Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.10, 2016

Polymorphism of *IGF-1* Promoter and the UTR Regions of Nigerian Locally Adapted Chickens

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Abstract

Growth traits which are controlled by many genes are important economic traits in the poultry industry. The insulin-like growth factor gene (*IGF-I*) is a candidate gene for growth, body composition and metabolism, skeletal characteristics and growth of adipose tissue and fat deposition in chickens. The promoter and the untranslated region (UTR) of Insulin growth factor *I* gene was investigated to identify single nucleotide polymorphism, their genetic diversity and evolutionary relationship among six locally adapted strains of chicken in Nigerian. In this study, blood samples were collected and a specific primer pair was designed for amplifying a fragment of *IGF-1* gene using polymerase chain reaction (PCR). The PCR products were sequenced while nucleotide sequences generated were edited and aligned using Mega v6.0 software. Nucleotide polymorphisms within each strain were detected using DNAsp v5 software. A total of eight SNPs were identified across the populations studied which were different already published SNPs associated with growth rate in chicken. Naked Neck chicken showed the highest genetic diversity from others with the highest number of polymorphic site, haplotype diversity and nucleotide diversity while the least were observed in Frizzle Feathered chicken. The phylogenic tree showed that small genetic differentiation exists among the chicken populations in this region. We reported the first genetic data from the promoter and the UTR regions of *IGF-I* gene in Nigerian native chickens and established baseline information regarding variation in insulin growth factor *I* which should inform continued investigations into production potentials of these chickens and formulation of appropriate selection and breeding programs

Keywords: SNPs, Genetic diversity, *IGF-1,* Chicken

1. Introduction

Indigenous chickens exhibit large variation in body size, plumage colours, feathering pattern, eggshell, earlobe and shank colour (Adene, 2004). Their meat and eggs are preferred widely by consumers because of their taste, leanness and suitability for special dishes (Horst, 1989). According to FAO (2007), animal genetic diversity allows farmers to select stocks or develop new breeds in response to environmental change, threat of disease, new knowledge of human nutrition requirement, changing market conditions and societal needs. Selection according to genotype has the power to increase productivity of farm animals as well as to enhance environmental adaptation and maintenance of genetic diversity (Hayes *et al*., 2009). There is a general consensus that introducing high yielding livestock breed into traditional and extensive production system can lead to a loss of genetic diversity in indigenous animals (Sere and Steinfeld, 1996). This may be due to genome dilution of "local hybrid" through crossbreeding during and after such program. To improve production traits and health simultaneously, it is appropriate to use molecular markers associated with one or two characteristics. The insulin-like growth factor gene (*IGF-1*) is a candidate gene for growth, body composition and metabolism, skeletal characteristics and growth of adipose tissue and fat deposition in chickens (Zhou *et al*., 2005). Expression of IGF system genes has been studied throughout development of mammals in many different tissues including liver, muscle, bone, reproductive organs, and the central nervous system (Allan *et al.,* 2001)

The insulin-like growth factor I is produced in the liver in response to the action of the growth hormone in the pituitary (Zhou *et al*., 2007). The subsequent isolation and amino acid sequence determination of somatomedin C (Klapper *et al*., 1983) and A (Enberg *et al*., 1984) has shown their identity with IGF-I. The use of the two designations somatomedin and IGF-I as synonyms is now generally accepted (Daughaday *et al*., 1987). The role IGF might play in the development and function of the nervous system has aroused considerable interest in recent years (Baskin *et al*., 1988). The discovery in the central nervous system of significant amounts

of IGF (Sara *et al*., 1986) of differential expression of *IGF-I* and 11 which is developmentally regulated (Rotwein *et al*., 1988), of IGF receptors and of release of immunoreactive IGF in brain explants (Binoux *et al*., 1981) has initiated the IGFs to the habitues of neurobiology.

