CHICKEN GROWTH HORMONE GENE POLYMORPHISM OF SELECTED CHICKEN BREEDS IN NIGERIA

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(M. Tech/SAAT/2019/9393)

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ABSTRACT

The study was carried out to investigate polymorphism at the Chicken Growth Hormone (*cGH*) gene of selected chicken breeds (Fulani ecotype, Noiler, FUNAAB Alpha, Frizzled feathered and Cobb 500 was used as the reference breed) in Nigeria. Genomic DNA was extracted from blood samples collected from 50 birds. Amplification of specific DNA fragments at intron 3 of the *cGH* gene yielded a product size of 715 bp and this was used to analyze for polymorphism using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique. Banding patterns of the gene investigated, revealed the occurrence of three RFLP variants: TT, CT and CC with genotype frequencies of 0.449, 0.245 and 0.306, respectively. The allele frequencies of the T and C alleles were 0.469 and 0.531, respectively indicating that the C allele was predominant. Chi-square test revealed that the chicken population investigated was in Hardy-Weinberg equilibrium. Forty seven (47) Single Nucleotide Variant (SNV) was observed at the intron 3 of the *cGH* gene for selected chicken breeds studied. Six numbers of Single Nucleotide Polymorphism (SNPs) were discovered at exons having four missense variant (locus 4036303, 4036384 and 4036921/4036922) with (8.51 %), two synomous variant (locus 4036325/4036326, 4036381) with (4.26%), one frame shift variant (locus 4036303) with (2.13 %) and one splic region variant (locus 4036384) with (2.13 %), while the rest were at the introns with (87.23 %). It was concluded that, the presence of observed genetic variations in the *cGH* gene, could be exploited because of its association with economically important traits.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

In the tropics, the high prevalence of diseases and limited effort to improve genetic quality are major factors limiting the productivity of local chickens (Alexander, 2001; Otim *et al.*, 2004). Domestic chickens have a huge potential to be developed. The genetic potential is reflected by the enormous genetic diversity especially with regard to the adaptive traits they possess, the ability to survive harsh conditions and under minimum feeding regimes (Sulandari *et al.*, 2009). The high diversity observed in Nigerian indigenous chicken traits, are genetic raw materials that can be utilized to create preeminent chickens through directional selection focused on the traits with high economic value such as meat and egg production, adaptability and immunity (Kansaku *et al.*, 2008).

According to the Food and Agriculture Organization (FAO, 2007), animal genetic diversity allows farmers to select stocks or develop new cross breeds in response to environmental change, threat of disease, new knowledge of human nutritional requirement, changing market conditions and societal needs. Selection according to genotype has the power to increase productivity of farm animals as well as to enhance environmental adaptation and maintenance of genetic diversity (Hayes *et al.*, 2009). It is generally believed that introducing high yielding livestock breeds into traditional and extensive production systems can lead to a loss of genetic diversity in indigenous animals (Sere and Steinfeld, 1996). This may be due to genome dilution of "local hybrid" through crossbreeding during and after such programmes. To improve production traits

and health simultaneously, it is appropriate to use molecular markers associated with one or two characteristics of interest. Egena and Alao (2014) reported that gene and genotypic frequencies through polymorphism study makes it easy to compare the gene stocks of animals and the possible effects of the genes on reproductive and performance traits. It can also be used to study genetic variability under any environmental conditions of selection.

The chicken growth hormone (*cGH*) is a polypeptide hormone synthesized and secreted by the pituitary gland. The genes of *cGH* are highly polymorphic and are involved in a wide variety of physiological functions such as growth, body composition, egg production, ageing and reproduction (Apa *et al.*, 1994) as well as immune responsiveness (Kelley and Felton, 1995). Chicken growth hormone gene contains 4,101 base pairs having five exons and four introns with its introns differing from similar mammalian gene because of its large size (Kansaku *et al.,* 2008; Su *et al.,* 2014). This gene encodes 191 amino acid residues which comprises of mature growth hormone protein in addition to 25 amino acid signal peptide (Nie *et al.,* 2005; Hrabia *et al.,* 2008). Chicken growth hormone gene has been used as a candidate gene for marker assisted selection for improved performance (Kuhnlein *et al.,* 1997).

1.2 Statement of the Research Problem

The genetic makeup of chicken has been characterized **(**Bingxue *et al.,* 2003). The results showed that c*GH* gene could be a genetic locus or could be linked to a major gene significantly affecting growth and carcass traits in chicken. Evidence of the *cGH* gene being an important candidate gene for productivity and a vitality trait was reported in Tolaki chicken (Muhammad *et al.,* 2018). This however has not been reported for the selected Nigeria indigenous chicken and cross reference with exotic breed.

The short term attempt to meet the protein demand of the ever increasing population has been to massively import exotic breeds of poultry into the country, has led to the indigenous chicken breeds remaining unimproved and unable to meet their productive capacities. There are limited literatures and information on *cGH* gene of Nigeria indigenous chickens and its possible relationship with body weight, growth-related traits and egg producing ability.

1.3 Justification for the Study

The inadequate researches carried out on the Nigerian indigenous chickens especially at the molecular level have ensured that latent potentials are largely ignored and untapped. Molecular research of the Nigerian indigenous chicken breeds is an emerging area of interest which if properly harnessed, could lead to the rapid improvement of the indigenous chicken breeds and serves as an essential synergy in bridging protein gap and malnutrition. The inadequate and scanty literature on the chicken growth hormone polymorphism of the Nigerian indigenous chicken breeds is a justification for the present study.

1.4 Aim and Objectives of the Study

The aim of the study is to identify the polymorphic variation at intron 3 of the chicken growth hormone of selected Nigerian indigenous chicken breeds. The objectives of the study are as follows; to:

i. evaluate polymorphism at the intron 3 of *cGH* of selected Nigerian indigenous breeds of chicken.

ii. determine the genotype and gene frequencies of the different PCR-RFLP fragments from the combination of various RFLP alleles generated based on presence and absence of one or more restriction sites.

iii. identify the variants and frequencies detected in the *cGH* of the selected Nigerian indigenous chicken breeds.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and Domestication of Chicken

Domestic chicken belong to the kingdom *Animalia*, phylum *Chordata*, class *Aves*, order *Galliformes*, family *Phasianidae* and genus *Gallus*. Domestic chickens are considered to be one of the most important and widely distributed avian species among poultry birds. It is believed to have descended from a single ancestor; the Red Jungle fowls (*Gallus gallus*) with Southeast Asia as the centre of origin (Yamashita *et al.,* 1994; Fumihito *et al.,* 1994, 1996). Chicken genetic resources comprise a wide range of breeds and populations including Red Jungle fowl, native and fancy breeds, middle-level food producers, industrial stocks and specialized lines (Wani *et al.,* 2014). Nigeria has many indigenous chicken breeds that together form a large proportion of the poultry population in the country, which are yet to be incorporated into breeding programmes to exploit their full genetic potentials. The indigenous chickens are able to better survive in low input systems, tolerant and/or resistant to disease challenges, and variable production and climatic conditions than their commercial counterparts (Muhammad *et al.,* 2018).

2.2 Genetic Characterization of Domestic Chicken

Characterization refers to the distillation of all knowledge, which contributes to the reliable predictions of genetic performance of an animal genetic resource in a defined environment and provides a basis for distinguishing between different animal genetic resources, and for assessing available diversity (Rege and Gibson, 2003). This accounts for the genetic attributes of the animal genetic resources and the environments to which they are adapted/not adapted to. The

driving force of characterization is to avail information for appropriate use to support human livelihood by improving productivity and adaptability of livestock (Cunningham, 1992).

In Africa, since the observable traits and genetic characteristics of breeds often have not been defined and validated, it is difficult to consider certain breeds as a priority for the purpose of conservation as much diversity functions are yet to be discovered as much as possible across breeds. This applies also to other developing countries where there is limited infrastructure to conserve the whole range of breeds. Information on genetic diversity is however, useful in optimizing both conservation and utilization strategies for farm animal genetic resources. If the chicken genetic diversity is known, a choice can be made as to which breeds to be conserved with the limited resources available. Characterization of indigenous chicken genetic resources in Africa and Nigeria in particular, will give an insight to distribution of genetic diversity among breeds of indigenous chickens (Muhammad *et al.,* 2018).

Economically important traits are strongly influenced by the environment and hence, are not appropriate for classification purposes. Genetic distance based on DNA sequences, is a more reliable measure of genetic variation between breeds since it is not influenced by the environment. The development of molecular biology techniques during the eighties, for the study of genetics led to improvement in genetic characterization. Classical approaches to the detection of genetic differences in farm animals have been based on the use of blood proteins (Hines, 1999). More recently, DNA polymorphisms have become more popular in genetic characterization of most farm animals (Nijman *et al.,* 2003). DNA fingerprints (Dunnington *et al.,* 1994; Ponsuksili *et al.,* 1998) and sequence have been utilized to determine genetic diversity in the chicken (Niu *et al.,* 2002; Liu *et al.,* 2004).

Knowledge-based phenotypic and genotypic data is essential for the characterization of indigenous animal genetic resources providing needed information for effective conservation of useful gene pool against future uncertainties (Liu *et al.,* 2004). Such uncertainties in the face of current global challenges include: agricultural, socio-economic, environmental, emerging diseases, population growth, and rising consumer demands. Recently, a review study was conducted to characterize the genetic diversity in chickens (Jian-Min *et al.,* 2010). Genetic variation is the basic raw material for animal breeding. Molecular studies bring complementary information to social surveys and phenotypic data, and allow for setting up of an integrated programme of characterization and conservation of indigenous animal populations (Bianchi *et al.,* 2011; Leroy *et al.,* 2012). There is therefore a growing interest in suitable genetic markers for assessing population differences in qualitative and quantitative traits.

