	-0.02	0.04
	BW5	691.93	-55.93	-58.61	55.93	-31.84	-9.37	22.46
	BW10	1126.57	-119.44	-116.98	119.44	-71.35	-21.01	50.34
	BW15	1670.65	-133.66	-111.55	133.66	-87.81	-25.85	61.95
	BW20	2014.68	-144.09	-138.71	144.09	-87.06	-25.63	61.42
	BW25	2348.04	-203.24	-192.66	203.24	-124.03	-36.52	87.51
M	**BW0**	38.53	-0.96	-0.48	0.96	-0.78	-0.24	0.54
	BW5	747.21	-14.78	-5.36	14.78	-12.70	-3.96	8.79
	BW10	1199.50	-51.64	1.77	51.64	-52.31	-16.24	36.07
	BW15	1667.79	-82.21	25.44	82.21	-91.85	-28.52	63.33
	BW20	1956.05	-66.05	32.71	66.05	-78.44	-24.36	54.08
	BW25	2704.20	-25.37	-59.27	25.37	-2.91	-0.90	2.00

4.4.5 Sequence alignment and gene regulation analysis

Five representative samples (605 bp PCR products) from each of the three genotypes AA, AC and CC were forward and reverse sequenced. The sequences were submitted to DDBJ/EMBL/GenBank databases and are available at DDBQ (Accession No : LC647795). The sequences obtained were aligned with the NCBI reference sequence (GenBank accession number: DQ912835). Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequence alignment. PCR amplicon sequence, aligned with the NCBI sequence starting from 877 to 1482 bp (Figure 11). Further, the alignment results indicated that there was an A>C transversion (interchange of purine for pyrimidine base) in the C allele at 1196 bp position with respect to DQ912835 reference sequence (i.e., 319 base pairs from starting nucleotide of sequenced sample). Such mutation resulted in loss of *Sca I* restriction site (5'AGT↓ACT3'). Gene regulation analysis carried out using Alibaba2 online tool (http://gene-regulation.com/pub/programs/ alibaba2/) indicated that in case of C allele due to the A>C transversion, the SRF transcription factor did not bind to its binding site i.e., from 309 to 318 bp of PCR product sequence (Figure 12).

In all fifteen samples of Punjab Brown birds that were sequenced, T>C transitional mutation was also observed at 1280 bp position (i.e., 403 base pair from starting nucleotide of sequenced sample) in both A and C allele. The GenBank reference sequence DQ912835, originally had T pyrimidine base at the corresponding position (Figure 13). Such mutation did not alter the binding of NF-1 transcription factor at its binding site (Figure 14). Though a silent mutation it could be considered as a candidate in developing breed specific signature.

Bhattacharya et al. (2019) concluded that by silencing of only ACVR2B receptor of myostatin gene, higher growth was observed in chicken during juvenile stage. Genxi et al. (2012) studied the myostatin (MSTN) gene polymorphism in 5'-upstream region in Bian chicken of China and observed one polymorphic locus (A1278T) with three genotypes (QQ, QR, and RR). Association analysis revealed that RR and QR genotypes had significantly higher ($p<0.05$) body weight from 14 to 18 weeks of age than the QQ genotype. Tanjung et al. (2019) reported Adenine insertion mutation at 2099-2100 position and T4842G substitution mutation to be having strong positive and strong negative association ($p<0.01$) with 49 day body weight in Gama



