

## Original Research Article

### Diversity of Ghrelin Gene in Nigeria's Fulani and Yoruba Ecotype Chickens

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#### ABSTRACT

Ghrelin (GHRL) is a novel 28-amino acid gut-brain peptide linked to a gene associated with regulation of growth hormones in birds. This study was carried out to investigate the polymorphism of Ghrelin gene in Fulani and Yoruba ecotypes chicken of Nigeria. Blood samples were collected from seventy-eight (78) Nigerian indigenous chickens comprising of 41 Yoruba ecotype and 37 Fulani ecotype. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was used and *MbolI* restriction (Grammatical correction) enzyme cut site 71 of the Ghrelin and genetic structure determined. Population structure was analysed using allele and genotype frequencies, heterozygosity and genetic variation metrics. Two alleles (C and T) and three genotypes (CC, CT and TT) were observed. In the Yoruba Ecotype, the allele frequencies were C (0.34) and T (0.66) respectively while C (0.45) and T (0.55) was observed in the Fulani Ecotype and the overall population C (0.39) and T (0.61). The genotype frequencies obtained were; *In* (Grammatical correction) Yoruba Ecotype, CC (0.10), CT (0.48), and TT (0.41) were observed. In Fulani Ecotype, CC (0.22), CT (0.45), and TT (0.32) were also observed, and in the overall population CC (0.15). CT (0.47) and TT (0.37) were observed. FIS values for the Yoruba Ecotype (-0.0847) and FE (0.00702) reflects random mating and inbreeding respectively. The effective number of alleles indicates that Fulani Ecotype has more effective alleles compared to Yoruba Ecotype. These results suggest that Yoruba Ecotype may be at Hardy-Weinberg Equilibrium, while Fulani Ecotype deviates for Ghrelin locus. In sum, our results may open opportunities for genetic improvement of Nigerian Indigenous Chicken due to the polymorphic nature of Ghrelin.

**Key words:** Ghrelin, Nigerian Ecotype, Genetic diversity

#### 1.

#### INTRODUCTION

Natural resource is one of the greatest endowments sustaining the tropical and subtropical economies globally. Indigenous animal genetic resource plays a pivotal role in maintaining the socio-economic climate of developing countries. Poultry farming is one the most attractive husbandry in rural economies due to their ease of rearing, low production cost, provision of quality protein and source of manure[1]. Diversity of genetic resource have been employed as a tool for genetic improvement, thus diversity of indigenous poultry species presents valuable resources for livestock development [2]. Since the genetic resources locked up in indigenous poultry is yet to be fully exploited, characterizing the Nigerian ecotype chickens becomes essential.

Chickens make up about 80% of the 120 million poultry found in Nigeria [3]. They have been characterised based on biochemical indices[4], plumage (normal or frizzled), body structure (naked neck and dwarf types), colour (black, white, brown and mottled)[5], body size or body weight (heavy and light ecotypes)[6] and location; Yoruba and Fulani ecotypes [7]. The Fulani ecotype chicken is found in the dry savannahs of Nigeria, known for their better meat and egg production[8]. They have been classified for their heavy mature body weight (0.9-2.55 kg) and are known for their growth and reproductive performance potentials[8]. In contrast, the Yoruba ecotype chickens are found in the swamp, rainforests and derived savannah agro-ecological zones known for their light mature body weight (0.68-1.5 kg). They have slower growth rate, flighty nature, and fancy colour pattern of mottling[9, 10]. Cross breeding of superior ecotypes for specific markers may be useful for enhancing the genetic potentials of our local poultry.

Characterizing valuable biological system is the first step for their genetic improvement[11]. Several efforts have been made to characterize Nigerian poultry resource using phenotypic traits[3, 4, 9]. This may be misleading because genetic composition does not necessarily translate into phenotype characteristics and phenotypes are complex interactions between genotype and the environment[11]. It therefore became expedient to uncover the Nigeria poultry local resource potentials from genetic or molecular level. Our previous study on turkey and those of others on guinea fowl have started exploring the genetic potentials of local poultry by characterizing genes associated with important economic traits[11,13].

