Chapter-1

Introduction

The indigenous chicken of Bangladesh show high levels of morphological and phenotypic variability and they have high fitness under natural conditions and they comprise about 90 percent of the total chicken population (Bhuiyan, 2011). Different kinds of indigenous chickens are Hilly, Naked Neck, Assel and Full feathered non-descriptive deshi. The variations of full feathered indigenous chickens are considered on the basis of plumage color, comb type, feather pattern and body shape. Commonly observed plumage color of indigenous chickens are red, white, black, black with red strips, white with red strips and red brownish. The predominant plumage color of the native chickens are black brownish (33.33%) followed by white with black tips (28.33 %) and red brownish (18.33%) (Faruque *et al*., 2010). The variations of the comb type and shank colors are also observed in indigenous chicken's population (Khan *et al*., 2004; Dutta *et al*., 2013).The comb types are mostly single (Faruque *et al*., 2010) and shank color are mainly yellowish (Faruque *et al*., 2010 and Bhuiyan *et al*., 2009).

Hen house egg production of deshi white chickens is 19.95% and black 17.65% and the yearly egg production of deshi white chicken is 90 no/year/chicken (Khan *et al*., 2017), which was highest than other types of deshi chickens. The egg weight varies from 41.27g to 43.85g for all types of Deshi chicken (Khan *et al*., 2017). Deshi chickens have average hatch weight is 29g; age at first egg was 175 days (Sazzad, 1992); weight of pullet (0.9 kg); mature body weight (1.3 kg); hatchability (52%); fertility (83%); 9-15% mortality up to 500 days of age (Bhuiyan *et al*., 2005, Khan *et al*., 2017). The egg color of non-descriptive deshi chicken was white (Khan *et al*., 2017).Number of egg/ hen from starting to ten months of laying are 108, 104 and 112, respectively in Non-Descriptive Deshi, Hilly and Naked Neck genotypes (Bhuiyan *et al*., 2009).

Nowadays DNA isolation is the important technique to recognize particular gene in different species including chicken for a specific traits. Gene isolation and identification has been widely used in chicken, whereas variation of plumage color, which are responsible for *PMEL17* gene (Keerje *et al*., 2004) in domestic chickens of Bangladesh are not studied yet.

DNA polymorphism of different traits is partially reported on indigenous chicken, naked neck (Mollah *et al*., 2009). Although the genetic characterization study using microsatellite of different native chicken population are available in literature (Fonteque *et al*., 2014; Zanetti *et al*., 2010; Nedup *et al*., 2012; Nassiri *et al*., 2007; Tadano *et al*., 2007 and Li *et al*., 2009) but these literatures lack information on plumage color inheritance of chickens.

PMEL17 is the plumage color affecting gene in the chicken, which causes variation in plumage color. *PMEL17* is a type I integral membrane protein present in the melanosome and is a component of the fibrous striations upon which melanin are polymerized (Berson *et al*., 2003). *PMEL17* gene is identified as a positional candidate gene for the phenotype in chicken. Dominant white locus encodes *PMEL17* protein which is specific protein and plays a key role in the development of melanocyte (Kerje *et al*., 2004). In the chicken plumage color variation; dominant white, dun and smoky are all caused by the mutations in *PMEL17* gene. Unique insertion/deletion polymorphisms (9-15bp in length) were also found in birds carrying these alleles.

This research will be helpful for realize the effects of genes that related with plumage color. This study also helps to know any mutation occur in the plumage color genes in the different types of indigenous chickens of Bangladesh. Measurement of the genetic diversity and gene variation were studied on each economic trait. In addition, the information of the current study can be incorporated in the genetic improvement programme of indigenous chickens. Therefore, the present study was conducted with the following objectives.

Objectives of the study

1. To study the performance of indigenous chickens in consideration of plumage color, body shape and comb types.
2. To study the genetic variation of plumage color gene in the indigenous chickens of Bangladesh.

Chapter-2

Review of literature

2.1 History and domestication of chicken

The domestic chicken's ancestry can be traced back to four species of wild jungle fowl from Southeast Asia (Xiang *et al*., 2015). Over 150 million years ago, first known bird called Archaeopteryx (ancient winged creature) took its birth. It was almost the size of crow and not like as day birds. It is not known as to when the first chicken was captured and domesticated. In 1400 B.C. Archaeological surveys indicated that fowls were domesticated in china (Eda *et al*., 2016). Conventionally wisdom has held that the chicken was domesticated in India but recent evidence suggests that domestication of the chicken was already underway in Vietnam over 10,000 years ago (Wikipedia, 2017). It is probably appear that people domesticated chickens over 5000 years ago, after centuries of hunting the wild jungle fowl. Centuries ago the Chinese began raising a variety of bird that was gradually brought to the west Asia, Greece and Rome (Moiseyeva *et al*., 2003). Chickens then probably spread through the Eastern Asia and they reached Persia about 1000 B.C. and played a role in their ancient religion. They were taken to Babylon from India in 600 B.C. and were introduced in Greece and Rome around 500 B.C (Kanginakudru *et al*., 2008). By around 500 B.C. chickens were raised by the Greeks for the 'sport' of Cock fighting. The sport of cockfighting had tremendous influence not only in the domestication of the chicken but also on the distribution of fowl throughout the world. After centuries of selection and breeding for numerous extremes, chickens now exist in many colors, sizes and shapes (Kanginakudru *et al*., 2008). Chickens are deliberated as one of the most important and widely distributed avian species among poultry birds. Of the four wild chicken species available viz., red jungle fowl (*Gallus gallus*), gray jungle fowl (*Gallus sonnerati*), Ceylon jungle fowl (*Gallus lafayettii*) and green jungle fowl (*Gallus rarius*), indigenous chickens locally known as Deshi (*Gallus domesticus*) are reported to be driven from *Gallus gallus* (Dutta *et al*., 2013 and Nasser *et al*., 2007) whereas *Gallus bankiva* is believed to be the major contributor to the development of modern commercial breeds (Lush, 1945). Geographic variation is very marked in red jungle fowl and this has been recognized by designating several sub-species for red jungle fowl (Moreng and Avens, 1985; Crawford, 1990a; Collias and Collias, 1996; Moiseyeva *et al*., 2003; USDA/ITIS,

2006a-j; Wikipedia, 2006). The geographic variation implies the adaptation of the different sub-species to certain environmental conditions.

2.2 Breeds, types and variation of indigenous chicken

Poultry population in Bangladesh is estimated about 304.17 million where chicken population is about 255.31 million (DLS, 2015). The growth rate of chicken for last 10 years was 3.75% (Hamid *et al.*, 2017). Chicken is the integral part of farming system in Bangladesh and has created direct, indirect employment opportunity including support service for about 6 million people (Ansarey, 2012). Different breeds and types of chicken are available in Bangladesh.

Chicken originated from a certain place with same or similar characteristics are of same class. For example, Asiatic class, European class, American class etc. Under class, chicken with same size, shape and characteristic similarity with each other are of same breed (Islam and Nishibori, 2009) like, Leghorn, Minorca etc. According to origin the chicken are of four types of classes were observed.

- Asiatic class: Brahma, Langshan, Cochin, Assel etc.
- English class: Austrolorp, Cornish, Dorking, Orpington etc.
- Mediterranean class: Leghorn, Minorca, Ancona, Fayoumi etc.
- American class: Rhode Island Red, New Hampshire, Plymouth Rock etc.

On the basis of production chicken are of three types.

Layer

Layer is for egg production and used as commercial purpose. Some popular layer breeds: Leghorn, Minorca, Ancona, Fayoumi, and strains: ISA brown, Star cross, Lohman etc.

Broiler

Broiler chickens are meat type chickens; they are mostly reared worldwide as commercial purpose. Star brow, Mini brow, Hi-line etc are popular broiler strain.

Dual (egg and meat) type

These types of breed are used for the purpose of both egg and meat production. Rhode Island Red, New Hampshire, Plymouth Rock etc. (Islam and Nishibori, 2009) are popular breeds for both meat and egg production.

The non-descript Deshi chicken is more acceptable by rural peoples as an important source of meat and eggs (Barua and Howlider, 1990) and cash income due to their lower nutritional demand and higher resistance to diseases and heat stress. Variation of the indigenous chickens of Bangladesh could be found on the basis of plumage color, comb type and feather pattern (Faruque *et al*., 2017 and Khan *et al*., 2017). Variation on morphological characteristics and production performance of Bangladeshi chickens has been reported by Howlider *et al*. (1995) and Islam and Nishibori, (2009).

Table 1: Types/breed in chicken found in Bangladesh

Sl. no	Breed	Characteristics
01	Rhode Island Red	Yellow skin[1,4,3]
		Single and rose comb[2,3]
		Medium size[1,2,4]
02	Fayoumi	Skin-yellow/white[2,4]
		Comb-single[1,2,3,4]
		Tight plumage[2,3]
03	Assel	Red earlobes[3,4]
		Massive size and loose plumage[1]
04	Naked neck	Medium size[1,2,3,4]
		Non-feather neck region[1,2,3,4]
05	Hilly chicken	Small size and round[1,2,3]
		Tight plumage[2,3]
06	Indigenous chicken	Plumage color black and red[1,3,4]
		Comb type mainly single**[1,3,4]**
		Egg size medium**[1,3,4]**

[1]Faruque *et al*. (2017), **[2]**Khan *et al*. (2017), **[3]**Faruque *et al*. (2010), **[4]**Bhuiyan *et al*. (2005)**,** **[5]**Khan *et al*. (2004).

In Bangladesh, considering the plumage color, comb type and shank color are distinct characteristics of indigenous chicken. Variation of the different comb type and shank colors is available in chickens. The comb types are available in single comb type and pea type, whereas shank color is yellowish, whitish and blackish color. The comb type of non-descriptive, hilly and naked neck was 100% single and in 100% cases, no feather was observed in the shank (Faruque *et al*., 2010). The shank colour of non-descriptive deshi

was 40% white or whitish. While, the shank colour of hilly was whitish 35%; yellowish 25% and others 35%. The highest proportion of shank colour of naked neck was 45% yellowish followed by whitish 30% and black 15%. The overall mean indicated that 35% of native chickens had whitish shank colour followed by yellowish 31.68%; black 11.66 % and others 21.67 % (Faruque *et al*., 2010).

Several researchers have been studied the plumage color variation in indigenous chickens. The predominant plumage colour of three types of native chickens was black brownish (33.33%) (Faruque *et al*., 2010). Non-descript Deshi chickens have no definite plumage color. Black brownish constituted the maximum proportion (40%) of plumage color followed by red brownish (35%). Hilly birds are covered with plumage of white with black tips (85%) followed by multicolor (15%) (Bhuiyan *et al*., 2005). Naked Neck birds are very colorful-black brownish, multicolor, red brownish and black feather combinations (Bhuiyan *et al*., 2005). In a study on plumage color, variation of plumage color were black, blackish red , blackish white, blackish yellow, yellow whitish yellow, reddish yellow ,white and blackish brown found in different region of Bangladesh (Islam *et al*., 2012). Tabassum *et al*. (2014), variation in plumage colors of indigenous chickens in Bangladesh, where multiple plumages color (24%) was prominent followed by others, black, black & white, red brown, red, white, yellow, grayish and white and red. In the findings of Daikwo *et al*. (2011) in chicken of Dekina (Brown/Black 35.5%, Black 10.25%, Black/White 6.5%, Brown/Black/White 3.25% and White 2.75%). Mengesha, (2012) reported that, plumage color of Ethiopian indigenous chicken is very much diversified, commonly observed plumage color of indigenous chicken are red, white, black, multicolor, black with red strips, white with red strips and red-brownish. In Ethiopia, a study by Dana *et al*. (2010) revealed that, white and red plumage colors were identified as the two important component traits used for selecting on the basis of body plumage. Red is the most favored plumage in the Benshangul-Gumuz (Mandura), Oromia (Horro), and Southern Regions (Konso and Sheka), whereas white is the body plumage color more favored by the Amhara community (Farta) irrespective of the sex of the birds. A study by Youssao, (2010) reported that, White (18%), red (21%), brown (22%), black (20%) and gray or golden (17%) was the possible plumage color of indigenous chickens available in the Benin.

2.3 Chicken production in Bangladesh

In Bangladesh, commercial poultry production has been growing rapidly since the early 1990 by using improved genetics, manufactured feeds and management (Raha, 2013). This improvement is done mainly in the private sector as a device for additional source of income and employment opportunities particularly in rural area. This process has been influenced by the programs of different NGO's and public sectors (Raha, 2013). Indigenous chicken is widely reared throughout the country by rural people since time of immemorial. Village poultry is still popular to millions, eight thousand years after domestication (Alders and Pym, 2009) and play a vital role to poor rural household. According to DLS (2017-18), the number of poultry were 3379.98 (in lakh) and the number of chicken were 2821.45 (in lakh), the national share of commercial strain of chicken and family poultry was 1552.00 (Core Number) in egg production while for meat production it was 72.60 lakh metric ton in Bangladesh (DLS, 2017). In addition to indigenous chicken a crossbred of RIR × Fayoumi with phenotypic appearance similar to local chicken called 'Sonali' was introduced in northern part of the country. Sonali rearing is easier than broiler due to suitable environment of the country (Saleque and Saha, 2013). Sonali comprised about 30% of the total broiler and layer production of the country (Haque *et al*., 2011). Traders use to sell Sonali in the name of local chicken at a higher price. Poultry industry contributes 1% the country's GDP while at least 70 lakh people are involved in the sector, but the industry lacks proper support from the government (Haque *et al*., 2013).

According to WHO – FAO joint survey, meat consumption per head in Bangladesh is 15.23 kg per year while the requirement is 43.8 kg per person. So, there is a deficit of 65.23 % to meet our domestic requirement. It may be noted that poultry contributes 35.25% of total meat supply (Akbar *et al*., 2013). On an average people consume 3.63 kg of poultry meat per year which is expected to be 5 kg by 2015 and 12 kg by 2021 (Bangladesh Economic Review 2016). Thus there is a need to increase the animal protein production to fulfill the demand of the people and subsequently to make them sound and healthy for increasing their working ability (Raha, 2013). According to Bangladesh Poultry Association (BPA) the growth domestic of consumption would not be sufficient to absorb domestic supply. It is reported that the country achieved self-sufficiency in production of chicken meat and eggs. There is a growing concern that excess production

of chicken meat and eggs leads to close down of poultry farms in the country (Shahin, 2014).

Chickens in developing countries provide nutrition for the family, a small cash flow reserve for times of celebrations or need and in some areas contribute to religious ceremonies and recreation (Roberts, 1995). Kaya and Yıldız, (2008) reported that, native chickens are known to be good foragers and efficient mothers, and they require minimal care to grow. They are, therefore, most suited for raising under rural conditions. Most of the chickens in Bangladesh are of nondescript except few game birds like Sarail, Aseel and Chittagong (Malay) (Faruque *et al*., 2010).

In free range scavenging condition, chicken is being reared in Bangladesh for a long time and it has contributed about 19.75% and 25.06% of total meat and egg production (Dutta *et al*., 2013). About 89 % of rural households keep chicken with an average flock size of 5.33 per holding under backyard scavenging system (Bhuiyan *et al*., 2013). The growth rate of livestock sector shows an upward trend over the reference periods, which were above the growth rates of other sub-sectors of agriculture. This high growth attributed to significant growth in poultry farm (BER, 2016). The poultry sector has emerged as a flourishing and promising commercial sector in Bangladesh during the recent years. The poultry sector registered a per holding increase of 38.8 percent and per capita increase of 64.8 percent for the period between 1983/84 and 2005 (Planning Commission, 2016). In fact, there has been a silent revolution in the poultry sector during the last decade. During the 2000/01-2008/09 decade poultry population registered a growth of over 5 per cent. Improving the poultry productivity would improve protein nutrition and could increase the income levels of the rural population. In addition, consumers prefer meat from indigenous chickens, because of its leanness. They also like the multi-colored plumage of these birds. The productivity of indigenous chickens can be improved by providing appropriate housing, disease control and good nutrition (Ndegwa *et al*., 2014).

