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SELECTION PRESSURE AND THE MATERNAL LINEAGE OF THREE LOCAL CHICKEN GENOTYPES IN SOUTH-SOUTH NIGERIA

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ABSTRACT
Local chicken are the most diversified among livestock species in Nigeria. They are sources of protein to man. In this study, cytochrome b region of mitochondrial DNA (mtDNA) was used to assess the genetic diversity of three (3) local chicken breeds (frizzle feathered, normal feathered and naked neck). Two ml (2ml) of blood was collected from their wing vein unto Flinders Technology Associates (FTA) cards for DNA extraction. The sequence fragments were viewed and edited using Bioedit software and subsequently analyzed for genetic diversity studies using various molecular software. The result obtained revealed a total of 9 haplotypes. Results for selection revealed positive, negative and neutral selection in the three populations. Normal feather occurred once with a positive selection pressure of 0.4644 and a negative selection pressure of -0.8411 with neutral selection occurring at 84 codon sites. In frizzle feather, positive selection occurred twice with synonymous substitution rate of 0.905. Naked neck showed neutral selection. The result obtained for maternal lineage showed that the Nigerian local chicken genotypes may have their origin from Asia and Europe. 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.
Keywords: Selection, maternal lineage, chicken, genotypes

INTRODUCTION
In Nigeria, chickens are the most widely distributed of all livestock species with a population of 166 million birds (FAO, 2007) Local chickens show large variation in body size, plumage colors, feathering pattern, earlobe, and eggshell and shank color (Adene, 2004). Chickens play very important role in the economy of most African countries. They provide mankind with egg and meat, and as such, requires more attention on the genetic variation that can be utilized in selective breeding. According to Hayes *et al.* (2009), 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. Genotype selection has the ability to increase productivity of livestock as well as enhance environmental adaptation and maintenance of genetic diversity (Nwosu, 1979). The term indigenous or local chicken is used interchangeably to denote a group of unimproved, un-pedigreed and unselected population of random breeding native chickens and are distributed throughout the rural areas of Nigeria (Ohwojakpo *et al*., 2012). Over the years, the demand for poultry and livestock products in general has increased significantly because of the dietary importance of meat and egg. There is limited information on the molecular genetic diversity of local chicken (Adebambo *et al*., 2010; Adeleke *et al*., 2011; Babatunde *et al*., 2016; Ajibike *et al*., 2017; Ekerette *et al*., 2018). For the purpose of conservation of 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 unveiling genetic information inherent in any species population and can be used to measure important genetic diversity indices (Adebambo *et al*., 2010). The amount of genetic variation among organisms leads to effective selection and

crossbreeding of livestock for conservation, genetic improvement and species adaptability (Adamu *et al*., 2016). Molecular characterization provides reliable information for assessing the level of genetic variation among the populations of animals (Niu *et al*., 2002). It unveils the rates of distribution of diversity between the populations. In addition, it allows the comparison of genetic variation within and between populations of individuals of the same species and reconstruction of phylogenetic tree to trace the history of ancestral populations (Groeneveld *et al*., 2010).

Nigeria 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 (Ohwojakpor *et al*., 2012). Local chicken populations in Nigeria include the frizzle feathered, naked neck, normal feather among others. The frizzle gene (F) is a genetic mutant in chickens and is responsible for the modified plumage conditions arising from the curving of the rachis of all feathers with curling of the barbs, in which the feathers grow so that they curve outward, instead of lying smoothly along the bird's body (Ohwojakpor *et al*., 2012). The naked neck gene (Na) is a genetic mutant which affects feather distribution on the neck. It can easily be identified by an isolated tuft of feather on the ventral side of the neck, while the normal feathered is the most common type, and it is neither frizzled nor featherless (Touchburn *et al.*, 1980). There is therefore the urgent need to characterize important genetic resources before they go into extinction. Multivariate base genetic diversity analysis of three chicken genotype of Nigeria revealed that the frizzle feather has the best performance (Ikpeme *et al*., 2016). South-south is a region in Nigeria where poverty is widespread and the traditional raising of local poultry therefore plays a crucial role in improving nutrition and ensuring food security.