2.3 Genetic Characterization of Nigeria Indigenous Chicken

Genetic resources embedded in the indigenous poultry awaits full exploitation that will provide basis for genetic improvement and diversification to produce breeds that are adapted to the local environment for the benefit of farmers in **developing countries** (Sonaiya *et al.*, 1999). The indigenous poultry species has valuable resources for livestock development because of their extensive **genetic diversity** which allows for rearing of poultry under varied environmental conditions, providing a range of products and functions (Muhammad *et al.*, 2018).

In Nigeria, indigenous chickens have been characterized largely along genetic lines of feather and plumage colour (normal or frizzled feathered), body structure (naked neck, dwarf types) and colour variants (black, white, brown and mottled). The frequency distribution of the normal feathered chicken was reported for instance, to be about 91.8 % while that of frizzled and naked

neck were 5.2 % and 3.0 %, respectively in Bayelsa State of Nigeria (Ajayi and Agaviezor, 2009). Classification has also been on the basis of location; there are various ecotypes of the local chicken found in the different agro ecological zones (Adedokun and Sonaiya, 2001; Ajayi, 2010). Most of the classification by the different agro ecological zones considered mainly the normal feathered indigenous chicken because they are the most prominent; the naked neck and frizzled feathered are rare and almost becoming endangered and the gene pool they represent may be lost if not characterized and conserved. Olori (1992) noted two ecotypes characterized as the forest and savannah or Yoruba and Fulani ecotypes, respectively. It was agreed that the Nigerian chicken is a light breed, often with a single comb and that black and brown plumage, laced with various colours such as mottling are common (Adebambo, 2005).

Recent works revealed that the different ecotypes can be grouped further into two major categories on the basis of their body size and body weight as the heavy and light ecotype (Momoh *et al.*, 2007). The heavy ecotype (also referred to as the Fulani ecotype) is found principally in the dry savannahs (Guinea and Sahel savannah), montane regions and cattle Kraals of the North and weigh about 0.9 - 2.5 kg at maturity. The light ecotype are the chicken types from the swamp, rainforest and derived savannah agro-ecological zones whose mature body weight ranges between 0.68 - 1.5 kg (Momoh *et al.*, 2007).

2.4 Growth Hormone

Growth Hormone (*GH*) is secreted by somatotropic cells of the anterior lobe of the pituitary. It has been documented that *GH* influences growth, lactation, reproduction and metabolism in animals (Baldi, 1999; Bauman, 1999; Breier, 1999). The *GH* gene, with its functional and positional potential, has therefore been widely used as a marker in several livestock species. The

intron 4 and exon 5 has accounted for the highest diversity and can be correlated with performance in animals (Malveiro *et al.,* 2001; Marques *et al.,* 2003; Gupta *et al.,* 2007; Gupta *et al.,* 2009; Hua *et al.,* 2009; Mousavizadeh *et al.,* 2009; An *et al.,* 2010; Wickramaratne *et al.,* 2010).The growth hormone gene is located on chromosome 27 and contains 5 exons and 4 introns with a total length equal to 4.35 kbp. It is a polypeptide hormone that is synthesized and secreted by the pituitary gland. It has been reported to affect growth traits in broiler production such as growth rates, body weight, maturation, metabolism rates, egg production, reproduction, appetite control and aging (Shaw *et al.,* 1991; Feng *et al.,* 1997; Harvey, 2013).

The Growth Hormone Receptor (*GHR*) gene which contains 10 exons and 9 introns with a total length equal to ~130.33 kbp, is located on the Z chromosome, which determine its homozygous state in hen birds. It was shown that several different types of SNP's affecting the intronic and exonic parts, as well as non translated 5` and 3` gene regions, are present (Nie *et al.,* 2005; Ouyang *et al.,* 2008; Khoa *et al.,* 2013). Reports has it that the allelic variants of the growth hormone receptor gene are associated with quality indices of shell and live weight, as well as with chicken dwarfism (Zhang *et al.,* 2008). Growth performance and carcass traits are very significant economic traits in broiler production, and are controlled by sets of complex genes. Growth is a complicated procedure, regulated by a wide variety of neuroendocrine pathways and for this reason, it is very difficult to make rapid progress using conventional methods of genetic selection within breeds (Zhang *et al.,* 2008).

2.4.1 Chickens growth hormone (*cGH*)

The *cGH* is a 22-kDa protein, containing 191 amino acid residues (Hrabia *et al.,* 2008). In poultry, *cGH* consists of 4,101 base pairs, having five exons and four introns (Kansaku *et al.,*

2008). The *cGH* is a polypeptide produced and secreted by the pituitary gland; it affects a variety of physiological functions and growth performance in the chicken (Byatt *et al.,* 1993; Apa *et al.,* 1994). It was reported to be one of the most important genes affecting chicken performance traits, and plays a critical role in both growth and metabolism rates (Vasilatos-Younken *et al.,* 2000). The chicken growth hormone gene plays a crucial role in controlling growth and metabolism as well as other physiological functions, such as promoting muscle growth, bone growth and development, regulation of fat content and metabolism (Zhang *et al.,* 2007; Ge *et al.,* 2012; Mazurowski *et al.,* 2015).

Studies indicate that *cGH* is also involved in the processes of sexual differentiation, pubertal maturation and participates actively in gonadal steroidogenesis, gametogenesis and ovulation (Hull and Harvey, 2001). In birds, *cGH* is also involved in a variety of secondary functions such as egg production, aging and reproduction (Kansaku *et al.,* 2003). Genetic diversity in the *cGH* gene has been reported to exits more in native chicken breeds when compared to commercial breeds (Nie *et al.,* 2002).

2.5 Polymorphism

Polymorphism was defined as the co-occurrence of two or more varieties in the same population at the same time in such proportions that the rarest of them cannot be maintained by mutation alone (Jafari *et al.,* 2015). Biochemical polymorphism is the occurrence of varieties attributed to biochemical differences, which are under genetic control. It has created a way for the genetic improvement of farm animals. This kind of diversity is a useful tool for characterization of farm animals. Moreover, it is possible to determine the degree of differences or similarities between

and within breeds and therefore, they are very useful raw materials for genetic improvement of farm animals (Hull and Harvey, 2001).

Genomic breeding is based on the study of polymorphism of target genes, the allelic variants of which are associated with productive properties of animals. The allelic variants of functional genes are a result of different modifications of nucleotide composition, such as point mutations (Single Nucleotide Polymorphism; SNP), insertions/deletions (indel), and so on. Polymorphism identification and subsequent study of its relationship with productivity characters provide a basis for further targeted breeding which is applicable to poultry farming (Fulton, 2008).

It is of interest to search for polymorphism in genes, the products of which are involved in the regulation of various biological functions, primarily, those associated with support of growth and differentiation (Enayati and Rahimi-Mianji, 2009). Such objects include the genes encoding regulatory proteins, in particular, hormones. In turn, the physiological effect of any hormone directly depends on its receptor, therefore, it is reasonable to study the polymorphism of the genes encoding both hormones themselves and their receptors.

2.5.1 Polymorphism of chicken growth hormone

Studies of polymorphisms of *cGH* gene carried out by new approaches such as RFLP technique and sequencing of DNA has been reported (Ip *et al.*, 2001; Yan *et al.*, 2003; Enayati and Rahimi-Mianji, 2009). Research on Chinese native Taihe Silkies chickens (Nie *et al.*, 2005) indicated that a 50 bp deletion occurred in intron 4 of the *cGH* gene. Assessment of intron 4 of the *cGH* gene demonstrated that polymorphisms were related to certain characteristics of the Chinese native chickens (Nie *et al.*, 2002). Substitution, deletion or insertion of a single nucleotide can lead to variations in a gene, called Single Nucleotide Polymorphisms (SNPs).

Thakur *et al.* (2009) in Kadakanath chicken of India observed three genotypes; AA, AB and BB at the *cGH1* locus. Bhattacharya *et al.* (2010) also reported three genotypes; AA, BB and AC in White Leghorn chickens. Wenming *et al*. (2010) while investigating the mutation characteristics of growth hormone (*GH*) gene in five Chinese indigenous goose populations, observed six SNPs per 1000 nucleotides in exons compared to two SNPs per 1000 nucleotides in intron regions. Four of the SNPs were detected by two primers and four genotypes; AA, BB, CC and DD were generated among the five populations studied.

Tanmankaurd *et al.* (2008) analyzed 776 bp amplicons of the chicken *GH* gene digested with *Msp*1 with the results revealing three different patterns (AA, AC and CC) created by a combination of polymorphic *Msp*1 cut sites present at exon-1 (one site) and at intron-1 (one site). A study of polymorphism at intron 4 of the *cGH* gene (Nie *et al*., 2002) of Chinese native chickens identified a total of eight restriction digestion profiles within the 1170 bp amplified PCR products by *Msp*I restriction endonuclease. Among the 20 populations studied, distinctly different allele numbers and fragments of intron 4 RFLPs between the native chickens were observed. A new allele D was identified at a high frequency in the slow growing Taihe Silkies and Beijing fatty strains.

In a study of native chicken population in Iran Makhsous *et al.* (2013), a total of 142 chicken blood samples were collected and a specific primer set was used to amplify a fragment of the growth hormone locus using PCR. The PCR products were then digested with *SacI* and *MspI* restriction endonucleases. Their results revealed the existence of three genotypes of the amplified fragments digested with *SacI* enzyme ($^{+}/^{+}$, $^{+}/^{-}$ and $^{-}/^{-}$) controlled by two wild type ($^{+}$) and normal type ($^{-}$) alleles with allelic frequency of 0.898 and 0.102, respectively; the genotype frequency for the three genotypes were 0.817, 0.162, and 0.021, respectively. The amplified fragments

digested with *MspI* enzyme revealed three alleles: A, B and C with frequency of 0.599, 0.102, and 0.299, respectively; six genotypes: AA, AB, AC, BB, BC, and CC were observed with genotype frequency of 0.338, 0.113, 0.409, 0.007, 0.070, and 0.063, respectively.

