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Comparative Investigation of Polymorphism in Growth hormones mRNA of FUNAAB Alpha and Ross 308 broiler Chickens

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Abstract

This study aimed to compare the polymorphisms of growth hormone mRNA of FUNAAB Alpha and Ross 308 broiler chickens. Liver tissues were harvested from 20 randomly selected birds (5 per sex comprising of 10 birds per breed)) from the population of 100 birds reared for the purpose of this study at Teaching and Research Unit of the Department of Animal Science, University of Uyo and a total RNA was extracted, reverse-transcribed to cDNA, and sequenced. Bioinformatics analyses were conducted to identify single nucleotide polymorphisms (SNPs), estimate genetic diversity, and assess differentiation between breeds. Results showed Polymorphic sites detected in the GH mRNA of both breeds. Ross-308 exhibited higher haplotype diversity (Hd = 0.833), while FUNAAB Alpha showed slightly greater nucleotide diversity (π = 0.00390). Genetic differentiation between the breeds was moderate (FST = 0.289), with limited gene flow (Nm ≈ 0.6), indicating distinct evolutionary trajectories. The findings confirmed that the GH gene is polymorphic, even in a highly selected commercial line, and a unique genetic architectures between the two breeds were observed. This study provides functional insight into GH mRNA variation and underscores the importance of transcriptional-level analysis in understanding the genetic basis of growth performance. The evidence of moderate differentiation and unique diversity patterns should be considered by animal breeders in their breeding programs. For the FUNAAB Alpha breed, deliberate management of its genetic diversity could be important to maintain its unique traits and avoid excessive inbreeding.

Keywords: Evolutionary relationship; FUNAAB Alpha; Genetic diversity; growth hormone mRNA; Ross 308 breed

1 Introduction

Ross-308 broiler is a high-performing hybrid breed developed by Aviagen and known for its exceptional growth rate and feed efficiency, breast muscle yield traits largely governed by the Somatotropic axis, with growth hormone (GH) playing a central role.

Poultry industry stands as a critical pillar of food security providing animal proteins through meat and eggs to a growing world populace. Broiler chicken production is characterized by intensive genetic selection for economically vital traits, specifically growth rate, feed efficiency, and breast muscle yield [1]. This accelerated growth phenotype is a complex polygenic trait, interplayed in various physiological systems, like digestion, metabolism, and endocrine signaling. At the heart of this regulatory network is the somatotrophic axis, comprising the hypothalamus, pituitary gland, and liver, which governs post natal growth. The central effector molecule of this axis is growth hormone (GH) or somatotrophin. GH is a single-chain polypeptide hormone synthesized and secreted by the somatotroph cells of the anterior pituitary gland [2]. Its physiological roles are multifaceted, extending beyond the classic promotion of linear bone growth to include profound effects on protein, carbohydrate, and lipid metabolism. The cGH gene is located on chromosome 27 and consists of five exons and four introns, a structure conserved across many vertebrate species [3]. The process of gene expression involves the transcription of DNA into a precursor messenger RNA (pre-mRNA) molecule, which

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includes both exons (coding) and introns (non-coding) sequences. This pre-mRNA undergoes a crucial maturation process called splicing, where introns are removed, and exons are ligated together to form the mature mRNA transcript. This mature mRNA is then translated into the functional GH protein [4].

Polymorphisms can occur in various forms: single nucleotide polymorphisms (SNPs), insertions, deletions variable number tandem repeats (VNTRs) [5]. They could be located in coding regions (exons), non-coding regions (introns, promoters, untranslated regions - UTRs), or at splice sites. The location and nature of a polymorphism dictate its potential functional consequences.

A polymorphism within an exon that changes the amino acid sequence (a non-synonymous SNP) could directly alter the structure, stability, or function of the resulting GH protein [6] while a synonymous SNP, one that does not change the amino acid sequence due to the degeneracy of the genetic code, can still have functional implications by influencing mRNA stability, secondary structure, or the rate and efficiency of translation [7]. For example, research has identified polymorphisms in the cGH gene associated with body weight, egg production, and carcass traits in various chicken breeds, including native breeds and some commercial lines [8]. A study by Nie *et al*. [9] reported significant associations between certain cGH gene SNPs and growth traits in commercial broilers.