Ghrelin (GHRL), an essential modulator of feed intake, growth factor release and fat deposition has captured our attention[14]. This is because diversity of this gene can help in marker assisted selection for improved growth in Nigerian chicken ecotypes. It functions to increase the secretion of growth hormone and control energy balance[15]. Ghrelin (GHRL) have been associated with neonatal development in rats, chickens, and coordinate the regulation of growth hormone in birds[16]. The chicken ghrelin (cGHRL) gene is encoded as a 116-amino acid Ghrelin precursor and a 26-amino acid in mature Ghrelin [17], located on chromosome 12 comprising of five exons and four introns[16].

The degree of diversity in any species is a strong determinant of the amount of genetic improvement that can be achieved, and little has been done to enhance the genetic progress and conservation of local chicken at molecular level in Nigeria. This is because attention has been directed towards commercialization using improved breeds[18], and thus may lead to the erosion of Nigerian local chicken genetic resource. This present study was carried out to investigate and identify the polymorphism of Ghrelin gene in Fulani and Yoruba ecotypes chicken raised under the same conditions using PCR-RFLP (polymerase chain reaction-Restriction Fragment length polymorphism).

## **2. 0**

## **MATERIAL AND METHODS**

This experiment was carried out at the Poultry Unit of the Teaching and Research Farm of Department of Animal Science, University of Ibadan, which is situated 6 kilometres to the North of the city of Ibadan (7°26'N and 3°54'E) Oyo state, South-Western Nigeria, at a mean altitude of 277 meters above sea level.

### **2.1 Experimental animals**

Seventy-eight (78) Nigerian indigenous chicken (NIC) which comprised of Fulani ecotype (FE) (37) and Yoruba ecotype (YE) (41) were used for this experiment.

### **2.2 Blood Experimental animals sample collection**

Blood samples were collected via the jugular vein of the chickens, using a needle and syringe and were transferred **unto** (Grammatical correction) FTA (Flinders Technology Associates) classical cards by dropping it on the cards. The samples were allowed to dry at room temperature and then put in sealed nylon envelopes. Extreme temperatures were avoided during storage and while the samples were transported to the Laboratory (ACUTIG Genetic services) for analysis.

### **2.3 DNA extraction**

DNA was extracted from air dried blood spotted on the FTA cards using the following procedure (Jena Bioscience); Punching out of the sample disc from the FTA (Flinders Technology Associates) cards containing the blood samples by using the Harris micro-punch card and then placed into the Eppendorf tubes (five discs per tube), 150 ml of Tris-SDS solution and 200 ml distilled water was added to each of the Eppendorf tubes and agitated for 10minutes. Then the distilled water was removed and another one added, and agitated for another 10 minutes and then heated at 95°C for 10 minutes, the obtained liquid is the DNA. The DNA quality was tested via agarose gel electrophoresis, using agarose at 0.15 g, 1X TBE i.e. 15 ml, and ethidium bromide, and ran for 10-20 minutes, bands were seen.

### **2.4 Polymerase Chain Reaction (PCR) Amplification**

The primer used for selective amplification of cGHRL (chicken Ghrelin) gene using reference sequence Genbank Accession No. AY303688 and Primer3 web program (Primer 3\_www.cgi v0.2) was synthesized by InqabaBiotec (Hartfield, Pretoria, South Africa). Polymerase chain reactions were performed in a 6.25 µl reaction volume containing 4 µl nuclease free water, 1.25 µl Fast Taq, 0.25 µl forward primer, 0.25 µl reverse primer and 0.5 µl DNA, making a total of 6.25 µl after the mixture the tubes were closed well to avoid evaporation of the contents and then vortexed and loaded into the PCR machine to run at an initial denaturation of 94°C x5mins, followed by 34 cycles of denaturation at 94°C x40s, annealing temperature of 57°C and 72°C for 1min and a final extension step of 72°C for 10mins. The amplification

of Ghrelin gene ran for 2 hours 18minutes. Amplification of the cGHRN (chicken Ghrelin) gene was confirmed by running the PCR (polymerase chain reaction) products on 2% agarose gel and visualizing under UV rays, bands were seen and pictures taken