Anh *et al.* (2015) studied crosses involving a commercial broiler Parent Stock (PS) with four Thai chicken breeds (Kaen Thong; KT, Khai Mool Esarn; KM, Soi Nin; SN and Soi Pet; SP dam lines) and observed the existence of three genotypes (AA, AG and GG) in all the crosses with the following gene frequencies for the A and G alleles: 0.20 and 0.80 for the PS × KM, 0.30 and 0.70 for the PS × KT, 0.29 and 0.71 for the PS × SN, and 0.27 and 0.73 for the PS × SP crosses, respectively.

The polymorphisms of growth hormone gene have been studied in many animals; the study of candidate genes is one of the primary methods used to find specific genes related to economic traits in farm animals (Moazeni *et al.,* 2016). In dairy cattle, it was observed that an *Mspl* (*Moraxella species*) polymorphism at intron 3 of the bovine Growth Hormone (*bGH*) gene is linked to milk protein (Lagziel *et al.,* 1999). Lagziel *et al.* (1999) also establish that, an association exist between growth hormone and reproductive performance of bulls used for artificial insemination using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) method. In relationship with other animals, the intron region of the *cGH* gene is highly polymorphic, and is linked with resistance to Marek's disease or avian leucosis, and also meat yield traits (Fotouhi *et al.,* 1993; Yan *et al.,* 2003). In addition, Lagziel *et al.* (1999) identified alleles that were involved in the selection of a series of egg layers for egg production and in the selection of the size of the abdominal fat pad in broilers. Mou *et al.* (1995) also reported the presence of 2 *Mspl* sites in chicken intron 1, with 1 *Mspl* RFLP being established.

2.6 Association between Chicken Growth Hormone and Economic Traits

Chicken growth hormone gene has been used as a candidate gene for marker assisted selection for improved performance (Muhammad *et al.,* 2018). The results showed that c*GH* gene could be linked to a major gene significantly affecting the growth and carcass traits in chicken. Fotouhi *et al*. (1993) observed significant ($p<0.05$) correlation of alleles of the growth hormone gene and growth hormone receptor gene with juvenile body weight, age at first egg, hen-day rate of egg production, egg specific gravity and egg weight in a strain of White Leghorns.

Refraction fragment length polymorphisms characterized in the introns of *cGH* gene of White leghorn chicken has been associated with egg production phenotype, resistance to Marek's disease and avian leukosis (Kuhnlein *et al.,* 1997). In various populations of Chinese native chickens, an allele in intron 1 was linked to laying performance (Mou *et al.,* 1995). Lei *et al.* (2007) reported that an SNP with G and A substitution of *cGH* was associated with abdominal fat pad weight, abdominal fat pad ratio and crude fat content of the breast muscle of chicken.

Makhsous *et al.* (2013) reported no significant effect of six *cGH* genotypes on egg number, laying percent and mean egg weight (g) at 28, 30 and 32 weeks of age in Iranian native chickens; the three *Sacl*-RFLP generated genotypes however, affected egg number and laying % with chickens having $^{+}/^{+}$ genotype laying more eggs. Laying rate was however similar in chickens having $^{+}/^{+}$ and $^{-}/^{-}$ genotypes.

Anh *et al.* (2015) observed no significant effect of the genotypes on body weight except at between week 2 and 4, and week 4 and 6 which were significant. Birds carrying the AG and GG genotypes were observed to have better body weight over those having AA genotype. Average

daily gain was significant only at 0-2 weeks, and 0-6 weeks age with birds carrying AG and GG genotypes having better performance compared to those having the AA genotype.

2.7 Intron

An intron is any nucleotide sequence within a gene that is removed by Ribonucleic Acid (RNA) splicing during maturation of the final RNA product (Stryer *et al.,* 2007; Albets and Bruce, 2008). Introns are found in the genes of most organisms and many viruses, and can be located in a wide range of genes, including those that generate proteins, ribosomal RNA (rRNA) and transfer RNA (tRNA). When proteins are generated from intron-containing genes, RNA splicing takes place as part of the RNA processing pathway that follows transcription and precede translation (Mou *et al.*, 1995).

2.7.1 Functions of intron

Introns impose a huge energy burden on the cell, considering that the density of introns is greater than that of exons in genomes. This means that the genic regions of introns consume large amounts of energy for nothing in terms of protein synthesis. According to Lynch (2007), introns are just selfish DNAs that invade protein-coding genes in eukaryotic genomes, and if deleterious, introns can be sustained due to severe population bottlenecks. Studies have shown that introns bring about selective advantages to the cell in eukaryotes, contributing to overcoming the energy disadvantage (Chorev and Carmal, 2012). However, the results derived from other studies are still controversial (Bergman and Kreitman, 2001).

Introns are crucial because the protein repertoire is greatly enhanced by alternative splicing in which introns take important roles. Alternative splicing is a controlled molecular mechanism

producing multiple variant proteins from a single gene in a eukaryotic cell (Lynch, 2007). One of the remarkable examples of the increasing protein repertoire by alternative splicing is the Drosophila Dscam gene, of which over 38000 isoforms can potentially be produced by alternative splicing. Pan *et al.* (2008) provided experimental evidence suggesting that approximately 95 % of multiexon genes in the human genome may undergo alternative splicing. Furthermore, very short introns are selected against because a minimal length of intron is required for the splicing reaction (Roy *et al.*, 2008).

Some studies have suggested that introns may have a role in chromatin assembly. Recent genome-wide mapping analyses of nucleosome positions have shown that nucleosomes are relatively depleted in intron regions compared to exonic regions (Schwartz and Meshorer, 2009; Spies *et al.*, 2009). Schwartz and Meshorer (2009) suggested that sequence elements of intron ends may be responsible for nucleosome depletion in introns by pushing the nucleosomes away toward the exons. The existence of introns in the genome is a real mystery, given the expensive energy cost a cell pays for copying the entire length of several introns in a gene and then excising them at the exact position, controlled by big RNA and protein complexes after transcription (Jo and Choi, 2015).

2.8 Exon

The term exon derives from the expressed region and was coined by the American biochemist Walter Gilbert in 1978: "The notion of the cistron must be replaced by that of a transcription unit containing regions which will be lost from the mature messenger – which was suggested we call introns (for intragenic regions) – alternating with regions which will be expressed – exons." (Gilbert, 1978). The term exon refers to both the DNA sequence within a gene and to the

corresponding sequence in RNA transcripts. In RNA splicing, introns are removed and exons are covalently joined to one another as part of generating the mature messenger RNA. Just as the entire set of genes for a species constitutes the genome, the entire set of exons constitutes the exome (Thanaraj and Robinson, 2000). An exon is a coding region of a gene that contains the information required to encode a protein. In eukaryotes, genes are made up of coding exons interspersed with non-coding introns. These introns are then removed to make a functioning messenger RNA (mRNA) that can be translated into a protein (Zhang, 1998).

Although unicellular eukaryotes such as yeast have either no introns or very few, metazoans and especially vertebrate genomes have a large fraction of non-coding DNA. For instance, in the human genome only 1.1 % of the genome is spanned by exons, whereas 24 % is in introns, with 75 % of the genome being intergenic DNA (Venter *et al.,* 2000). This can provide a practical advantage in omics-aided health care (such as precision medicine) because it makes commercialized whole exome sequencing a smaller and less expensive challenge than commercialized whole genome sequencing. The large variation in genome size and C-value across life forms has posed an interesting challenge called the C-value enigma. Across all eukaryotic genes in GenBank there are (in 2002), on average, 5.48 exons per gene. The average exon encoded 30-36 amino acids (Sakharkar *et al.,* 2002). While the longest exon in the human genome is 11555 bp long, several exons have been found to be only 2 bp long (Sakharkar *et al.,* 2004). A single-nucleotide exon has been reported from the Arabidopsis genome (Lei and Chun-Ming 2015).

2.8.1 Functions of exon

Exons is a messenger RNA precursor (pre-mRNA). Exons can include both sequences that code for amino acids (red) and untranslated sequences (grey). Introns -those parts of the pre-mRNA that are not in the mRNA -(blue) are removed, and the exons are joined together (spliced) to form the final functional mRNA. The 5′ and 3′ ends of the mRNA are marked to differentiate the two untranslated regions (grey). In protein-coding genes, the exons include both the protein-coding sequence and the 5′- and 3′- untranslated regions (UTR). Often the first exon includes both the 5′-UTR and the first part of the coding sequence, but exons containing only regions of 5′-UTR or (more rarely) 3′-UTR occur in some genes, that is, the UTRs may contain introns (Bicknell, 2012). Some non-coding RNA transcripts also have exons and introns.

Exons are pieces of coding DNA that encode proteins (Brown, 2012). Different exons code for different domains of a protein. The domains may be encoded by a single exon or multiple exons spliced together. The presence of exons and introns allows for greater molecular evolution through the process of exon shuffling. Exon shuffling occurs when exons on sister chromosomes are exchanged during recombination. This allows for the formation of new genes. (Klug and Cummings, 1994).

Exons also allow for multiple proteins to be translated from the same gene through alternative splicing (Brown, 2012). This process allows the exons to be arranged in different combinations when the introns are removed. The different configurations can include the complete removal of an exon, the inclusion of part of an exon, or the inclusion of part of an intron. Alternative splicing can occur in the same location to produce different variants of a gene with a similar role, such as the human gene, or it can occur in different cell or tissue types, such as the mouse alpha-

amylase gene. Alternative splicing, and defects in alternative splicing, can result in a number of diseases including alcoholism and cancer (Klug and Cummings, 1994).