At DNA-level polymorphisms in cGH have been studied in various chickens, a focused investigation on the polymorphism present in the actual expressed mRNA of the high-performing Ross-308 line is needed and the FUNAAB Alpha chickens. This approach will provide a functional information of the gene's status, identifying any variation in the final message that codes for the hormone, which could have direct implications for protein function, growth efficiency, and the continued genetic management of this vital agricultural animal. In view of this knowledge is required in comparing of Polymorphism in Growth hormones mRNA of FUNAAB Alpha and Ross 308 broiler Chickens in this study.

2 Materials and methods

2.1 Experimental site

The study was conducted at the Poultry Breeding Unit of the Teaching and Research Farm of the University of Uyo, Uyo. Uyo is situated in Akwa Ibom State, the South-South region of Nigeria. It is located within latitude 4°31'N and 4°50'N, and longitude 7°31'E and 4°50'W. The area is a tropical Zone. It has an annual rainfall 3000 mm. Its elevation is approximately 38m above sea level, relative humidity of 79%, and an average temperature of 28°C [10].

2.2 Experimental animals

A total of 100 day old chicks, 100 FUNAA/B Alpha of 50 male and 50 female were purchased from the Federal University of Agriculture, Abeokuta, Ogun State.

2.3 Housing and management of experimental animals

These birds were reared intensively but partitioned into different pens according to their sex and breed. The brooding pen was constructed 30cm x 50cm each pen could accommodate 5 chicks till termination of the period of 8 weeks. The brooders were cleaned, washed, disinfected and fumigated for one week before the arrival of the chicks. Wood shavings were laid for beddings and changed weekly. Electricity and lamps were used as heat sources. The pen was fully ventilated. Drinkers and feeders were washed, disinfected, rinsed, and dried before usage. On the arrival, the chicks were given glucose water to boost their energy level, they were vaccinated against Newcastle disease. The chicks were winged-tagged for easy identification before they were placed in their allotted pens. The chicks were brood for 3 weeks and starter ration (22% Crude Protein and 2900 Kcal/Kg Metabolizeable energy) was given with clean water *ad libitum* on the 4th week birds were taken to the rearing pen and grouped according to breed and sex, finishers feed (19.41% Crude Protein and 3200 Kcal/Kg Metabolizable energy) were given to the birds till termination period of 8 weeks. At day one, I/O (Intraocular vaccine) was administered against Newcastle disease, then Gumboro vaccine was given at 3rd weeks to fight viral diseases. Antibiotics like Antiviral, Coccidiostat were administered to birds against through Coccidiosis.

2.4 Data Collection

2.4.1 Growth Data

On arrival, the chicks' were weighed for initial body weight (BW) with SF-400 sensitive scale (with 10000×10g/35oz×0.1oz capacity) before water and feed was given. Canny electronic weighing scale (with a maximum capacity of 150kg) as used after the brooding stage. This was done weekly for 8 weeks of the termination of the research. Linear body parameters were measured weekly with tailor's tape. The parameters were: BL, body length (distance from neck joint to the tip of the tail); SL; shank length (distance from the knee joint to the tip phalanges); SC; shank circumference (circumference of the shank at its widest point); BG; breast girth (circumference of the chest at the widest part); KL; keel length (distance from the base of the breast to the end of the sternum); DS; drumstick length (distance from the hook joint to the end of the knee); DC; drumstick circumference (circumference of the drumstick at its widest point) and WL wing length (from the shoulder joint to the tip of the wing).

2.4.2 Genomics Data

Tissue Harvesting

A total of 20 chickens, comprising of 5 males and 5 females of 10 birds from the population of FUNAAB Alpha and Ross 308 broiler breeds of chickens were randomly selected for tissue harvesting (liver) at 8 weeks for gene expression profiling of Chicken Growth Hormone (GH) gene. The randomly selected chickens were slaughtered, a piece of liver sample was quickly cut and immediately dropped into a labelled *Eppendorf* tube containing RNA/DNA shield to avoid RNA degradation and stored in ice box of -4°C, then taken to Inqaba biotec™ Subunit at Ibadan for RNA extraction and qPCR Amplification.