Over the years, sequence data have proven to be the most direct and reliable method of assessing the pattern of variation among species (Adamu *et al*., 2016). 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 its fast rate of mutation in the regions (Wilson *et al*., 1985;Abdul *et al.,* 2015) and specificity as it is inherited only through maternal cell line (Brown *et al*., 1993). Mitochondrial DNA (mtDNA) is commonly used as molecular marker in population genetics because of its high copy number in cells, maternal inheritance and its evolutionary rate (Sofla *et al*., 2017). The first genetic study on mtDNA suggest that Indo Chinese Red Jungle flow subspecies *Gallus gallus gallus is* the primary ancestor of the domestic chicken (*Gallus gallus* d*omesticus*) (Eriksson, 2008). Other studies revealed that at least three sub-species of *Gallus gallus* were enrolled in the origin of domestic chicken breeds, and that there may be at least two domestic center: one in Southeast Africa and one in the Indian subcontinent (Nighibori *et al*., 2005). Chicken mtDNA has 16,775 base pairs (Desjardins and Morais, 1990). Different regions of the mtDNA evolve at different rates making it a marker of choice for studying genetic diversity within as well as between species. MtDNA is maternally inherited in most species and does not undergo recombination (Hayashi *et al*., 1985). These features mean that each molecule as a whole usually has a single genealogical history through maternal lineage. The cytochrome b (*cyt-b*) gene is one of the important coding genes in mtDNA with a length of about 1.2kb (Sawaimul *et al.,* 2014). Because of its maternal inheritance, its well known gene structure and sequence, the occurrence of low recombination and other characteristics, the *cyt-b* gene has been widely used for phylogenetic studies of several animal species (Patwardhan *et al*., 201). The sequence variability of *cyt-b* makes it very useful for comparison of species in the same genus or the same family. The results obtained in several phylogenetic studies in which this gene has been used have led to new classification schemes that better reflects the phylogenetic relationships among the species studied (Arnason *et al.,* 1995).

MATERIALS AND METHODS

Study location and sample collection

A total of thirty-four (34) matured local chickens comprising of 13 naked neck, 11 normal feathered

and 10 frizzle feathered were randomly collected from 10 villages in three states (Akwa Ibom, Rivers and Delta States) of South South Nigeria (Fig.1)**.**

Blood sample collection

One month after the chickens were purchased, about 2 ml of blood was collected from all their wing vein using sterile needle and syringe for each individual to avoid cross contamination. The blood collected was taken to the laboratory and 2ml was transferred into the Flinders Technology Associates (FTA) cards by dropping on the concentric circular motion. 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, extreme temperature was avoided.

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. 200µl of biofluid, cell buffer and 20µl of proteinase k was added to 200µl of sample in a centrifuge tube. It was thoroughly mixed and then the tubes were incubated at 55ºC for 10 minutes. 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 ≥ 12,000 × g for one minute. The collection tubes were discarded while the zymo spin column was transferred to a new collection tube. 400µl of DNA pre wash buffer was added to the spin column, followed by one minute centrifugation at 10,000 × g. 700µl of g-DNA wash buffer was added to the spin column followed by centrifugation for one minute at 10,000 × g. After this the spin column was then transferred to a micro centrifuge tube. 50µl 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 seconds for DNA elution. The eluted DNA in the micro centrifuge tube was stored at less than 20ºC pending the amplification.

Fig. 1: Map of Nigeria showing the geographical locations of sample

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 and H15173 (5^1-CCCCTCAGAATGATATTTGTCCTCA -3^1) as reverse primer were used (Hayashi *et al*., 1985). PCR amplification was carried out using 25µl reaction volume containing 1µl genomic DNA, 2Mm $MgCl_2$, 200µM of dNTP, 2.5µl of 10xPCR buffer comprising 10mM tris-Hcl (pH 8.3) and 50mM kcl and 1µM of each primer and two units of SFABVIDA proprietary taq polymerase. This was performed using the GeneAmp$^{(R)}$ PCR system (9700) thermal cycler (Applied Biosystems, Foster city, USA) programmed as follows: initial denaturation step at 90^{o}C for 5 minutes, 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 50 ^{o}C for 45 seconds, extension at 72 ^{o}C for 45 seconds and final extension at 72 ^{o}C for seven minutes. PCR products were purified using exofast protocol according to the manufacturer's instruction.

Sequencing of cytochrome b regions of mtDNA

Two primers, L14816 (5^1-CCATCCAACATCTCAGCATGATGAAA-3^1) as the forward primer and H15173 (5^1-CCCCTCAGAATGATATTTGTCCTCA -3^1) as reverse primer (Desjardins, and Morais, 1990) were used for cytochrome b region. Sequencing reaction was performed in STABVIDA Laboratory, Quinta de torre, Portugal with AB13730 x L sequencer using 20µl reaction comprising approximately 20ng of purified PCR product as template DNA, 8µl of Big Dye Terminator Reaction mix (dNTPs, ddNTPs, buffer, enzyme and $MgCl_2$), 8µl of deionize water, 2µl of primer programmed as 35 cycles at 94^{o}C for 30 seconds, 60^{o}C for five second, 60^{o}C for four minutes.