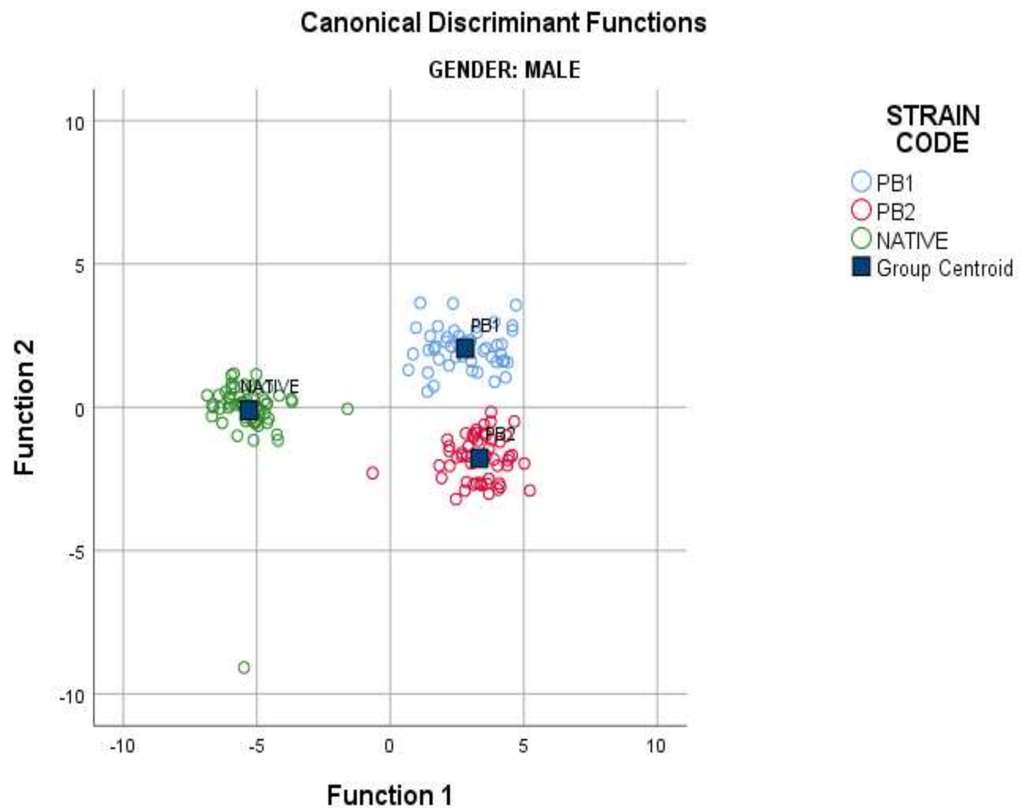


Figure 6: Scatter plot of sex wise discriminant function at 15 weeks age with three levels of outcome variable indicating spread of observations of different genetic groups around centroid mean

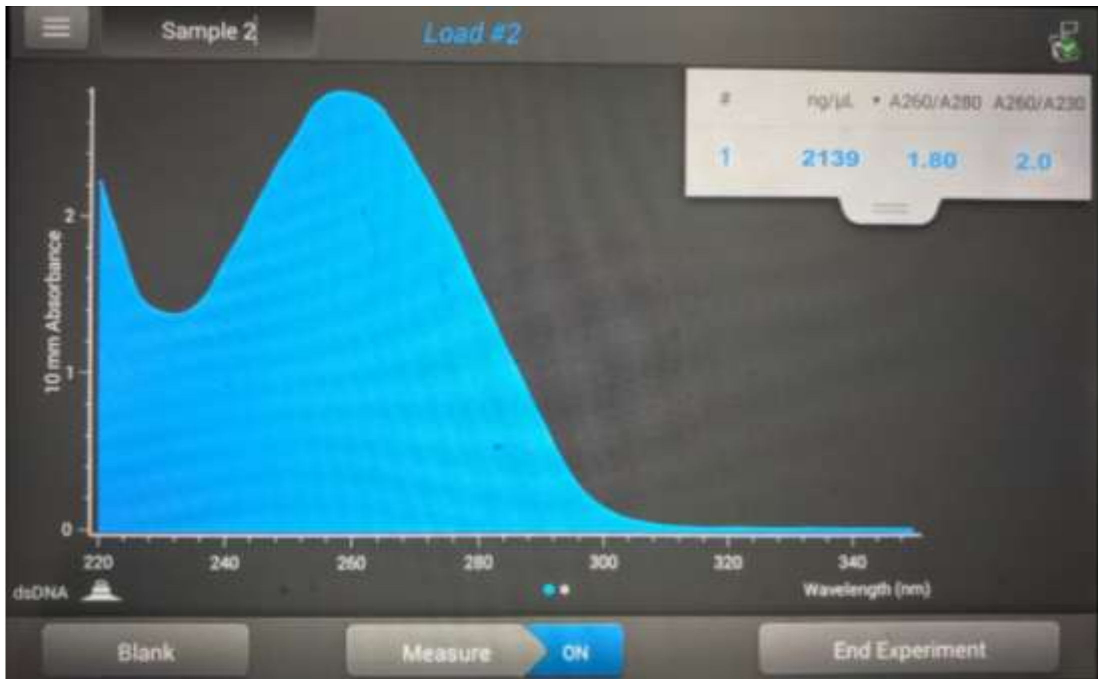


Figure 7: Quantification of DNA in Nano drop spectrophotometer showing DNA concentration (ng/µL) and OD ratio (260/280 and 260/230)