RNA Extraction

The manufacturer's protocol guide was followed strictly for the extraction, an animal tissue RNA purification kit (Zymo Research Quick-RNA MiniPrep Plus Kit) was used. In preparation for the extraction, the RNA wash buffer was prepared by mixing 48 mls of RNA wash buffer concentrate with 208 (ml) of 95% ethanol. Lyophilized DNase 1 was reconstituted in RNase-free water by gently vortexing 250U with 275µl DNase/RNase-free water and was then placed on ice. Lyophilised Proteinase K was immediately placed on ice after being reconstituted at a concentration of 20 mg/ml using 1.04 ml of Proteinase K storage buffer. DNA/RNA shield (2× concentrate) and Nuclease-free water at 1:1 ratio to make a 1× solution. Four fundamental steps were followed in performing the RNA isolation which include: Sample Homogenization and lysis, Cell lysis, sample clearing, and gDNA elimination, DNase 1 Treatment, and only Purification and elution of total RNA. Efficient disruption of the starting materials (liver tissues) was achieved using RNase-free mortar and pestle. The RNase-free mortar and pestle was pre-chilled in liquid nitrogen. The frozen and preserved tissues were thawed, weighed (25mg) and ground in 500µl of RNA shield. After adding 15 µl of the reconstituted proteinase K and 30 µl of PK digestion buffer, the samples were vortexed quickly. After that, samples were incubated for 30 minutes at 20 - 30°C. Samples were centrifuged for 30 seconds at 16,000 rpm to eliminate particle matter from homogenized tissue, and the resulting supernatants were then moved into a tube devoid of nuclease. To ensure adequate mixing, RNA lysis buffer was added to the supernatant at a 1:1 ratio and vortexed. To eliminate most of the genomic DNA, samples lysed in RNA lysis solution were put onto a yellow Spin-away filter and centrifuged. The flow-through was retained in situ and 95-100% ethanol was added to it after which it was vortexed for proper mixing. The samples were then transferred into the Zymo-Spin IIIG column (green) in a collection tube and centrifuged. Flow-through was discarded and green collection tubes were transferred into well labelled nuclease-free tubes. The green column was centrifuged after being washed with 400 µl of RNA wash buffer. Flow through was thrown away. 75µl DNA digestion buffer and 5µl DNase 1 (1U/µl) were thoroughly mixed together and added into the column matrix, it was then incubated for 15 minutes at 20-30°C. 400 µl RNA prep buffer was added to the column and centrifuged and flow-through discarded. 700 µl RNA wash buffer was added to the column and centrifuged and flow-through discarded. 400µl RNA wash buffer was added again to the column and centrifuged for 1 minute to ensure complete removal of the wash buffer. Column containing RNA was transferred into a nuclease - free tube. After adding 50 µl of DNase/RNase-Free water to the column matrix, it was centrifuged for one minute at 16,000 x g. To ensure optimal elution, this procedure was carried out twice.

RNA purity and concentrations check of GH gene

The Nano-drop spectrophotometer was used to measure the concentration and purity of the RNA samples. After using lint-free tissue to gently clean and buff the pedestals, 2µl f blanking solution (Nuclease Free Water) were put onto each pedestal, and the blank measured. Following the measurement of the blank, the pedestal was cleaned, and 2µl of RNA sample were put onto it. The optical density ratios of 260/280nm and 260/230nm were then determined for each

sample. The ideal 260/280nm value is (geq 1.70). The 260/280nm ratio indicates the extent to which samples are free from contaminating protein. Protein contamination is indicated by values below 1.70. A sample's purity from salts and other impurities can be determined using the 260/230nm ratio. Values (geq 2.0) is the ideal 260/230nm value, less than 2.0 values suggest the presence of contaminants [11].

Reverse transcription (cDNA synthesis)

Reverse transcription reaction of the RNA samples using primer for cGH gene was performed by converting only the RNA nucleotides that corresponded to protein-encoding genes into complementary DNA (cDNA). As a fraction of total cellular RNA from liver, messenger RNA (mRNA) which is the RNA that codes for the protein sequence, was isolated and purified. With the aid of an RNA-dependent DNA polymerase enzyme known as reverse-transcriptase, mRNA was converted into a cDNA template during the reverse-transcription process. Reverse transcription (RT) was performed using the LumScript RT Super Mix Kit in accordance with the manufacturer's convention. The RT was performed on the extracted tissue sample as indicated in Tables 1 and 2

Table 1 LumScript RT Supermix kit Reaction Methodology for cDNA Synthesis

Components	Per 20µl RXN	Final concentration
LunaScript RT	4µL	1×
SuperMix		(5×)
RNA sample	1µL	(up to 1 µg)
Nuclease-free water	15µL	