Several studies have shown that circulating *IGF-I* affects growth rate, body composition and lipid metabolism in poultry (Ballard *et al*., 1990; Beccavin *et al*., 2001; Zhou *et al*., 2005). The chicken IGF-I gene has been cloned and sequenced and is composed of four exons and three introns (Kajimoto and Rotwein, 1991). It has been shown that a SNP of IGF-I gene (PstI-RFLP) was associated with body weight at 2 and 4 months of age in the Wanzhai Yellow breed chickens (Wang *et al*., 2004). In serum of different species, over 99% of IGF molecules circulate as complexes to at least 7 specific and high affinity-binding proteins, which regulate bio-activity of IGF (Lei *et al*., 2005). Studies have shown that there is no direct dependence between the levels of growth hormone and the growth rate in chickens, therefore the need to study insulin-like growth factors (Beccavin *et al*., 2001) as mediators of the functions of the growth hormone (Lei *et al*., 2005). The IGF-I especially the promoter and the UTR regions have been studied widely across different geographical regions of the world because a polymorphism within the region (A507C) is associated with feed intake, growth rate, carcass traits and lipid metabolism in poultry (Nagaraja 2000; Baccavin *et al*., 2001; Amills *et al*., 2003; Zhou *et al*., 2005). For Nigerian native fowls characterized with slower growth rate and relatively poor performance, promoter and the UTR regions provide a practical starting point to investigate polymorphism within the *IGF-I*.

The chicken populations of Nigeria comprise of Normal feathered, Frizzle feathered and Naked neck; these are the primary indigenous populations that represent important genetic resources of Nigeria. Through crossbreeding and intensive selection over many generations, other populations have also been developed including the FUNAAB Alpha 1 and FUNAAB Alpha 2 at the Federal University of Agriculture of Agriculture, Abeokuta, Nigeria for improved meat and egg production without sacrificing adaptation to tropical environment characterized with heat stress and infectious diseases. The native chickens of Nigeria are of special interest because they serve as a major source of livelihood for the majority of the rural households especially women in Nigeria that are into their production. Our objectives here were to characterize the promoter and the UTR regions of Nigerian native chickens and to compare the pattern of variation with that of commercial broiler which has been selected for improved growth rate. Ultimately we aim to report the first data on *IGF-I* diversity in Nigerian native chickens and establish a baseline for comparison for future studies investigating the genetics of production potentials in Nigerian native fowls

2. Materials and Methods

2.1 Experimental Bird and Study location

The experimental birds comprise the three Nigerian indigenous ecotypes (Frizzle Feathered, Normal Feather and Naked Neck), the FUNAAB Alpha 1, the FUNAAB Alpha 2 and the Arbor Arce commercial broiler chicken. The experimental birds comprise 360 chicken representing 60 each from each population. The experiment was carried out at the Poultry Breeding Unit of Directorate of University Farms, Federal University of Agriculture, Abeokuta, Nigeria. The University is located within latitude 7° 10'N and longitude 3°2'E and lies in the south western part of Nigeria. It has an average temperature of 33.7^{o}C and relative humidity of 80% with rainfall of about 1037mm. the vegetation in the University represents an interphase between the tropical rainforest and the derived savannah (Goggle Earth, 2016).

2.2 Blood collection

Blood samples were collected from all the chickens using a syringe from the right jugular vein with a new needle and syringe for each individual to avoid cross contamination. The blood collected was transferred into Ethylene Di-amine Tetra-acetic Acid (EDTA) bottle to avoid coagulation by forming a complex with the oxygen in the environment which can have a negative effect on further analysis.

2.3 Genomic DNA Extraction and Quantification

DNA was extracted from the whole blood using Qiagen DNA extraction kit following the manufacturer protocol. The purity and concentration of the extracted DNA was carried out using Nano-drop spectrophotometer.

2.4 DNA Amplification and Sequencing

The genomic sequence and primer characteristics reported by Zhou *et al.* (2005) and Nagaraja *et al*. (2000) were used to for this research (Table 1). The amplification product was 813 bp involving the promoter and the 5' UTR regions of the chicken *IGF-I* gene based on reference sequence with accession number M74176 (Zhou *et al*.,

2006). The PCR mixture consisted of 1µl of 50ng chicken DNA template, 0.25 µl of 10 µM of forward and reverse primers, 3.2 µl dNTP mixture, 0.2 µl Taq polymerase (Promega, USA), 2.0 µl 10X Buffer and ddH_2O to a final volume of 20µl. The amplification procedure was as follows; 5 min at 94°C for initiation of denaturation, then 45s at 94°C (final denaturation), 45 s at 58°C (annealing), 60 s at 72°C for extension (30 cycles), followed by 5 min at 72°C for final extension on Agilent Surecycler 8800. PCR products were analysed by gel electrophoresis on a 1.2 % agarose gel containing ethidium bromide. The PCR products were purified and read in a 3730XL sequencer from Applied Biosystems (STAB Vida, Oelras, Portugal).