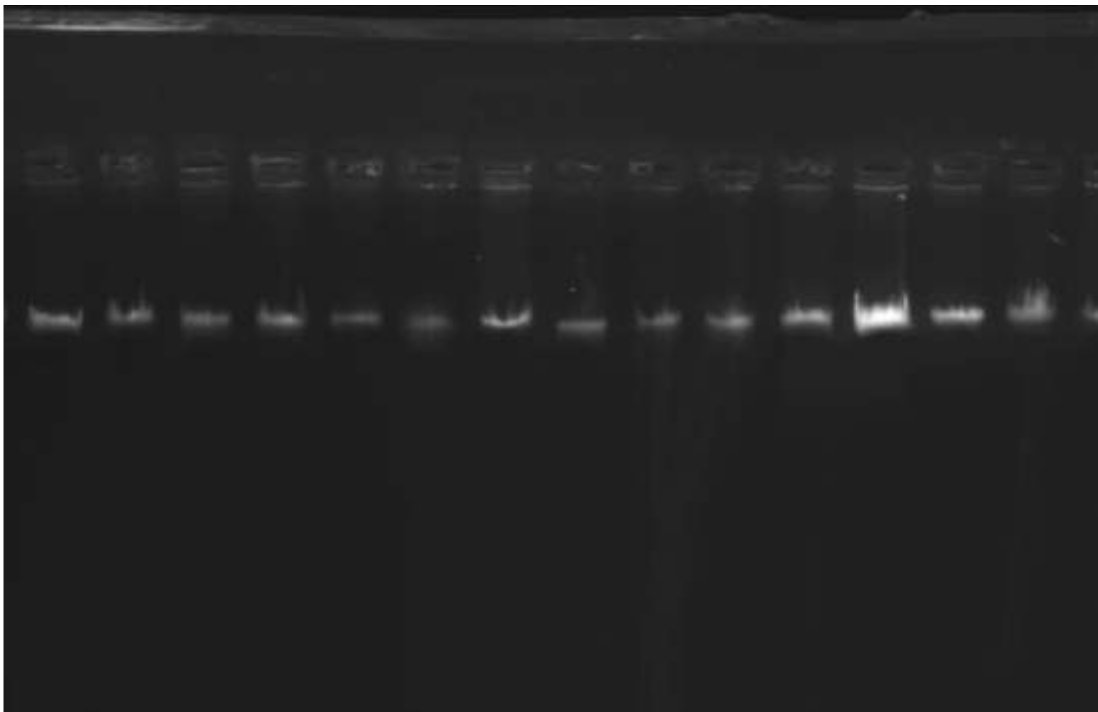


Figure 8: Gel photographed under gel documentation system (Syngene, UK) showing extracted genomic DNA of 14 samples

Figure 9: **Gel photographed under gel documentation system (Syngene, UK) showing extracted showing bands at 605 bp for 12 samples and no band in negative control (NTC)**

Figure 10: **PCR-RFLP indicating three genotypes (AA, AC and CC). Allele A is characterized by two fragments of size 321bp and 284bp and allele C is characterized by single uncut fragment of 605bp**