Table 2 Performance of indigenous chicken

Traits	Types of indigenous chicken		
	Deshi	**Necked neck**	**Hilly**
Hatch weight (g)	29[1,2]	30[2,6]	29[1,6,]
Age at first egg (d)	175[2,4]	234[2,7,]	240-300[6,2,8]
Mature body weight (kg)	1 - 1.3[2,6,8]	1.171[1,2,6]	1.7-2.50[6,8]
Egg production/hen/year (no.)	45-50[2,3,5]	50-55[2,3]	33[2,6,8]
No. of eggs/clutch	10 – 16[1,6]	10-12[3,4,6]	8-10[3,6,7]
No. of clutch/year	10.15[6]	-	-
Egg weight (g)	35-39[2,6]	42[7,8]	42.6[1,7,8]
Fertility (%)	83 %[2,5]	80%[6,7,8]	96.33%[2,3,4]
Hatchability (%)	75 – 87[2,6]	70-80[2,6,7]	91[1,2,3]

[1]Hoque *et al*. (2013), [2]Bhuiyan *et al.* (2004), [3]Barua (1992), [4]Sazzad (1986), [5]Haque (2011), [6]Khan *et al*. (2017); [7]Haque and Assaduzzaman (1990), [8]Ahmed and Islam (1985).

2.4 Characterization of chicken genetic resources

Genetic characterization is important for distinguishing among different animal genetic resources and for assessing the available diversity (FAO, 1998). However, inadequate attention has been given to evaluating these resources or to setting up realistic and optimum breeding goals for their improvement. It is also stated that an increasing loss of genetic diversity has been observed for all agriculturally used species (Dettelaff *et al*., 1991) and poultry genetic resources are considered to be the most endangered (Crawford, 1990). Globally over 6379 documented breed populations of some 30 species of livestock have been developed in the 12,000 years since the first livestock species were domesticated (FAO, 2000). The majority of livestock genetic diversity is found in the developing world where documentation is scarce and risk of extinction is highest and increasing. More particularly, it is estimated that 35% of mammalian breeds and 63% of avian breeds are at risk of extinction, and that two breeds are lost every week (FAO, 2000). The current breeding strategies for commercial poultry concentrate on specialized production lines, derived by intense selection from a few breeds and very large populations with a great genetic uniformity of traits under selection (Notter, 1999). However, there are numerous local chickens that are characterized by medium or low

performance and maintained in small populations (Groene *et al*., 1999). These local chickens face genetic erosion which may lead to the loss of valuable genetic variability in specific characteristics. The local breeds contain genes and alleles pertinent to their adaptation to a particular environments and local breeding goals (Pandey *et al*., 2002). Characterization, conservation and use of indigenous animal resources under low levels of input in the tropics are usually more productive than is the case with exotic breeds. The locally adapted animals are also more readily available to resource-poor farmers and they can be productive without high disease-control inputs. Therefore, characterization, utilization and conservation of these indigenous genetic resources are of paramount importance.

2.5 Relationship of plumage color and different gene

Identification of plumage color gene was studied by number of researchers in different literature (Kerje *et al*., 2004, Vaez *et al*., 2008, Pandey *et al*., 2002). Kerje *et al*. (2004) reported that, Dominant white, Dun, and Smoky type's plumage color whereas *PMEL17* gene was the chief plumage color affecting gene. However, linkage analysis of *PMEL17* and dominant white using a red jungle fowl/White Leghorn intercross revealed no recombination between these loci. Sequence analysis showed that the dominant white allele was exclusively associated with a 9-bp insertion in exon 10 (Kerje *et al*., 2004), leading to an insertion of three amino acids in the *PMEL17* transmembrane region. Similarly, a deletion of five amino acids in the transmembrane region occurs in the protein encoded by Dun (Kerje *et al*., 2004). The Smoky allele shared the 9-bp insertion in exon 10 with dominant white, as expected from its origin, but also had a deletion of 12 nucleotides in exon 6, eliminating four amino acids from the mature protein (Kerje *et al*., 2004). These mutations are, together with the recessive silver mutation in the mouse, the only *PMEL17* mutations with phenotypic effects that have been described so far in any species. Vaez *et al*. (2008) revealed that, the lavender phenotype in the chicken *MLPH* gene which affecting the dilution of plumage color. Whereas *MLPH* was located in chicken chromosome 7 and to identify the mutation underlying the lavender phenotypes which dilute the plumage color in MLPH exon 1 region. Gunnarsson *et al*. (2006) assessed that *SLC45A2* gene also affecting the plumage color of chicken and Japanese quail, where affecting color S*S (Silver), S*N (wild-type/gold) and S*AL (sex-linked imperfect albinism). *MC1R* gene is associated with extended black feather color in Duck reported in Yu *et al*. (2012). The wild type plumage pattern has minor variations, which are

responsible for the distinct features and appearance in some duck breeds (Lancaster, 1990). In this study, the four pigmentation phenotypes of duck breeds were deduced by classical genetics from MC1R genotyping according to the color and pattern of plumage, and the E locus (Lancaster, 1990). For genotyping the following sub section is essential.

2.6 Molecular marker

During the last two decades several DNA markers such as RAPD, AFLP, RFLP and microsatellites have been developed and utilized in genetic diversity analysis (Wu *et al*., 2004). In contrast to using morphological traits and/or measurements for characterization, DNA-based methods are independent of environmental factors and provide useful information about genetic diversity (Mollah *et al*., 2009). This holds particularly true for DNA-profiling methods, which is based on the polymerase chain reaction (PCR). Microsatellites are tandem repeated loci with a core motif of 1 to 6 bp repeated several times (Yu *et al*., 2012). The application of microsatellite markers are currently thought to be more useful than the other markers, since they are numerous and randomly distributed in the genome, seem highly polymorphic and show co-dominant inheritance (Smith and Smith, 1993; Crooijmans *et al*., 1996). They have been useful in determining genetic variation and phylogenic relationships among populations of the same species (Crooijmans *et al*., 1996). Microsatellite markers have been successfully used in chicken diversity studies (Crooijmans *et al*., 1996; Ponsuksili *et al*., 1996). Prior studies have used microsatellites as genetic markers for mapping purposes to estimate gene flow, effective population size and inbreeding as well as in parentage determination and forensics (Weigend *et al*., 2001).

2.7 Genetic diversity

Genetic characterization through the use of molecular markers associated with powerful statistical approaches is providing new avenues for decision making choices for the conservation and rational management of AnGRs (Okabayashi *et al*., 1998). Genetic distances are metrics which have been developed to summarize allele frequency differences among populations. So far, no general consensus exists as to which of the many genetic distance estimates would be the best for the analysis of variation within and between populations. However, the standard genetic distances (DS) of Nei (1972; 1978), the chord distance (DA) of Nei *et al.* (1983) measure of genetic structure (FST, in which its values can range from 0 to 1) were chosen among the many available genetic distance

estimating methods, because they are all relatively popular and have distinct properties to measure the genetic distance between populations. The standard genetic distance (Ds) of Nei (1978), is formulated as:

$Ds = (1- Jx\ y)-1/2\ \{(1- Jx) + (1- Jy)\}$

Where: $JX = (2nx\ \Sigma x2i - 1)/ 2nx -1)$

$Jy = (2ny\ \Sigma y2i - 1)/ 2ny -1)$

$Jxy = \Sigma\ xy$

n = Number of individual sample size per population

XiYi =Allele frequencies for X^{th} allele in population x and y.

This remains to be the most commonly used method to measure the genetic distances between populations. Mollah *et al*. (2009) reported that, the alleles were analyzed to determine the mean number of alleles per locus and the observed (Ho) and expected (He) heterogygosity. The breed coancestry (Fij) was analyzed to assess the Hardy-Weinberg Equilibrium using computer software (Khan *et al*., 2017).

Relationships among the individuals were further investigated by phylogenetic analysis of 14, 554, 492 SNPs and NJ tree was constructed with 100 bootstrap iteracions (Ulfah *et al.*, 2016). GJFm and GJFj formed a cluster, showing that GJF is phylogenetically distinct (Ulfah *et al*., 2016), with negligible genetic contribution to other varieties. In addition, GJFm birds had long branches more than other species did, indicating high genetic variation within the species. In contrast, wild RJF from Sumatra (RJFs) and Java (RJFj), as well as the reference RJF, did not form a monophyletic group, indicating diverging genetic backgrounds. RJFj formed a monophyletic group that was sister to the GJF group, which in turn formed a monophyletic group with RJFs (Ulfah *et al*., 2016).

Summary of literature review

- There were various types of indigenous chickens are available throughout the country. The types are considered on the basis of plumage color, comb type, feather pattern and body shape of the chickens.
- Among the types, based on plumage color types are more visible and different available plumage color chickens are black, blackish red, blackish white, blackish yellow, yellow whitish yellow, reddish yellow, white and blackish brown

- Several genes such as *PMEL17, MC1R, SLC45A2* and *MLPH* are responsible for plumage color variation of chickens.*PMEL17*gene is major plumage color affecting gene, which is responsible for white, black and reddish color plumage color. In this current research different plumage color chickens performance was studied, however, for genetic study only black-white, black-brown and spotted types chickens were considered therefore, *PMEL17* gene has chosen for this research.
- The polymerase chain reaction (PCR) technique is explosively increases the systematic and population genetic research and microsatellites have been the most widely used markers for genetic diversity estimation.
- Gene sequencing is the method, which has been measuring scale gene expression patterns.

Chapter 3

Materials and Methods

3.1 Study area

The study was conducted in the Patiya Upzilla (Sub-district) of Chittagong district of Bangladesh, Poultry Research and Training Center (PRTC) and in the Department of Genetics and Animal Breeding at Chittagong Veterinary and Animal Sciences University, Khulshi, Chittagong.

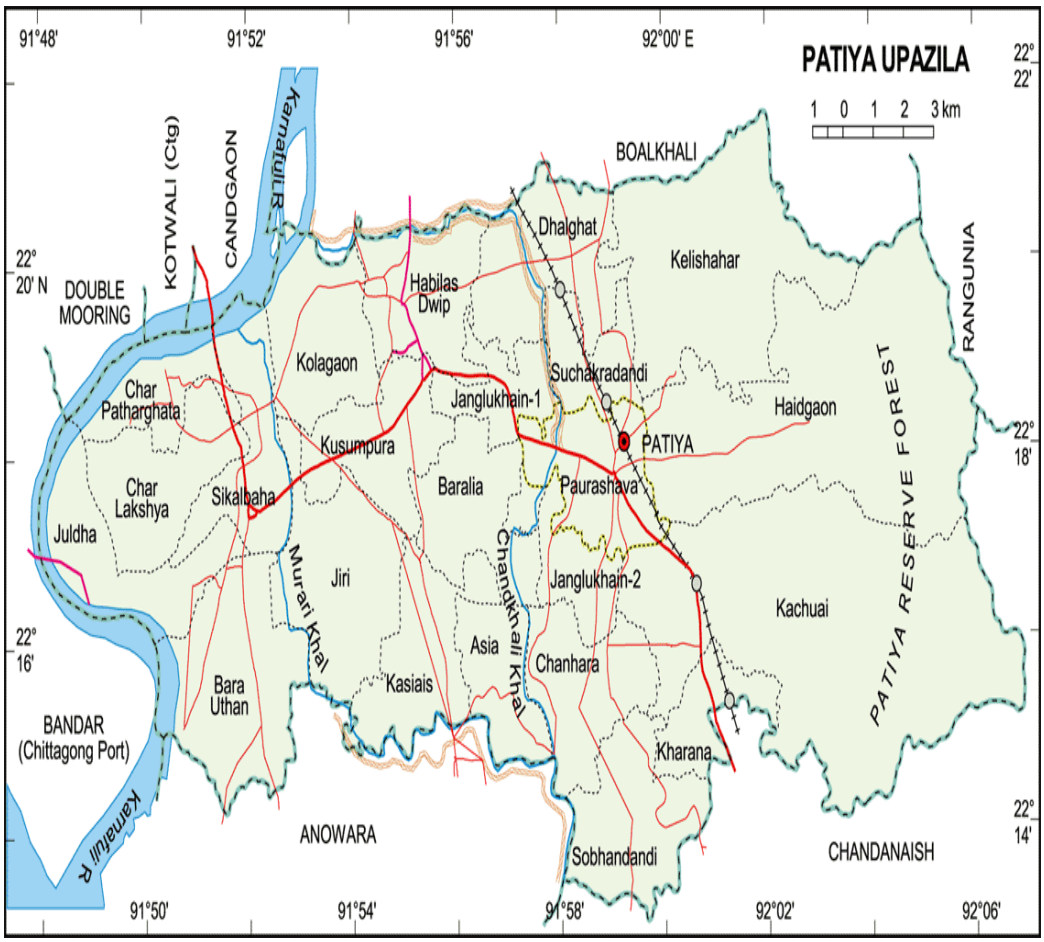


Figure 1: Study area in Patiya

3.2 Study period

The study was conducted from March, 2017 to May, 2018.

3.3 Baseline survey and typing of the chicken

A baseline survey was conducted in the Patiya Upzila (Sub-district) of Chittagong district with a pre-tested designed questionnaire. A total of 55 households (those farmers who have at least 3 chickens) from the Badamtal (location 1), Kusumpura (location 2) and Kolagaon (location 3) under this Upazilla (sub-district) of Chittagong district were surveyed directly. The phenotypic and morphological features of the chickens were recorded. Chickens were chosen on the basis of different criteria for categorized them, which included plumage color, comb type and body shape.

3.4 Tagging and recording of the chicken's character

All of these identified chickens were tagged by using leg ring tag. Different traits of chickens were recorded according to types, which included mature body weight, body shape, monthly egg production (egg no), clutch size, egg weight and plumage length.

3.5 Recorded of different external and internal characteristics of egg

Eggs from different types of chicken in selected areas were collected and different external characteristics like egg weight, egg length, egg wide, shell color, shell thickness, shell weight, shape index, surface area and different internal characteristics like albumen weight, albumen length, albumen wide, albumen ratio, yolk weight, yolk length, yolk wide, yolk ratio, shell membrane thickness and Haugh unit etc. Egg quality traits were measured following standard procedures of Reddy *et al*. (1979); Monira *et al*. (2003) and Fayeye *et al*. (2005).

3.5.1 External characteristics of egg

Egg weight, length and wide

Twenty eggs were considered for each type of chicken. Collected eggs were cleaned using a paper towel and eggs weight was measured with an electronic digital balance and recorded. Egg wide and egg length were measured by using Vernier calipers.

Shape index

Egg shape index was calculated as a ratio of the egg width to the egg length as followed by Yakubu *et al*. (2008):

$$\text{Shape index} = \frac{\text{Egg wide}}{\text{Egg length}} \times 100$$

Egg shell weight and surface area

Eggs were broken and shell weight was measured with an electronic digital balance and then the shell surface area (SSA) was determined from the expression according to Carter (1975):

Surface area = 3.9782 × SW× 0.7062,

Where SW = Shell weight; and 3.9782 and 0.7062 values were constant.

Shell thickness

The thickness of egg shell was determined using a micrometer screw gauge. Accuracy of shell thickness was ensured by measuring shell sample at the broad end, middle portion and narrow end of the shell.

3.5.2 Internal characteristics of egg

Albumen and yolk weight

Twenty eggs were considered for each type of chickens. Each egg was later carefully broken and the yolk and the albumen were then placed in separate petri dishes, which had initially been weighed by using digital weighing balance. The difference in the weight of each petri dish after and before the introduction of the yolk and albumen was taking as the weight of the yolk and albumen, respectively (Yakubu *et al*., 2008).

