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Research Article
Genetic Relationship among Three Nigerian Chicken (*Gallus gallus*) Genotypes Based on Cytochrome b of Mitochondrial DNA

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Abstract

Background and Objective: Demand for poultry and livestock products in general has increased significantly because of the dietary importance of meat and egg that they produced. In this study, the genetic relationship of three local chicken breeds in South-South Nigeria (frizzle feather, normal feather and naked neck) was evaluated. **Materials and Methods:** Two milliliter of blood was collected from their wing vein using sterile needle and syringe and stored in FTA cards for DNA extraction, PCR amplification and sequencing. **Results:** The result obtained revealed a total of 9 haplotypes. Frizzle feather had the highest haplotype and nucleotide diversity while the lowest haplotype and nucleotide diversity was from naked neck. High sequence conservation was observed across the three genotypes. Genetic distance estimates revealed that naked neck and frizzle feather were more closely related while the furthest genetic distance was between normal feather and frizzle feather. The phylogenetic tree revealed two clusters. Cluster one had three individuals from the same population (normal feather). All other samples were grouped under cluster 2. **Conclusion:** The study revealed the existence of low genetic diversity among the three populations of chicken studied and also showed that the Cytochrome b gene is highly conserved.

Key words: Local chicken, *Gallus gallus*, genotypes, genetic diversity, Cytochrome b, mtDNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Although the advancements made in agricultural research have helped increased food availability, many households living in underdeveloped regions of the world are still faced with malnutrition due to poor finance[1]. The world population is expected to increase from 5.4 to at least 7.2 billion within the next two decades, mainly in developing countries. This increase in human population will have a major effect on both available natural resources and future demands for livestock. Thus careful analysis is required so that local chicken development strategies can be oriented towards better use of the genetic material in them to contribute effectively to food security, improve the living standard of the poor farmers and ensure sustainable development of the chicken breeds.

In recent decades, the demand for poultry and livestock products in general has increased significantly because of the dietary importance of meat and egg that they produced. Among the different food sources, poultry products contribute significantly to the Nigerian protein demand. Indigenous breeds of chicken plays an important role in rural and urban economic gains in most developing and under developing countries. Nigeria, arguably the largest country in Africa is divided into six geo-political zones. One of these zones is the South-South region. Most of the plant and animal genetic resources are at risk of extinction because of the terrain of this region[2]. According to a research[3], local chickens show large variation in body size, plumage colors, feathering pattern, earlobe and eggshell. Animal genetic diversity allows farmers to select stocks or develop new breeds in response to environmental changes, threat of diseases, new knowledge of human nutritional requirement, changing market value conditions and societal needs. The term local chicken is used interchangeably to denote a group of unimproved, un-pedigreed and unselected population of random breeding native chickens[4] and spread throughout the rural areas of Nigeria.

There is limited information on the molecular genetic diversity of local chicken[2,5-8]. For the purpose of conserving important livestock species it is advisable to use modern molecular markers like the mitochondrial *D loop* and *cyt B* region of mitochondrial DNA. These markers are capable of revealing all the genetic information inherent in any species population and can be used to measure important genetic diversity indices. The advent of genetic markers such as RAPD, RFLP and microsatellite in conjunction with statistical tools has revolutionized population studies which tend to explore the phylogenetic relationship within and between populations[9]. The amount of genetic variation among organisms leads to effective selection and crossbreeding of livestock for conservation, genetic improvement and species adaptability[10-12].

In recent times, sequence data have proven to be the most direct and reliable method of assessing the pattern of variation among species[9]. Sequence data may be obtained from mtDNA or nuclear DNA, however, mtDNA found within the cytoplasm of a cell remains a good choice over nuclear DNA due to the fast rate of mutation found there in[13,14] and specificity as it is inherited only through maternal cell line[15]. It is easy to amplify, as it appears in multiple copies in the cells and the mitochondrial gene content is strongly conserved across generations. Chicken mtDNA has 16,775 base pairs[16]. Cytochrome b (*cyt-b*) gene is one of the important coding genes in mtDNA with a length of about 1.2 kb and has been widely used for phylogenetic studies of several animal species[17,18]. The sequence variability of *cyt b* makes it very useful for comparison of species in the same genus or the same family. Therefore, Cytochrome b region of mitochondrial DNA (mtDNA) was used to assess the genetic relationship of three (3) local chicken breeds in South-South Nigeria (frizzle feather, normal feather and naked neck).

