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Original scientific article

SEQUENCE ANALYSES OF INSULIN-LIKE GROWTH FACTOR 1 GENE IN NIGERIAN INDIGENOUS AND ARBOR ACRE CHICKENS

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The chicken *Insulin-like growth factor 1* (*IGF1*) is a candidate gene for growth, body composition and metabolism, skeletal characteristics and growth of adipose tissue and fat deposition in chickens. It is mapped to 165.95 cM on chromosome 1 and composed of four exons and three introns, spanning more than 50 kb. Genomic DNA was extracted from blood samples collected from the experimental birds using Qiagen DNA extraction kits. Polymersae chain reaction (PCR) was carried out using established primers. The PCR amplicon involving 5'untranslated region were sequenced. The sequences were analysed to identify polymorphisms, their genetic diversities and evolutionary relationships among three strains of Nigerian indigenous chickens [Frizzle Feathered (7), Normal Feathered (19) and Naked Neck (19), and the Arbor Acre broiler chicken (17)]. Nucleotide sequences generated were edited and aligned using Codon Code Aligner. Diversity analysis was done using DnaSp while MEGA6 software was used to plot phylogenetic tree using maximum likelihood method. A total of nineteen single nucleotide polymorphisms (SNPs) were detected from 560 bp portions of the 5'UTR among the four chicken populations studied with none detected in the Frizzle feathered chicken. The Naked neck chicken had the highest number of SNP's (13), haplotypes (6), haplotype

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diversity (0.778), nucleotide diversity (0.00487), average number of nucleotide differences (2.725), highest number of polymorphic (segregating) sites (13), parsimony informative site (5) and singleton variable site (8). The Naked neck chicken therefore had the highest rate of mutation and degree of allelic variation compared to other chicken strains used in this study. The phylogenetic tree showed that small genetic differentiation exists among the chicken populations studied. Some of the SNPs are newly discovered; hence, association between these alleles and productive traits in Nigerian native chickens is desirable in future studies.

Keywords: IGF1, FUNAAB Alpha, Indigenous chicken, Gene, Sequence.

INTRODUCTION

Nigerian indigenous chickens constitute about 80 % of the 166 million poultry birds in Nigeria (FAO, 2007). They are hardy and generally reported to adapt favourably to the local environment. The chickens are light in nature, resistant to some tropical diseases and parasites and lay small-sized eggs with relatively thick shells (PETERS *et al*., 2007). ODUBOTE (2015) reported that the Nigerian indigenous chicken exhibited large variation in the body size, plumage colour, and feather characteristics. These chickens can be categorized into various genetic groups having verifiable genes that have direct and indirect effects on productive and reproductive traits (FAYEYE *et al*., 2006). The three major genes, which include the Normal feathered, the naked neck and the Frizzled feathered are associated with heat tolerance traits and possess productive adaptability (FAYEYE *et al*., 2006).

In an effort to address the problem of low productivity in local chickens, high-yield exotic breeds have been introduced into Nigeria; one of these breeds is the Arbor Acre chicken. Arbor Acre is a broiler chicken that has high growth rate, feed conversion ratio (FCR), liveability, and meat yield which are consistently improved with continued genetic advances; also being made in bird welfare, leg health, cardiovascular fitness, and robustness (www.aviagen.com, 2014). Broiler chickens are raised specifically for meat production (LIU, 2009). They are important sources of high quality protein. The growth rate and body weight of broiler chickens had been increased remarkably by decades of genetic selection (HAVENSTEIN *et al*., 2003).

Insulin-like growth factor 1 (*IGF 1*) also called somatomedin is a growth hormone which was identified in 1957 by Salmon and Daughaday and designated "sulphation factor" by their ability to stimulate 35-sulphate incorporation into rat cartilage (LARON, 2001). In the plasma, 99% of I*GFs* are complexes to a family of binding proteins, which modulate the availability of free *IGF 1* to the tissues. *IGF 1* is secreted by many tissues and the secretory site seems to determine its actions. Major part of *IGF 1* is secreted by the liver and transported to other tissues, acting as an endocrine hormone. *IGF 1* is also secreted by other tissues, including cartilaginous cells, and acts locally as a paracrine hormone. It is also assumed that *IGF 1* can act in an autocrine manner as an oncogene. As reviewed by KADLEC *et al*. (2012), IGFs belong to the family of polypeptide hormones; they are structural homologues of insulin and also have a similar function. Hormones such as the growth hormone, *IGF*, thyroid hormones and insulin, play important and diverse roles in chicken's growth and carcass characteristics (ZHOU *et al*., 2005; LI *et al*., 2008).