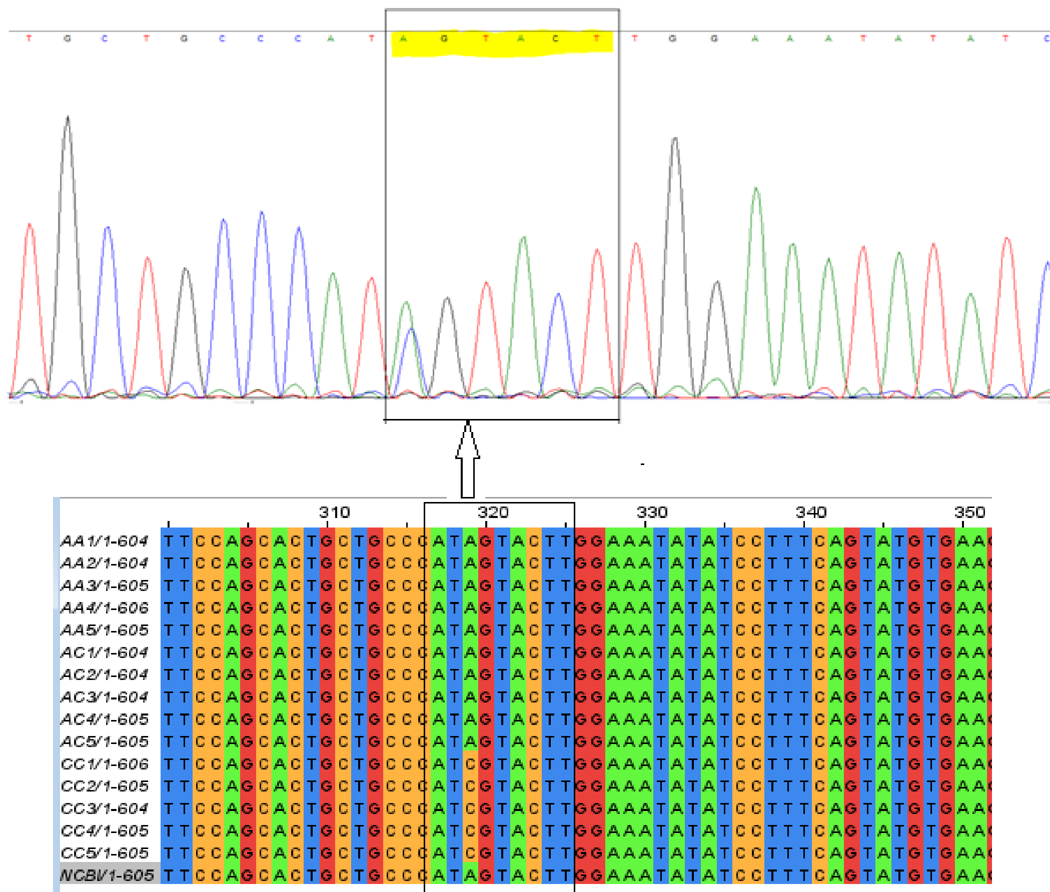


Figure 11: **C allele having A>C transversion at 1196 bp position with respect to DQ912835 reference sequence (i.e. 319 base pairs from starting nucleotide of sequenced sample).**

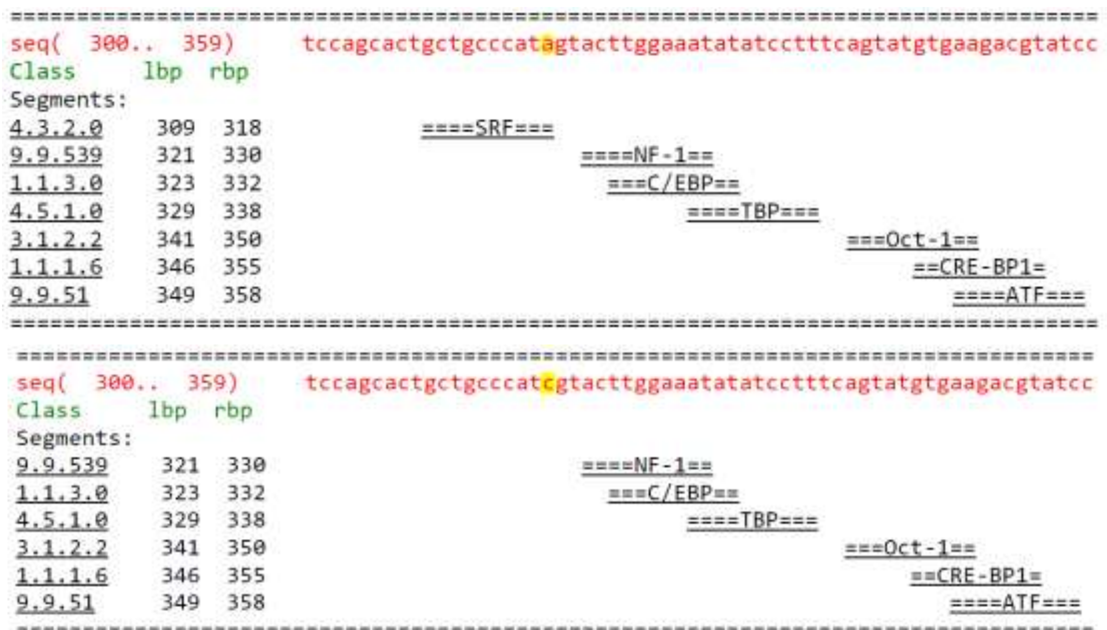


Figure 12: **Gene regulation analysis indicating non binding of SRF transcription factor due A>C transversion at 1196 bp position in CC genotype individuals**