MATERIALS AND METHODS

Study area and sample collection: This research was carried out in the Molecular Laboratory of Department of Genetics and Biotechnology University of Calabar, Calabar between August, 2018 to January, 2019. A total of thirty-four (34) matured local chickens comprising of 13 naked neck, 11 normal feather and 10 frizzle feathered genes were randomly collected from 10 villages in three states of South South Nigeria (Akwa Ibom State, Rivers State and Delta State) (Fig. 1).

Blood sample collection: One month after the chickens were purchased, approximately 2 mL of blood was obtained by venipuncture of the brachial vein from each of the birds using sterile needle and syringe for each individual to avoid cross contamination. The blood collected was taken to the laboratory and about 2 mL was transferred into the Flinders Technology Associates (FTA) classical cards by dropping on the concentric circular motion within printed circle area to avoid coagulation. The blood sample in the FTA cards were allowed to dry at room temperature and then placed in a sealed plastic bag containing silica gel beads and extreme temperature was avoided.

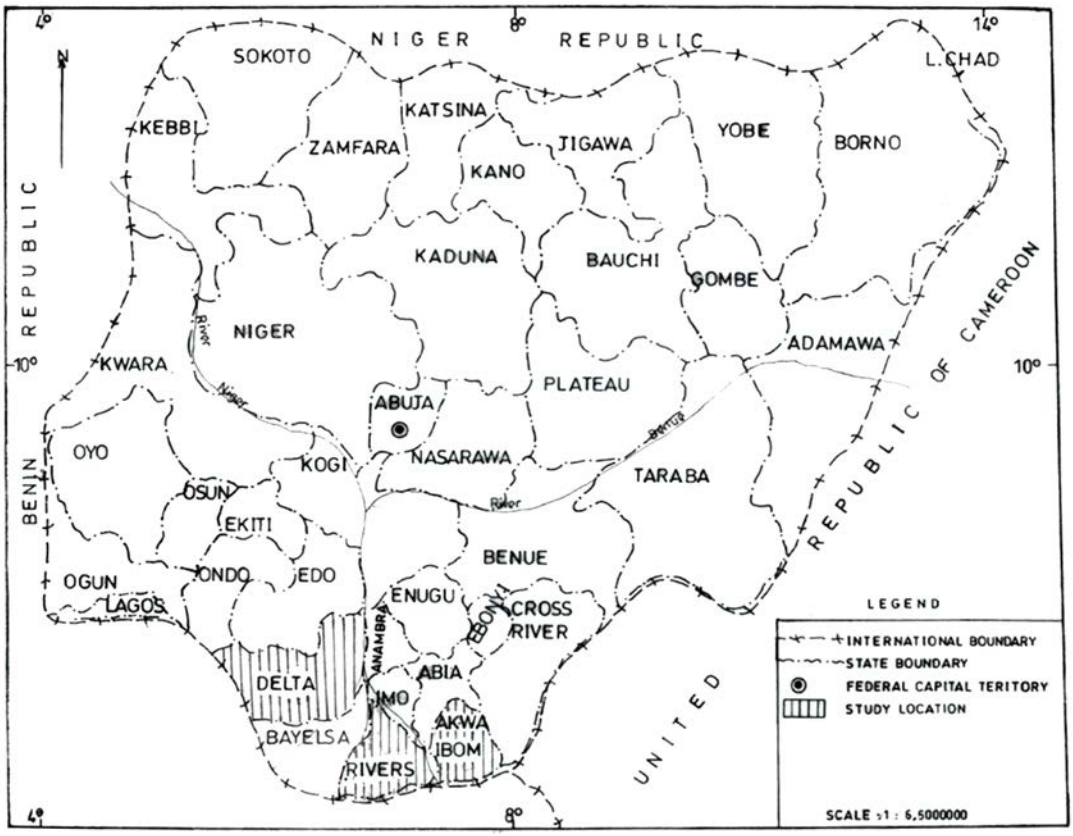

Fig. 1: Map of Nigeria showing the geographical locations of sample
Source: Department of Geography and Regional Planning, University of Calabar