2.9 Polymerase Chain Reaction (PCR)

The technique was developed in 1983 by Kary Mullis in California and involves the use of a single genetic material called DNA to generate over 100 billion identical molecules within a very short time. The reaction is very simple to execute and requires simple laboratory tools; only very simple reagents and source of heat are required. The core ingredient of PCR is a thermostable DNA polymerase (enzyme), particularly Taq and Vent polymerases. Both can work effectively even at high temperatures to cause the separation of the two DNA strands in the Watson-Crick DNA double helix structure (Delidow *et al.,* 1993). The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. The key components of this reaction are the primers or short DNA fragments containing sequences complementary to the target region, and a DNA polymerase or replicating enzyme in cells. These key components of the reaction enable selective and repeated amplification to occur. As PCR progresses, the DNA generated is itself used as a template for replication (the process by which more copies of DNA are produced from pre-existing DNA template), setting in motion a chain reaction in which the DNA template is exponentially amplified. The PCR can easily be performed and when carefully carried out, leads to the amplification of specific DNA sequences (Schorderet, 1994).

2.9.1 Mechanism of polymerase chain reaction

PCR involves several cycles (about 20-45 cycles or repeated temperature changes). There are three steps involved in one complete cycle. These steps are: denaturation, primer annealing and

primer extension, elongation or polymerization. Prior to these three steps however, there is what is called the initialization step. In this step, the reaction mixture is heated to a temperature of 94 - 96 °C (or 98 °C) if thermostable polymerases are used, which is held for 1 - 9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR (Delidow *et al.*, 1993).

2.9.1.1 Denaturation

This is the melting of target DNA and it is the first regular step of the cycling event. It consists of heating the reaction mixture to 94 - 98 °C for 25 seconds (that is the target DNA to be amplified is heat denatured to separate the complementary strands). It causes DNA melting by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules or strands which then acts as templates for DNA synthesis (Delidow *et al.*, 1993).

2.9.1.2 Primer annealing

During this stage, primers are added in excess and the temperature is lowered to about 68 °C for 60 seconds. In this step, there is annealing or hybridisation of the primers to the single-stranded DNA template on both ends. The primers form hydrogen bonds (i.e. anneal to the DNA on both sides of the DNA sequence). Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis. The annealing temperature for this reaction varies, but too low temperature enhances mispairing. Hybridization step is usually performed at 50 °C below the melting temperature (Tm) for perfectly matched sequences. For every mismatched base pair, a further 50 °C reduction is necessary to maintain hybrid stability. The

annealing temperature at 0 °C can be calculated by using the relation; Tm = 4 (number of GC base pairs) + 2 (number of AT base pairs) (Zhong and Bajay, 1993).

2.9.1.3 Polymerisation

When polymerization step is ongoing, the nucleoside triphosphate (dNTPs), DNA polymerase and Mg^{2+} are added to the reaction mixture. The DNA polymerase speeds up the polymerisation process of the primers and also extends the primers at 68 – 72 °C resulting in the synthesis of copies of target DNA sequences. Two popular polymerases or enzymes (Taq and Vent polymerases) can be used. The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75 – 80 °C and commonly a temperature of 72 °C is used with this enzyme (Delidow *et al.*, 1993). At this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs. The $dNTP_S$ are complementary to the template in a 5' to 3' direction, condensing the 5' phosphate group of the dNTPs with the 3' hydroxyl group at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. The DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting reagents, at each extension step, the amount of DNA target is doubled, leading to exponential or geometric amplification of the specific DNA fragment (Schorderet, 1994).

2.10 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique invented in 1984 by the English scientist Alec Jeffreys during research into hereditary diseases. It is used for the analysis of unique patterns in DNA fragments in order to genetically differentiate between organisms –

these patterns are called Variable Number of Tandem Repeats (VNTRs). RFLP has been used for several genetic analysis applications since its invention. Some of these key applications of RFLP are listed below: To determine the status of genetic diseases such as Cystic Fibrosis in an individual. To determine or confirm the source of a DNA sample such as in paternity tests or criminal investigations. In genetic mapping to determine recombination rates that show the genetic distance between the loci. To identify a carrier of a disease-causing mutation in a family (Zhong and Bajay, 1993).

The RFLP technique is however, faster and less expensive than other kinds of DNA fragment analyses; it can be used with any quantity of DNA. It is even suitable for work on unknown genomes. The method is also capable of sampling genome randomly. It had also been used to develop genome maps (Levin *et al.,* 1993). Several authors (Smith *et al.,* 1996; Zhang *et al.,* 2002; Olowofeso *et al.,* 2005) have tested the usefulness of the assay method with chickens, Rahman *et al.* (2006); El Gaali and Satti, (2009); Khaldi *et al.* (2010) in goats, and also in buffalo (Puttalakshmamma *et al.,* 2014).

2.11 Electrophoresis

Electrophoresis is a method of separating charged macromolecules such as nucleic acids (DNA and RNA) or protein molecules using an electric field applied to a gel matrix. Electrophoresis is usually carried out for analytical purposes, after amplification of DNA via PCR. Electrophoresis is the electromotive force that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an appropriate electric field, the molecules will move through the matrix at different rates, determined by their mass when the charge to mass ratio of all species is uniform. If the molecule is negatively charged, it moves toward the anode

and if positively charged, toward the cathode. DNA molecules have negative charges and migrate towards the anode (Moretti *et al.,* 1998).

The electric field applied across the gel, causes the negatively charged proteins or nucleic acids to migrate across the gel towards the positive (+) electrode (anode). Depending on their size, biomolecules move differently through the gel matrix; small molecules more easily and larger ones with more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. Biomolecules may therefore be separated roughly according to their size, which depends mainly on molecular weight under denaturing conditions, but also depending on higher-order conformation under native conditions. However, certain glycoproteins behave anomalously on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS gels) (SDS-PAGE, 2009).

Stains are used during electrophoresis to aid visualization of the biomolecules. Common gel stains include Coomassie Brilliant Blue R-250 for proteins, ethidium bromide for nucleic acids and silver stain for either. After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of the unknown biomolecules by comparing the distance travelled relative to the marker (Moretti *et al.,* 1998).

2.11.1 Agarose gel electrophoresis (component and function)

Agarose gel electrophoresis is one of several physical methods for determining the size of DNA. Here, DNA is forced to migrate through a highly cross-linked agarose matrix in response to an

electric current. In solution, the phosphates on the DNA are negatively charged, and the molecule will therefore migrate to the positive (red) pole. There are three factors that affect migration rate through a gel; size of the DNA, conformation of the DNA, and ionic strength of the running buffer. Electrophoresis is essentially a sieving process; larger fragments of DNA are easily entangled in the gel matrix and, therefore, migrate slowly. Smaller fragments run more quickly at a rate proportional to their size. The relationship of size to migration rate is linear throughout most of the gel, except for the very largest fragments. Large fragments have a more difficult time penetrating the gel and their migration does not have a linear relationship to size. The matrix can be adjusted, though, by increasing the concentration of agarose (tighter matrix) or decreasing it (looser matrix). A standard 1 % agarose gel can resolve DNA from 0.2 – 30 kb in length (Moretti *et al.*, 1998).

This type of gel is composed mainly of agarose (a linear polymer of D-galactose and 3,6-anhydro-L-galactose obtained from seaweeds). Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores which allows for the separation of macromolecules and macromolecular complexes. Agarose gels are more porous than polyacrylamide gels, and hence, can be used to separate large sized macromolecules like DNA. An agarose gel is a complex network of polymeric molecules whose average pore size depends on the buffer composition and the type and concentration of agarose used. DNA bands in agarose gel can be visualize by soaking the gel in a solution of ethidium bromide (EtBr), which quickly complexes with DNA by intercalating between stacked base pairs and photographing the orange fluorescence which results upon ultraviolet irradiation (Moretti *et al.*, 1998).

The mechanism responsible for the separation of DNA molecules by molecular weight during gel electrophoresis is still quite obscured. Originally, DNA movement in gel was thought to resemble motion of a snake (repetition); however, recent studies have shown that DNA exhibit elastic behaviour by stretching in the direction of the applied field and then contracting into the dense balls. The larger the pore size of the gel, the greater the ball of DNA which can penetrate and hence, the larger the molecules which can be separated. In conventional electrophoresis, a single constant electric field is applied.

The complete chamber (apparatus) where this type of electrophoresis is carried out is called submarine agarose unit which often contain 15 x 10 cm white tray. Agarose gels are referred to as submarine gels because the slab is laid horizontally and is completely covered by running buffer. The buffer commonly used is the 0.5 x TBE, and λ DNA/Hind III + EcoRI is a suitable DNA ladder to estimate the size of amplified bands. This type of electrophoresis is now popularly known as Submerged Agarose Gel Electrophoresis (SAGE). Gel electrophoresis of large DNA or RNA is usually done by agarose gel electrophoresis (Schorderet, 1994).

2.12 Common DNA extraction methods

Different extraction methods result in different yields and purity of DNA. Some of the extraction methods have been systematically evaluated for specific applications such as soil and sediment samples, human microbiome and faecal samples.

2.12.1 Organic extraction

In this widely used method, cells are lysed and cell debris is usually removed by centrifugation. Then, proteins are denatured/digested using a protease and precipitated with organic solvents

such as phenol, or a 1:1 mixture of phenol and chloroform, and the protein precipitate is removed by centrifugation. Purified DNA is usually recovered by precipitation using ethanol or isopropanol. In the presence of monovalent cations such as Na+, and at a temperature of –20 °C, absolute ethanol efficiently precipitates polymeric nucleic acids and leaves behind short-chain and monomeric nucleic acid components including the ribonucleotides from RNase treatment in solution. This method uses hazardous organic solvents, is relatively time-consuming, and residual phenol or chloroform may affect downstream applications such as PCR. Easy-DNA® Kit (Invitrogen) is an example (Frey, 1998).

2.12.2 Silica based technology

This is the most widely employed method in most of the current kits. DNA adsorbs specifically to silica membrane/beads/particles in the presence of certain salts and at a particular pH. The cellular contaminants are removed by wash steps. DNA is eluted in a low salt buffer or elution buffer. Chaotropic salts are included to aid in protein denaturation and extraction of the DNA. This method can be incorporated in spin columns and microchips, is cost effective, has a simpler and faster procedure than the organic extraction, and is suitable for automation. Kits based on this method include Purelink Genomic DNA extraction kit (Invitrogen) and Qiagen DNeasy Blood and Tissue Kit (Frey, 1998).