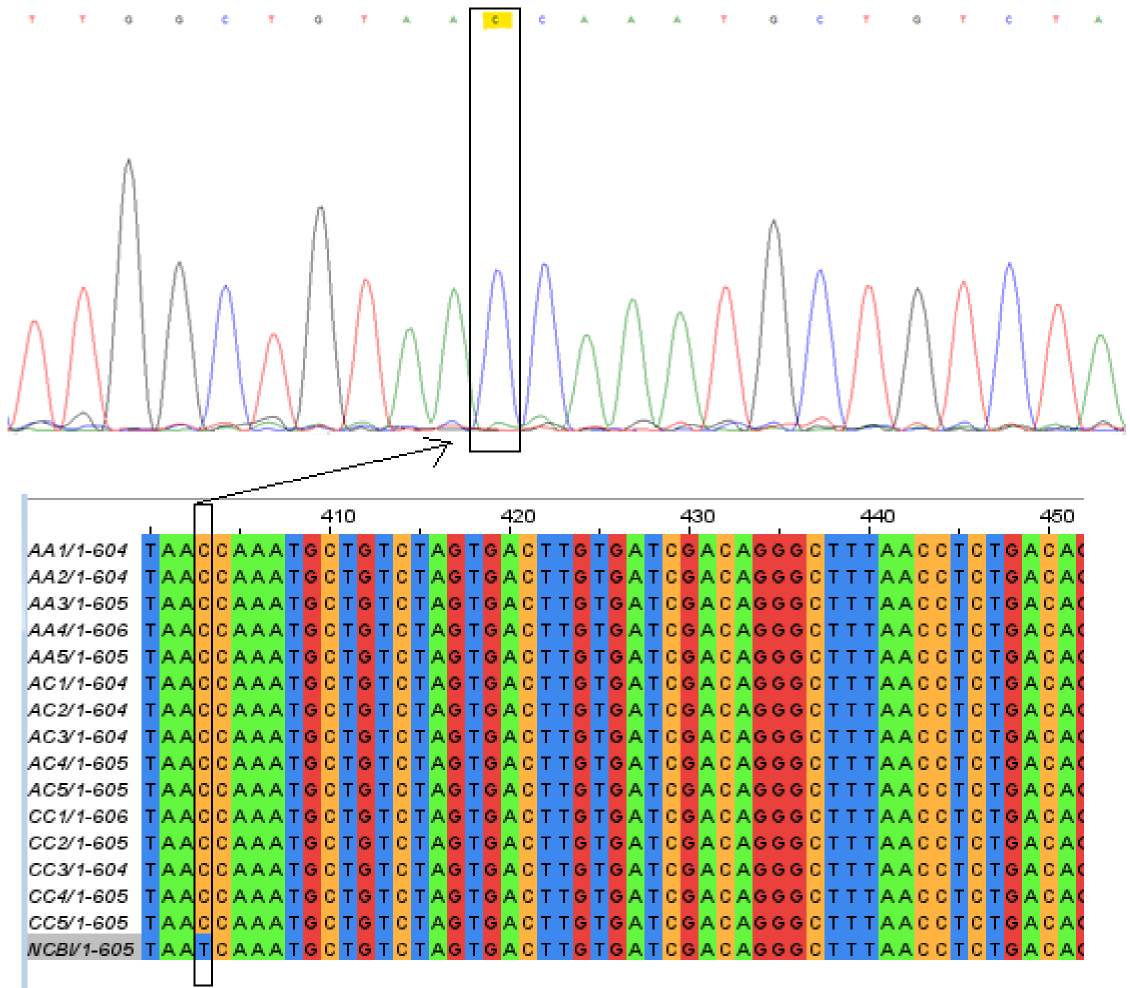


Figure 13: **T>C breed specific transitional mutation observed at 1280 bp position w.r.t. NCBI reference sequence.**