Sequence analysis

Bioedit software version 7.2.5 (Hall *et al*., 1999) was used to view and edit the sequences. MEGA 6.06 was used for multiple sequence alignment of all the samples (Tamura *et al*., 2013). To classify the species into maternal lineage based on the cytochrome b region, cytochrome b sequences of other indigenous chicken species were retrieved from GenBank database available on National Centre for Biotechnology Information (NCBI) with the following accession numbers; KF650606.1, HQ122606.1, EU826429.1, KM224424.1, KP231500.1, DQ512917.1, AY724764.1, G027618.1, FM205718.1 and AJ971340.1. These were all aligned with the query sequences obtained from indigenous chickens in the studied populations to construct a maximum likelihood tree using MEGA 6.06 with 1000 bootstrap replication (Tamura *et al*., 2013). The genetic distance within and between populations as well as estimation of selection types were performed using MEGA 6.06 (Tamura *et al*., 2013). NETWORK 4.6.1.1 was used for network analysis of the different haplotypes (Librado and Rozas, 2009).

RESULTS

Selection analysis

The result showing selective forces in the *cyt-b* sequence of the three chicken genotypes is presented in Table 1. In the normal feather genotype, positives selective force occurred one time with a positive non-synonymous substitution of 0.4644. Similarly, negative selective force occurred at a single site index with d_N-d_S of -0.8411, while neutral selection occurred at the remaining sites. The only selective force that was observed in the *cyt-b* sequences of the frizzle feather genotype was positive which took place at 2 site indexes with d_N-d_S of 0.905. Naked neck genotypes did not experience any positive or negative selective force as all the sites were observed as neutral (Table 1).

Haplotype distribution

Analysis of haplotype distribution showing haplotype sharing across the genotypes is presented in Table 2. There were 4 haplotypes observed across the genotypes. Haplotype 1 (Hap_1) was shared by 30 individuals cutting across the three genotypes with a frequency of 0.882 (88.2%). Hap_2 and Hap_4 were not shared as they were only observed in normal feather genotypes (NF_107

and NF_110) respectively. Hap_3 was shared by two individuals belonging to the naked neck. Haplotype network of all the genotypes is presented in figure 2.

Maternal lineage

Maximum likelihood phylogenetic tree showing the maternal lineage of chicken genotypes based on mitochondrial *cyt-b* using the query sequence of Nigerian chicken genotype within the population with 10 reference sequences obtained from GenBank (KF650606.1, HQ122606.1, EU826429.1, KM224424.1, KP231500.1, DQ512917.1, AY724764.1, G027618.1, FM 205718.1 and AJ 971340.1) is presented in Figure 3. From the lineage analysis, Nigerian chicken genotypes within the population used in this study shared the same lineage with *Gallus gallus* (red jungle fowl) from the GeneBank.

Table 1: Analysis of selection pressure in chicken genotypes

Genotype	Selection type	d_N	d_S	d_n-d_s	Site-index	P-value
Normal Feather (NF)	Positive	0.4644	0.00	0.4644	1	0.718
	Negative	0.00	0.8411	-0.8411	1	1.000
	Neutral	0.00	0.00	0.00	83	0.00
Frizzle Feather(FF)	Positive	0.905	0.00	0.905	2	0.807
	Negative	0.00	0.00	0.00	0.00	0.00
	Neutral	0.00	0.00	0.00	88	0.00
Naked Neck (NN)	Positive	0.00	0.00	0.00	0.00	0.00
	Negative	0.00	0.00	0.00	0.00	0.00
	Neutral	0.00	0.00	0.00	85	0.00

d_N (non- synonymous)

Table 2: Haplotype distribution in three Nigeria chicken genotypes

Haplotypes	Number of samples	Chicken genotypes	Haplotype frequency (%)
	30	NF_{130}, NF_{140}, NF_{105}	
Hap. 1		NF_{106}, NF_{108} NF_{112} NN_{115}, NN_6, NN_{12}, NN_{23}, NN_{26}, NN_{28} NN_{29}, NN_{32}, NN_{33} NN_{34}, NN_{35}, NN_{36} NN_{38}, NN_{40}, NN_{41} FF_{49}, FF_{50}, FF_{51} FF_{56}, FF_{57}, FF_{62} FF_{75}, FF_{102}, FF_{25}	0.882 (88.2%)
Hap_2	1	NF_{107}	0.029 (2.90%)
Hap $_3$	2	NF_{109}, NF_{111}	0.059 (5.90%)
Hap.4	1	NF_{110}	0.029 (2.90%)