Albumen and yolk height and wide

The albumen and yolk height were determined using a spirometer albumen height was measured in the middle of the thick albumen equidistant from the outer edge of the albumen and the yolk. Albumen and yolk width were measured around the widest horizontal circumference using vernier slide calipers.

Albumen and yolk ratio/index

Albumen and yolk ratio was determined as:

$$\text{Albumen or yolk ratio} = \frac{\text{Albumen or yolk height}}{\text{Albumen or yolk width}}$$

Haugh unit

In order to correct for the difference in egg weight, the albumen height was converted into Haugh unit. (Haugh, 1937) as follows:

$$HU = 100 \log (H + 7.6 - 1.7W^{0.37})$$

Where, HU = Haugh unit

H = Observed height of the albumen in millimeter.

W = Weight of egg in grams.

3.6 Blood sample collection for molecular study

PMEL17 gene is major plumage color affecting gene, which is responsible for white, black and reddish color plumage color. In this current research different plumage color chickens performance was studied, however, for genetic study only black-white, black-brown and spotted types chickens were considered therefore, *PMEL17* gene has chosen for this research. To know the effects of this gene on these genotype (type 1: 20, type 2: 10, type 3: 30). Blood samples were collected from jugular vein and wing vein of 35 laying hens and supplemented with vacutainer tube containing 0.5M EDTA (pH=8). All samples were delivered back to the laboratory in an ice box and transferred to the laboratory freezer (−20°C) by the use of aseptic means.

3.7 DNA extraction

DNA was extracted from the whole blood samples using FavorPrepTM blood genomic DNA extraction mini kit. At first 20µl whole blood sample added with 180µl phosphate buffer saline (PBS) and this sample was transferred to a micro-centrifuge tube with 20µl proteinase-k and 200µl FABG buffer and mixed thoroughly by pulse-vortexing. Then, incubated the sample for 15 minutes at 60ºC temperature and keep vortexing the sample for every 3-5 minutes interval for lysate. Added 200 µl of 100% ethanol and mixed through by pulse vortexing for 10 seconds and briefly spine the tube for removed the drops from the inside of the tube. After that mixture was transferred to FABG column with collection tube and centrifuge at 6000×g for 1 minute. Spine the FABG mini column after added 400 µl W1 buffer at 18000×g (full speed) for 30 seconds and 750 µl wash buffer at 18000×g for 30 seconds respectively and discarded the flow through in a consecutive manner. Centrifuge was continued for additional 3 minutes to dry the column and finally

transferred FABG mini column to the elution tube where 120 µl elution buffers added to center of the column. Stored the total DNA at -20ºC after completed the final centrifugation for 1 minute at full speed.

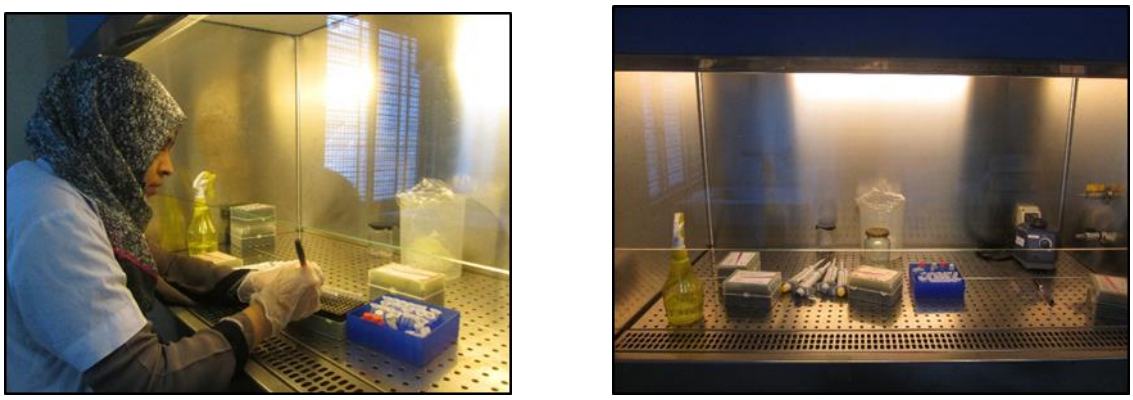


Figure 2: DNA extraction from blood

3.8 *PMEL17* gene

The phenotypic traits are closely related to genetic makeup of this trait, to investigate the genetic makeup of a trait DNA extraction and other molecular techniques can be used. The plumage color including other color pattern is the interactions of color factors.

PMEL17 is the plumage color affecting gene in the chicken, which causes variation in plumage color. *PMEL17* is a type I integral membrane protein present in the melanosome and is a component of the fibrous striations upon which melanin are polymerized (Berson *et al*., 2003). *PMEL17* gene is identified as a positional candidate gene for the phenotype in chicken. Dominant white locus encodes *PMEL17* protein which is specific protein and plays a key role in the development of melanocyte (Kerje *et al*., 2004). In the chicken plumage color variation; dominant white, dun and smoky are all caused by the mutations in *PMEL17* gene. Unique insertion/deletion polymorphisms (9-15bp in length) were also found in birds carrying these alleles.

3.9 Primer selection

The primer was selected on the basis of plumage color gene *PMEL17* and primer to amplify the complete *PMEL17* gene was designed on the basis of a chicken cDNA sequence (GenBank D88348). The gene was sequenced in five parts using the primer pairs P1fwd/P1rev, P2fwd/P2rev, P3fwd/P3rev, P4fwd/P4rev and P6fwd/P6rev (Karje *et al*., 2004). The P6 primer was used to amplify genomic chicken DNA. Forward primer

sequence of *PMEL17* gene was F-5´-GTGGATGTGACACAGCTGGA-3´ and reverse was R-5´-CCGGAGCATCACCACCTGA -3' whereas the amplified region was Exon 6 and annealing temperature was 65ºC. After PCR amplification, the PCR product was found at 542bp.

3.10 PCR and agar gel electrophoresis

A total of 25µl polymerase chain reactions (PCR) composed of 12.5µl master mix, 2.5µlM of each primer (forward and reverse), buffer 5µl and 2.5µl DNA template were prepared (FavorPrepTM). The PCR amplification was conducted in a MJ PTC-200 per liter. Thermal Cycler or a Bio-Red C 1000 Thermal Cycler with initial denaturation at 95ºC for 10 minutes followed by 50 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds at the 65ºC (Karje *et al*., 2004) and extension at 72ºC for 2 min, and a final extension at 72ºC for 10 minutes. The PCR products were electrophoresis on 2.5% agarose gel (Lonza USA) at 90 V for 1.5 to 2h and stained with ethidium bromide and their sizes were estimated using a 100-bp DNA ladder. The amplified PCR band pattern were visualized by on a UV trans-illuminator and photographed in computer.

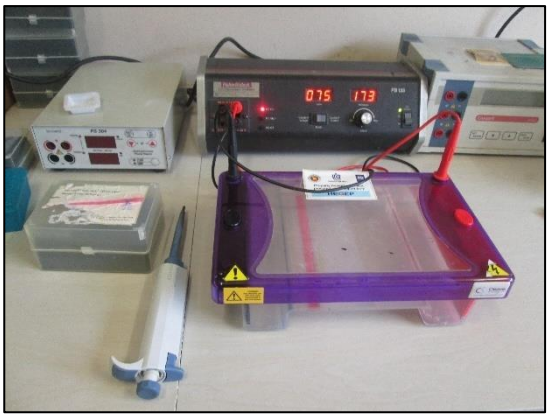


Figure 3: PCR and gel electrophoresis

3.11 Gene sequencing and alignment

3.11.1. PCR product purification

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

3.11.2. Sequencing

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

3.11.3. Analytical method and DNA information from gene banks

One of the most informative methods used in sequence data analysis was similarity searching. For DNAs, similarity at the sequence level implies some structural or functional similarity between the protein products or regulatory elements of gene expression. Searching a database with an uncharacterized gene sequence can identify homologues in other species or sequence elements that encode structural domains within the protein. Searches can be conducted with either nucleotide sequences. However, detection of similarity at the nucleotide level is difficult unless the sequences are closely related. A commonly used tool for similarity searching is BLAST (Basic Local Alignment Search Tool) because of its practical balance of speed, sensitivity and selectivity. In the current study,8 sequences of the *PMEL17* gene, including DNA, were taken from the NCBI information gene bank, and using the tool BLAST on the website http://ncbi.nlm.nih.gov, similar sequences, their similarities (nucleotide) and possible mutations were investigated.

3.11.4. Phylogeny and determination of evolutionary direction

In order to draw the phylogenetic tree, the nucleotide sequence of the *PMEL17* gene was predicted for the species being studied using MEGA6 software program (Tamura *et al*., 2013). After editing the sequences and deleting the noncoding regions, the phylogenetic tree was drawn using neighbor-joining (NJ) method. In this method, a matrix (Q) was used, so that in this matrix, all the branches were used and the lowest value which represents high similarity between two branches were selected and employed in a branching of the phylogenetic tree. Bootstrap values were obtained through 100 times of re-sampling. The phylogenetic tree was drawn using NJ method.

$$Q(i, j) = (r - 2)\, d(i, j) - \Sigma d(i, k) - \Sigma d(j, k)$$

Where,

d (i, j): distance between branches i and j.

k: k^{th} branch of the tree.

r: total number of the branches.

Q(i, j): numerical value of branches i and j.

3.11.5 Mutation in the plumage color gene

MEGA6 is a commonly used program for multiple sequence alignment. It uses a progressive algorithm to align sequences in successively larger groups, beginning with the most closely related sequences. Detection of mutation based on sequence alignment by using muscle (MEGA6 software).

3.12 Statistical analysis

Least square means were estimated for the different parameters on the basis of chicken types and were analyzed by PROC GLM and PROC MIXED of SAS (SAS, 2008) using randomized block design (RBD). The mean differences were compared using least significant difference (lsd) (Steel *et al*., 1997) at 5% level of significance.

The nucleotide sequences were analyzed to determine the nucleotide sequences comparison, evolutionary relationship and maximum composite likelihood estimation by using NCBI BLAST (Madden, 2013) and MEGA6 software (Tamura *et al*., 2013).

Chapter-4

Results

4.1 Phenotypic characteristics of indigenous chicken

The mean with standard error values of different traits of indigenous chickens' according to types and locations are presented in Table 3.

The egg production (number/clutch/chicken) did not differ between locations but they were differed significantly ($p<0.05$) among the types within the location (Table 3). Among the types the highest egg production (number/clutch) was recorded for spotted single round type chicken (13.5 numbers) and the lowest was for brown pea cylindrical type chicken (5.25 numbers). However, in type comparisons within location, the egg production per clutch was significantly different ($p<0.05$) between the types. Among the types the highest and lowest clutch size in studied locations was 15.05±0.28 days for brown single round and 5.88±0.44 days for brown pea cylindrical type, respectively. However, no significant differences were observed between locations (Table 3).

The live weight (kg) of all types of chickens were significantly differed ($p<0.05$) between locations. In the first location, among the type no significant differences found within the location, however, significant differences were observed between location 2 and 3. Among the types, the highest live weight (1.6kg) was recorded in the black-white pea round and spotted single cylindrical types in location 3 and the lowest (0.89 kg) was observed in the case of black single round in the location 2.

The plumage length of different types of chickens was significantly different between locations (Table 3). Among the types, the highest (18cm) plumage length was recorded for brown pea cylindrical and black-white pea round and lowest (15 cm) was observed for brown pea cylindrical type (Table 3).

Table 3: Different traits (Mean ± Standard Error) of indigenous chickens according to types and locations

Location	Types	No of observation	Traits							
			Egg no. (per clutch)	Location average	Clutch size	Location average	Live weight (kg)	Location average	Plumage length (avg. cm)	Location average
Location-1	BLPR	12	7.83^{b}±0.6	7.56±0.77	10.0^{c}±0.5	10.36±0.81	1.05±0.08	1.09^{b}±0.03	15.5^{b}±0.55	16.49^{b}±0.39
	BLSR	10	8.0^{b}±0.22		12.0^{b}±0.75		0.98±1.2		17.0^{ab}±0.02	
	BRPCL	8	5.25^{c}±1.0		8.83^{d}±0.6		1.07±0.02		15.0^{b}±0.89	
	BRSR	15	12.5^{a}±0.2		15.0^{a}±0.9		1.05±0.04		18.25^{a}±0.7	
	BWPR	18	6.25^{bc}±0.3		8.0^{d}±0.57		1.2±0.065		16.5^{b}±0.55	
	BWSR	12	6.5^{bc}±0.75		9.75^{cd}±0.25		1.0±0.88		15.5^{b}±0.90	
	SPSCL	9	6.5^{bc}±0.5		10.0^{c}±0.99		1.12±0.27		17.5^{ab}±0.49	
	SPSR	7	7.67^{b}±0.4		10.3^{c}±0.88		1.26±0.13		16.7^{ab}±0.88	
Location-2	BLPR	10	5.83^{b}±0.4	7.58±0.79	12.8^{b}±0.75	10.18±1.28	1.2^{ab}±0.75	1.14^{ab}±0.07	17.0^{b}±0.07	17.5^{a}±0.19
	BLSR	16	8.0^{ab}±0.9		10^{c}±0.99		0.89^{c}±0.08		17.2^{b}±0.25	
	BRPCL	10	5.5^{b}±0.5		5.88^{d}±0.44		1.04^{bc}±0.04		18.0^{a}±0.12	
	BRSR	7	10.0^{a}±0.5		15.05^{a}±0.3		1.09^{b}±0.88		17.0^{b}±0.43	
	BWPR	11	6.5^{b}±1.5		10.5^{bc}±0.22		1.4^{a}±0.023		18.0^{a}±0.01	
	SPSR	13	9.67^{a}±0.8		11.67^{b}±0.8		1.23^{ab}±0.08		17.8^{ab}±0.33	
Location-3	BBPCL	18	9.0^{c}±0.08	8.49±0.76	12.0^{b}±0.51	11.13±0.78	1.09^{b}±0.25	1.3^{a}±0.06	16.7^{ab}±0.14	16.73^{b}±0.16
	BBSCL	12	8.6^{c}±1.69		11.0^{bc}±0.37		1.13^{b}±0.11		17.3^{a}±0.32	
	BBSR	8	9.57^{c}±0.7		12.4^{b}±0.89		1.3^{ab}±0.11		17.3^{a}±0.38	
	BLPR	10	6.67^{cd}±2.7		9.0^{c}±0.99		1.18^{b}±0.19		16.5^{ab}±0.75	
	BLSR	9	5.5^{d}±0.49		8.5^{c}±0.49		1.3^{ab}±0.14		16.8^{ab}±0.14	
	BRSR	13	11.5^{b}±0.5		14.0^{a}±0.3		1.2^{b}±0.045		15.5^{b}±0.73	
	BWPR	10	7.0^{cd}±0.99		9.0^{c}±0.99		1.6^{a}±0.14		17.2^{a}±0.67	
	BWSR	14	7.6^{cd}±0.50		10.4^{c}±0.63		1.2^{b}±0.16		16.5^{ab}±0.23	
	SPSCL	11	6.0^{cd}±0.99		9.0^{c}±0.89		1.6^{a}±0.14		16.9^{ab}±0.09	
	SPSR	5	13.5^{a}±0.4		14.0^{a}±0.99		1.06^{b}±0.25		16.6^{ab}±0.14	
	Mean value	**268**		**8.3**		**10.9**		**1.21**		**16.98**
	SEM value			**0.3**		**0.4**		**0.63**		**0.205**
	P value			**0.4**		**0.4**		**0.003**		**0.02**

Legends: BLPR=Black pea round, BLSR= Black single round, BRPCL=Brown pea cylindrical, BRSR=Brown single round, BWPR=Black white pea round, BWSR=Black white single round, SPSCL=Spotted single cylindrical, SPSR=Spotted single round, BBPCL=Black brown pea cylindrical, BBSCL=Black brown single cylindrical, BBSR=Black brown single round, SPSCL=Spotted single cylindrical. SEM= Standard Error of Mean.