2.12.3 Magnetic separation

This method is based on reversibly binding DNA to a magnetic solid surface/bead/particles, which have been coated with a DNA binding antibody or a functional group that interacts specifically with DNA. After DNA binding, beads are separated from other contaminating cellular components, washed and finally the purified DNA is eluted using ethanol extraction.

This method is rapid and can be automated. However, it can be costlier than other methodologies. Examples are AgencourtDNAdvance Kit (Beckman Coulter) and Magnetic Beads Genomic DNA Extraction Kit (Geneaid) (Frey, 1998).

2.12.4 Anion exchange technology

This method is based on the specific interaction between negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds specifically to the substrate in the presence of low salt, contaminants are removed by wash steps using low or medium salt buffer, and the purified DNA is eluted using a high salt buffer. This technology is more commonly employed in plasmid isolation kits such as PureLink®HiPure Plasmid DNA Purification Kits (Invitrogen), Qiagen plasmid mini/midi kits and Genomic-tip, and NucleoBond® PC kits (Macherey Nagel) (Frey, 1998).

CHARTER THREE

3.0 MATERIALS AND METHODS

3.1 Source of Experimental Birds

The study was carried out using two Nigerian indigenous chickens' population (Fulani ecotype and Fizzle feathered), two improved Nigerian indigenous chicken breeds (Noiler and FUNAAB Alfa broilers) and, an exotic commercial broiler breed (Cobb 500). The Nigerian indigenous chicken breeds were sourced from different locations across Niger State, Nigeria. The improved Nigerian indigenous chicken breeds (Noiler chickens and FUNAAB Alpha broilers) were gotten from Amo hatchery and Federal University of Agriculture, Abeokuta respectively. The exotic breed was used as the reference breed for the experiment and was purccused from Olam hatchery.

3.2 Blood Sample Collection

Blood samples were collected from the brachial (wing) vein of fifty chickens used for the study using 2 ml syringes; with new needles and syringes used for individual birds to avoid cross contamination. The blood samples (10 each from the breeds stated above) were collected into Ethylene Di-amine Tetra-acetic Acid (EDTA) bottles to avoid coagulation by forming a complex with oxygen in the environment as this would have had negative effect on further analysis. Collected samples were stored on ice packs for onward transfer to the laboratory where they were stored until when needed for subsequent genomic DNA extraction. The genomic DNA extraction was done at African Biosciences Ltd., Ibadan, Oyo State, Nigeria. After genomic DNA extraction one sample was contaminated and could not be proceed further.

3.3 DNA Extraction

The process begins with absolute ethanol being used to wash the buffer which was then mixed by shaking for a few seconds; ddH_2O was added to Proteinase K and then vortexed to ensure it was completely dissolved. Once this was achieved, it was centrifuged for a few seconds to spin down the mixture. The ddH_2O and Proteinase K mixture was then stored at 4 ^oC. Whole blood (200 µl) was transferred into 1.5 ml micro centrifuge tube to which was added 20 µl of Proteinase K, mixed by pipetting, and incubated at 60 ^oC for 5 minutes. Two hundred (200 µl) of GSB buffer was then added and mixed by shaking vigorously. This was then Incubated at 60 ^oC for 30 minutes. During incubation, a required volume of elution buffer (200 µl/ sample) was transferred to a 1.5 ml microcentrifuge tube and heated to 60 ^oC for the elution step. Two hundred (200 µl) of absolute ethanol was added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. A GS column tube was placed in a 2 ml collection tube to which were transferred all the mixtures including all insoluble precipitate. The mixture was taken to be centrifuged (14-16,000 x g for 1 minute). The 2 ml collecting tube containing the flow-through were then transferred to a new 2 ml collection tube; 400 µl of W1 buffer was added to the GS column tube and centrifuged at 14-16,000 x g for 30 seconds; the flow-through was discarded. The GS column tube was placed back into the 2 ml collection tube and 600 µl of wash buffer was added to make sure that the absolute ethanol was added to the GS column tube. This was centrifuged at 14-6,000 x g for 30 second, followed by the discarding of the flow-through. The GS column tube was placed back into the 2 ml collection tube and centrifuge again for 3 minutes to dry the column matrix. The GS column tube was then transferred into a clean 1.5 ml microcentrifuge tube. To this was added 100 µl of pre-heated elution buffer into the center of the column matrix, followed by a waiting period of at least 3 minutes to allow for the complete

absorption of the elution buffer. Finally, this was centrifuged at 14-16,000 x g for 30 seconds to elute the purified DNA.

3.4 Chicken Growth Hormone (*cGH*) Gene Primers

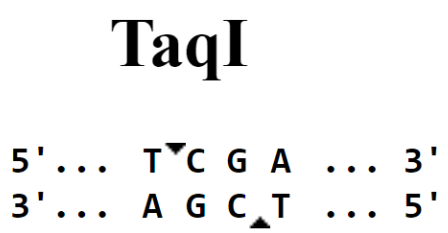
The primers used for the study was specific to *cGH* gene and was designed using the National Centre for Biotechnology Information (NCBI) website. *cGH* primer 5`…TCAGTACGCAGACCTACCCTC…3` (forward) and 5`…TGCACATCATGTCCCACGTTT…3` (reverse) were used for the study.

3.5 Polymerase Chain Reaction (PCR)

The extracted genomic DNA was diluted with autoclaved distilled water to 30 ng per µl. A total of 21 µl of reaction mixture for the primer was mixed in master cycler gradient (eppendorf tube). The reaction volume (21 µl) contained the primers (both forward and reverse), each of 1.5 µl, PCR master mix 10 µl, water 2.7 µl and 5.3 µl (90 ng) diluted DNA. The reaction mixture was subjected to an initial denaturation at 95 °C for 4 minutes, followed by 50 cycles at 94 °C for 25 seconds. Annealing then followed at 56 °C for 45 seconds, an extension at 72 °C for 40 seconds and a final extension was done for 10 min at 72 °C.

3.6 Restriction Enzyme Digestion

The intron 3 *cGH* (forward and reverse primers) was amplified using PCR-RFLP method. Restriction digestion was done using the enzyme endonuclease *TaqI* for *cGH*. This enzyme which is (from the bacterium *Thermus aquaticus* species), was used to cut the DNA molecules into smaller pieces at specific locations as shown overleaf;



3.7 Agarose Gel Electrophoresis

The PCR products digested with *TaqI* restriction enzyme were analyzed on 2.5 % agarose gel. For genomic DNA, 1.0 % agarose gel was used while for RFLP, 3.0 % agarose gel was used (15 µl of PCR product mixed with 1 µl of gel loading dye). The electrophoresis was conducted at a constant voltage of 75 volts for 45 minutes at 37 °C using 1.0 x TAE buffer. The mass ruler DNA ladder (100 bp) was used for sizing of the DNA bands. The DNA fragments were then stained with ethidium bromide (Sambrook and Russel, 2000) and photographed using a Blue Light (BL) transilluminator (Biologix®) to visualize the bands.

3.8 DNA Sequencing

The PCR products (2 samples per breed) were sequenced and then aligned against the chicken genome with the BLAST programme to verify their identity. Prior to sequencing, the DNA were cleaned to ensure that the gene is not contaminated with impurities. This was done using DNA clean and concentrator kit according to the manufacturers protocol (ZYMO RESEARCH).

3.9 Statistical Analysis

Genotypic frequencies of the different PCR-RFLP patterns were estimated from the combination of various RFLP alleles generated based on the presence or absence of one or more restriction sites. Different genotypes were identified on the basis of the different patterns observed. Gene frequencies were calculated from genotypic frequencies. The allele frequencies were calculated

using standard methods. The Chi-square (χ^2) test for goodness of fit was used to find out the difference among various genotypes and tested for Hardy-Weinberg equilibrium. Sequences were blasted against database on NCBI and Reference Sequences acquired. Sequence trimming was carried out on BioEdit. Multiple Sequence Alignment by MAFFT online sequence alignment tool was used to align the sequence data. Allele frequencies were computed on Genalex 6.503. Variants effects were predicted on VEP (Variants Effect Predictor), as deployed on Ensembl genome browser. Restriction enzyme for genotyping was identified using NebCutter. Gel images were scored using PyElph1.4.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Genomic DNA isolation of selected chicken breeds in Nigeria

The agarose gel electrophoresis result of isolated genomic DNA of selection chicken breeds is presented in Plate 4.1. The isolated genomic DNA of the selected Nigeria indigenous chicken breeds indicated high quality and appeared as single bands without sheared fragments.

4.1.2 PCR amplified product of *cGH* of selected chicken breeds

The agarose gel electrophoresis results of the PCR amplified *cGH* of selected chickens in Nigeria is presented in Plate 4.2. Amplification of specific DNA fragments of the *cGH* gene was successfully performed for the total samples using the designed primer pair (forward and reverse) under PCR condition explained earlier. In the entire samples tested, the amplified products appeared as clear single bands, and occupied the appropriate position with approximately 700 bp of amplicon size, with no variation in sizes between the chicken breeds studied.

4.1.3 PCR-RFLP amplified product of *cGH* of selected chicken breeds

The agarose gel electrophoresis result of the PCR-RFLP analysis amplified products of intron 3 region of *cGH* of selected chicken breeds is presented in Plate 4.3. The banding pattern of the present investigation revealed three RFLP variants which were arbitrarily assigned as CC, CT

and TT genotypes. The CC, CT and TT genotypes were detected by the presence of two bands, three bands and one band, respectively.

4.1.4 Frequency of RFLP patterns of *cGH* gene in selected chicken breeds

The frequencies of the different RFLP fragments at intron 3 of the *cGH* gene of selected Nigerian chicken breeds are presented in Table 4.1. It was found that out of the 49 chickens, for which readings were obtained, 15 birds had CC genotype, 22 birds had CT genotype and 12 birds had TT genotype, respectively. It was observed from the investigation that 30.6 % of the studied birds had CC genotype, 44.9 % had CT genotype while 24.5 % had TT genotype.