### **2.5 Primers used for selective amplification of ghrelin gene**

Forward primer 5'- 3' CATTCTAAGCTTTTGCCAGTT

Reverse primer 5'- 3' CACTGTTATTGTCATCTTCTC (Grammatical correction)

### **2.6 Restriction Digestion**

The PCR products were digested with *Mbol*lenzyme (Grammatical correction), using 1 µl buffer, 0.5 µl of the amplicon (PCR product) from each tube, 7.5 µl of H<sub>2</sub>O and 0.5µl of *Mbol*lin (Grammatical correction) manufacturer's recommended assay, in a final reaction volume of 9.5 µl. All the components were added and mixed properly before adding the restriction enzyme. The reaction mixture was incubated at 37°C for 10 minutes, and the enzyme was inactivated by heating for 20 minutes at 65°C.

#### **2.6.1 Agarose Gel Preparation and Staining (Grammatical correction)**

To make a concentration of 2% agarose, 10 ml of 1 × TBE buffer was added to 0.2 g of agarose powder in a flat-bottomed Erlenmeyer flask. The mixture was swirled briefly and then put in a microwave oven for 40 seconds to dissolve the agarose. To accelerate the dissolution, the slurry was swirled at intervals. After all the agarose had dissolved, the solution was allowed to cool on the bench. Meanwhile, as the solution cooled, the casting tray was prepared for casting of the gel. The edges of the tray were sealed using a masking tape and the comb(s) with the number of teeth that correspond to the number of samples slotted in position above the tray. The tray was set horizontally on the leveling stand on top of the bench.

When the gel solution was cold enough (~55°C), the combs were removed to create wells into which the digested products are loaded.

#### **2.6.2 Staining and loading of electrophoresis samples**

Before loading, the PCR products were first mixed with the gel loading dye. The dye SYBR<sup>®</sup> GREEN (0.2 µl)was (Grammatical correction) added to stain 4 µl of the DNA, and then loaded into the wells formed. The staining of the gel with SYBR<sup>®</sup> GREEN is important in the detection of DNA (Grammatical correction) under UV light and also because it increases the density of the sample making it possible for the DNA to drop evenly into the well and also enables one to follow the migration front. A 3000 bp (base pairs) ladder was loaded into the first well. 100 V of electric current was applied for the migration of DNA to start and electrophoresis was allowed to continue for 20 minutes so that the amplicons form clear

bands, and visible bands were seen and photographs were taken for each set of samples loaded on the gel.

### **2.7 Statistical analysis**

Genotype and allele frequencies were determined by direct gene counting method[19]. Allele and genotype frequencies of each gene in each strain of chicken was tested for fit to Hardy-Weinberg equilibrium (HWE), as well as genetic diversity parameters using POPGENE 1.32 software package[20].

## **3.0**

## **RESULTS AND DISCUSSION**

### **3.1 Results**

Our study examined the polymorphism of Ghrelin gene in Nigerian ecotype chicken which were raised in an intensive system. After DNA extraction from blood samples, amplification and digestion with *MbolI* restriction enzyme cleaved a 369bp fragment resulting into Alleles C and T consequently producing homozygous and heterozygous genotypes (CC, TT and CT). Table 1 presents the allelic and genotypic frequencies, table 2 shows the summary of heterozygosity while table 3 represents the summary of the genetic variation statistics of Ghrelin gene in Nigerian indigenous chickens.