DNA extraction: Genomic DNA extracted from the air-dried blood preserved on FTA card (Whatman Bioscience) was carried out in the Molecular Laboratory of Department of Genetics and Biotechnology University of Calabar, Calabar, Nigeria using quick-DNA™ miniprep plus kit protocol. The sample disc was punched out from the FTA cards containing the blood samples using Harris micro-punch card and then placed into micro centrifuge tubes. The 200 µL of biofluid, cell buffer and 20 µL of proteinase k were added to 200 µL of sample in a centrifuge tube. It was thoroughly mixed and then the tubes were incubated at 55°C for 10 min. Almost 1 volume of genomic binding buffer was added to the digested sample and mixed thoroughly. The mixtures were then transferred to a Zymo-Spin™ IIC-XL column in a collection tube and centrifuge at $\geq$12,000×g for 1 min. The collection tubes were discarded while the zymo spin column was transferred to a new collection tube. The 400 µL of DNA pre wash buffer was added to the spin column, followed by 1 min centrifugation at 10,000×g. The 700 µL of g-DNA wash buffer was added to the spin column followed by centrifugation for 1 min at 10,000×g. After this the spin column was then transferred to a micro centrifuge tube. The 50 µL of DNA elution buffer was added to the spin column and incubated for five minutes at room temperature. This was followed by final centrifugation at top speed (14,000×g) for 30 sec for DNA elution. The eluted DNA in the micro centrifuge tube was stored at less than 20°C pending the amplification.

Polymerase chain reaction (PCR) amplification: Polymerase Chain Reaction (PCR) amplification was carried out in STABVIDA Laboratory, Quinta de torre, Portugal. The primers L14816 (5^1-CCATCCAACATCTCAGCATGATGAAA-3^1) as the forward primer (23) and H15173 (5^1-CCCCTCAGAATGATAT TTGTCCTCA-3^1) as reverse primer were used. The PCR amplification was carried out using 25 µL reaction volume containing 1 µL genomic DNA, 2 Mm $MgCl_2$, 200 µM of dNTP, 2.5 µL of 10×PCR buffer comprising 10 mM tris-HCl (PH 8.3) and 50 mM KCl and 1 µM of each primer and two units of SFABVIDA proprietary taq polymerase. This was performed using the Gene Amp$^{(R)}$ PCR system (9700) thermal cycler (Applied Biosystems, Foster city, USA) programmed as follows initial denaturation step at 90°C for 5 min, 35 cycles of

denaturation at 94°C for 30 sec, annealing at 50°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 7 min. PCR products were purified using exofast protocol according to the manufacturer's instruction.

Sequencing of Cytochrome b: Two primers, L14816 (5[1]-CCAT CCAACATCTCAGCATGATGAAA-3[1]) as the forward primer [22] and H15173 (5[1]-CCCCTCAGAATGATATTTGTCCTCA-3[1]) as reverse primer were used for Cytochrome b region. Sequencing reaction was performed in STABVIDA Laboratory, Quinta de torre, Portugal with AB13730×L sequencer using 20 µL reaction comprising approximately 20 ng of purified PCR product as template DNA, 8 µL of Big Dye Terminator Reaction mix (dNTPs, ddNTPs, buffer, enzyme and $MgCl_2$), 8 µL of deionize water, 2 µL of primer programmed as 35 cycles at 94°C for 30 sec, 60°C for 5 sec, 60°C for 4 min.