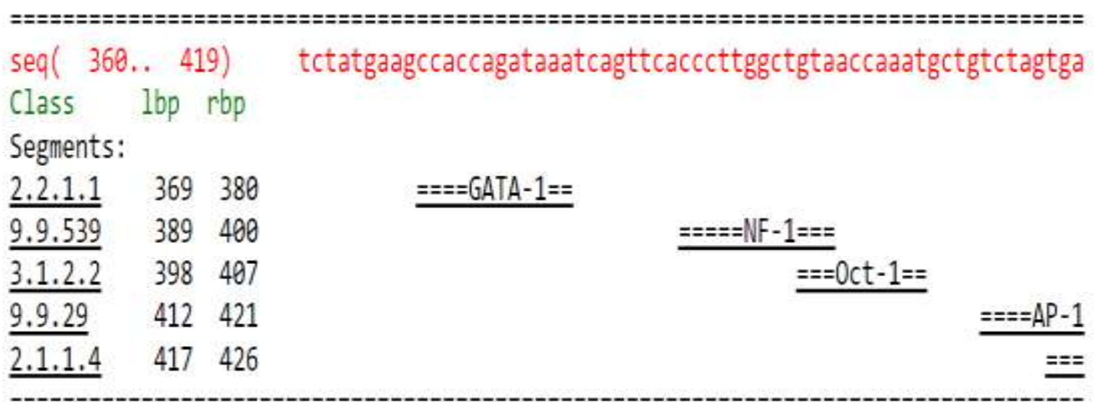


Figure 14: **Gene regulation analysis indicating binding NF1 transcription factor in individuals with T>C synonymous**

chicken of Indonesia. Bhattacharya & Chatterjee (2013) did single-stranded conformation polymorphism (SNP) and sequencing of exons 1, 2 and 3 of myostatin gene in PB-1 and CB line broiler chicken in India. They observed polymorphism in MSTN gene and reported that it had significant association with 28, 42, and 49 day body weight in the PB-1 line and 14th and 49th day weight variables in the CB line. Paswan et al. (2014) reported transition SNPs in the promoter region of myostatin (GDF-8) gene in Indian broiler chicken, significantly affecting the body weight at hatching. They used PCR-SSCP followed by sequencing and observed 2 alleles A and B and 3 genotypes; with allele A having T23C, G150A, and A214T allele-specific nucleotide substitution mutations, similarly allele B having A241T mutation. Zhang et al. (2015) used PCR-RFLP for study of polymorphism in exon 1 of MSTN gene and identified three genotypes (AA, AG and GG) in Bian chicken of China. Their sequencing results revealed 234G>A substitution in myostatin (MSTN) gene as the cause of polymorphism. Functional effects of which were further studied by gene expression analysis; indicating that selection for AA genotype resulted in fast growing broiler, as the 234G>A substitution resulted in higher expression of MSTN gene. Mitrofanova et al. (2017) used PCR-RFLP and reported rs313744840 SNP in the myostatin receptor genes in Pushkin breed chickens. Three genotypes were observed, with AA genotypes having significantly ($P<0.01$) larger at 7 weeks than AG and GG genotypes. Khaerunnisa et al. (2016) used PCR-RFLP followed by sequencing and observed three genotypes TT, TG and GG in Indonesian broilers. They observed significant association of T4842G MSTN gene polymorphism with body weight and carcass traits with TT genotype having significantly higher estimates for all variables considered for their study. Kumar et al. (2007) reported G976A and A1196C polymorphisms in myostatin promoter in Punjab Brown breed, using PCR-RFLP approach. Z. Gu et al. (2004); Z. L. Gu et al. (2002) have also reported polymorphisms of myostatin gene and their association with different carcass traits in Wenling grass and broiler × silky F2 cross chickens, respectively.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present investigation was carried out with two objectives, firstly, morphological characterization and discriminant function analysis for Punjab Brown native poultry vis-à-vis parent lines broiler variety and secondly, to study the polymorphisms in promoter region of myostatin gene in Punjab Brown native poultry. The body weight and morphological variables were recorded in both sexes of birds, of three genetic groups viz. Punjab Brown native chicken (PBN), and broiler parent lines viz. PB1 (sire line) and PB2 (dam line), maintained under the ICAR-AICRP Poultry Breeding Project, at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. On each individual of the three genetic group variables were recorded from birth till 25 weeks of age at every 5 weeks interval. At different age, the variables recorded were body weight (BW), body length (BL), wing length (WL), shank length (SL), thigh length (TL), breast girth (BG), keel length (KL), respectively. On date of hatch to avoid any stress to the chicks only BW variable was recorded.