Means with different superscripts in the same row differ significantly ($p< 0.05$).

4.2 External and internal characteristic of chicken egg

4.2.1 External characteristics of chicken egg

The external characteristics of eggs (mean± SE) of different types of chickens under the three locations are depicted in Table 4.

The egg weight (g) of all types of chickens were significantly differed ($p<0.05$) between locations. In all locations, among the types, the egg weight ranged from 31.6 to 44.83g. The highest egg weight (44.83± 0.54 g) was recorded in the case of black pea round type in location 1 and lowest (31.6±0.72 g) was in location 3 for the same type of chicken (Table 4).

Significant differences were found between the locations for egg length (Table 4) however, the egg length of different types of chickens was significantly varied between types within the location. Egg length of different types of chicken ranged from 4.43 to 6.3cm.

Egg width and egg shell weight of different types of chicken did not differ between locations, but significantly differed ($p<0.05$) between types in location 1 only and in all locations, respectively (Table 4).

Between the locations all types of chicken were significantly different ($p<0.05$) in the case of egg shell membrane thickness. Significantly higher (1.1±0.52 mm) shell membrane thickness was observed in the case of spotted single cylindrical type chicken than the brown single round type chicken (0.2 mm). The significant difference ($p<0.05$) was recorded between the types within the locations.

On the other hand the shape index was not shown any differences between the locations, but different types of chicken were showed variation among the type in location 1 and 2.

The surface area of different types of chicken eggs did not differ between locations, but significantly differed ($p<0.05$) between types within the location. Among all types, the highest (14.60) surface area was observed in the case of black-white pea round type chicken and the lowest (10.49) was recorded in case of black single round (Table 4).

Table-4: External characteristics (Mean ± Standard error) of eggs of different types of chickens under the three locations

Location	Types	No. of observation	Traits													
			Egg weight (g)	Average	Egg length (cm)	Average	Egg wide (cm)	Average	Shell weight (g)	Average	Shell thickness (mm)	Average	Shape index	Average	Surface area	Average
Location 1	BLPR	12	$44.83^{a}\pm0.5$	$38.06^{b}\pm0.30$	$4.79^{ab}\pm0.52$	$5.05^{a}\pm0.19$	$3.76^{ab}\pm0.8$	3.75 ± 0.12	$4.49^{ab}\pm0.05$	4.41 ± 0.18	$0.9^{a}\pm0.033$	$0.83^{a}\pm0.07$	$78.4^{b}\pm0.88$	74.73 ± 1.91	$12.61^{b}\pm2.04$	12.04 ± 0.51
	BLSR	10	$38.17^{bc}\pm0.7$		$5.17^{a}\pm0.01$		$3.88^{ab}\pm0.4$		$5.12^{a}\pm0.03$		$0.7^{ab}\pm0.50$		$75.04^{b}\pm1.08$		$14.38^{a}\pm0.03$	
	BRPCL	8	$36.27^{c}\pm0.4$		$4.85^{ab}\pm0.43$		$3.71^{ab}\pm0.6$		$4.8^{ab}\pm0.021$		$0.8^{ab}\pm0.21$		$76.4^{b}\pm0.90$		$13.48^{a}\pm0.87$	
	BRSR	15	$40.4^{b}\pm0.41$		$4.87^{ab}\pm0.004$		$3.64^{b}\pm0.1$		$3.79^{b}\pm0.81$		$0.47^{b}\pm0.27$		$74.7^{b}\pm2.4$		$10.7^{bc}\pm2.3$	
	BWPR	18	$32.87^{e}\pm0.2$		$4.57^{b}\pm0.85$		$3.14^{b}\pm0.7$		$3.82^{b}\pm0.01$		$0.7^{ab}\pm0.65$		$68.7^{c}\pm2.76$		$10.73^{bc}\pm2.09$	
	BWSR	12	$34.91^{d}\pm0.4$		$5.15^{a}\pm0.05$		$4.3^{a}\pm0.12$		$4.84^{a}\pm0.02$		$0.9^{a}\pm0.03$		$83.4^{a}\pm0.01$		$10.59^{c}\pm1.08$	
	SPSCL	9	$36.4^{c}\pm0.69$		$4.7^{b}\pm0.10$		$3.57^{b}\pm0.1$		$3.93^{b}\pm0.14$		$1.1^{a}\pm0.19$		$75.3^{b}\pm4.04$		$11.03^{bc}\pm0.42$	
	SPSR	7	$40.7^{b}\pm0.83$		$6.3^{a}\pm1.27$		$4.05^{a}\pm0.4$		$4.5^{ab}\pm0.02$		$1.1^{a}\pm0.52$		$65.9^{c}\pm5.8$		$12.8^{b}\pm0.07$	
Location 2	BLPR	10	$44.32^{a}\pm0.7$	$38.4^{a}\pm0.32$	5.17 ± 0.012	$4.79^{b}\pm0.1$	3.68 ± 0.4	3.58 ± 0.06	$4.4^{b}\pm0.06$	4.55 ± 0.14	$0.8^{a}\pm0.67$	$0.6^{b}\pm0.08$	$71.1^{b}\pm0.43$	74.6 ± 1.97	$12.36^{c}\pm0.56$	12.8 ± 0.39
	BLSR	16	$34.4^{c}\pm0.5$		4.9 ± 0.31		3.39 ± 0.2		$4.4^{b}\pm0.45$		$0.5^{ab}\pm0.24$		$68^{b}\pm1.19$		$12.4^{c}\pm1.3$	
	BRPCL	10	$37.8^{b}\pm0.01$		4.43 ± 0.88		3.58 ± 0.6		$4.7^{b}\pm0.30$		$0.7^{a}\pm0.04$		$80.8^{a}\pm0.03$		$13.20^{b}\pm0.44$	
	BRSR	7	$37.31^{b}\pm0.7$		4.59 ± 0.75		3.42 ± 0.7		$4.33^{b}\pm0.54$		$0.2^{b}\pm0.78$		$74.5^{ab}\pm3.01$		$12.16^{c}\pm1.09$	
	BWPR	11	$37.96^{b}\pm0.8$		4.65 ± 0.88		3.69 ± 0.03		$5.2^{a}\pm0.06$		$0.7^{a}\pm0.07$		$79.3^{b}\pm0.4$		$14.60^{a}\pm0.07$	
	SPSR	13	$38.6^{b}\pm1.88$		5.05 ± 0.2		3.74 ± 0.3		$4.3^{b}\pm0.19$		$0.7^{a}\pm0.04$		$73.9^{ab}\pm0.82$		$12.08^{c}\pm0.55$	
Location 3	BBPCL	18	$33.6^{d}\pm0.8$	$36.11^{b}\pm0.85$	$4.78^{b}\pm0.07$	$4.77^{b}\pm0.08$	3.47 ± 0.06	3.45 ± 0.05	$4.6^{a}\pm0.11$	4.55 ± 0.09	$0.76^{a}\pm0.03$	$0.67^{b}\pm0.03$	72.5 ± 2.09	72.28 ± 0.67	$13.2^{a}\pm0.32$	12.8 ± 0.28
	BBSCL	12	$35.9^{c}\pm0.42$		$4.79^{b}\pm0.15$		3.52 ± 0.09		$4.63^{a}\pm0.23$		$0.6^{ab}\pm0.06$		73.7 ± 1.6		$13.002^{a}\pm0.67$	
	BBSR	8	$35.2^{c}\pm0.3$		$4.98^{a}\pm0.13$		3.56 ± 0.11		$4.5^{a}\pm0.17$		$0.7^{a}\pm0.08$		71.6 ± 2.3		$12.74^{ab}\pm0.47$	
	BLPR	10	$31.6^{d}\pm0.72$		$4.9^{a}\pm0.15$		3.56 ± 0.26		$4.7^{a}\pm0.15$		$0.73^{a}\pm0.08$		71.1 ± 3.27		$13.22^{a}\pm0.44$	
	BLSR	9	$35.3^{c}\pm0.38$		$5.2^{a}\pm0.20$		3.65 ± 0.3		$3.7^{b}\pm0.35$		$0.75^{a}\pm0.04$		69.7 ± 2.04		$10.49^{b}\pm1.0$	
	BRSR	13	$35.75^{c}\pm0.7$		$4.23^{b}\pm0.65$		3.13 ± 0.8		$4.7^{a}\pm0.33$		$0.5^{b}\pm0.88$		73.9 ± 2.08		$13.42^{a}\pm0.64$	
	BWPR	10	$38.8^{b}\pm0.84$		$4.49^{b}\pm0.38$		3.33 ± 0.16		$4.67^{a}\pm0.37$		$0.55^{b}\pm0.04$		75 ± 10.2		$13.13^{a}\pm1.04$	
	BWSR	14	$38.4^{b}\pm0.09$		$4.98^{a}\pm0.23$		3.55 ± 0.11		$4.7^{a}\pm0.05$		$0.68^{ab}\pm0.04$		71.4 ± 2.57		$13.32^{a}\pm0.16$	
	SPSCL	11	$35.5^{c}\pm0.48$		$4.66^{b}\pm0.21$		3.50 ± 0.16		$4.8^{a}\pm0.22$		$0.79^{a}\pm0.04$		75.1 ± 0.09		$13.73^{a}\pm0.64$	
	SPSR	5	$41.08^{a}\pm0.8$		$4.77^{b}\pm0.26$		3.27 ± 0.06		$4.5^{a}\pm0.15$		$0.75^{a}\pm0.14$		68.8 ± 2.59		$12.64^{ab}\pm0.45$	
	mean	**268**		**37.4**		**4.9**		**3.58**		**4.52**		**0.73**		**72.6**		**12.71**
	SEM			**0.54**		**0.69**		**0.004**		**0.006**		**0.74**		**1.18**		**0.24**
	P value			**0.02**		**0.01**		**0.65**		**0.23**		**0.003**		**0.74**		**0.56**

Legends: Chicken type description is presented under the Table 3; Means with different superscripts in the same row differ significantly ($p< 0.05$).

4.2.2 Internal characteristics of chicken egg

The mean and standard error of internal characteristics of eggs of different types of chickens under the three locations are presented in the Table 5.

The albumen height of all types of chicken was observed significantly ($p<0.05$) difference between the locations, however, albumen height of different types of chickens were varied between types within the location (Table 5). For the albumen width, between the locations all types of chicken were significantly different ($p<0.05$). Albumen width ranged from 2.67 to 6.52cm and there were significant differences observed among the types within all the locations (Table 5). The highest (5.43±0.01g) albumen weight was obtained in brown single round type and lowest (3.16±1.6g) was found in spotted single round type. There were significant variations ($p<0.05$) observed between the types within the locations and also noticeable differences were found between locations. In case of albumen ratio there were no significant differences ($p<0.05$) between the location. Among all types of chicken significantly variation was observed between types in location 1 and 3 (Table 5).

In the case of yolk height, significant differences ($p<0.05$) were found between the types of the chicken within the location. The maximum (13.8±0.06cm) value was observed in the black pea round type chicken and the minimum value (5.67±3.7cm) was observed in brown single round type chicken. Yolk width of egg were significantly ($p<0.05$) varied between the locations and types of chickens and the yolk width varied from 5.28 to 1.30 for all types of chicken (Table 5). Yolk weight were significant ($p<0.05$) differed between the locations. However, yolk weight of different types of chickens was significantly varied between types within the location (Table 5).The highest yolk weight (26.4g) was recorded in the case of spotted single round type chicken and lowest (10.04g) was recorded in the case of black white pea round type chicken.

Between the locations all types of chicken were significantly different ($p<0.05$) in case of yolk ratio. However, the yolk ratio of different types of chickens was significantly differed between types within the location (Table 5).

The haugh unit of all types of chickens significantly differed ($p<0.05$) between locations. Among the types of chicken significant differences were observed between types within locations. Haugh unit was observed lowest (53.6) in black single round type chicken in location 1 and the highest (97.99) in brown single round type chickens in location 2.

Shell membrane thickness was not significantly different between locations, however, shell membrane thickness were significantly differed between types within the locations. Shell membrane thickness ranged from 0.10 to 0.30mm (Table 5).

Table-5: Internal characteristics (Mean ± Standard error) of eggs of different types of chickens under the three locations