Two alleles (C and T) were observed for Yoruba, Fulani and the overall (table 1) populations with varying frequencies. Frequencies of 0.34 and 0.66 were observed for Yoruba ecotype, 0.45 and 0.55 for Fulani ecotype and the 0.39 and 0.61 for overall. Similarly, genotypes CC, CT and TT were also observed for Ghrelin locus. We observed frequencies of 0.10, 0.49 and 0.41 in Yoruba ecotype while frequencies of 0.22, 0.46 and 0.32 was observed in Fulani ecotype and 0.15, 0.47 and 0.37 for the overall. Hardy-Weinberg test values ( $X^2$  and  $G^2$ ) for this sampled population revealed Ghrelin locus is at equilibrium. Alleles T and genotype CT were found to be in highest frequency for Ghrelin locus in Nigerian ecotype chicken. The heterozygosity indices for ghrelin locus in Nigerian chicken (table 2) showed that the observed homozygosity for Yoruba, Fulani and overall is 0.51, 0.54 and 0.53 while the expected homozygosity 0.54, 0.50, 0.52 respectively. Observed heterozygosity was lower for Fulani ecotype (0.46) in comparison to expected heterozygosity (0.50). Nei's genetic distance ranges from 0.45-0.49 with average heterozygosity for all population is 0.47. The measure of inbreeding coefficient (FIS) for this population was negative for Yoruba ecotype (-0.0847) while it is positive for Fulani (0.00702) and overall (0.0040). The summary of genetic variation of Ghrelin gene showed (table 3) that 2 alleles ( $N_a$ ) were observed in the sampled population (C and T). However, the number of alleles expected to attain the levels of heterozygosity expected for this sampled population based on HWE ( $N_e$ ) ranged from 1.82-1.98 with the Yoruba (1.82) having the least while Fulani (1.98) is greater than the

overall (1.91). The Shannon index showed that the Fulani ecotype may be more diverse (0.69) than the Yoruba ecotype (0.64).

**Table 1: Observed Allele and Genotype Frequencies for Ghrelin Locus in Nigerian Ecotype Chickens**

Ecotype	Allele	Frequency	Genotype	Frequency	X <sup>2*</sup>	G <sup>2*</sup>	DF*
Yoruba	C	0.34	CC	0.10	0.64	0.64	2
	T	0.66	CT	0.49			
			TT	0.41			
Fulani	C	0.45	CC	0.22	0.61	0.61	2
	T	0.55	CT	0.46			
			TT	0.32			
Overall	C	0.39	CC	0.15	0.93	0.93	2
	T	0.61	CT	0.47			
			TT	0.37			

\*X<sup>2</sup> and \*G<sup>2</sup> = Chi-square and Likelihood ratio test for Hardy-Weinberg equilibrium respectively \*DF = degree of freedom for genotype

**Table 2: Summary of Heterozygosity for Ghrelin Locus in Nigerian Ecotype Chickens**

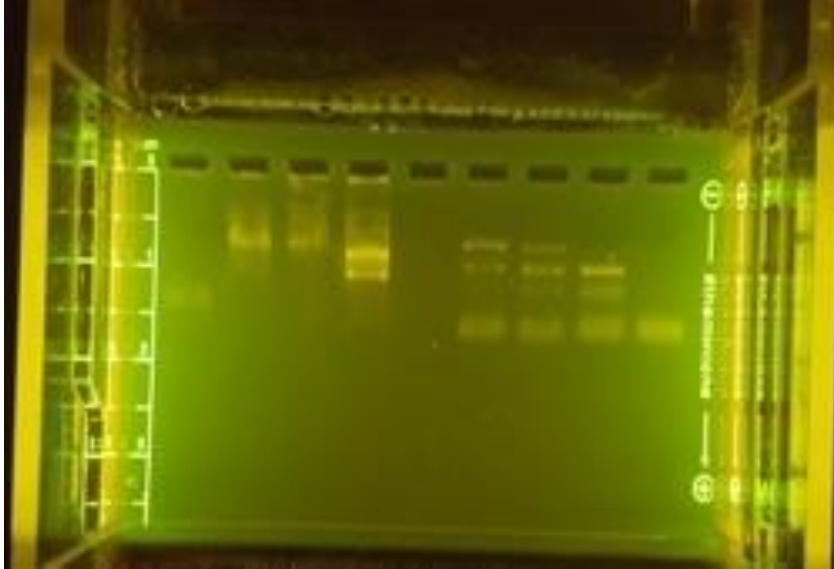
Ecotype	Obs <sub>hom</sub> *	Obs <sub>het</sub> *	Exp <sub>hom</sub> *	Exp <sub>het</sub> *	Nei's*	Ave <sub>het</sub> *	FIS*
Yoruba	0.51	0.49	0.54	0.46	0.45	0.47	-0.0847
Fulani	0.54	0.46	0.50	0.50	0.49	0.47	0.00702
Overall	0.53	0.47	0.52	0.48	0.48	0.47	0.0040