Table 2 cDNA Synthesis Incubation Steps

Step	Temperature	Time
Primer annealing	25 °C	2 minutes
cDNA synthesis	55 °C	10 minutes
Heat inactivation	95 °C	1 minute

Primer Design for PCR Amplification

Primers were designed and were used to amplify the GH gene and housekeeping gene (GAPDH gene). The sequences of the primers for the amplification are presented in Fig.1

Product Name Sequence of Forward (F) and Reverse (R) Primers

GH	(F: (5′ATCCCCAGGCAAACACCCTC-3′)
	R: (5′CCTCGACATCCAGCTCACAT-3′)
GAPDH	F: (5′GAGAAATTGTGCGTGACATCA-3′)
	R: (5′CCTGAACCTCTCATTGCCA-3′)

Figure 1 GH and GAPDH primer sequences

qPCR Reaction of GH gene

Quantitation of reverse transcription product (cDNA) for the GH gene was done using Luna Universal qPCR master mix. The qPCR reaction was also prepared according to manufacturer's instruction as stated in tables 3 and 4. The Bio Rad CFX96 Real-time thermal Cycler was used to run the qPCR reaction. The critical result was the final quantity of Amplicon produced after the process which was read following fluorescent detection in the Real-time mainline. The RT-PCR uses

the linearity of DNA amplification to determine absolute or relative amounts of a known sequence in a sample. By using a fluorescent reporter in the reaction, the amplicons were detected.

Table 3 qPCR Protocol of Luna Universal qPCR Master Mix

Components	20 µl reaction	Final concentration
Luna universal qPCR mix	10 µl	1×
Forward primer (10µM)	0.5 µl	0.25 µM
Reverse primer (10µM)	0.5 µl	0.25 µM
Template DNA	1 µl	<100ng
Nuclease free water	8 µl	

Table 4 Real-Time qPCR (RT-qPCR) incubation steps

Cycle Step	Temperature	Time	Cycles
Initial denaturation	95°C	60 Secs	1
Denaturation	95°C	15 Secs	40±5
Annealing/Extension	60°C	30 secs	(+plate read)
Melt curve	60-95°C	Varied	1

Bioinformatics analysis

After sequencing of GH mRNA, the sequences were viewed in Bioedit and BLAST against GenBank at the National Centre for Biotechnology Information, U.S. This was done to check quality and similarity of the sequences with cGH sequences from other breeds of chicken. The sequences were trimmed and filtered for quality control before identification of single nucleotide polymorphism. Reference sequence of cGH gene was downloaded and aligned against GH sequences from this study to identify single nucleotide polymorphism presence among the sequences. The sequences were submitted to Genbank and accession numbers were given as: PV392654, PV392665, PV392656, PV392657 PV392658, PV392659, PV392660 PV392661. The frequency and distribution of the identified single nucleotide polymorphisms were estimated using DNAsp software. Genetic diversity indices of the sequences were estimated using R software. The single nucleotide polymorphisms identified were used in variant prediction effect using ENSEMBL. The sequences were translated to protein sequences.

2.5 Statistical Analysis

2.5.1 Descriptive statistics

Descriptive statistics, including measures like mean, median, standard deviation, and range, was computed to summarize the characteristics of the experimental data. All data collected on growth performance traits were presented as means ± SEM (standard error of the mean).

2.5.2 Association analysis

After a preliminary analysis of 2 by 2 factorial experiments to account for effects of sex, breed and SNPs and their interactions on growth performance traits, interaction effects (First and Second levels) were not significant. The data were then subjected to three-way analysis of variance using the Generalized Linear Model procedure of SAS ver. 9.2 [12]. Differences between means was determined using Least square means (General Linear Model Procedure) and Duncan's multiple range test as a post hoc test at a significance level of 0.001.

The statistical Model used was:

$$Y_{ijkl} = + B_i + S_j + S_k + BS_{ij} + SS_{ik} + BSS_{ijk} + \varepsilon_{ijkl}$$

Where: Y_{ijkl} is the individual observed trait,

μ = population mean,
B_i = the fixed effect of ith breed (2- FUNAAB Alpha and Ross 308),
S_j = the fixed effect of the jth sex (2- male and female)
S_k = the fixed effect of the kth SNP haplotype (4- Hap. 1, Hap. 2, Hap. 3 and Hap. 4)
BS_{ij} =Interactive effect of breed and sex
SS_i = Interactive effect of sex and SNPs
BSS_{ijk} = Interactive effect of breed sex and SNPs
ε_{ijkl} = the random residual errors.