Locations	Types	Traits																			
		Albumen height (cm)	Average	Albumen wide (cm)	Average	Albumen weight (g)	Average	Albumen ratio	Average	Yolk height (cm)	Average	Yolk wide (cm)	Average	Yolk weight (g)	Average	Yolk ratio	Average	Haugh unit	Average	Shell memb. Thick. (mm)	Average
Location-1	BLPR	4.67^{ab}±0.32	4.01^{ab}±0.6	5.45^{a}±0.08	3.68^{ab}±0.68	19.2^{b}±0.8	13.001^{a}±2.3	1.16^{a}±0.04	0.76±0.14	13.8^{a}±0.06	7.16±1.5	4.9^{a}±0.65	3.30^{ab}±0.7	23.42^{a}±0.03	14.4^{b}±2.6	0.35^{d}±0.44	0.46^{a}±0.11	69.11^{d}±8.6	45.5^{c}±2.85	0.20^{a}±0.02	0.19±0.03
	BLSR	4.2^{ab}±0.55		5.63^{a}±0.02		14.4^{c}±2.3		1.3^{a}±0.03		5.83^{bc}±2.7		5.86^{a}±0.3		16.98^{c}±1.5		1.0^{a}±0.05		53.6^{c}±9.06		0.20^{a}±0.05	
	BRPCL	0		0		0		0		0		0		0		0		0		0.30^{a}±0.01	
	BRSR	5.43^{a}±0.01		4.13^{b}±0.46		15.8^{c}±0.28		0.75^{bc}±0.08		8.87^{b}±3.37		4.01^{b}±0.12		16.1^{c}±1.08		0.51^{c}±0.2		84.37^{a}±1.1		0.20^{a}±0.15	
	BWPR	4.75^{a}±0.03		4.36^{b}±0.76		21.0^{a}±0.03		0.92^{b}±0.56		4.36^{c}±4.02		3.26^{bc}±0.6		10.04^{d}±2.5		0.75^{b}±0.04		83.42^{a}±2.1		0.30^{a}±0.07	
	BWSR	4.87^{a}±0.5		3.65^{bc}±1.02		9.91^{d}±1.7		0.74^{bc}±0.02		5.78^{bc}±1.0		3.43^{bc}±0.8		21.08^{b}±0.04		0.59^{c}±0.43		76.23^{b}±2.7		0.10^{b}±0.35	
	SPSCL	5.04^{a}±0.1		4.61^{b}±0.51		11.9^{c}±0.30		0.91^{b}±0.06		9.64^{ab}±4.25		3.66^{b}±0.23		16.9^{c}±7.04		0.45^{c}±0.2		82.23^{a}±1.92		0.10^{b}±0.50	
	SPSR	3.16^{b}±1.6		2.67^{c}±1.6		11.8^{c}±6.0		0.37^{c}±0.3		9.06^{ab}±4.56		1.30^{c}±1.3		10.7^{d}±5.4		0.1^{d}±0.09		74.6^{b}±5.76		0.14^{b}±0.4	
Location-2	BLPR	4.33^{b}±0.43	2.38^{b}±1.07	3.87^{b}±0.87	2.40^{b}±1.10	13.8^{b}±0.6	6.96^{c}±3.1	0.89±0.07	0.50±0.2	8.97^{b}±1.8	5.24±2.4	3.56^{b}±0.8	2.04^{b}±0.9	11.45^{c}±0.3	8.24^{c}±3.9	0.34^{ab}±0.65	0.16^{b}±0.08	82.5^{b}±0.88	92.01^{a}±1.17	0.10^{b}±0.33	0.14±0.02
	BLSR	5.42^{a}±0.03		4.59^{b}±0.23		15.5^{a}±0.03		0.84±0.67		12.25^{a}±0.4		4.14^{ab}±0.03		15.11^{b}±2.6		0.2^{b}±0.08		97.99^{a}±0.03		0.15^{b}±0.05	
	BRPCL	0		0		0		0		0		0		0		0		0		0.10±0.07	
	BRSR	0		0		0		0		0		0		0		0		0		0.20^{a}±0.07	
	BWPR	0		0		0		0		0		0		0		0		0		0.20^{a}±0.40	
	SPSR	4.56^{b}±0.1		5.96^{a}±0.56		12.5^{b}±0.21		1.31±0.12		10.24^{ab}±1.5		4.55^{a}±0.47		22.9^{a}±2.6		0.47^{a}±0.1		71.6^{c}±2.3		0.13^{b}±0.03	
Location-3	BBPCL	4.55^{ab}±0.1	4.56^{a}±0.10	5.83^{a}±0.8	5.58^{a}±0.2	11.03^{b}±1.3	12.4^{b}±0.5	1.29^{a}±0.24	1.22±0.04	10.09^{b}±2.4	9.68±0.7	4.48^{ab}±0.49	4.40^{a}±0.2	22.6^{ab}±2.4	23.6^{a}±0.54	0.48^{ab}±0.08	0.47^{a}±0.02	77.8^{b}±2.83	74.7^{b}±1.86	0.20^{a}±0.45	0.18±0.01
	BBSCL	4.49^{ab}±0.2		5.59^{b}±0.4		13.4^{a}±0.88		1.24^{ab}±0.13		8.83^{b}±1.16		4.46^{ab}±0.43		22.5^{ab}±0.81		0.51^{ab}±0.03		73.9^{b}±2.06		0.22^{a}±0.01	
	BBSR	4.36^{b}±0.3		5.51^{b}±0.36		11.3^{b}±0.47		1.29^{a}±0.18		9.99^{b}±1.24		4.58^{ab}±0.3		23.8^{a}±1.3		0.47^{b}±0.03		71.6^{b}±5.64		0.17^{b}±0.02	
	BLPR	4.61^{a}±0.29		6.52^{a}±0.4		11.4^{b}±1.00		1.42^{a}±0.12		10.2^{ab}±2.6		4.67^{ab}±0.85		20.1^{b}±1.7		0.49^{ab}±0.06		81.1^{a}±1.68		0.16^{b}±0.03	
	BLSR	4.93^{a}±0.26		6.16^{a}±0.8		10.7^{b}±0.89		1.24^{ab}±0.11		7.42^{bc}±1.54		3.91^{b}±0.86		24.6^{a}±1.2		0.52^{a}±0.004		81.92^{a}±4.72		0.15^{b}±0.05	
	BRSR	3.88^{b}±0.56		5.09^{b}±0.45		10.87^{b}±76		1.31^{a}±0.01		5.67^{c}±3.7		3.02^{b}±1.4		24.13^{a}±0.5		0.53^{a}±0.05		63.8^{c}±4.07		0.20^{a}±0.02	
	BWPR	5.10^{a}±0.19		5.8^{b}±0.9		14.05^{a}±1.8		1.13^{b}±0.15		11.1^{ab}±0.29		4.3^{b}±0.39		24.8^{a}±0.96		0.38^{c}±0.02		81.005^{a}±4.1		0.20^{a}±1.0	
	BWSR	4.44^{ab}±0.2		5.63^{a}±0.51		14.3^{a}±0.69		1.27^{a}±0.12		8.43^{bc}±1.55		4.26^{b}±0.31		24.1^{a}±1.58		0.55^{a}±0.06		69.82^{bc}±3.8		0.16^{b}±0.02	
	SPSCL	4.60^{a}±0.29		5.3^{b}±0.49		12.9^{ab}±1.95		1.14^{b}±0.03		12.2^{a}±1.20		5.08^{a}±0.51		22.6^{ab}±1.52		0.41^{bc}±0.34		76.0^{b}±9.04		0.15^{b}±0.05	
	SPSR	4.66^{a}±0.67		4.38^{c}±0.48		14.6^{a}±1.17		0.94^{c}±0.03		12.9^{a}±0.68		5.28^{a}±0.1		26.4^{a}±0.3		0.41^{bc}±0.009		70.7^{b}±10.5		0.25^{a}±0.05	
	Mean value		**4.5**		**5.22**		**12.9**		**1.14**		**9.5**		**4.22**		**21.2**		**0.46**		**71.2**		**0.17**
	SEM value		**0.69**		**0.05**		**0.36**		**0.003**		**0.5**		**0.03**		**0.66**		**0.0005**		**28.3**		**0.0001**
	P value		**0.02**		**0.03**		**0.38**		**0.07**		**0.9**		**0.05**		**0.01**		**0.002**		**0.05**		**0.41**

Legends: Chicken type description is presented under the Table 3;

Means with different superscripts in the same row differ significantly ($p < 0.05$).

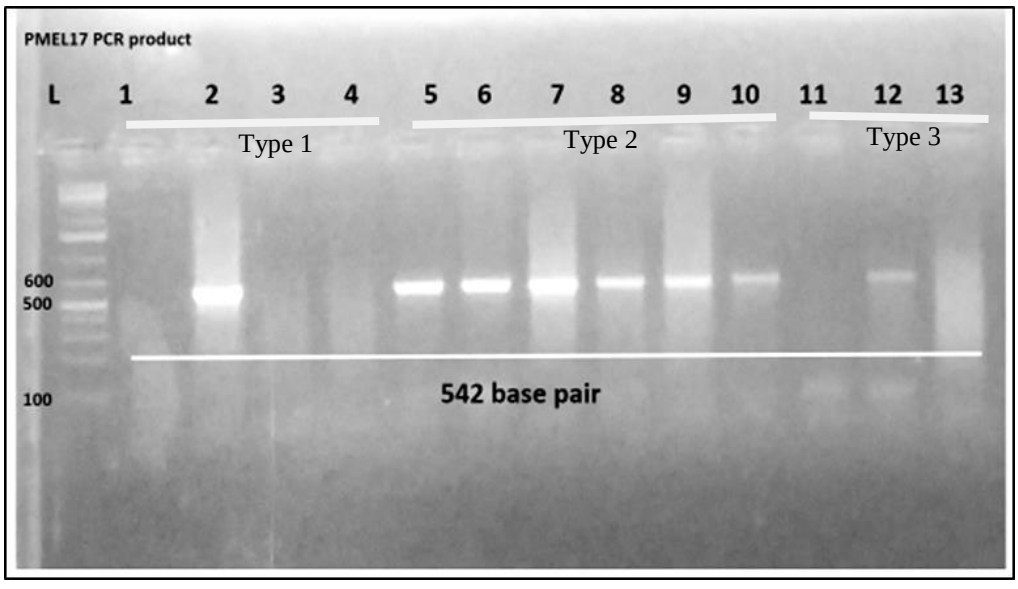
4.4 Molecular study of *PMEL17* gene

A total of 35 samples were confirmed for the presence of *PMEL17* gene whose amplicon sizes was 542bp. Among the all samples 8 samples were positive for *PMEL17* genes where typical amplicon sizes of the gene products measured by PCR, which shown in Table 6 and Figure 4. Entirely 55% samples were positive for *PMEL17* genes for all groups on the other hand, type 3 groups were 73.33% positive.

Table 6: Percentage of plumage color gene (*PMEL17*)

Plumage color gene	Amplicon size	No. of sample tested			Positive tested			Positive %			Total Positive (%)
		Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type3	
PMEL17	542bp	10	5	15	5	2	11	50	40	73.33	55%

Legends: Type-1: Black white, Type-2: Spotted, Type-3: Black Brown



Legends: L-Ladder, PCR-polymer chain reaction

Figure 4: PCR product of *PMEL17* gene

4.5 Nucleotide sequencing

4.5.1 Comparisons of Nucleotide sequence of *PMEL17*

Nucleotide sequence of three different types of chickens (spotted, black brown and black white) was compared for plumage color gene (*PMEL17*). The sequences under this investigation significantly aligned with the available sequences of NCBI/BLAST that was shown in the Figure 5, 6, 7, 8, 9 and 10, respectively. The result of comparing similarity, were rank first for Max Score/Total Score and the least E-values respectively. Score of the pairwise comparison between query DNA sequence and the desired DNA sequence in the NCBI database.

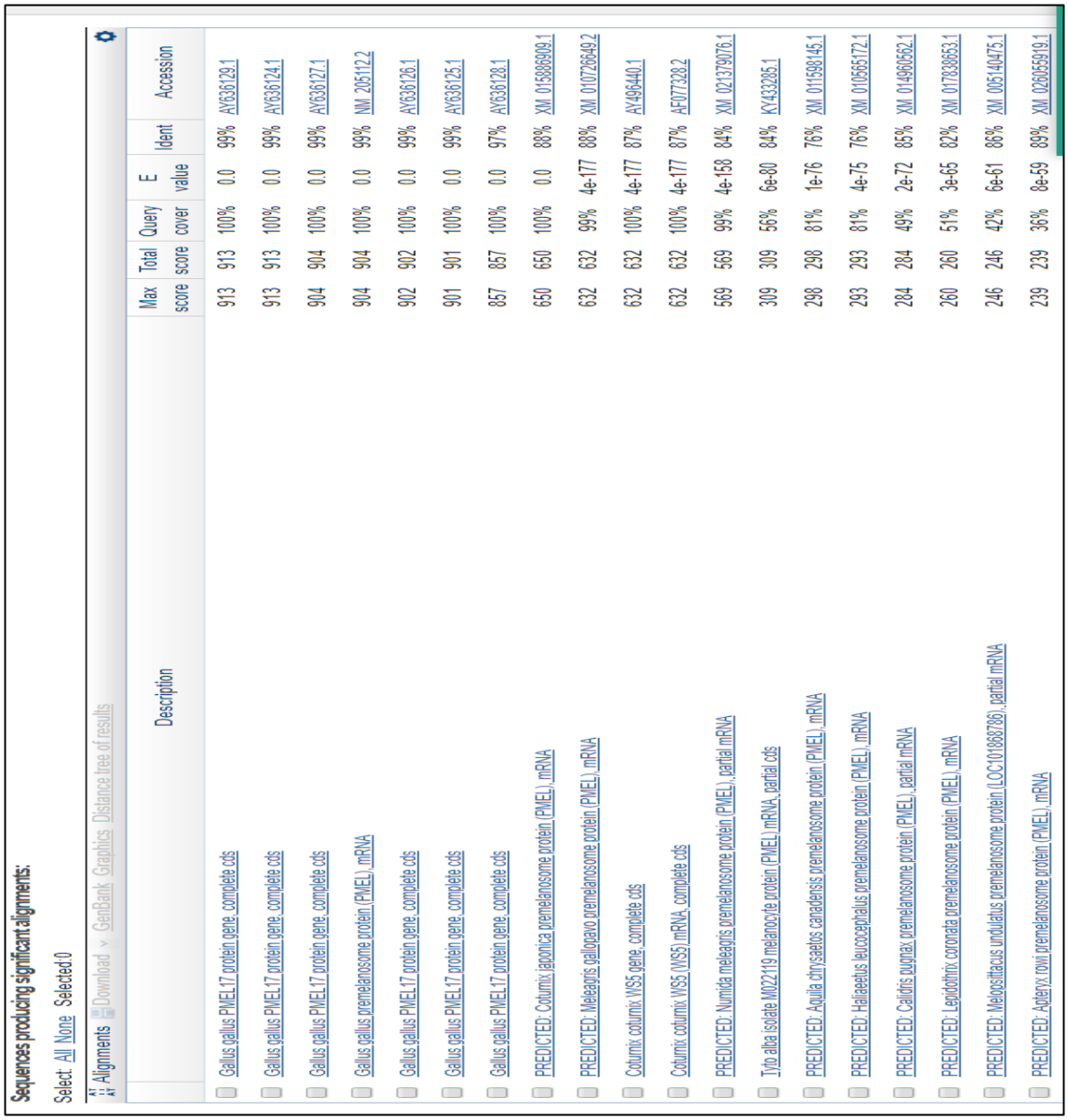
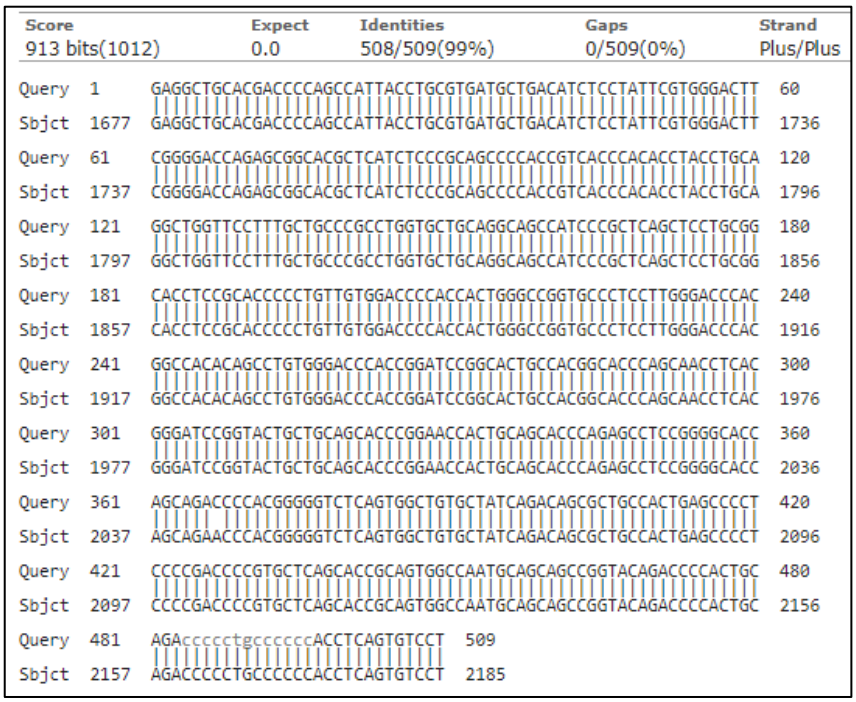


Figure 5: The scoring of the similarity and matching rate of sequences (spotted type)



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Query  61    CGGGGACCAGAGCGGCACGCTCATCTCCCGCAGCCCCACCGTCACCCACACCTACCTGCA  120
Sbjct  1737  CGGGGACCAGAGCGGCACGCTCATCTCCCGCAGCCCCACCGTCACCCACACCTACCTGCA  1796