2.5 Sequence analyses

Sequence alignment, trimming and polymorphism detection were carried out using Chromas v2.33. (http://www.technelysium.com.au/chromas.html) and BioEdit (Hall, 1999). ClustalX (www.clustalx) and MEGA 6.0 software (Tamura et al., 2013) were also used. Haplotype diversity, nucleotide diversity, number of segregating site were estimated using DnaSP v5 (www.ub.edu/dnasp). The indices of diversity measured include number of haplotype, haplotype diversity, nucleotide diversity, number of polymorphic site, singleton variable site and parsimony informative site for each population of the chicken. Relationships among the Nigerian native chickens and two sets of alleles representing other chicken assessed from the GenBank were determined using Neighbor-joining phylogenies of maximum composite likelihood distances and tested 10,000 boostrap replicates based on *IGF-I* gene using MEGA 6.0 (Tamura *et al*., 2013).

3. Results and Discussion

Two DNA sequences of the promoter and the UTR of IGF-I gene were obtained from the GenBank. The two DNA sequences were the same for the blast result of the Arbor acre and Frizzle Feathered chicken in our populations. The DNA sequences retrieved were for Rizhao Partridge chicken with the length of 580bp and Luxi Game chicken of 622bp as presented in Table 2 and were included in the drawing of phylogenetic relationships among the chicken populations.

After removal of sequences differed by only one nucleotide, trimming of the ends of the sequences to remove artefacts and some sequences that were noisy, eight distinct alleles or polymorphisms from 549bp portion of the promoter and UTR regions of chicken *IGF-I* were identified among the six chicken populations and each chicken population was found to contain at most two alleles, consistent with a single locus. Polymorphism and allelic frequencies with the different population carrying it are shown in Table 3a and 3b. The results showed that Arbor Arce which is a commercial and exotic broiler in Nigeria had 5 SNPs out of the eight discovered in these populations with allele frequency ranging from 0.07 (C201T, T314C) to 0.22 (G291T). This indicated that the popular allele (A507C) being referred to as 'broiler allele' (Zhou *et al*., 2005) and which is associated with feed intake, growth rate, carcass traits and lipid metabolism in poultry (Nagaraja 2000, Zhou *et al*., 2005; Amills *et al*., 2003; Baccavin *et al.,* 2001) in the previous studies was not covered by our sequencing or might have fallen in the region trimmed off due to background noise at the ends of the sequences. It has been shown that chickens inheriting *IGF-I* broiler alleles have heavier body weight at all ages to market weight and average daily gain than birds with the leghorn allele and that plasma chicken *IGF-I* concentration was greater in genetic lines selected for high growth rate compared with that in slower growing lines (Scanes *et al*., 1989; Zhou *et al*., 2005). Identification of other polymorphisms that have not been previously described although still segregating indicates high genetic diversity within the region and any or some of this gene could be in linkage disequilibrium with associated SNP with growth performance in chicken. The naked neck chicken has all the polymorphisms with allele frequency ranging from 0.07 (C541T) to 0.57 (A120G), the normal feathered chicken has only three polymorphisms with allele frequency ranging from 0.1 (C541T) to 0.63 (T341C). On the other hand, the frizzle feathered chicken did not have any polymorphism which might suggest that the region is relative conserved in the strain. For the other two strains that were developed through selection within the normal feathered chicken and series of crossbreeding with the arbor acre commercial broiler, the FUNAAB Alpha 1 which is a dihybrid cross has three polymorphisms with allele frequency ranging from 0.07 (T457C) to 0.5 (T341C) while the FUNAAB Alpha 2, a trihybrid cross has five polymorphisms with allele frequency ranging from 0.03 (C201T) to 0.27 (T481C). The highest number of polymorphism was observed in Naked Neck chicken with nothing observed in Frizzle Feathered chicken. This suggested that there is diversity in *IGF-I* gene in the different populations of our locally adapted chickens. All these polymorphisms with low to moderately high frequencies will need to be examined through association studies to detect their influence on production performance if any. The lack of fixed allele or the presence of other alleles in our native chickens corroborate that the individuals have not been selected as a breeder hen for improved growth rate in chicken of future

generation.