3 Results and discussion

This findings present the molecular analysis of the growth hormone (GH) mRNA in FUNAAB Alpha and Ross-308 broiler chickens.

3.1 Identification and Comparison of Polymorphic Sites in FUNAAB Alpha and Ross-308

The findings of this study confirmed that the GH gene is polymorphic at the mRNA level in both FUNAAB Alpha (locally selected) and Ross-308 broiler (commercially selected) Chicken breeds. Distinct patterns of variation between the two broiler breed studied reflected in the multiple sequence alignment observed which confirmed genetic variations within this GH gene in both broiler breeds as showed in Fig 1. The Sequence of FUNAAB Alpha breed revealed 3 Polymorphic Site which are the specific Nucleotide positions observed among the four samples sequenced. These three variable sites combined to define two distinct haplotypes, which are unique combinations of alleles along a chromosome while the sequence from Ross 308 broiler chickens breed showed 2 Polymorphic sites with 3 unique haplotypes detected from the same number of samples sequenced. The presence of Polymorphisms in both breeds is a significant finding in this study. The higher number of segregating sites (3 versus 2) in the FUNAAB Alpha breed suggests a broader spectrum of underlying nucleotide variation within the sampled population of this local breed. This aligned with the expectation that local breeds, subjected to less intense and more multifaceted selection pressures (including adaptation to local environments and diseases), might retain a wider array of historical mutations while Ross 308 breed which are intensively selected over years for a particular traits showed a refined genetic structure which exhibited fewer variable nucleotide positions but greater number of unique haplotype combinations from this fewer site (2 versus 3).

However, the result of this study agrees with the result of De Leon *et al*. [13], who, while studying a Cobb-500 broiler line a direct competitor to the Ross-308—found that within the hyper selected commercial population, a limited number of segregating sites gave rise to more haplotypes. Nevertheless, the result of this study disagrees with the finding of Hassan *et al*. [14], who reported a significantly higher number of both polymorphic sites and haplotypes in the commercial line compared to the local breed conducted in a study of comparative analysis of GH gene polymorphism between a commercial broiler line and an indigenous breed. Hassan *et al.* [14]. The differences found in this result could be attributed to differences in breeding history which embodied around breed composition and genetic background as well as selection criteria between these broiler breeds compared in Hassan's study and that of this study (Ross-308; FUNAAB Alpha) which capitalized on heterosis and aggregate favourable alleles. Secondly, the FUNAAB Alpha is not a primitive indigenous breed but a locally developed and selected strain, its breeding history might involve a different founding population and selection criteria, leading to a unique pattern of genetic variation where a few, but highly divergent, mutations are preserved.

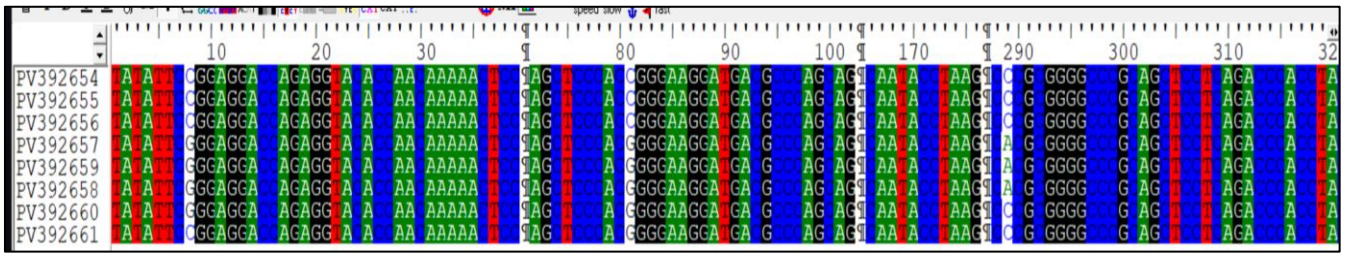

Figure 2 FUNAAB Alpha and Ross 308 mRNA sequences (the first four sequences are for FUNAAB Alpha broiler and the second four sequence are Ross 308)

3.2 Estimation of Genetic Diversity Indices in FUNAAB Alpha and Ross-308

The result present in Table 3.2 present the two indices calculated in this study which were Haplotype Diversity (Hd) and Nucleotide Diversity (π) in the two broiler breeds (FUNAAB Alpha and Ross-308).