Query  121   GGCTGGTTCCTTTGCTGCCCGCCTGGTGCTGCAGGCAGCCATCCCGCTCAGCTCCTGCGG  180
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Query  181   CACCTCCGCACCCCCTGTTGTGGACCCCACCACTGGGCCGGTGCCCTCCTTGGGACCCAC  240
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Query  481   AGAccccctgccccccACCTCAGTGTCCT  509
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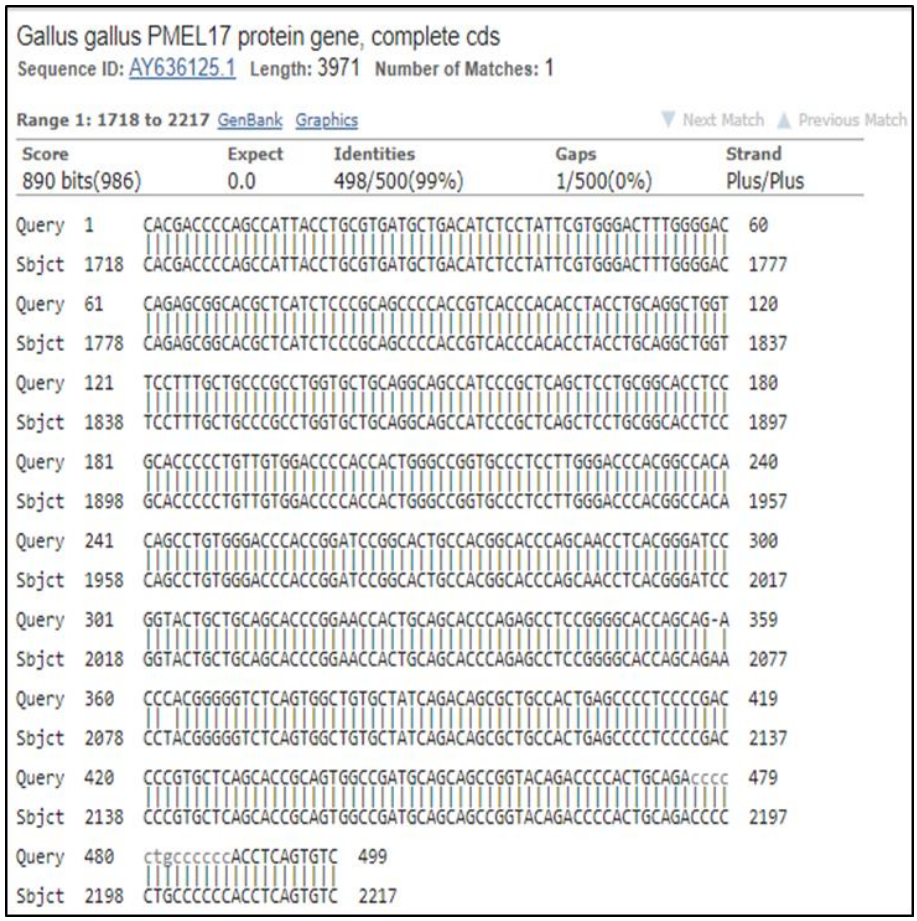
Figure 6: The sequence of *Gallus gallus PMEL17*gene for spotted



Gallus gallus PMEL17 protein gene, complete cds

Sequence ID: AY636125.1 Length: 3971 Number of Matches: 1

Range 1: 1718 to 2217 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
890 bits(986)	0.0	498/500(99%)	1/500(0%)	Plus/Plus

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Query  181   GCACCCCCTGTTGTGGACCCCACCACTGGGCCGGTGCCCTCCTTGGGACCCACGGCCACA  240
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Query  241   CAGCCTGTGGGACCCACCGGATCCGGCACTGCCACGGCACCCAGCAACCTCACGGGATCC  300
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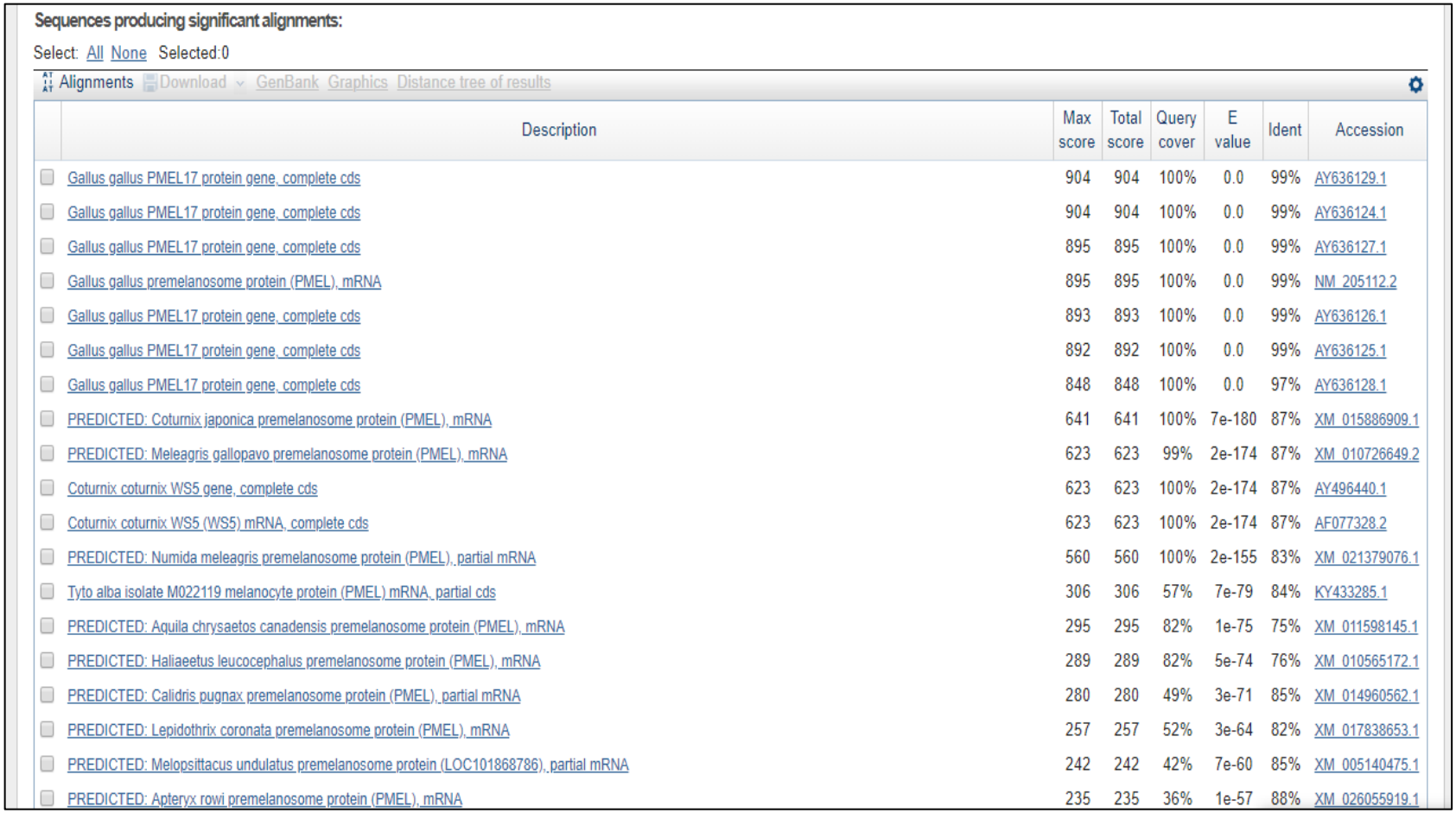
Figure 7: The sequence of *Gallus gallus PMEL17* gene for black brown type



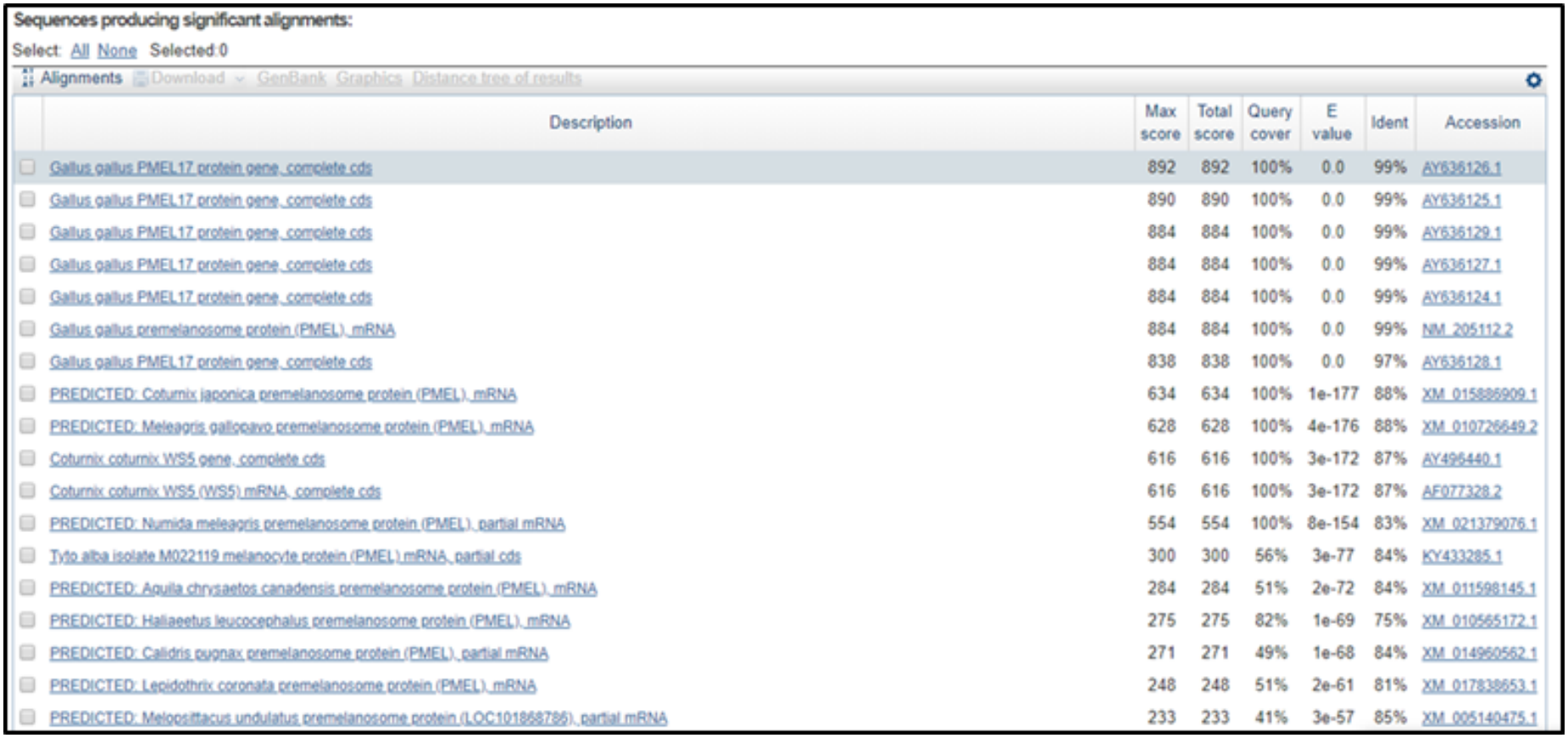
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Gallus gallus PMEL17 protein gene, complete cds	904	904	100%	0.0	99%	AY636129.1
Gallus gallus PMEL17 protein gene, complete cds	904	904	100%	0.0	99%	AY636124.1
Gallus gallus PMEL17 protein gene, complete cds	895	895	100%	0.0	99%	AY636127.1
Gallus gallus premelanosome protein (PMEL), mRNA	895	895	100%	0.0	99%	NM_205112.2
Gallus gallus PMEL17 protein gene, complete cds	893	893	100%	0.0	99%	AY636126.1
Gallus gallus PMEL17 protein gene, complete cds	892	892	100%	0.0	99%	AY636125.1
Gallus gallus PMEL17 protein gene, complete cds	848	848	100%	0.0	97%	AY636128.1
PREDICTED: Coturnix japonica premelanosome protein (PMEL), mRNA	641	641	100%	7e-180	87%	XM_015886909.1
PREDICTED: Meleagris gallopavo premelanosome protein (PMEL), mRNA	623	623	99%	2e-174	87%	XM_010726649.2
Coturnix coturnix WS5 gene, complete cds	623	623	100%	2e-174	87%	AY496440.1
Coturnix coturnix WS5 (WS5) mRNA, complete cds	623	623	100%	2e-174	87%	AF077328.2
PREDICTED: Numida meleagris premelanosome protein (PMEL), partial mRNA	560	560	100%	2e-155	83%	XM_021379076.1
Tyto alba isolate M022119 melanocyte protein (PMEL) mRNA, partial cds	306	306	57%	7e-79	84%	KY433285.1
PREDICTED: Aquila chrysaetos canadensis premelanosome protein (PMEL), mRNA	295	295	82%	1e-75	75%	XM_011598145.1
PREDICTED: Haliaeetus leucocephalus premelanosome protein (PMEL), mRNA	289	289	82%	5e-74	76%	XM_010565172.1
PREDICTED: Calidris pugnax premelanosome protein (PMEL), partial mRNA	280	280	49%	3e-71	85%	XM_014960562.1
PREDICTED: Lepidothrix coronata premelanosome protein (PMEL), mRNA	257	257	52%	3e-64	82%	XM_017838653.1
PREDICTED: Melopsittacus undulatus premelanosome protein (LOC101868786), partial mRNA	242	242	42%	7e-60	85%	XM_005140475.1
PREDICTED: Apteryx rowi premelanosome protein (PMEL), mRNA	235	235	36%	1e-57	88%	XM_026055919.1

Figure 8: The scoring of the similarity and matching rate of sequences (black brown type)