Most of the functions of the growth hormone in chickens are mediated by insulin-like growth factors (LEI *et al*., 2005) which stimulate amino acid uptake, glucose metabolism, DNA synthesis, protein synthesis, and the proliferation of different cell types. They are also involved in the regulation of growth and carcass characteristics. As reviewed by LI *et al.* (2008), studies have shown that the growth hormone - insulin-like growth factor-1 (*GH-IGF 1*) system affects productive traits and its polymorphism has been discovered in various chicken breeds as related to growth. Hence, the *IGF 1* gene polymorphism could be a potential marker for use in marker-assisted selection programmes for improving performance of Nigerian indigenous chickens. Most variations in the *IGF 1* gene have been found at the promoter and 5' flanking regions. Therefore, sequence variations in this portion of *IGF 1* gene can potentially interfere with the normal relationship in protein expression. This provides a basis for considering diversity of *IGF 1* gene sequence in Nigerian indigenous and Arbor Acre chickens and to establish a baseline for comparison in investigating the genetics of their production potentials.

MATERIALS AND METHODS

Experimental Site

The research was conducted at the Poultry Breeding Unit of Directorate of University Farms (DUFARMS) of Federal University of Agriculture, off Alabata road, Abeokuta, Ogun State, Nigeria (7º10'N and 3º2'E). The area lies in the south-western part of Nigeria with a prevailing tropical climate and a mean annual rainfall of about 1037 mm. The mean ambient temperature ranges from 21.8ºC during the coldest period (July-September) to 33.2ºC during the hot period (February-April). Relative humidity ranges from 60% in January to 94% in August with a year average of about 82% (www.accuweather.com).

Experimental Chickens

A total of one hundred experimental birds (25 from each genotype) comprising three genotypes of Nigerian indigenous chickens (Frizzle Feathered, Normal Feathered and Naked Neck), and the Arbor Acre broiler chicken were utilized in the study.

Blood Sample collection

Blood samples were collected from the wing vein of all the birds using 2ml disposable syringe. The area of the skin was disinfected with methylated spirit. 1mls of the blood taken was transferred into a labeled test tube containing an anti-coagulant ethylene diamine tetra-acetic acid (EDTA) to avoid coagulation by forming a complex with the oxygen in the environment which can have a negative effect on further analysis.

DNA Extraction

DNA was extracted from the whole blood collected from all the birds using Zymo Research DNA extraction kit following the manufacturer's protocol. The purity and concentration of the extracted DNA was carried out using Nano-drop spectrophotometer.

DNA Amplification

The primers reported by ZHOU *et al*. (2005) were used for the amplification (Table 1). The amplification product involved the 5' UTR of the chicken *IGF 1* gene. The PCR

amplification protocol include 94ºC for 5 minutes and 35 cycles of 94ºC for 1 minutes, annealing at 60ºC for 45 seconds, extension at 72ºC for 1 minute and final extension at 72ºC for 10 minutes.

Table 1. IGF 1 gene primer for 5'UTR

Primer	AT (ºC)	PCR product (bp)
5′-CATTGCGCAGGCTCTATCTG-3′ (forward)	60	813
5′-TCAAGAGAAGCCCTTCAAGC-3′ (reverse)		

Source: Zhou *et al*. (2005)

Agarose gel electrophoresis

After the PCR, the amplified product was electrophoresed on a 1.5% (w/v) agarose gel stained with ethidium bromide in a 1 × TBE buffer at 100 volts for 1 hour. Alongside the sample fragments, a molecular size marker with different fragment sizes was electrophoresed on the same gel. The inclusion of a marker was important in order to estimate the size of the fragments as a confirmation of having amplified the right DNA fragment. The detection of the fragments amplified was done under ultra violet light (UV light) using a trans-illuminator and photographs taken to show the samples and markers in their lanes.