The variables recorded were subject to statistical analysis using multivariate ANOVA to study the between group differences w.r.t. the variables. The results indicated that PB1 (45.57 grams) was having significantly higher estimates for BW0 variables followed by PB2 (39.94 grams) and PBN strains (37.66 grams). The 5 week estimates of all variables, of PB1 were significantly higher ($p<0.01$) than PB2 and PBN birds. For BW10, BW15, BW20 and BW25 there was no significant difference between the weight variables of PB1 and PB2 birds in both sexes. BL variables at 10, 20 and 25 weeks age were higher in PB2 birds in either sex. BL10, BL15 and BL20 variables were least in Punjab brown native (PBN) birds. Wing length (WL) and shank length (SL) of Punjab Brown females were significantly higher from PB1 and PB2 females across age groups. In either sex the mean estimates of thigh length (TL) were least in PB2 birds among the three genetic groups. At different ages and in either sex the keel length (KL) estimates were least in Punjab brown birds in comparison to broiler birds. Post 5 weeks age the estimates of BG10, BG15 and BG20 (28.55±0.10, 29.44±0.11 and 29.86±0.12 centimetres) of PB2 females were higher ($p<0.05$) than other groups.

The correlation estimates between the variables indicated that BW was strongly correlated with BL and WL variables and all other estimates of correlation were medium to low. BW had mostly moderate correlation with TL, KL and BG. Morphometric variables WL, SL and TL were weekly correlated.

Three and six levels of outcome variable were used for discriminant analysis using data on body weight and morphometric variables. Age wise and gender wise, discriminant analysis with three levels of outcome variable, extracted two functions with the first function explaining variance in the range of 71.86 to 88.18%. Pooled sexes data was used for discriminant analysis at 5 weeks and 10 weeks onwards, the analysis was done sex wise and age wise. Classification analysis revealed that such discriminant functions had high accuracy in the range of 92.2% to mostly 100% in correctly classifying the cases.

Age wise discriminant analysis with six levels of outcome variable extracted 5 functions with the first function explaining 56.60 to 72.20% of variance. Classification analysis revealed that the discriminant functions (with 6 levels of outcome variable) of 10, 15, 20 and 25 weeks age groups of birds had 90.2% to 93.6% accuracy for correctly classifying the original grouped cases.

Blood samples from 180 Punjab Brown native birds (90 males and 90 females) were collected and genomic DNA was extracted using phenol chloroform method. The extracted genomic DNA had good average yield (3000 ng/µL) and quality with OD ratio ranging from 1.80 to 1.85 and 2.00 to 2.05 for A260/A280 and A260/230, respectively. Using Standardized PCR protocol, 605 bp product was produced and subjected to *Sca I* restriction enzyme digestion. *Sca I* digested PCR product, produced two bands of 321 bp and 284 bp sizes. PCR-RFLP analysis helped in identifying three genotypes in both sexes followed by estimation of genotype and gene frequency. AA, AC, CC genotype frequencies were 0.46, 0.50, 0.04 in females and 0.45, 0.47, 0.07 in males, respectively. Gene frequency of A and C alleles were estimated to be 0.71 and 0.29 in females and 0.69 and 0.31 in males, respectively.

The effect of genotype was not significant on day old weight in both male and female Punjab Brown birds. Among both sexes the effect of genotype was significant in females as the CC females had significantly ($p<0.05$) higher body weight followed by AC and AA genotypes. Though the CC males had higher body weight followed by

males with AC and AA genotype, but the effect of genotype was not significant in males.

Average effect estimates indicated that with increasing age A (α_1) allele had an increasing negative effect, whereas C (α_2) allele had an increasing positive effect on body weight variables. These estimates indicated that when substituted the C allele, the A allele reduced the population mean or the performance of the individuals carrying A allele.

Sequencing results indicated that in case of C allele there was A>C transversion (interchange of purine for pyrimidine base) at 1196 bp position and such mutation resulted in loss of *Sca I* restriction site (5'AGT↓ACT3') and also resulted in non binding of SRF transcription factor at its binding site. Another T>C transitional mutation was observed at 1280 bp position. It was a silent mutation as there was no loss or creation of restriction site and also it did not affect the binding of NF-1 transcription factor at its binding site. However, this specific mutation was observed in all the samples of Punjab Brown birds that were sequenced.