4.1.5 Genotype and gene frequencies of *cGH* gene in selected chicken breeds

In the population of the selected chicken breeds studied, the genotype and gene frequencies with respect to intron 3 of the *cGH* gene were calculated and shown in Table 4.2. The genotype frequencies were found to be 0.306, 0.449 and 0.245 for CC, CT and TT genotype, respectively. The allele frequencies of the C and T allele were 0.531 and 0.469, respectively.

4.1.6 Hardy-Weinberg equilibrium at intro 3 of *cGH* gene

Hardy-Weinberg equilibrium at intro 3 of *cGH* gene were calculated and depicted in Table 4.3. The genotype CC, CT and TT having 15, 22 and 12 for the observed individual and 14.028, 23.944 and 11.028 for the expected individual respectively. The calcalated Chi-square value (0.312).

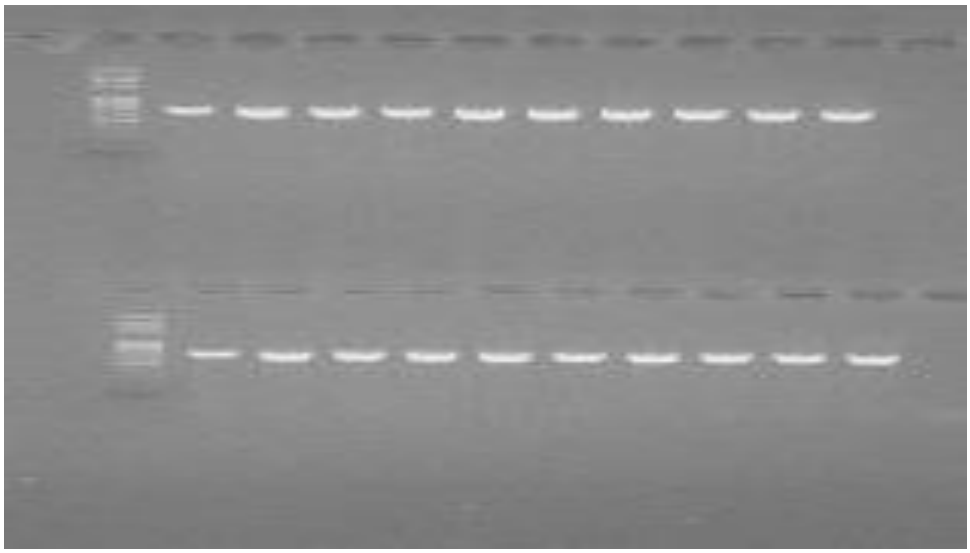


Plate 4.1: Genomic DNA isolated from selected chicken breeds in Nigeria

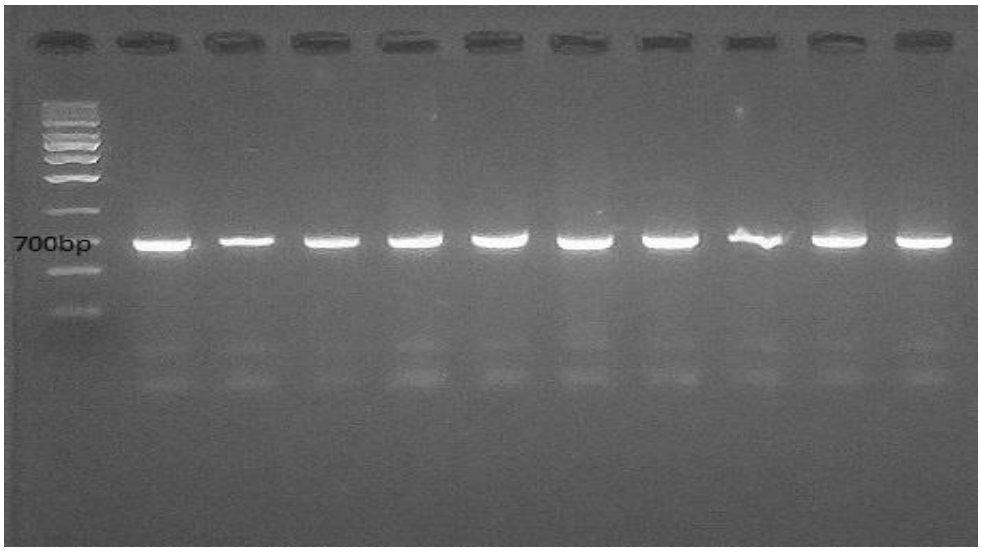


Plate 4.2: PCR amplified products of intron 3 region of *cGH* of selected Nigerian chicken breeds

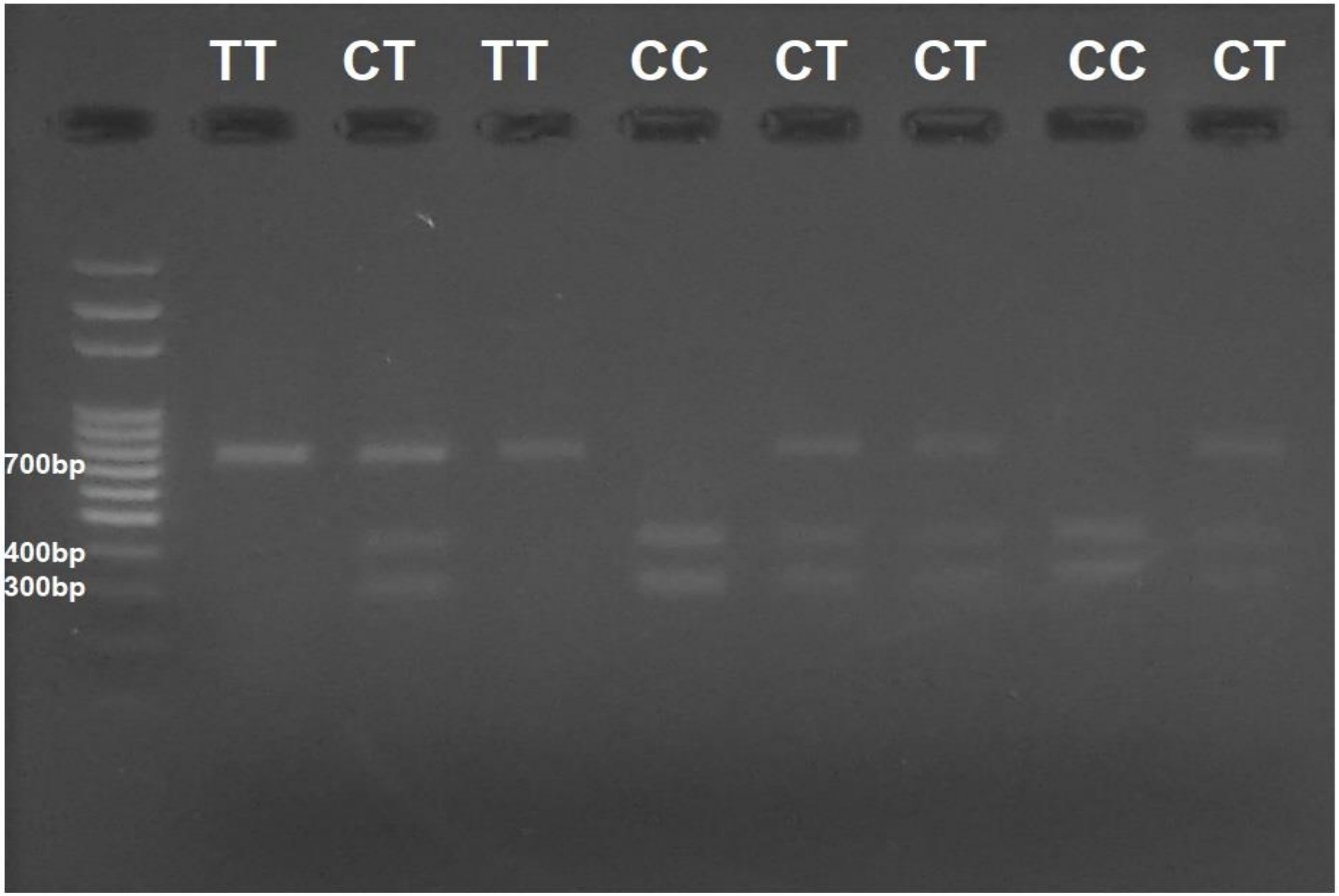


Plate 4.3: Genotype of amplified products of intron 3 region of cGH of Selected Nigerian chicken breeds

Table 4.1: Frequency of RFLP patterns at intron 3 region of *cGH* gene in selected chicken breeds

Pattern	Genotype	Frequency	Percentage
Two bands	CC	15	30.6
Three bands	CT	22	44.9
One band	TT	12	24.5

Table 4.2: Genotype and allelic frequencies at intron 3 region of *cGH* gene in selected chicken breeds

Genotype	Numbers	Frequency
CC	15	0.306
CT	22	0.449
TT	12	0.245
Allele		
C	52	0.531
T	46	0.469

Table 4.3: Hardy-Weinberg equilibrium at intro 3 of *cGH* gene

Genotype	Observed Individuals	Expected Individuals	$\frac{(O-E)^2}{E}$	X^2
CC	15	14.028	0.067	0.312
CT	22	23.944	0.158	

TT	12	11.028	0.087

4.1.7 Pattern of single nucleotide variants (SNVs) and frequencies across regions of interest

The pattern of SNVs and haploid frequencies across regions of interest is presented in Figure 4.1. The pattern indicated that *cGH* for the selected Nigerian chicken breeds were observed in exon 4, intron 3, exon 3 and intron 2. The exons were located on chromosome 4036303 and 4036899, while a large proportion of the introns were located on chromosome 4036558.