Sequencing of Amplified product

The amplicons were purified and sequenced according to the protocol of the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Life Technologies). Sequencing reactions were purified and applied in the automated ABI PRISM 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence Analysis

Each genotype sequences were aligned after trimming and editing in Codon Code Aligner. Some sequences were removed because they have much noise and were too short compared to others. A total of sixty-three (62) sequences were used for the research comprising seventeen (17) Arbor Acre, nineteen (19) Naked neck, twenty (19) Normal feathered and seven (7) Frizzled feathered. Single Nucleotide Polymorphisms (SNPs) were detected from the whole sequences using the Codon Code Aligner software. The aligned sequences were also separated based on genotypes and were tested for single nucleotide polymorphisms (SNPs). The locations of the SNPs were identified from the main sequence using the Chromas v2.33 (http://www.technelysium.com.au/chromas.html). The aligned sequences were loaded into Mega 6.0 (TAMURA *et al*., 2013) and saved in FASTA format for diversity analysis. Genetic diversity within each of the genotypes was estimated in DnaSP v5 (www.ub.edu/dnasp). Genetic diversity parameters, such as haplotype diversity (Hd), nucleotide diversity (Pi), number of segregating site (S), average number of nucleotide differences (k), singleton variable site (SP) and parsimony informative sites (PIP) were estimated. A consensus sequence was determined for the sequences within each of the genotypes using BioEdit (HALL, 1999). The consensus sequences were used in Genbank to search for similar sequences using Blastn. The similar sequences were identified based on their accession number and were downloaded in FASTA format. The retrieved

sequences were aligned with the consensus sequences from the four genotypes using Clustalw (www.clustalx) and MEGA 6.0 was used to plot phylogenetic tree using maximum likelihood. The DNA sequences retrieved from the Genbank are for Rizhao Partridge chicken with the Accession Number EF488284.1 and length 580bp and Luxi Game chicken with Accession Number EF198877.1 and length 622bp, respectively.

RESULTS

Detection of Single Nucleotide Polymorphisms (SNPs)

Nineteen (19) SNPs were detected from 560 bp portions of the 5'UTR regions of chicken *IGF 1* among four chicken populations and each chicken population was found to contain at most two alleles, consistent with a single locus when the sequences were pooled. The same result was observed when the analysis was done based on genotypes. The only exception was found at 551 bp which had three (3) alleles in Arbor Acre chicken. The polymorphism, its location, genotype and frequencies are shown in Table 2. The results revealed that the Naked neck (NK) had the highest SNPs of thirteen (13); Arbor Acre (AB) had nine (9) SNPs; the Normal feather (NM) had four (4) SNPs while the Frizzle feathered (FZ) had no SNP.

Table 2. Single Nucleotide Polymorphisms of 5'UTR region of IGF 1 gene in Nigerian Indigenous and Arbor Acre Chickens

No	Position	SNP	Genotype	Allele Frequency
1	21	T>A	NM	0.02
2	29	T>A	NM	0.02
3	57	A>C	NK	0.02
4	61	T>A	NK,	0.02
5	101	T>C	NK	0.02
6	106	C>A	NK	0.02
7	123	A>G	NK, NM	0.05, 0.14
8	205	C>T	AB, NK	0.05, 0.08
9	270	C>T	AB, NK	0.05, 0.08
10	295	G>T	NK	0.02
11	345	T>C	AB, NK, NM	0.05, 0.08, 0.14
12	461	T>C	AB, NK	0.05, 0.08
13	485	T>C	AB, NK	0.03, 0.02
14	503	A>C	NK	0.02
15	520	T>G	NK	0.02
16	551	A>G	AB	0.05
17	551	A>T	AB	0.02
18	552	C>G	AB	0.02
19	557	G>A	AB	0.05

NM= Normal feathered, AB= Arbor Acre, FZ= Frizzled feathered, NK= Naked neck.