NN =Naked neck, FF= Frizzle feather and NF= Normal feather

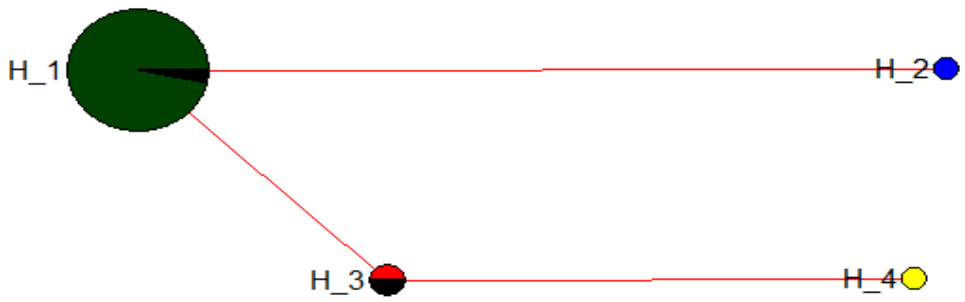

Fig. 2: Haplotype network of the three chicken genotypes

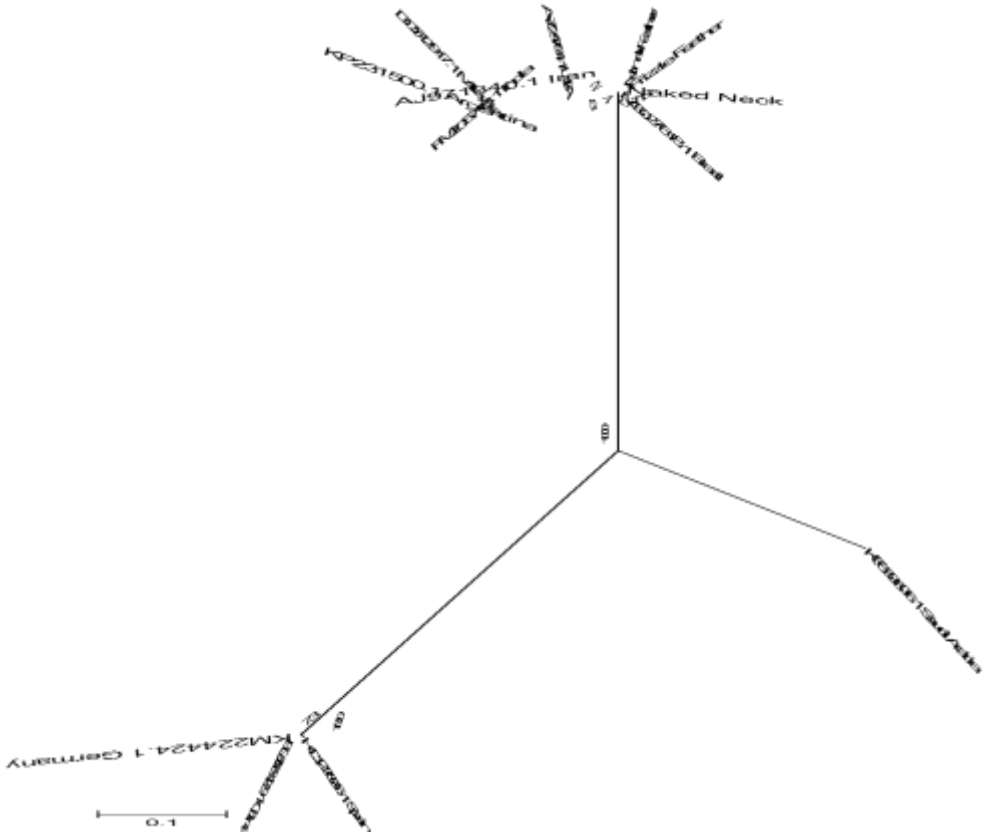

Fig. 3: Maximum likelihood phylogenetic tree showing maternal lineage of chicken genotypes in Nigeria