\*Obs<sub>hom</sub>=observed homozygosity, Obs<sub>het</sub>=observed heterozygosity, Exp<sub>hom</sub>=expected homozygosity, Exp<sub>het</sub>= expected heterozygosity, Nei's =Nei's expected heterozygosity, Ave<sub>het</sub>= average heterozygosity, FIS=Wright's Fixation index

**Table 3: Summary of Genetic Variation of Ghrelin Locus in Nigerian Ecotype Chickens**

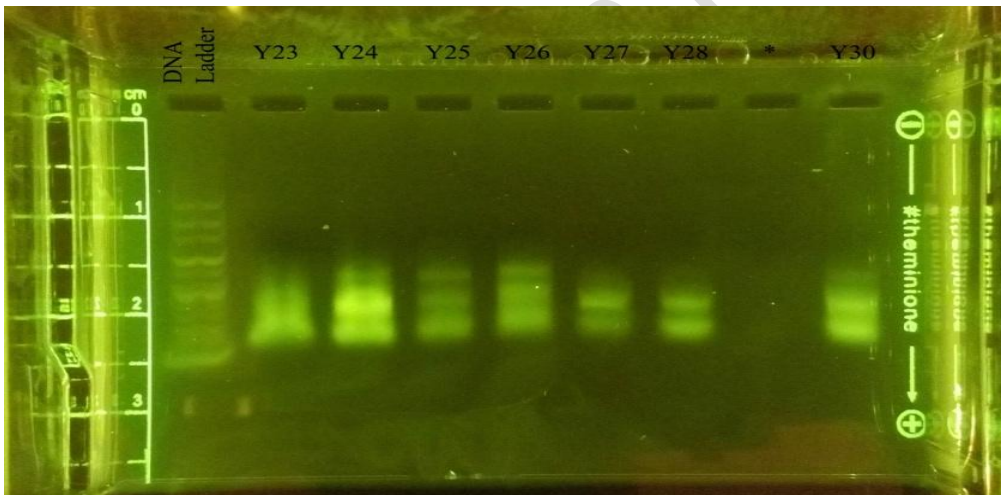
Ecotype	Na*	Ne*	I*
Yoruba	2.00	1.82	0.64
Fulani	2.00	1.98	0.69
Overall	2.00	1.91	0.67

Na\* = observed number of alleles, Ne = Effective number of alleles, I = Shannon's information index



Lane 1-8 represents PCR products (amplicons) of Ghrelin gene

**Figure 1:** Electrophoresis pattern of site 71 of the Ghrelin gene in Nigeria indigenous chickens obtained by Polymerase chain reaction (PCR).



Band 1:3000bp ladder, band 2 genotype: TT, band 3 genotype: CT, band 4 genotype: CT, band 5 genotype: CT, band 6 genotype: TT, band 7 genotype: TT, band 8 genotype: TT.

**Figure 2:** electrophoresis product of site 71 of ghrelin gene in nigeria indigenous chicken obtained by digesting with *mboi*.

### 3.2 Discussion

Our study successfully characterized the GHRL (Ghrelin) locus of the Nigerian ecotype chickens for its diversity using the PCR-RFLP method. Diversity of GHRL (Ghrelin) receptors and gene have been characterized showing different variations linked growth related traits[21-24]. The present study was geared at characterizing a major gene encoding an essential modulator of feed intake, growth factor release and fat deposition in chicken[25].