Ross-308 breed recorded haplotype diversity (Hd = 0.833) in this study while FUNAAB Alpha recorded (Hd = 0.500). Haplotype Diversity (Hd) measures the uniqueness and frequency of haplotypes in a population, which was considered higher in the Ross-308 breed (Hd = 0.833) than in FUNAAB Alpha (Hd = 0.500). From the number of haplotype obtained in Ross-308 population, a more diverse set of GH gene sequence variants within this samples were observed. This high Hd indicates that it is unlikely to randomly pick two identical GH mRNA sequences from the Ross-308 samples while the lower Hd in FUNAAB Alpha suggests chance of sampling identical haplotypes, reflecting the presence of only two haplotypes in a small sample size.

Ross-308 breed recorded nucleotide diversity (π = 0.00303) in this study and FUNAAB Alpha recorded (π = 0.00390). FUNAAB Alpha showed a slightly higher π value (0.00390) than Ross-308 (0.00303). Nucleotide Diversity measures the average degree of genetic difference per site between any two sequences randomly chosen from the population. The results implies that the haplotypes in Ross-308 are likely more similar to each other, differing at only a couple of few positions while in FUNAAB Alpha, the fewer haplotypes are, on average, more genetically distant from one another. The mutations present in FUNAAB Alpha are more divergent, perhaps due to being older and accumulated more nucleotide changes, or originated from more distinct ancestral lineages.

The high Hd for Ross 308 in this finding agrees with the observation of Adeleke *et al*. [15] who, in a review on genetic resilience, observed that well-managed commercial poultry populations can retain significant haplotype diversity at key loci despite intense unidirectional selection for production traits. However, the result of this study disagrees with the finding of Li *et al*. [16], who observed significantly higher nucleotide diversity (π > 0.008) in the promoter region of the GH gene in Huangshan chicken breed compared to the commercial line. This could be due to differences in area of analysis. In this study, analysis was based on mRNA expression which is at exotic coding sequence (more constrained area), while Li *et al*.[16] focused on the promoter region (less constrained area) in genomic DNA. Secondly, it could be due to the specific genetic history of the FUNAAB Alpha chickens breed of this study.

The analysis of genetic differentiation between the two broiler breeds yielded critical insights into their evolutionary relationship (Table 5). The Chi-square test of neutrality was not statistically significant (p = 0.149) in this study. This suggests that, at this locus and with this sample size, we cannot reject the null hypothesis that the polymorphisms are not evolving under strong selection. The Fixation Index (FST) value of 0.289 obtained in this study indicates moderate to great genetic differentiation between the breeds at the GH locus. FST range from 0 (no differentiation) to 1 (complete differentiation), and values above 0.25 are often considered to indicate great genetic differentiation. This is further supported by the estimated number of migrants (Nm) per generation, which was approximately 0.6 as recorded in this findings. An Nm value less than 1 is an indication of limited gene flow, sufficient to allow populations to diverge through genetic drift.

The result of this study agrees with the result of Adeleke *et al. [15]* who observed a similarly moderate FST value (0.25) between two Nigerian indigenous chicken ecotypes, attributing it to geographic isolation and the action of genetic drift in small, separated populations. FST value of 0.289 of this study strongly suggests that the FUNAAB Alpha and Ross-308 breeds are on separate evolutionary trajectories with respect to the GH gene. This evolutionary separation is a direct consequence of their completely isolated and distinct breeding programs. The Ross-308 is developed and maintained in a closed, global breeding pyramid by Aviagen, with no genetic exchange with Nigerian local breeds. The FUNAAB Alpha, developed within Nigeria, has its own unique selection path. This reproductive isolation allows for the independent accumulation of breed-specific mutations through genetic drift and potentially, differential selection pressures, leading to the observed level of differentiation.