DISCUSSION

Knowledge of the genetic structure of species in a population is very necessary in developing conservation and management strategies for threatened species (Ekerette *et al.*, 2018). Local chicken are the most diverse of all livestock species. They provide mankind good source of protein from their meat and egg. Local chickens are independent and hardy birds with the ability to withstand harsh weather conditions and adapt to adverse environmental conditions. They have the ability to hatch on their own and possess immunity from endemic diseases. Therefore, it is necessary to assess their genetic variations for

possible selective breeding and genetic improvement. A scientific proof that the local chicken responds positively to genetic improvement strategies could stimulate public or private sector investment to improve the local chicken. There is a growing interest in research involving the quantification of selection pressure and their role to genetic polymorphism in populations. In this perspective, several methods have been proposed (Bandelt *et al*., 1999; Yang, 2002; Nielsen, 2002) to determine new mutations which may have a selective advantage over others in a population. Positive selection occurs when an extreme phenotype is favoured over other phenotypes in a population (Huelsenbeck and Dyer, 2004) while negative selection occurs when the effect of deleterious genes are gradually removed or selected against in a population until all the associated alleles are removed. Neutral selection on the other hand does not affect organism's ability to survive and reproduce (Molles, 2010). In this study, the rate of synonymous to non-synonymous substitution (d_N-d_S) for positive selection site index was high in frizzle feather but low in normal feather and naked neck indicating the presence of positive selection pressure in the populations. It was reported that positive selection occurs when populations experienced new environmental pressures as a result of migration from one environment to another leading to rapid changes in allelic frequency and speciation (Ekerette *et al*., 2018). Thus, the positive selection pressure identified in the frizzle feather populations may not be unconnected with the high level of d_N-d_S substitution rate for the positive site index considering that local chicken are known to be independent and migratory birds. This therefore is an indication that many alleles in the population are under the positive selection advantage of perpetuity which may eventually lead to population structuring and speciation over time. It may also be an indication that certain haplotypes in the population are under selective advantage that may positively influenced their adaptation. Negative selection pressure was also recorded in the population but in a lower d_N-d_S and negative site index. This is an indication that the rate of negative/purifying selection in the population may be low. As documented earlier, negative selection reduces the rate of deleterious genes in population (Fay *et al*., 2002). Thus, it is possible that the selection pressure arising from negative selection in the local chicken populations may act to remove the effect of deleterious mutations in their habitat. This may have positive impact on the survivability of these species in their habitats. The negative selection pressure may be effective in mitigating the effect of deleterious homozygosis arising from genetic drift and inbreeding activities among the populations. Haplotype distribution of mtDNA showed low genetic variation. Charlesworth (2006) 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 our study. According to Liu *et al.* (2006), haplotype diversity are key parameters for assessing population polymorphisms and genetic differentiation. The low frequency of haplotype observed in this study may suggest the existence of low molecular differences within and between the populations of local chicken used. 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 our study was nine (9) which was lower than 31 haplotypes reported by Ajibike *et al*. (2017), 20 haplotypes by Hassaballah *et al*. (2015), 11 haplotypes by Adamu *et al*. (2016) and 28 haplotypes by Gao *et al*. (2017). The observed haplotype was also lower than that reported by Muchadeyi *et al.* (2008) and Hoque *et al*. (2013). The terrain of the South South zone might have contributed to the low genetic diversity in the present study. It might be attributed to the loss of chickens due to environmental disaster such as flooding, environmental pollution and oil spillage (Ohwojakpor *et al*., 2012), which occur often in this area making the environment unconducive for local chicken to strive maximally. Maximum likelihood phylogenetic tree showing the maternal lineage of chicken genotypes based on mitochondrial *cyt-b* using the query sequence of Nigerian chicken genotype within the population

with 10 reference sequences obtained from GeneBank indicated that local chicken originate from Asia and may also share lineage with Europe. Our study revealed a multiple maternal lineage to local chicken. This result is compatible to reports by Muchadeyi *et al.* (2008), Oka *et al.* (2007) and Mtileni *et al.* (2011) who all reported multiple maternal lineages among local chickens.

CONCLUSION

The findings of this study revealed that positive, negative and neutral selection pressures were detected in the local chicken populations which must have played a critical role in the evolution of the chicken mitochondrial DNA. Selection pressures help to modify alleles in individuals and make them more fit in their natural environments. It is highly recommended that further studies are carried out to select chicken genotypes with advantageous alleles under selection pressure for hybridization and breeds improvements. Multiple maternal lineage was found from the maternal lineage analysis.

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