Sequence analysis: Bioedit software version[19] 7.2.5 was used to view and edit the sequences. MEGA 6.06 was used for multiple sequence alignment of all the samples[20] excluding all the gaps. Estimation of polymorphism in the aligned regions including nucleotide diversity (n) and haplotype diversity (Hd) values was carried out using DNASp 5.1 software[21]. The genetic distance within and between populations was performed[20] using MEGA 6.06.

RESULTS

Mitochondrial *cyt b* polymorphism in chicken: The mitochondrial *cyt b* polymorphism in chicken is presented in Table 1. The number of sequences for normal feather, naked neck and frizzle feather were 11, 13 and 10, respectively. There were 3 segregating sites in normal feather, 2 in naked neck and 4 in frizzle feather genotype. The number of haplotypes in normal feather was 4 with haplotype diversity of 0.600±0.024. Naked neck had 1 haplotype with 0.0013±0.0004 haplotype diversity, while frizzle feather genotype had four haplotypes with 0.644±0.023 haplotype diversity. Nucleotide diversity was highest in frizzle feather (0.0038±0.0029) followed by normal feather (0.0029±0.001) and naked neck (0.0024±0.0001). *Cyt b* sequences were more conserved in naked neck genotypes with 99.3% conservation rate while the lowest conservation rate was observed in frizzle feather genotypes (94.8%).

Genetic distance: Genetic distance between the three genotypes of chicken is presented in Table 2. The highest genetic distance was between normal and frizzle feather genotypes (0.010), followed by normal feather and naked neck (0.007) while naked neck and frizzle feather was the lowest (0.003). Within the normal feather genotypes, the highest genetic distance was observed between NF_5 and NF_8 as 0.011 as showed in Table 3. The highest genetic distance in frizzle feather was 0.010 as presented in Table 4. In the naked neck genotypes all the chickens showed no genetic distance among them as presented in Table 5.

Phylogenetic relationship among the chicken genotypes: Results of the phylogenetic analysis among the chicken genotype are presented in Fig. 2. There were 2 major clusters observed in the phylogenetic tree. Cluster 1 had three individuals all from normal feather genotype (NF_109, NF_110 and NF_111). All other samples from the three genotypes were grouped under cluster 2 with only a single normal feather genotype (NF_107) separated into a single sub-clade.

Table 1: Genetic polymorphism of three Nigerian chicken genotypes

Diversity parameters	NF	NN	FF
Number of sequences	11	13	10
Number of sites	295	295	295
Monomorphic sites	292	293	291
Polymorphic sites	3	2	4
Parsimony information site	1	0	2
Singleton variable site	2	2	2
Number of haplotypes	4	1	4
Haplotype diversity (Hd)	0.600±0.024	0.013±0.0004	0.644±0.023
Nucleotide diversity (n)	0.0029±0.001	0.0024±0.0001	0.0038±0.0029
Sequence conservation	0.955 (95.5%)	0.993 (99.3%)	0.948 (94.8%)

NF: Normal feather, NN: Normal feather, FF: Frizzle feather

Table 2: Genetic distance between three genotypes of Nigerian local chickens

Specimen	NF	NN	FF
NF	0	-	-
NN	0.007	0	-
FF	0.010	0.003	0.00

Overall average genetic distance = 0.007, NF: Normal feather, NN: Normal feather, FF: Frizzle feather