4.1.8 Identification of restriction enzyme for genotyping *cGH*

The possible restriction digestion enzymes that could be used in genotyping the *cGH* were identified and presented in Figure 4.2. This indicated all the enzymes that could be used across the region of interest and also at locus (27_4036554) (C/G/T).

4.1.9 Single nucleotide variants and frequencies detected in the *cGH* of the selected chicken breeds

The variants and frequencies detected in the *cGH* of the selected chicken breeds are presented in Tables 4.4a and 4.4b. Forty seven SNVs were identified. Most of the SNVs were located in intron 3. Six of the exons having two missense variants, two synonymous variants, one frame shift variant and one missense splice region variant. The frequencies of the variants ranged from 0.091 to 0.909.

4.1.10 Genotypes on sequencing chromatogram

The genotypes on sequencing using chromatogram are presented in Figure 4.3. This showed the sequence patterns at which the locus of interest (27_4036554) was cut. These indicate homozygote sites for C, heterozygote site for CT, and homozygote sites for T, respectively.

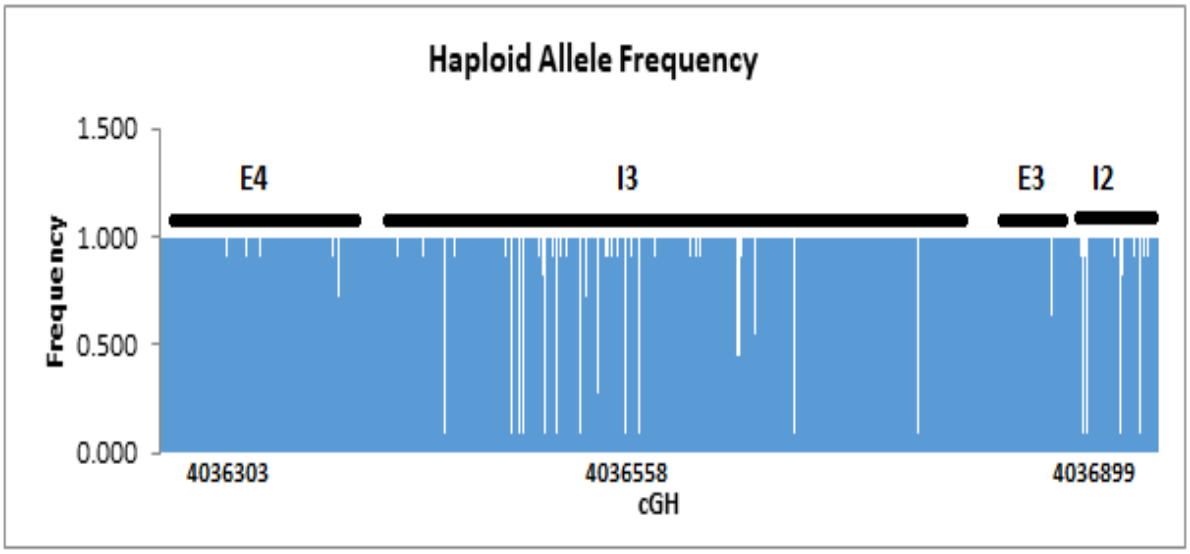


Figure 4.1: Pattern of SNVs and frequencies across region of interest

Key: E4 – exon 4, I3 – intron 3, E3 – exon 3, I2 – intron 2.

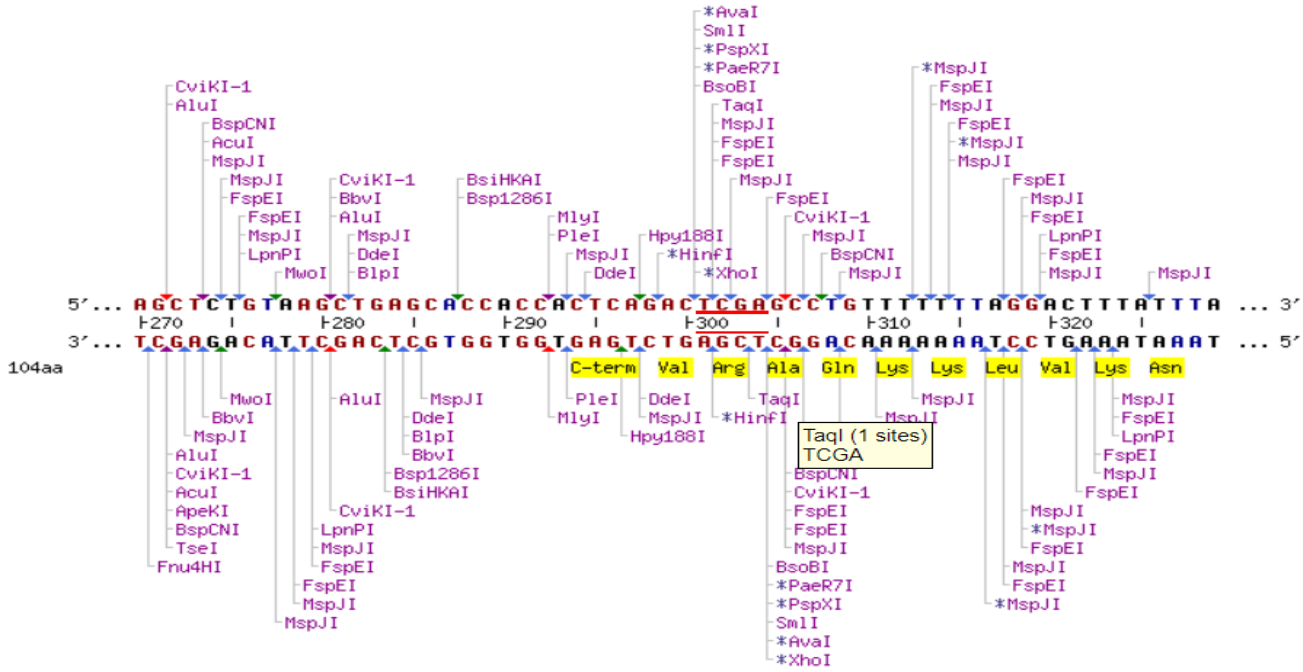


Figure 4.2: Possible restriction enzyme that could be used for genotyping

Table 4.4a: Single nucleotide polymorphisms of *cGH* of selected indigenous chicken breeds in Nigeria

Locus	Type of mutation	SNP	Allele frequencies
4036303	missense_variant	C /A	0.909/0.091
4036317	frameshift_variant	A/-	0.909/0.091
4036325/4036326	synonymous_variant	-/ T	0.909/0.091
4036381	synonymous_variant	G/A	0.909/0.091
4036384	missense_variant,splice_region_variant	G/ C	0.727/0.273
4036429	intron_variant	G/T	0.909/0.091
4036448	intron_variant	A/T	0.909/0.091
4036463	intron_variant	A/ C	0.909/0.091
4036470	intron_variant	G/ C	0.909/0.091
4036509	intron_variant	G/ A	0.909/0.091
4036512	intron_variant	G/A/ C	0.818/0.091/0.091

4036517	intron_variant	G/ A	0.909/0.091
4036519	intron_variant	G/ A	0.909/0.091
4036530	intron_variant	A/ T	0.909/0.091
4036532	intron_variant	G/ C/ T	0.818/0.091/0.091
4036537	intron_variant	G/ C	0.909/0.091
4036539	intron_variant	A/ C	0.909/0.091
4036542	intron_variant	A/ C	0.909/0.091
4036545	intron_variant	A/ C	0.909/0.091
4036554	intron_variant	C/ G/ T	0.545/0.091/0.364
4036558	intron_variant	C/ T	0.727/0.273
4036566	intron_variant	T/ C	0.727/0.273
4036570	intron_variant	G/ A	0.909/0.091
4036571	intron_variant	G/ A	0.909/0.091
4036573	intron_variant	C/ T	0.909/0.091
4036577	intron_variant	A/ T	0.909/0.091
4036581	intron_variant	A/ T	0.909/0.091
4036585	intron_variant	T/ G	0.909/0.091
4036591	intron_variant	T/ A	0.909/0.091
4036601	intron_variant	T/ C	0.909/0.091

Table 4.4b: Single nucleotide polymorphisms of *cGH* of selected indigenous chicken breeds

Locus	Type of mutation	SNP	Allele frequencies
4036628	intron_variant	G/ C	0.909/0.091
4036631	intron_variant	T/ G	0.909/0.091
4036633	intron_variant	T/ G	0.909/0.091
4036662	intron_variant	A/ C/ G	0.455/0.091/0.455
4036663	intron_variant	G/ T	0.909/0.091
4036672	intron_variant	C/ T	0.455/0.545
4036702	intron_variant	G/ A	0.909/0.091
4036797	intron_variant	G/ A	0.909/0.091
4036921	intron_variant	C/ G	0.909/0.091
4036921/4036922	missense_variant	-/ T	0.909/0.091
4036922	intron_variant	T/ C/ G	0.545/0.364/0.091
4036942	intron_variant	G/ T	0.909/0.091
4036945	intron_variant	G/ A/ C	0.818/0.091/0.091
4036954	intron_variant	G/A	0.909/0.091
4036957	intron_variant	C/ G	0.909/0.091
4036959	intron_variant	T/ G	0.909/0.091
4036960/4036961	intron_variant	-/ G	0.909/0.091