Table 5 Genetic Diversity Metrics of FUNAAB Alpha and Ross 308 broiler chickens

Metric	FUNAAB Alpha	Ross-308
Haplotype diversity (Hd)	0.500	0.833
Average differences (K)	1.500	1.167
Nucleotide diversity (π)	0.00390	0.00303
π (JC corrected)	0.00392	0.00304

The analysis of genetic differentiation between the two broiler breeds yielded critical insights into their evolutionary relationship (Table 6). The Chi-square test of neutrality was not statistically significant (p = 0.149) in this study. This suggests that, at this locus and with this sample size, we cannot reject the null hypothesis that the polymorphisms are not evolving under strong selection. The Fixation Index (FST) value of 0.289 obtained in this study indicates moderate to great genetic differentiation between the breeds at the GH locus. FST range from 0 (no differentiation) to 1 (complete differentiation), and values above 0.25 are often considered to indicate great genetic differentiation. This is further supported by the estimated number of migrants (Nm) per generation, which was approximately 0.6 as recorded in this findings. An Nm value less than 1 is an indication of limited gene flow, sufficient to allow populations to diverge through genetic drift.

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Table 6: Genetic Differentiation (Between Breeds)

Metric	Value	Interpretation
Chi-square test	p = 0.149	Not significant; no strong differentiation
Hst (haplotype-based)	0.151	Moderate differentiation
Kst (sequence-based)	0.188	Moderate sequence differentiation
Kst*, Z, Z*	Moderate	Suggest some evolutionary structure
Snn	0.625	Some clustering of sequences within populations
Fst	0.289	Moderate genetic differentiation
Nm	~0.6	Indicates limited gene flow between breeds

(Nei, 1982)

3.3 Comparison of Evolutionary Patterns in FUNAAB Alpha and Ross-308

A phylogenetic analysis was conducted using the Neighbor-Joining method to visualize the evolutionary relationships of the two broiler breeds implied by the genetic differences. This analysis constructed a tree that represents a hypothesis about the evolutionary history of the individual GH mRNA sequences (fig 3.2). The tree was built from alignment of 448 nucleotide positions, and the sum of all branch lengths which was 1.228. This indicated a low-to-moderate level of overall genetic divergence among all sequences sampled from both breeds in this study. This is consistent with the low π values observed in this study and the fact that all chickens, regardless of breed, share a relatively recent common ancestor. The topology, or the specific branching pattern interpret the relationships. While the text should provide details of the clustering of sequences, the moderate FST value of 0.289 predicts that sequences from the same breed would generally cluster together more closely on the tree than with sequences from the other breed. However, due to the shared ancestry of all domestic chickens and the potential for shared ancestral polymorphisms, some intermingling of sequences from the two breeds within the tree is also a better outcome. In a broader sense the evolutionary analysis supports the conclusion of moderate differentiation measured by the FST in this study.

Conversely, the result of this study agrees with the result of Kühnlein *et al*. [17] in their early, yet foundational, Restriction Fragment Length Polymorphism (RFLP)-based study of the GH gene. In which a diverse set of chicken populations were analyzed and found that certain GH gene alleles were shared across different, and often distantly related, populations. This made them suggest that some GH gene variants are ancient and were present in the common ancestor of these recent populations, persisting through time. This phenomenon of shared ancestral polymorphism is a strong explanation for why this study's phylogenetic tree might not show perfectly distinct, monophyletic clusters for each breed.

The haplotypes found in both Ross-308 and FUNAAB Alpha may not have arisen independently in each breed but might have been inherited from a common ancestral pool and maintained in both lineages. The purifying force of selection on this gene of interest would act to conserve these functional ancient variants, while genetic drift in the isolated breeding programs led to the differences in their frequencies, as captured by the FST value. This interplay between conserved ancestral variation and population-specific drift creates the complex pattern of diversity and differentiation we have uncovered.

This finding is not in line with the phylogenetic structure of Zhang *et al*. [18] for other economically important genes in chickens which was discovered in their genome-wide study of selection signatures, they constructed phylogenetic trees for genes related to muscle development and metabolism and found clear, distinct clustering by breed with high bootstrap support (a measure of the robustness of the tree branches). This meant that all sequences from one breed formed a distinct group separate from all sequences of another breed. The less pronounced or intermingled clustering predicted in this study for the GH gene could suggest that it is under a different evolutionary pattern. The GH gene may be under such strong purifying selection that its evolutionary rate is exceptionally slow. Many of the polymorphisms we detected might be shared ancestral variations ancient mutations that predate the formation of the modern Ross-308 and FUNAAB Alpha breeds rather than recent, breed-specific mutations. When polymorphisms are shared between breeds, it can cause their sequences to cluster together based on the shared allele rather than their breed origin, blurring the phylogenetic distinction. The specific branching pattern of this Phalogenetic tree would be key to confirming whether the moderate genetic differentiation measured by FST is visibly and clearly reflected in the evolutionary relationships of the individual sequences (Fig 3).