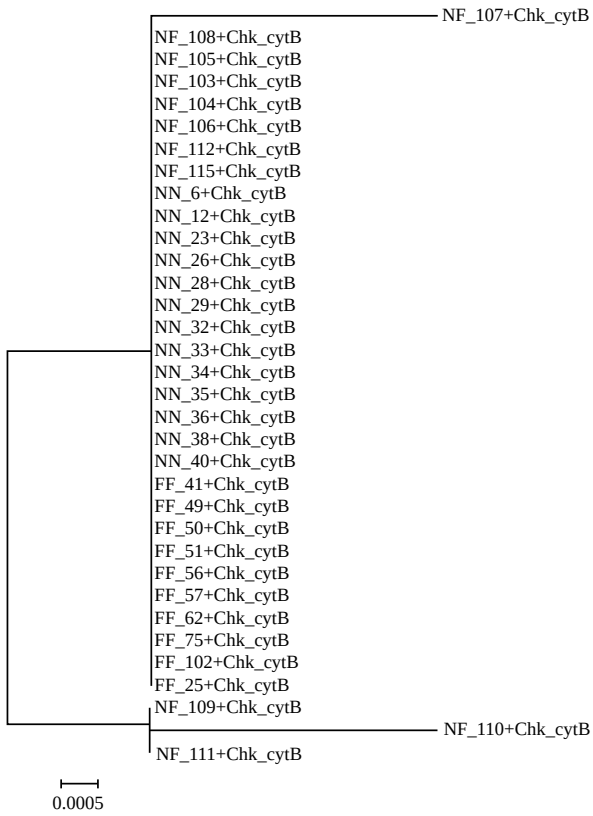

Fig. 2: Phylogenetic tree showing the relationship between Nigerian chicken genotypes

Table 3: Genetic distance within the normal feather chicken genotypes

Specimen	NF_1	NF_2	NF_3	NF_4	NF_5	NF_6	NF_7	NF_8	NF_9	NF_10	NF_11
NF_1	0.00										
NF_2	0.000	0.00									
NF_3	0.000	0.000	0.00								
NF_4	0.000	0.000	0.000	0.00							
NF_5	0.004	0.004	0.004	0.004	0.00						
NF_6	0.000	0.000	0.000	0.000	0.004	0.00					
NF_7	0.004	0.004	0.004	0.004	0.007	0.004	0.00				
NF_8	0.007	0.007	0.007	0.007	0.011	0.007	0.004	0.00			
NF_9	0.004	0.004	0.004	0.004	0.007	0.004	0.000	0.004	0.00		
NF_10	0.000	0.000	0.000	0.000	0.004	0.000	0.004	0.007	0.004	0.00	
NF_11	0.000	0.000	0.000	0.000	0.004	0.000	0.004	0.007	0.004	0.000	0.000

NF: Normal feather

Table 4: Genetic distance within the frizzle feather chicken genotypes

Specimen	FF_1	FF_2	FF_3	FF_4	FF_5	FF_6	FF_7	FF_8	FF_9	FF_10
FF_1	0.000									
FF_2	0.007	0.000								
FF_3	0.000	0.007	0.000							
FF_4	0.007	0.000	0.007	0.000						
FF_5	0.003	0.010	0.003	0.010	0.000					
FF_6	0.000	0.007	0.000	0.007	0.003	0.000				
FF_7	0.000	0.007	0.000	0.007	0.003	0.000	0.000			
FF_8	0.000	0.007	0.000	0.007	0.003	0.000	0.000	0.000		
FF_9	0.003	0.010	0.003	0.010	0.007	0.003	0.003	0.003	0.000	
FF_10	0.003	0.010	0.003	0.010	0.007	0.003	0.003	0.003	0.007	0.000

FF: Frizzle feather

Table 5: Genetic distance within the naked neck chicken genotypes

Specimen	NN_1	NN_2	NN_3	NN_4	NN_5	NN_6	NN_7	NN_8	NN_9	NN_10	NN_11	NN_12	NN_13
NN_1	0.000												
NN_2	0.000	0.000											
NN_3	0.000	0.000	0.000										
NN_4	0.000	0.000	0.000	0.000									
NN_5	0.000	0.000	0.000	0.000	0.000								
NN_6	0.000	0.000	0.000	0.000	0.000	0.000							
NN_7	0.000	0.000	0.000	0.000	0.000	0.000	0.000						
NN_8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
NN_9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
NN_10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
NN_11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
NN_12	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
NN_13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