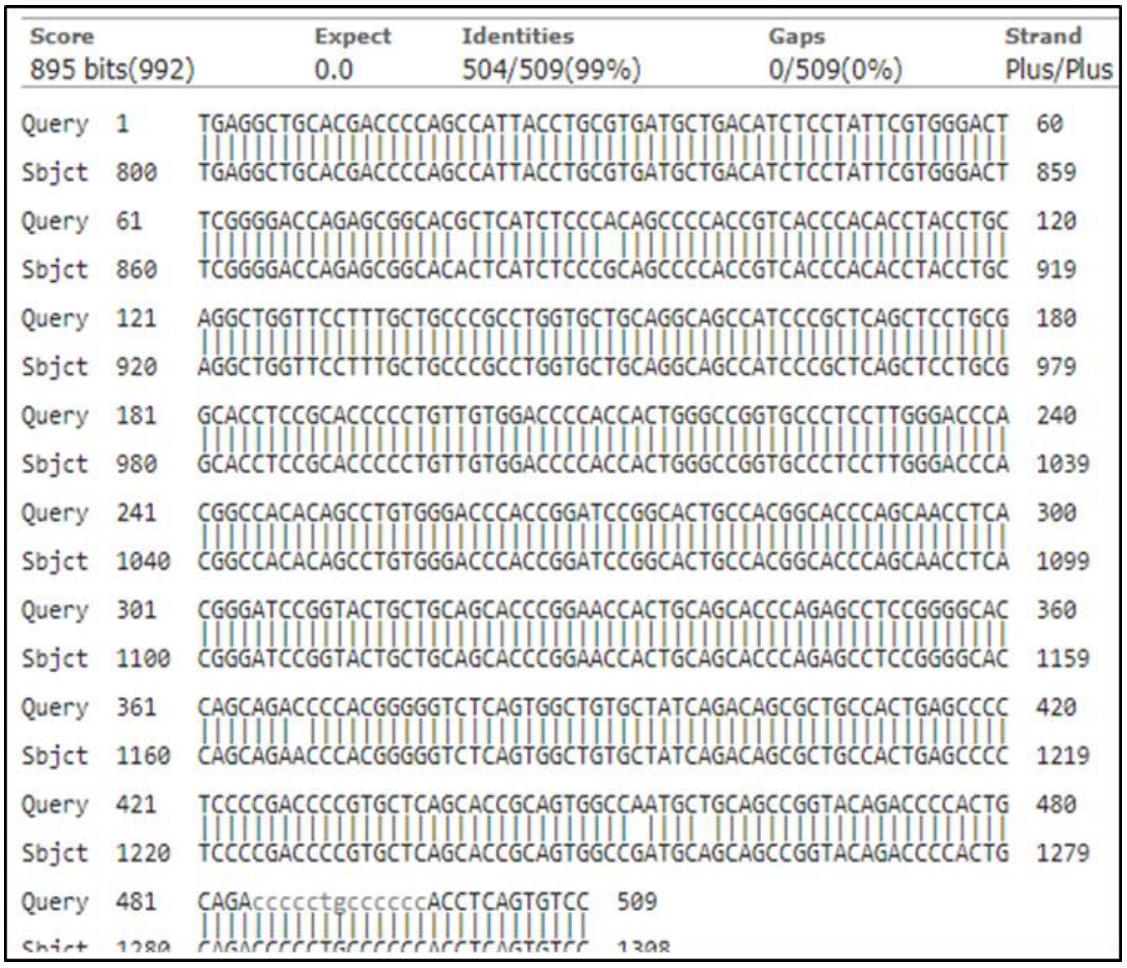
Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Gallus gallus PMEL17 protein gene, complete cds	892	892	100%	0.0	99%	AY636126.1
Gallus gallus PMEL17 protein gene, complete cds	890	890	100%	0.0	99%	AY636125.1
Gallus gallus PMEL17 protein gene, complete cds	884	884	100%	0.0	99%	AY636129.1
Gallus gallus PMEL17 protein gene, complete cds	884	884	100%	0.0	99%	AY636127.1
Gallus gallus PMEL17 protein gene, complete cds	884	884	100%	0.0	99%	AY636124.1
Gallus gallus premelanosome protein (PMEL), mRNA	884	884	100%	0.0	99%	NM_205112.2
Gallus gallus PMEL17 protein gene, complete cds	838	838	100%	0.0	97%	AY636128.1
PREDICTED: Coturnix japonica premelanosome protein (PMEL), mRNA	634	634	100%	1e-177	88%	XM_015886909.1
PREDICTED: Meleagris gallopavo premelanosome protein (PMEL), mRNA	628	628	100%	4e-176	88%	XM_010726649.2
Coturnix coturnix WS5 gene, complete cds	616	616	100%	3e-172	87%	AY496440.1
Coturnix coturnix WS5 (WS5) mRNA, complete cds	616	616	100%	3e-172	87%	AF077328.2
PREDICTED: Numida meleagris premelanosome protein (PMEL), partial mRNA	554	554	100%	8e-154	83%	XM_021379076.1
Tyto alba isolate M022119 melanocyte protein (PMEL) mRNA, partial cds	300	300	56%	3e-77	84%	KY433285.1
PREDICTED: Aquila chrysaetos canadensis premelanosome protein (PMEL), mRNA	284	284	51%	2e-72	84%	XM_011598145.1
PREDICTED: Haliaeetus leucocephalus premelanosome protein (PMEL), mRNA	275	275	82%	1e-69	75%	XM_010565172.1
PREDICTED: Calidris pugnax premelanosome protein (PMEL), partial mRNA	271	271	49%	1e-68	84%	XM_014960562.1
PREDICTED: Lepidothrix coronata premelanosome protein (PMEL), mRNA	248	248	51%	2e-61	81%	XM_017838653.1
PREDICTED: Melopsittacus undulatus premelanosome protein (LOC101868786), partial mRNA	233	233	41%	3e-57	85%	XM_005140475.1

Figure 9: The scoring of the similarity and matching rate (black white type)



Score	Expect	Identities	Gaps	Strand
895 bits(992)	0.0	504/509(99%)	0/509(0%)	Plus/Plus

```
Query  1     TGAGGCTGCACGACCCCAGCCATTACCTGCGTGATGCTGACATCTCCTATTCGTGGGACT  60
Sbjct  800   TGAGGCTGCACGACCCCAGCCATTACCTGCGTGATGCTGACATCTCCTATTCGTGGGACT  859

Query  61    TCGGGGACCAGAGCGGCACGCTCATCTCCCACAGCCCCACCGTCACCCACACCTACCTGC  120
Sbjct  860   TCGGGGACCAGAGCGGCACACTCATCTCCCGCAGCCCCACCGTCACCCACACCTACCTGC  919

Query  121   AGGCTGGTTCCTTTGCTGCCCGCCTGGTGCTGCAGGCAGCCATCCCGCTCAGCTCCTGCG  180
Sbjct  920   AGGCTGGTTCCTTTGCTGCCCGCCTGGTGCTGCAGGCAGCCATCCCGCTCAGCTCCTGCG  979

Query  181   GCACCTCCGCACCCCCTGTTGTGGACCCCACCACTGGGCCGGTGCCCTCCTTGGGACCCA  240
Sbjct  980   GCACCTCCGCACCCCCTGTTGTGGACCCCACCACTGGGCCGGTGCCCTCCTTGGGACCCA  1039

Query  241   CGGCCACACAGCCTGTGGGACCCACCGGATCCGGCACTGCCACGGCACCCAGCAACCTCA  300
Sbjct  1040  CGGCCACACAGCCTGTGGGACCCACCGGATCCGGCACTGCCACGGCACCCAGCAACCTCA  1099

Query  301   CGGGATCCGGTACTGCTGCAGCACCCGGAACCACTGCAGCACCCAGAGCCTCCGGGGCAC  360
Sbjct  1100  CGGGATCCGGTACTGCTGCAGCACCCGGAACCACTGCAGCACCCAGAGCCTCCGGGGCAC  1159

Query  361   CAGCAGACCCCACGGGGGTCTCAGTGGCTGTGCTATCAGACAGCGCTGCCACTGAGCCCC  420
Sbjct  1160  CAGCAGAACCCACGGGGGTCTCAGTGGCTGTGCTATCAGACAGCGCTGCCACTGAGCCCC  1219

Query  421   TCCCCGACCCCGTGCTCAGCACCGCAGTGGCCAATGCTGCAGCCGGTACAGACCCCACTG  480
Sbjct  1220  TCCCCGACCCCGTGCTCAGCACCGCAGTGGCCGATGCAGCAGCCGGTACAGACCCCACTG  1279

Query  481   CAGAccccctgccccccACCTCAGTGTCC  509
Sbjct  1280  CAGACCCCCTGCCCCCCACCTCAGTGTCC  1308
```

Figure 10: The sequence of *Gallus gallus PMEL17* gene for black white types chicken

4.5.2 Evolutionary relationship taxa of *PMEL17* gene

The evolutionary history was inferred using the Neighbor-Joining method. The branching pattern of the tree was used to determine the most closely related pair of the sequences. The optimal tree with the sum of branch length = 2.16545591 is shown. (Next to the branches). Among them nucleotide sequence of indigenous chicken were closely related with *PMEL17* gene (NCBI accession no: AY636124.1, AY636125.1, NM_205112.2) sequence. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 499 positions in the final dataset. In three samples like black white, black brown and spotted type chicken were closely related to the reference sequence NM 205112.2 (Figure 11). The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

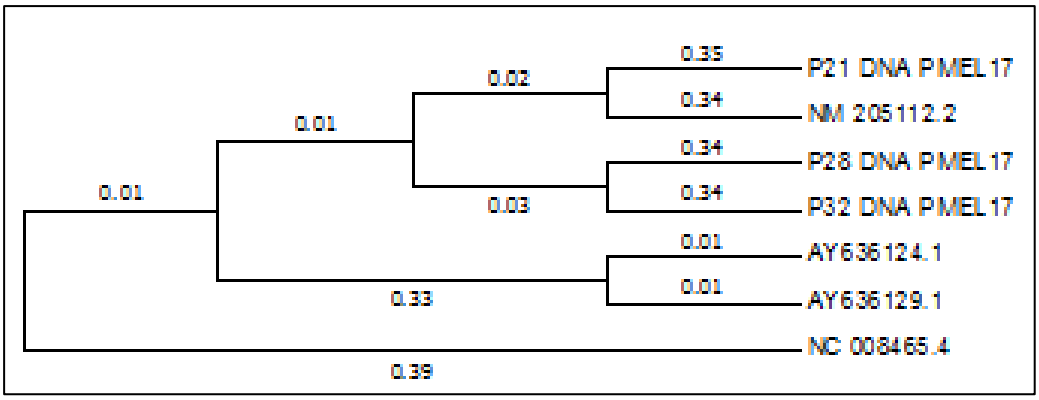


Figure 11: Phylogenetic tree drawn based on nucleotide sequences of the *PMEL17* gene

4.5.3 Mutation of plumage color, *PMEL17* gene

Among three types of indigenous chicken namely spotted, black brown and black white type showed mutation in the plumage color (*PMEL17*) gene. Based on the sequences alignment analysis the changes of sequence was occurred at position 64bp (C replace T) (Figure 12) in white black type chicken whereas the GenBank accession numberAY636129.1 was used as a reference sequence (Kerje *et al*., 2004). The nucleotide at position 91bp was shifted from G to A at the following sequence in case of black brown type chicken that shown Figure 12. The GenBank accession number AY636129.1 was used as a reference sequence (Kerje *et al*., 2004). In compartment of spotted type chicken, there was no mutation occur in the nucleotide sequences alignment. The mutation was observed in the amino acid sequence which found after protein alignment using Mega6 software that leads to changes the protein.

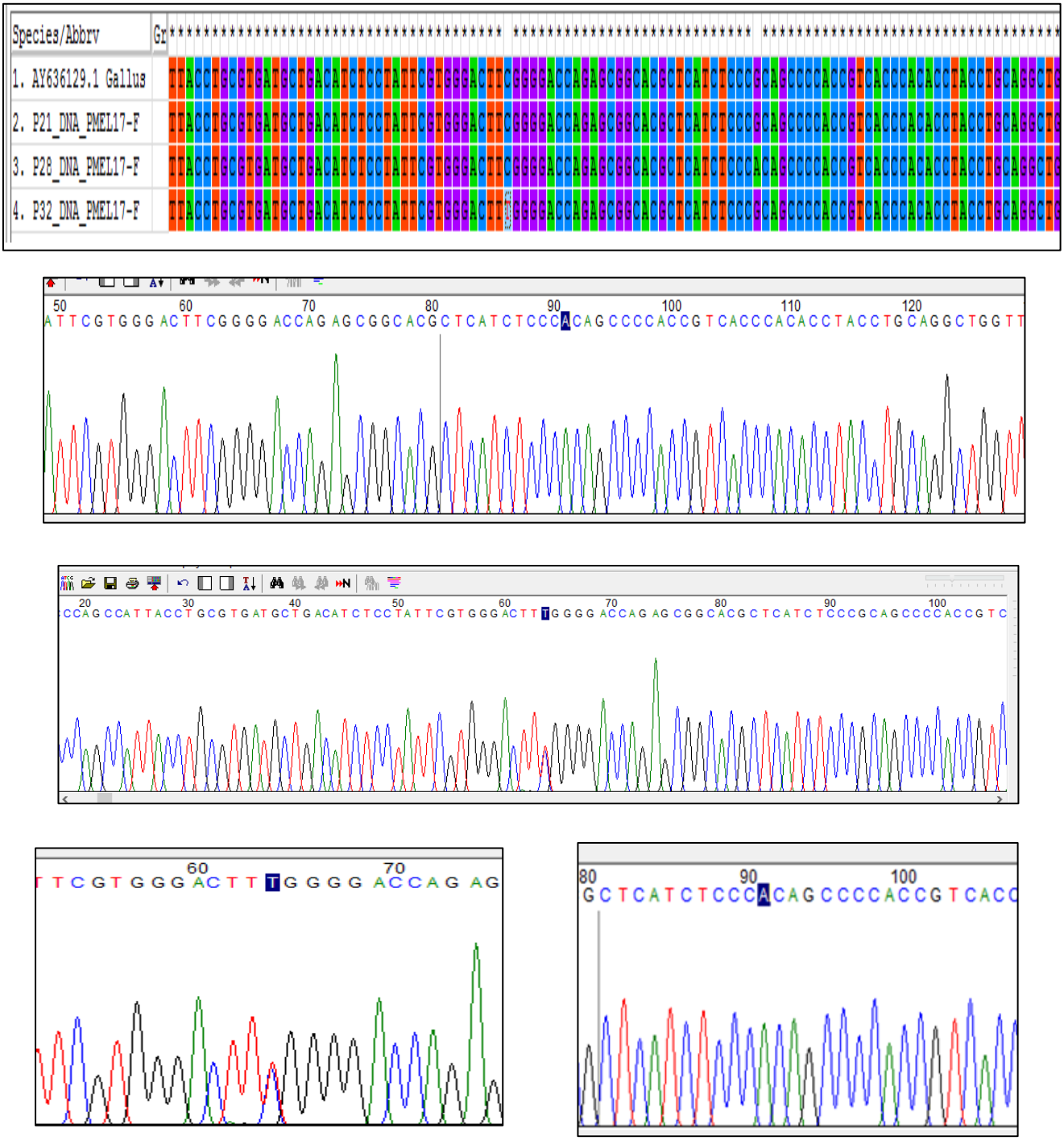


Figure 12: Mutation of *PMEL17* gene

4.5.4 Maximum composite likelihood estimate of the pattern of nucleotide substitution of *PMEL17*

The probability of substitution (r) from one base (row) to another base (column) of each nucleotide. Substitution pattern of nucleotide and rates were estimated. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions were shown in italics (Table 7). Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100. The nucleotide frequencies were A = 16.91%, T/U = 15.79%, C = 40.69%, and G = 26.62%. The maximum Log likelihood for this computation was -3731.771. The analysis involved 7 nucleotide sequences and there were a total of 409 positions in the final dataset.

Table 7: Maximum Likelihood estimate of the pattern of nucleotide Substitution

	A	**T**	**C**	**G**
A	-	*6.22*	*16.02*	**9.00**
T	*6.66*	-	**4.70**	*10.48*
C	*6.66*	**1.83**	-	*10.48*
G	**5.72**	*6.22*	*16.02*	-

Legends: A= Adenine, T=Thiamine, C=cytosine, G=Guanine.

Chapter-5

Discussion

5.1 Phenotypic characteristics of chicken

5.1.1 Egg production

In the studied area different types of indigenous chicken were identified and studied there egg production per clutch. In this investigation, egg production of spotted single round type was higher (13.5/clutch) than brown pea cylindrical (5.25/clutch) which showed significantly different ($p<0.05$) from each other within the breed (Table 3). The results were similar with the findings of Yakubu *et al*. (2008), who found that significantly higher eggs per clutch in naked neck (11.63 no) than the fully feathered chickens (9.71 no). The current findings were compared favorably with the results of Mapiye and Sibanda (2005) who observed on average, the village chickens laid and incubated 10±2 and 8±1 eggs per clutch. Khan *et al*. (2017) observed that, hilly chickens produced more eggs than deshi chicken. The deshi white chickens was produced (90no/year/chicken)which were significantly higher than spotted hilly (83.4 no/year)but reddish brown hilly type produced more number (100.80 no/year/chicken) of eggs than all types (Khan *et al*., 2017) whose results were higher than the present study. Variation of egg production could be due to the broodiness in hen if it can be minimized that could yield more eggs, which could be achieved by simply changing the location and bedding of the hen at brooding. Variation of egg production can be found due to the effect of management intervention. Khan *et al*. (2017) and Deng *et al*. (2012) also investigate that variation of egg production, the higher egg production was observed in case of lighting duration from 10 to 14 h and lower egg production was due to heat stress.

5.1.2 Clutch size

Highest clutch size was obtained for Brown single round (15.05 no/day) and lowest was found in brown pea cylindrical (5.88 no/day) in different locations. Daikwo *et al*. (2011) suggested that, the mean clutch size of indigenous chicken of Nigeria was 7.20 no/day which were lower than the current study. Ssewannyana *et al*. (2008) studied that, most hens produced 2.2no/day clutches of eggs per year and the inter-clutch interval averaged 2.7 months. Variation of clutch size might be due to the genetic and also management.