Haplotypes and Haplotype frequencies

When all the sequences were pooled and analysed using DnaSp software, nine (9) haplotypes were discovered. Table 3 shows the haplotypes, the frequencies/ genotype and sequences that made up the haplotypes. In summary, Arbor Acre had three haplotypes (1, 3 and 6), Naked neck had six haplotypes (1, 3, 4, 5, 8 and 9), Normal feathered had three haplotypes (1, 2 and 7) and Frizzle feathered had one haplotype (1). The first haplotype had the highest frequency of thirty-six and it is made up of all the genotypes with Arbor Acre having the highest of fourteen, Naked neck, eight, Normal feathered, seven and frizzled feathered seven. The second haplotype is made up of only Normal feathered sequences. The third haplotype had a frequency of five with two from Arbor Acre and three from Naked neck. The fourth, sixth, seventh and ninth, had a frequency of one from Naked neck, Arbor Acre, Normal feathered and Naked neck. The fifth and eighth had frequencies of two and four from Naked neck. Two haplotypes were shared between the genotypes; these were haplotypes 1 and 3. Haplotype 1 had all the genotypes while haplotype 3 had only naked neck and Normal feathered.

Table 3. Haplotypes and haplotype frequencies among Nigerian Indigenous and Arbor Acre Chickens

Haplotype	Frequencies or Number of samples					
	AB	NK	NM	FZ	TOTAL	Sequence
1	14	8	7	7	36	CATTGATT
2			11		11	TTTCATAG
3	2	3			5	TTAGTAAG
4		1			1	CGTGTTGC
5		2			2	AAACGTGT
6	1				1	TTATTAAG
7			1		1	CTTTGATT
8		4			4	AGAGGTAT
9		1			1	AGAACCAG
TOTAL				62		

NM= Normal feathered, AB= Arbor Acre, FZ= Frizzled feathered, NK= Naked neck.

Genetic diversity

Genetic diversity analysis showed that the Naked neck had the highest number of polymorphic sites, haplotype diversity, nucleotide diversity, average number of nucleotide differences, singleton variable site and parsimony informative sites when the sequences were pooled: This was followed by the Normal feathered then and Arbor Acre. Frizzled feathered had no value for these parameters. The total values obtained for those parameters were 14, 0.629, 0.00917, 1.420, 9 and 5, respectively (Table 4).

Table 4. Genetic diversity of IGF 1 gene in Nigerian Indigenous and Arbor Acre Chickens

Genotype	S	h	Hd	K	Pi	SP	PIP
Arbor Acre	3	3	0.324	0.838	0.00150	0	3
Naked neck	13	6	0.778	2.725	0.00487	8	5
Normal feathered	2	3	0.556	0.596	0.00107	1	1
Frizzled feathered	0	1	0	0	0	0	
Total	14	9	0.629	1.420	0.00254	9	5

h= Number of Haplotype, S= Number of Polymorphic (Segregating) Site, Hd= Haplotype Diversity, k= Average number of nucleotide differences, Pi= Nucleotide Diversity, SP = Singleton Variable Site, PIP = Parsimony Informative Sites

Phylogenetic analysis

The phylogeny of the Nigerian indigenous and Arbor Acre chickens showed that Normal feathered chicken was more diverse from others genotypes (Figure 1). The phylogenetic tree further revealed that the six chicken genotypes can be separated into two main clusters with the first cluster consisting of Rizhao Partridge as a distinct strain on the evolutionary scale while other strains: Luxi Game chicken, Frizzle feathered, Arbor Acre, Naked Neck and Frizzled feathered chickens diverged together. The second cluster further diverged into two sub-clusters which composed of Luxi Game chicken, on one hand, and Frizzle Feathered, Arbor Acre, Naked Neck chicken, and Normal Feathered chicken, on the other hand (Figure 2).