This attempt is to understand the polymorphic nature of GHRL (Ghrelin) gene and how we can assess it for marker assisted selection for improved chicken production in Nigeria. We showed that this locus is polymorphic with varying frequencies for alleles and genotypes. Allele C is shown to be in lower frequency compared to T in both ecotypes. We suppose that T allele may have less selective constraints for it to be genetically and environmentally selected for in both ecotypes. Similarly, the CC genotype experienced higher selective constraints alluding to their lower frequencies across the ecotypes, however, we did not find a corresponding higher frequency of TT across the ecotypes but observed the heterozygote genotype (CT) to be at a higher frequency. TT genotype has been linked to lower feed consumption in pigs[26], which may pose a danger for the survival of animal. Thus, our study reveals that allele C and T are essential for the conservation of Nigerian ecotype chicken. Similarly, allele and genotype frequencies obtained from 12 Chinese chicken breeds for the GHRL (Ghrelin) locus attest to the presence of low frequencies for the C allele with a corresponding higher T allele[24]. However, they found a corresponding higher TT genotype frequency for all breeds greater than CT different GHRL (Ghrelin) sites. The similarity may be attributed to an evolutionary conservation mechanism that is maintained across generation and environmental climes. The differences in between the studies may be due to different markers, primers, and differences in breed which be confounded by environment-by-genotype interactions. Thus, genotype CT may be more favourable for establishing Nigerian ecotype chickens in different environment.

Heterozygosity is an important metric for assessing the genetic diversity within a population and as such a good management metric for threatened taxa [27]. Our data for heterozygosity in GHRL (Ghrelin) locus (Table 2) reveals that observed heterozygosity ( $Obs_{het}$ ) is higher than expected heterozygosity ( $Exp_{het}$ ) implying that random mating is observed for the Yoruba ecotype while reverse is presented in the Fulani ecotype. Thus, since  $Exp_{het}$  and  $Obs_{het}$  is dependent on allele frequencies and individual genotypes (also depends on the genetic variation and inbreeding) respectively, Inbreeding can be derived from these metrics[28]. When  $Exp_{het} > Obs_{het}$ , inbreeding is at work in the population with  $FIS > 0$ [27]. FIS value for the Yoruba is negative and  $< 1$  and that of the Fulani population is  $> 1$ . In sum, the Yoruba ecotype may be heterozygous due to some randomness of alleles at the locus while the Fulani ecotype may be homozygous due to inbreeding for the GRHL locus and thus shows departure from HWE.

The allelic diversity reflected by the mean number of alleles, effective number of alleles and Shannon's information index is presented in Table 3. Numbers of alleles for the ecotypes is 2, however,  $N_e$  and  $I$  indicates that T allele may have more impact on the Fulani in comparison to Yoruba ecotype[29]. This shows there is little variation in the GHRL (Ghrelin)

locus for the sampled population. Fijabiet *al.* (Grammatical correction) [14] found similar result in Nigerian turkey using similar methods. Our study provided baseline information to understand the genetic diversity of local chicken breeds in Nigeria and its implication for genetic improvement by assessing the GRHL (Ghrelin) locus. Metrics measured here are sample size sensitive and thus larger sample size and natural flocks can also be exploited in other to maximize the influence of DNA polymorphisms on GHRL (Ghrelin) activity and function.

#### 4. CONCLUSION

The aim of this study was to determine the genetic diversity between and within Fulani and Yoruba ecotype chickens in Nigeria at the ghrelin gene locus using PCR-RFLP. The results of the study revealed that the sampled populations were polymorphic for ghrelin gene locus, two alleles (C and T) and three genotypes (CC, CT, and TT) were observed. Conclusively, the results obtained in this research may open opportunities for genetic improvement of Nigerian Indigenous Chicken due to the polymorphic nature of Ghrelin.

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