5.1.3 Live weight

There was a significant differences ($p<0.05$) found in the body weights of the different types of chicken within breed between locations, with heavy weight (1.60kg) recorded for spotted single cylindrical type and black brown pea round type compared to black single round types (0.89kg) (Table 3). The present result on different types of indigenous chicken compared with the value of 1.30kg reported for native hens under the farmer management in a selected area of Bangladesh (Ershad, 2005). Other investigation revealed that, mature live weight were significantly different between hilly chicken (1.4kg) and deshi chicken (1.3kg) under three condition (lighting, heat and control) in Bangladesh (Khan *et al*., 2017) which was matched with the present investigation. Tabassum *et al*. (2014) reported that, the mean body weight of indigenous chickens of Bangladesh was 0.96kg which was lower than the present study but very close to the findings of Islam *et al*. (2012), Semakula *et al*. (2011) and Ssewannyana *et al*. (2003). However, the higher live weight of 1.55 kg (Naked neck) and 1.49kg (Baladi) reported for Sudanese indigenous chicken types (Mohammed *et al*., 2005). In a similar fashion, Safty *et al*. (2006) reported values of 1.58 and 1.45kg as mature body weights for naked neck and normal feathered birds under low ambient temperature in Egypt. Most of the studies showed that the live weight of indigenous chicken was varied between management system and breed. Local types chicken average body weights could be due to genetical and phonotypical variations within those types (Mohammed *et al*., 2005). Also variation of live weight of indigenous chicken may be found due to heat stress and feed intake.

5.1.4 Plumage length

The average plumage length of indigenous chicken were found significantly ($p<0.005$) difference under different locations. Comparing chicken type within indigenous chicken brown single round type showed highest average plumage length (Table 3). Yeasmin *et al*. (1998) reported that, plumage length of normal deshi hen was 16.3cm which was lower than the current study. Variation of plumage length might be occurred due to genetically variation.

5.2 External characteristics of chicken egg

The external characteristics like egg weight, egg height, egg wide, shell weight, shell thickness, shape index were discussed as:

The egg weight of indigenous chicken were found superior in the case of black pea round type (44.83g) than the black brown pea cylindrical type (33.6g). Within location and types of the chickens significant differences ($p<0.05$) were observed (Table 4). Mohammed *et al*. (2005) reported that, the mean egg weight of Sudanese indigenous chicken types was 37.95, 38.46 and 39.89g which was lower than the studied chicken type, black pea round type and higher than the black brown pea cylindrical type. Similar observation was found for naked neck and normal feathered chickens in coastal region Kenya (Njenga, 2005). These results are in agreement with other reports from different African countries on local birds where the egg weights were reported as ranging from 30–49g (Missohou, 2002). Under semi-scavenging system at Bangladesh, Khan *et al*. (2004) reported that the egg weight of different chicken genotypes ranged from 42.3 to 55.0g. Other studied reveled that, the egg weight of different deshi chicken types varied from 41.27 to 43.85g (Khan *et al*., 2017) this findings were similar with the present study. Although egg weight is largely affected by environmental factors, feed restriction (Cary *et al*., 1993) and parental average body weight; and also genetics. Variation of egg weight can be found due to effect of lighting. In laying hen, egg weight was decreased due to heat stress (Ebeid *et al*., 2013). Variation of egg weight might be ensued due to genetics and management

The egg length of different types of indigenous chicken was significantly differed between the locations and types. Superior egg length was recorded in the case of spotted single round (6.3cm) than the brown pea cylindrical (4.43cm) types. The finding of this study were higher than the results of Yakubu *et al*. (2008) whereas lower than the result of Daikwo *et al*. (2011). Monira *et al*. (2003) reported that, egg length was found statistically significant due to the breed characteristics, holding period and the interaction effect. Genetic difference in egg length was also elucidated and accentuated by Monira *et al*. (2011). Fayeye *et al*. (2005) also explained the egg length was higher in the presence of Na gene in the case of Fulani-ecotype chicken. This variation in the measurement may be due to the variation in different breed and age variations.

The egg wide was highest in the black white single round type chicken (4.3cm) and lowest in brown single round (3.13 cm). The findings of this investigation were lower than the findings of Daikwo *et al*. (2011). It may be genetic variation is the factor that affects the variation of egg wide.

Comparing chicken type, the shell weight was highest in the black single round than other types of indigenous chicken. The findings of this study were in line with the findings of Iqbal *et al*. (2008) and Yakubu *et al*. (2008). The mean shell weight obtained in the present study were comparable to that reported for the two genotypes in a similar study of Safty *et al*. (2006) and higher than the study of Ershad (2005). Chicken breed, age, moulting and nutritional factor were the major factor, which affect the variation of egg shell weight.

Shell quality particularly shell thickness, is an important bioeconomic trait that primarily breeder of egg laying flock incorporated in their breeding programmes to reduce egg shell breakages. In this research, highest shell thickness was recorded in case of spotted single cylindrical and lowest in brown single round. However, different trend was realized for shell thickness, where the Na genotype had an edge over the fully feathered birds. This finding was inconsistent with the findings of Safty *et al*. (2006). Ikeobi *et al*. (2004) also reported a higher average shell thickness value in normal plumage genotype, although the difference was not significant. Additionally, Khan *et al*. (2004) reported that the eggshell thickness is an important character for hatchability, hence for the best result of hatchability, therefore the eggshell thickness should be in between 0.33 and 0.35 mm and few eggs with a shell thickness less than 0.27 mm would be hatched.

The shape index was highest in black white single round type (83.4) and lowest in spotted single round (65.9). There were significant differences were found among the types within the breed. The outcome of the investigation was higher than the results of Monira *et al*. (2003). Khan *et al*. (2004) revealed that the shape index trait varied from 72% to 82% using different Bangladesh chicken genotypes. Accordingly, the genetic factor plays an important role in the shape index trait of the chicken eggs. Egg shape index is a good indicator of external egg quality. The higher value obtained in naked neck further consolidated their superiority over the normal plumage birds (Yakubu *et al*., 2008). The genetic differences between the strains which have major effect in shape index (Monira *et al*., 2011). The decrease shape index which increasing the egg weight was supported by Reddy *et al*. (1979). The difference in shape has been suggested to be hereditary (Hutt, 1949) only that the number of genes was not known. Shape index variation may be occurred due to breed variation and management factor.

5.3 Internal characteristics of chicken egg

Outcome of this investigation revealed that the mean albumen height was highest in black single round and lowest in spotted single round type chicken. However, there were significantly differences observed between the locations within the breed. Monira *et al*. (2003) found albumen height of White rock, White leghorn and Rhode Island Red egg was 4.66, 4.33 and 3.60mm, respectively; which were similar with the present observation. The mean value of albumen height was similar with the investigation which reported by Fayeye *et al*. (2005) in case of naked neck chicken. The present study was similar with the findings of Olawumi *et al*. (2006). The albumen height variation might be related to the fact that was a great variation between the nutritional, genetic and environmental factor.

Albumen weight was recorded in higher for black white pea round type and lower in black single round. The mean albumen weight recorded in the current study is in agreement with those reported by Fayeye *et al*. (2005), Nonga *et al*. (2012) and Yakubu *et al*. (2008). Albumen is normally sensitive to diseases, nutrition and poor storage and may easily be affected compared to yolk reported from Nonga *et al*. (2012). Albumen weight variation was reported to be more closely associated with egg weight than yolk weight (Harms and Hussein, 1993). Variation of albumen weight may be found due to eco-type of chicken (Jones and Musgrove, 2005; Zaman *et al.,* 2005), age, storage conditions and type of feed in scavenging farming condition.

Different types of chickens showed different yolk height in the different locations (Table 5). The highest yolk height was recorded for black pea round (13.8), which were lower than the findings of Zaman *et al*. (2005) and higher than Nonga *et al*. (2012) and Yakubu *et al*. (2008). Yolk height was increased with the increases of age and had a tendency to decrease over time (Nonga *et al*., 2012). Variation of yolk height might be occurred due to age and breed variation. In the present study, yolk weight was superior in the case of black white pea round type than other type of indigenous chicken. Significantly variation between the types within the breed and locations were observed in the current study. The findings in the study were higher than the study of Yakubu *et al*. (2008) and Nonga *et al*. (2012) but similar result was obtained in layer breeders (Olawumi *et al*., 2006). Yolk weight might be varied due to nutrition.

Haugh unit have been reported to be the best indicators of internal egg quality (Ihekoronye and Ngoddy, 1985). In this investigation, highest haugh unit was found in case of brown

single round type (84.37) and lowest in the brown pea cylindrical type chicken. In this study, there were significant differences found between types within breed and locations ($p<0.005$). The current results were similar with the findings of Yeasmin *et al*. (1998) and Zaman *et al*. (2005).The outcome of the present investigation was higher than the findings of Nonga *et al*. (2012); Monira *et al*. (2003); Zaman *et al*. (2005) and Akbas *et al*. (1996) has shown that haugh unit decreased with the increases of hen age. Variation of haugh unit might be occurred due to the breed variation and management factor.

Genetics was closely associated with phenotypic study. Any molecular study, the genetic variation was assisted to find out the expression of phenotype. It helps to find out the pinning mechanism of plumage color and its impact on quantitative traits. In current investigation, molecular study of plumage color genes was studied.

5.4 Gene sequencing

5.4.1 Nucleotide sequence comparisons of PMEL17

The sequence of same region was imperiled to close similarity with the other chicken sequences. The designated sequences were revealed 99% homology (NCBI accession no: AY 636126.1, AY636129.1 respectively) with the sequence of *Gallus gallus* reported by Kerje *et al*. (2004), on the other hand those sequences were 99% similar with designated sequences of domestic chicken (*Gallus domesticus*) (Kerje *et al*., 2004 and Kuliawa *et al*., 2009). The sequences of *PMEL17* gene were also similar with the other chicken nucleotide sequences (NCBI accession no: NM205112.2, AY636128.1, respectively) reported by Kuliawa *et al*. (2009).

5.4.2 Phylogenetic tree analysis of *PMEL17* gene

Phylogenetic trees are significant tools for establishing knowledge of biological diversity, and they link hypothesized evolutionary relationships among nested groups of taxa that were supported by shared traits known as synapomorphies (Novick *et al.*, 2011). Usually, most recent common ancestry was used to interpret taxa similarity. Taxa that share a more recent common ancestor must be more closely related to each other than to another taxon with a less recent common ancestor (Dees *et al*., 2014). MEGA6 software is an ordinarily applied program for interpret the taxa relatedness in case of multiple sequence alignment (Tamura *et al*., 2013). It uses a progressive algorithm to align sequences in successively larger groups, beginning with the most closely related sequences. Using MEGA6 software,

sequences were studied for compared and a tentative measurement of relatedness, characterized by a distance matrix. This was used to produce a phylogenetic guide tree, using the neighbour-joining (NJ) method (Saitou and Nei, 1987). The branching pattern of the tree was used to determine the most closely related pair of the sequences. A final alignment was gained by repeating this procedure until it reaches the root of a tree. Comparative studies of sequences were used in a wide range of taxonomic levels, to evaluate phylogenetic relationships. Results showed different regions and intragenic distances of the DNA varied among species within a *PMEL17* gene sequences. The phylogeny results was based on nucleotide and amino acid sequences of *PMEL17*, which related with clustering of sequences among the various species that obtained in this research, although there was some intermingling between the species.

5.4.3 Mutation of *PMEL17* gene

In this investigation, mutation was found in two types of chicken. The change of sequence was occurred at position 64bp (C exchanges T) in the case of white black type chicken and the nucleotide at position 94bp was shifted from G to A at the following sequence in the compartment of black brown type chicken. The GenBank accession number AY636129.1 was used as a reference sequence (Kerje *et al.*, 2004). Mutation was observed in the amino acid sequence. Similar findings were found by Kerje *et al*. (2004). Sequence analysis showed that the dominant white and black allele were exclusively associated with an insertion and deletion of amino acids in the *PMEL17* transmembrane region. Vaez *et al*. (2008) also found, mutation was together with the recessive to silver polymorphism in the mouse, the only *PMEL17* gene polymorphism gaunt with phenotypic effects that has been described so far in any species.

5.4.4. Maximum composite likelihood estimate of the pattern of nucleotide substitution of *PMEL17* gene

In this investigation, transitional and transversional parameters represents a measure of the biochemical similarity of bases and transitional substitutions were occurred more often than the transversional substitutions, which was strongly, coincide with the findings of Palero and Crandall (2008). Rates of different transitional substitutions were used to denote changes in the nucleotide substitutions from (A ↔G, C ↔T) and transversions were used to changes in pyrine to pyrimidine base pair that's denotes the point mutation.

Conclusions

From this study, it was seen that the variation of quantitative traits of different types of chicken observed under different locations. The external and internal characteristics of egg of different types of chicken were all varied under the locations. In comparison of types of chickens, three types: spotted single round, black-white pea round and black single round type chicken were better for egg production, live weight and external and internal quality of eggs. Therefore, farmers can be suggested to rear these chickens under traditional system. However, further research is needed to confirm this finding. Phenotypic analysis was closely involved to genetics of animals. Among the types, based on plumage color types were more visible and it was the major phenotypic character in the chicken. Different types of gene were responsible for plumage color, whereas *PMEL17* gene was the main gene affecting plumage color of the chickens. The evolutionary history of branching pattern of *PMEL17* gene showed the relatedness of the nucleotide sequence. In this investigation, between the two types chickens (white black and black brown type chicken) mutation was detected, which indicates that changes the amino acid sequence leads to protein change and ultimately variation of the phenotype (plumage color) that were expressed. Finally, it may be concluded that various types of chicken plumage color were found with the potential role of all possible mutation occurred in the plumage color gene, *PMEL17*. Further investigations including DNA re-sequencing complete gene sequencing and molecular marker analysis can be done for confirmation of the current findings. These genomic thoughts will be helpful for the genetic constitutions and could assist in structured genetic improvement programme on indigenous chickens of Bangladesh.

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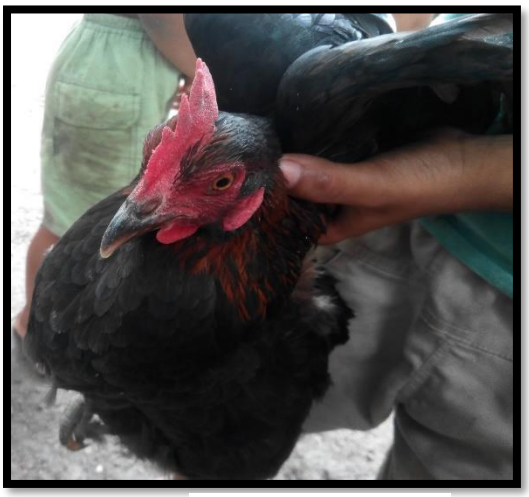
Photo Gallery

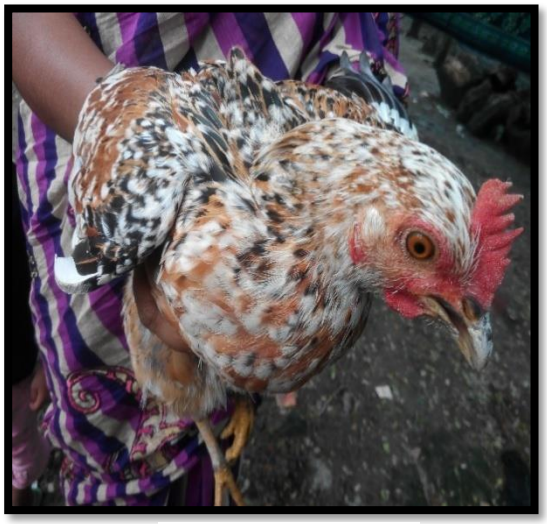
Brown black type

White black type

Brown black type



Black type



Spotted type

Figure 13: Different types of indigenous chicken