The highest number of polymorphic site was discovered in naked Neck (Table 4) with Frizzle Feathered chicken having no polymorphic site. Haplotype and nucleotide diversity with average number of nucleotide differences followed the same trend with naked neck chicken having the highest while the frizzle feathered chicken had the least. The highest number of haplotype was also observed in naked neck. Our overall calculated nucleotide diversity was 0.00237 with naked neck with highest diversity having 0.00395 which decreased in FUNAAB Alpha 2, arbor acre, normal feathered, FUNAAB Alpha 1 and frizzled feathered in that order. The generally low diversity suggested low mutation rate in this region. The native chicken especially the naked neck had a higher diversity than others native chicken and the exotic counterpart. This may be as a result of difference in their effective population size. It has been reported that SNP frequency and nucleotide diversity are affected by several factors, including selection, mutation rates, mating system, effective population size, and demography, gene flow between populations, introgression from hybridisation and historical effects on these factors (e.g., population bottlenecks) (Nei, 1987; Frankham *et al*., 2002). High nucleotide diversity has been reported in the avian genome (Primmer *et al*., 2002), the low level observed in our study might be connected to the length of the sequence used. The significance of the gene in determining the growth performance of chicken also might prevent unnecessary mutations that might impair its function.

Twenty two haplotypes were identified in the study with ten being shared between the populations and twelve specific to different populations (Tables 5 and 6). Highest number of haplotypes and haplotype diversity were detected in naked neck chicken compared to other chickens used in the study. This showed that the Naked Neck *IGF-I* gene has the highest degree of allelic variation compared to the other breed of chicken used in this study. The degree of the allelic variation extends to the genetic diversity of the gene. High diversity should enhance the adaptation of these species as it could provide the evolutionary potential to adapt to the rapidly changing environmental condition of the tropical climatic conditions.

The phylogenic tree showed that the eight strains of chicken can be separated into two cluster with first cluster consisting of Rizhao Partridge as a distinct strain on the evolutionary scale while other strains diverged progressively. The second cluster composed of Luxi Game chicken followed by FUNAAB Alpha 2, Frizzle Feathered, Arbor Arce, Naked Neck chicken, FUNAAB Alpha and Normal Feathered chicken as a sub-cluster. The phylogenic tree showed that FUNAAB Alpha 2 and Luxi Game chickens are closed to each other with bootstrap value of 98. This suggests that they share a recent common ancestor as reported by Okamoto *et al.* (1999) that small genetic differentiation exists among the chicken populations. While the strains of chicken tend to group together loosely phylogenetically, the inclusion of the Luxi Game chicken in the trend illuminates the tendency of alleles to be shared past strains not minding the geographical barrier although this is not well supported by bootstrap value probably due to length of the sequence analyzed.

4. Conclusion

Analysis of the promoter and the UTR regions in our locally adapted chickens showed the existence of polymorphisms except in frizzle feathered chicken, the level of which varied according to genotype. The naked neck chicken had the highest number of polymorphism with higher nucleotide and haplotype diversity. Number of polymorphic site, haplotype diversity and nucleotide diversity show the level of allelic variation in the *IGF-I* gene of chicken which result in the genetic diversity of the *IGF-I* gene in our native chickens. The phylogenetic tree showed that small genetic differentiation exists among the chicken population studied. For the final confirmation of the observed new allelic pattern, the association between the alleles and our native chickens should be considered to see whether they are associated with production traits. Whether the new variants observed here are unique to this population remains to be seen. It would be possible to direct our selection schemes to favor the desired genotypes for improved growth rate and other related parameters in chicken

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Table 1: *IGF-I* primers used in the study

Primer	AT (^{o}C)	PCR product (bp)
5′-TCAAGAGAAGCCCTTCAAGC-3′ 5′-CATTGCGCAGGCTCTATCTG-3′	60	813

Source: Nagaraja *et al*. (2000)

Table 2: Sequence retrieved from the public domain (GenBank)

GenBank Accession number for *IGF-I* gene	gene Name	Number of base pair	Chicken Name
EF488284.1	*IGF-I* gene	580bp	Rizhao Partridge
EF198877.1	*IGF-I* gene	620bp	Luxi Game

Table 3a: Single nucleotide polymorphism of *IGF-I* of Nigerian native fowls

No	Position	SNP	Strain	Allele frequency
1	120	A>G	NK	0.57
2	201	C>T	AA	0.07
	201	C>T	NK	0.2
	201	C>T	Fα2	0.03
3	291	G>T	AA	0.22
	291	G>T	NK	0.53
	291	G>T	Fα2	0.13
4	341	T>C	AA	0.07
	341	T>C	NK	0.3
	341	T>C	NM	0.63
	341	T>C	Fα1	0.5
	341	T>C	Fα2	0.22
5	457	T>C	AA	0.1
	457	T>C	NK	0.23
	457	T>C	Fα1	0.07
	457	T>C	Fα2	0.07

AA = Arbor acre, NK = Naked neck, NM = Normal feathered, Fα1 = FUNAAB Alpha 1, Fα2 = FUNAAB Alpha 2