NN: Naked neck

DISCUSSION

Understanding the genetic blue print of species in a population is very necessary in developing conservation and management strategies for threatened individuals[9]. Overcoming the ever increasing demands for poultry products calls for intensified efforts and interest in local chicken research as the genetic resources from them remain underutilized. In this research, local chickens from three populations in South-South were used for genetic analysis. The Cytochrome b region of mtDNA was sequenced for all populations. This was to assess if local chickens from the three populations are genetically similar or different. The results for genetic diversity indices including haplotype and nucleotide diversity regions of mtDNA showed low genetic variation. The research by Ajibike *et al*.[8] reported a haplotype diversity of 0.05 in local chicken using the hyper variable region of mitochondrial DNA which is lower than the findings in this study. Haplotype and nucleotide diversity of 0.893 and 0.00591 were reported by Gao *et al*.[22] in Jiangxi using mitochondrial D- loop. According to Ekerette *et al*.[9], nucleotide and haplotype diversity are key parameters for assessing population polymorphisms and genetic differentiation. The low level of nucleotide and haplotype diversity observed in the present study may suggest the existence of low molecular differences within and between the chicken genotypes. Low genetic diversity compromises the ability of populations to evolve and thus reduces their chances of survival under environmental changes. The total number of haplotypes observed in this study was nine (9) which was lower than that reported by references[8,10,22-25]. However, the haplotype diversity in this study was higher than that reported by Mtileni *et al*.[26]. Very high conservation percentages were recorded in the *cytb* gene of the local chicken with naked neck having the highest 99.3% which implies that the *cyt b* region is much conserved. With this information, genetic diversity of the mtDNA *cytb* gene could be a useful approach in strategizing conservation and management of local chicken. The generally low genetic diversity may be due to low mutation rated in *cyt b*. The frizzle feather had a higher diversity among other local chickens. This may be as a result of differences in their effective population size since nucleotide diversity is affected by several factors including selection, mutation rates, mating system, genetic drift, effective population size and demographic gene flow between populations. The terrain of the South region such as environmental disaster including flooding, environmental pollution and oil spillage, which occur commonly in this area may also contribute to the loss of variants genetic resources of local chicken over time.

Genetic distance measures the genetic differences between species and populations with similar alleles are expected to have low genetic distance as an indication of their genetic similarity[9]. Therefore, genetic distance between the populations may be further indication of the molecular divergence between native chicken. In this study, the genetic distance among the three populations was moderate with the highest genetic distance between normal feather and frizzle feather. The high genetic distance between both genotypes may be due to a lot of modifying genes associated with frizzle feather, which contributed to high genetic variation observed in earlier research[27]. Genetic distance estimates revealed that naked neck and frizzle feather are more closely related suggesting that they may share common lineage.

Phylogenetic tree constructed for chicken samples from the three populations clearly separated them into two major clusters based on genotypes. Phylogenetic analysis according to Adamu *et al*.[10] provides important guideline for making conservation initiatives among indigenous chickens. All samples of naked neck and frizzle feather were in the same

cluster, while some samples from normal feather genotypes had separate clusters. This further affirms the existence of a higher genetic similarity between naked neck and frizzle feather. The uniqueness in the feather pattern of the normal chicken genotypes has great influence in distinguishing them[28]. Information within this study will be very useful in planning for conservation and genetic improvement of Nigerian local chicken through selection and hybridization.

CONCLUSION

From the findings of this study, it was concluded that there is a higher genetic similarity between naked neck and frizzle feather than they relate to normal chicken genotypes based on variations in mtDNA Cytochrome b.

SIGNIFICANCE STATEMENT

This study has clearly unveiled that based on *cyt b* gene of mtDNA, frizzle feather chickens have higher genetic variation within genotypes than naked neck and normal feather. It also revealed that naked neck and frizzle feather are more closely related, which may share a more common ancestral origin. Generally there was low genetic variation among the three chicken genotypes based on *cyt b* gene. The findings here will help breeders to select this genotypes for conservation, hybridization and genetic improvement.

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