On the whole, this investigation into the GH mRNA of FUNAAB Alpha and Ross-308 chickens reveals a landscape of genetic variation that is more complex than might be assumed. The Ross-308 commercial line demonstrates a strategy of maintaining high haplotype diversity from a limited set of segregating sites, a finding supported by De Leon *et al*. [13] and consistent with the concept of managed genetic resilience proposed by Adeleke *et al*. [15]. The FUNAAB Alpha breed, while having fewer haplotypes, shows a tendency towards more divergent nucleotide sequences, though not to the extreme degree reported in some other indigenous breeds like the Huangshan chicken [16].

The populations are significantly differentiated (FST = 0.289), as reported in other isolated poultry populations yet the evolutionary history of the GH gene were characterized by strong purifying selection and shared ancestral polymorphisms [17]. This might have disrupted phylogenetic split between the breeds which was of a different patterns with other genes as observed by [18].

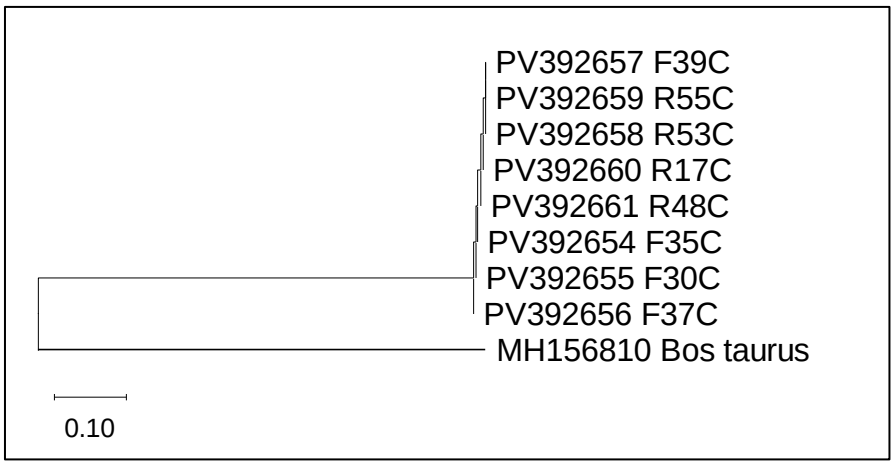

Figure 3 Phalogenetic tree of 5 taxa

4 Conclusion

In Conclusion Both Ross-308 and FUNAAB Alpha chickens possess low genetic variation (polymorphism) within the GH mRNA sequence. The two breeds exhibit distinct patterns of diversity. The Ross-308 breed demonstrates greater haplotype diversity (Hd), indicating a wider variety of genetic combinations, while the FUNAAB Alpha breed shows slightly higher nucleotide diversity (π), suggesting more divergent mutations at the nucleotide level.

Moderate genetic differentiation (FST ≈ 0.289) between the two breeds at the GH locus was observed.

Limited estimated gene flow (Nm ≈ 0.6) was observed indicating separate evolutionary trajectories, likely driven by their isolated breeding programs and genetic drift.

GH gene is relatively conserved, significant and informative genetic differences existed between the commercial Ross-308 and the local FUNAAB Alpha breeds. These differences highlighted the unique genetic architecture of each breed and their response to selection for growth traits.

Recommendation

The evidence of moderate differentiation and unique diversity patterns should be considered by animal breeders in their breeding programs. For the FUNAAB Alpha breed, deliberate management of its genetic diversity could be important to maintain its unique traits and avoid excessive inbreeding. For commercial lines, understanding this variation can help in marker-assisted selection for further genetic improvement.

Compliance with ethical standards

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Disclosure of conflict of interest

I declare that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in this manuscript.

Statement of ethical approval

This research was conducted in accordance with ethical guidelines governing the use of animals and genetic materials in scientific research as stipulated by the Ethical Committee of Animal Science, University of Uyo.

Statement of Informed consent

Author has given approval for this research to be published for there is no competing interest and nor published anywhere in the World.

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