Table 3b: Single nucleotide polymorphism of *IGF-I* of Nigerian native fowls

No	Position	SNP	Strain	Allele frequency
6	481	T>C	AA	0.2
	481	T>C	NK	0.37
	481	T>C	Fα1	0.27
	481	T>C	Fα2	0.27
7	541	C>T	NK	0.07
	541	C>T	NM	0.1
	545	T>A	NK	0.1
	545	T>A	NM	0.2

AA = Arbor acre, NK = Naked neck, NM = Normal feathered, Fα1 = FUNAAB Alpha 1, Fα2 = FUNAAB Alpha 2

Table 4: Haplotype and nucleotide diversity of the of the promoter and the UTR regions of *IGF-I* gene of Nigerian native fowls

Strain	S	No of H	Haplotype diversity	K	Pi
AA	5	5	0.295	0.909	0.00166
NK	8	13	0.757	2.168	0.00395
FF	0	1	0.000	0.000	0.00000
NM	3	6	0.610	0.833	0.00152
Fα1	3	5	0.673	1.093	0.00199
Fα2	5	7	0.516	0.978	0.00178
Total	8	22	0.624	1.301	0.00237

H = Haplotype, K = Average number of nucleotide differences, Pi = Nucleotide diversity, S = number of segregating site

Table 5: *IGF-I* gene haplotypes in the six chicken populations

Haplotype	Frequency	Sequence
Hap_1	145	ACGTTTCT
Hap_2	8	A.TC.C..
Hap_3	6	.TT.CC..
Hap_4	1	..T..C..
Hap_5	3	..T.CC..
Hap_6	16	G…….
Hap_7	1	.TTC.C..
Hap_8	1	.TT..C..
Hap_9	1	..TC..TA
Hap_10	1	G.TC..TA
Hap_11	1	G.T….A
Hap_12	1	..TCCC..
Hap_13	1	..TCC…
Hap_14	1	.TT.C…
Hap_15	2	……TA
Hap_16	1	…C..TA
Hap_17	2	…….A
Hap_18	32	…C….
Hap_19	1	…C…A
Hap_20	14	…C..C.
Hap_21	4	…CCC..
Hap_22	2	…..C..

Table 6: *IGF-I* haplotypes shared within and between the six chicken populations

Haplotype	Frequency	AA	NK	FF	NM	Fα1	Fα2
1	145	31	19	14	24	23	34
2	8	2	4				2
3	6	2	3				1
4	1	1					
5	3	1	1				1
6	16		16				
7	1		1				
8	1		1				
9	1		1				
10	1		1				
11	1		1				
12	1		1				
13	1		1				
14	1		1				
15	2			2			
16	1			1			
17	2			2			
18	32				17	9	6
19	1			1			
20	14					9	5
21	4				4		
22	2				1	1	

AA = Arbor acre, NK = Naked neck, FF = Frizzle feathered, NM = Normal feathered, Fα1 = FUNAAB Alpha 1, Fα2 = FUNAAB Alpha 2

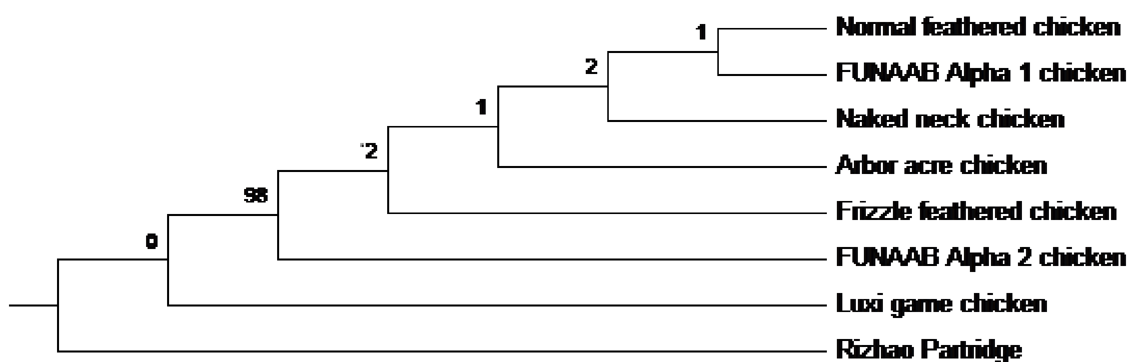

Figure 1: Neighbor-joining phylogenies among the six chicken populations and other chickens from GenBank