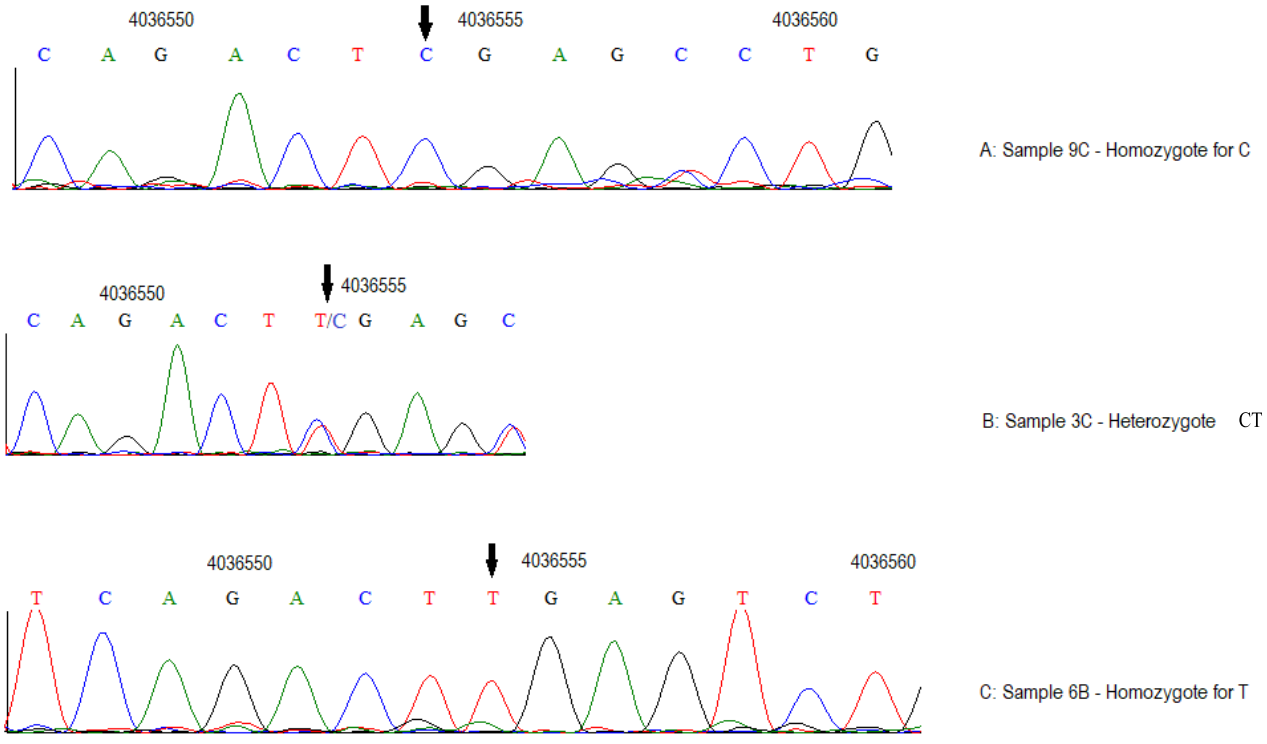


Figure 4.3: Genotypes on Sequencing Chromatogram

4.2 Discussion

4.2.1 PCR-RFLP amplified products of *cGH* of selected Nigerian chicken breeds

The amplified products were consistent with the target fragments and had a good specificity which could be directly analyzed through PCR-RFLP technique. The present findings at intron 3 of the *cGH* gene regarding the base pair size is in agreement with the reports of Mu and Lumatauw (2013) who reported on intron 4 of Indonesia native chickens population, and Bingxue *et al*. (2003) in F_2 chickens derived from broilers x Silky crossing in China, respectively. However, Shahnaz *et al*. (2008) reported 1200 bp in Bantam and White Leghorn chickens of India; Thakur *et al.* (2009) reported 1216 bp in Kadaknath chicken; Khoa *et al.* (2013) reported 563 bp in Vietnamese local breeds of chicken; Makhsous *et al*. (2013) reported 1164 bp in Fars native chicken of Iran; Rahmadani *et al.* (2014) reported 367 bp in Indonesia local chicken breeds and Saikhom *et al.* (2017) reported 713 bp in Haringhata Black chicken. The differences observed in the bps of the PCR fragments suggest the possibility of insertion/duplication of the genetic bases, which can only be confirmed by sequencing.

4.2.2 Frequency of RFLP patterns of *cGH* gene in selected Nigerian chicken breeds

The frequencies (30.6 % for CC genotype, 44.90 % for CT genotype, 24.50 % for TT genotype) of the different RFLP fragments observed at intron 3 of the *cGH* gene (Table 4.1) is in agreement

with the reports of Bingxue *et al*. (2003) who reported three genotypes: AA, AB and BB in a population of hybrid chickens (broilers x Silky) in China. Also, Tanmankaurd *et al.* (2008) analyzed a 776 bp amplicons of *cGH* gene digested with *Msp*1 with their results revealing three different genotype patterns (AA, AC and CC) created by a combination of polymorphic *Msp*1 cut sites present in exon-1 and intron-1, respectively. The result however is in contrast with the findings of Thakur *et al.* (2009) and Kulibaba (2015) who observed only two genotypes (AA and AB) in Kadaknath chickens, and AB and BB in local chicken breeds of Vietnam respectively. The differences observed in the frequencies and genotypes of the variants could be adduced to the chickens' environment. Different environments mean that the chickens will have to mutate to better suit themselves in other survive.

4.2.3 Genotype and gene frequencies of *cGH* Gene in selected Nigerian chicken breeds

The genotypic frequencies observed for the birds (0.306 for CC, 0.449 for CT and 0.245 for TT, respectively) is not in agreement with the reports of Bingxue *et al*. (2003) who also reported three distinct genotypes (AA, AB, BB) with genotypic frequencies of 0.759, 0.213 and 0.027, respectively in a population of hybrid chickens. Thakur *et al.* (2009) and Kulibaba, (2015) however, reported two genotypes (AA, AB) in Kadaknath, and AB and BB in local chicken breeds of Vietnam, respectively; the genotype frequencies of the Kadaknath and local Vietnam chickens were 0.4151 and 0.5849, and 0.070 and 0.930, respectively. The results of Makhsous *et al.* (2013) showed that six genotypes (AA, AB, AC, BB, BC, CC) exist in native chickens of Iran. The genotypic frequencies of 0.221, 0.576, and 0.203, were reported for AA, AG, and GG genotypes in Gushi and Anka native chickens of China (Wang *et al.*, 2015)**.** Wang *et al.* (2015) also reported that, the frequencies of the alleles A and G, and that the AA genotype frequency was as the GG genotype.

4.2.4 Single nucleotide polymorphisms and frequencies detected in the *cGH* of selected chicken breeds

Forty seven (47) SNVs was observed at the intron 3 of the *cGH* gene for selected chicken breeds studied. The result is in agreement with the reports Nie *et al.* (2005) who found forty six SNPs in the Leghorn (L), White Recessive Rock (WRR), Taihe Silkies (TS) and Xinghua (X) chicken breeds they studied. Most of the SNPs (36 of 46) were also located in the introns, with four in the 5#UTR, one in the 3#UTR, and five in the coding exons. Two of the five coding SNPs led to amino acid changes. All 46 SNPs were nucleotide substitutions and transitions; 38 occurred more frequently than transversions (8). One of two nonsynonymous coding SNPs (Gþ951A) altered an amino acid in the *cGH* precursor (A13T), and the other (Gþ1532A) changed an amino acid in the mature *cGH* (R59H). Unfortunately, the current study did not extend to amino acids changes brought about by to the SNPs changes due to indels. The high diversity of the *cGH* gene appears to be confirmed in a preliminary genome-wide scan for chicken SNPs that reported 2.8 million SNPs across the whole chicken genome, with 23 SNPs located in the region (chr27: 141644–145748) of the *cGH* gene (International Chicken Polymorphism Map Consortium, 2004) (ICPMC, 2004).

CHAPTER FIVE

5.0 CONCLUSION, RECOMMENDATIONS AND CONTRIBUTIONS TO KNOWLEDGE

5.1 Conclusion

Based on the results obtained from the study, the following conclusions could be drawn:

The study revealed three RFLP variants which were arbitrarily assigned as CC (homozygote dominant), CT (heterozygote) and TT (homozygote recessive) genotypes.

The genotypic frequencies were found to be 0.306, 0.449 and 0.245 for the CC, CT and TT genotype, while allelic frequencies of the C and T allele were 0.531 and 0.469, respectively.

Forty seven (47) SNVs was observed at the intron 3 of the *cGH* gene for selected chicken breeds studied. Six numbers of SNPs were discovered at exons having four missense variant (locus 4036303, 4036384 and 4036921/4036922) with (8.51 %), two synomous variant (locus 4036325/4036326, 4036381) with (4.26%), one frame shift variant (locus 4036303) with (2.13 %) and one splic region variant (locus 4036384) with (2.13 %), while the rest SNPs were at the introns with (87.23 %).

5.2 Recommendations

From the research studied, it can be recommended that:

The obtained data can be used in targeted breeding of Nigerian indigenous chickens in order to get offspring's with desirable genotypes, which in combination with classical breeding methods; will make it possible to unlock the productive potential of the chickens to maximum effect.

The association between the genotypes of the chickens with productive traits of interest should be studied with the aim of using them as markers for effective selection. This way, it will be possible to direct selection schemes to favour the desired genotypes for improved growth rate and other related parameters in chicken.

5.3 Contributions to Knowledge

The study revealed three RFLP variants which were arbitrarily assigned as CC (homozygote dominant), CT (heterozygote) and TT (homozygote recessive) genotypes. The genotypic frequencies were found to be 0.306, 0.449 and 0.245 for the CC, CT and TT genotypes. The allelic frequencies for the C and T alleles were 0.531 and 0.469 respectively. Forty seven (47) Single Nucleotide Variants (SNVs) were observed by the intron 3 of the *cGH* gene of the selected chicken breeds studied. Six Single Nucleotide Polymorphisms (SNPs) were discovered at the exon studied having four missense variants (at locus 4036303, 4036384, 4036921/4036922) representing 8.51 % of the total polymorphism; two synomous variants (at locus 4036325/4036326, 4036381) representing 4.26 % of the total polymorphism; one frame shift variant (at locus 4036303) representing 2.13 % of the total polymorphism, and one splice region variant (at locus 4036384) also representing 2.13 % of the total polymorphism. The remaining SNPs were observed at the intrinsic (87.23 %).

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