The following conclusion can be drawn from the present study;

1. The body weight and morphometric variables had clear differences between Punjab brown native and PB1 and PB2 broiler lines.
2. In comparison to Punjab Brown native chicken the higher estimates for variables in case of broiler lines (PB1 and PB2) indicated clear effect of selection for increased growth.
3. Discriminant analysis with body weight and morphometric variables was successful in classification of individuals to different genetic groups.
4. Due to the lesser covariance between the variables, the discriminant functions were significant ($p<0.01$).
5. Age wise and gender wise discriminant functions having 3 levels of outcome variable, explained higher variance with least classification errors.
6. The discriminant function considering 6 levels of outcome variable explained lesser variance, and also had higher classification errors.
7. In both sexes, the estimates of gene and genotype frequencies indicated that the C allele existed mostly in the form of AC heterozygote.

8. Average effect estimates indicated that A allele had increasing negative effect and C allele had increasing positive effect with increasing age.
9. A>C transversion at 1196 bp position affected gene regulation, by non-binding of SRF transcription factor at its binding site.
10. CC genotype individuals had higher body weight and this allele could be used in marker assisted selection.
11. Though a silent mutation, T>C transitional mutation, observed at 1280 bp position, could be considered as a candidate in developing breed specific signature.

FUTURE PROSPECT

Discriminant analysis can be conducted in a larger sample and a function can be developed which can be used for selection of Punjab Brown birds from field areas. In addition to body weight variables the morphometric variables can be included in poultry selection programme through development of multi-trait selection strategies. The CC genotype had higher body weight in comparison to AA genotype, thus C allele can be used for developing marker assisted selection strategies in Punjab Brown native birds. Role of SRF transcription factor in growth, differentiation and its association with other economic traits could be explored. In addition to T>C transitional mutation, observed at 1280 bp position, more such breed specific SNPs are required to be identified in Punjab Brown birds for developing breed specific signatures.

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ANNEXURE-I

Chemicals	Company
Agarose Special	Himedia, Mumbai
Chloroform- Isoamyl Alcohol (24:1)	SRL, India
dNTP	Promega, USA
Isopropanol	SRL, Mumbai
Phenol-Chloroform-Isoamyl alcohol (pH=8)	Sigma Aldrich, USA
Phenol Tris Saturated (pH=8)	Amresco, USA
Ethanol	Amresco, USA

Laboratory Wares	Company
Eppendorf tubes (1.5ml, 2ml)	Tarson, India
15ml Centrifuge tube	Tarson, India
Glass wares	Borosil, India
Plastic wares	Genaxy, USA and Tarson, India

Equipment	Company
Dry Bath	Labnet, USA
Gel Documentation System	Syngene, UK
High Precision Water Bath	Thermoscientific
Horizontal electrophoresis assemblies	Bio-Rad, USA
Laminar Air Flow	NSW, India
Centrifuge	Thermoscientific
Nanodrop	Thermoscientific
Thermocycler	Veriti, Applied Biosystem,

ANNEXURE-II

Enzymes	Company
Sca I-HF®	New England Biolabs, USA
Proteinase K (20mg/ µl)	Qiagen, Germany
RNase	Thermo Scientific

Buffers	Compositions
1X TE (10:1)	1M Tris (10) : 0.5M EDTA (1)
Poultry Lysis Buffer	1M Tris, 0.5M EDTA, 5M NaCl, 10% SDS
1X TAE	Tris: Glacial Acetic Acid: EDTA
10X buffer R	10 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1mg/ml BSA
10X PCR Buffer	200 mM Tris-HCl (pH 8.4), 500 mM KCl.

Solutions	Compositions
0.5M EDTA	EDTA disodium salt and double distilled water
5M NaCl	Sodium chloride and double distilled water
10% SDS (Sodium Dodecyl Sulfate)	Sodium Dodecyl Sulfate and double distilled water
3M Sodium Acetate (pH 5.5)	Sodium acetate and double distilled water
70% Ethanol	70ml Ethanol and 30ml double distilled water
1M Tris	Tris HCl and double distilled water

VITA

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