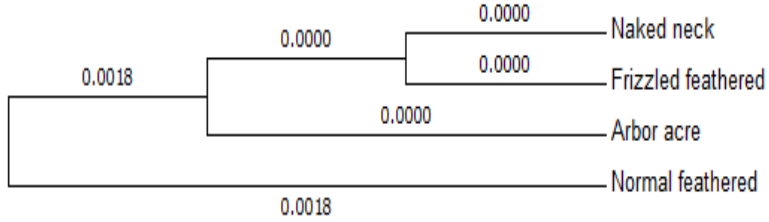

Fig. 1. Phylogenies among the four study populations of chickens

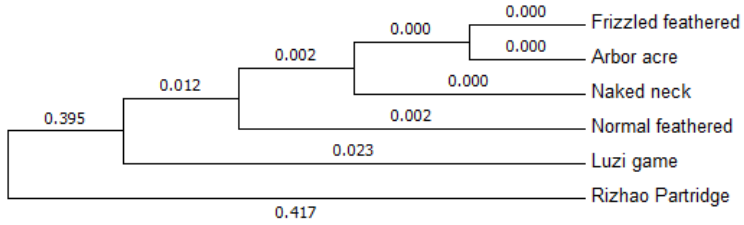

Fig. 2. Phylogenies among chicken populations in the Genbank

DISCUSSION

A total of nineteen (19) SNPs were detected in 560 bp portions of the 5'UTR regions of chicken *IGF 1* among the four chicken populations. This was different from the results obtained by ILORI *et al.* (2016), who detected eight (8) SNPs from 549 bp portion of the promoter and UTR regions of chicken *IGF 1* among six chicken populations using the three Nigerian indigenous chickens (Frizzle Feathered, Normal Feather and Naked Neck), the FUNAAB Alpha 1, the FUNAAB Alpha 2 and the Arbor Acre commercial broiler chicken. However, six out of the eight single nucleotide polymorphisms (SNPs) discovered by ILORI *et al.* (2016) corresponded with those discovered in this research which are SNP 7 (A>G), 9 (C>T), 12 (G>T), 14 (T>C), 15 (T>C) and 16 (T>C) located at 123 (NK, NM), 205 (AB, NK), 295 (NK), 345 (AB, NK, NM), 461 (AB, NK) and 485 (AB, NK) respectively. Also, the fact that no SNPs was observed in frizzled feathered agreed with ILORI *et al.* (2016). Other SNPs observed in this research must have been due to different forms of mutations that have occurred in codon particularly the replacement of certain nucleotides by others as well as the consideration of different portion of 5' UTR and / or number of base pairs (bp). It can also be due to differences in populations sampled and sample size in the two studies. Previous study indicated the presence of one (1) SNP in 5'UTR of the *IGF 1* gene sequences near a putative TATA box, and consisted of one A>C substitution at 570 position using two strains of black Penedesenca chicken (AMILLS *et al.*, 2003). The same SNP was identified by ZHOU *et al*. (2005) in F_2 population of Leghorn and Fayoumi which had a significant association with growth, body composition and skeletal integrity. Two A>C substitutions were observed in the present study. They were found in Naked neck at 57 and 503 bp. The A>C substitution at 520 bp in this research corresponded with that reported by the AMILLS *et al*. (2003) above researchers. ABBASI and KAZEMI (2011) also reported one SNP (C>T) in the promoter and 5'UTR of *IGF 1* in Mazandaran native chicken. Two C>T substitutions were detected at 205 bp and 270 bp of Arbor Acre and Naked neck. Also, AL-HASSANI *et al*. (2015) found T>C mutation at the site 279 bp of 5'UTR in *IGF 1* gene in Cobb500 and Hubbard F-15 chickens. Four T>C mutations were observed at 101 bp, 345bp, 461bp and 485bp. Perhaps, one of these might correspond with the mutation observed by AL-HASSANI *et al*. (2015). Some other researchers who identified SNP(s) in 5'UTR of *IGF 1* gene are NAGARAJA *et al*. (2000); KADLEC *et al*. (2012); SHAH *et al.* (2012); PANDEY *et al*. (2013); PROMWATEE *et al*. (2013) and BABAYI *et al*. (2014). ABDALHAL *et al*. (2016) reported a single mutation in exon one of *IGF 1* gene found to be significantly associated with growth and reproductive traits in Jinghai yellow chickens.

A total of nine (9) haplotypes were discovered in this research when the sequences were pooled (i.e. among genotypes) with the Naked neck birds having the highest number of haplotypes (6). This was similar to the results obtained by ILORI *et al*. (2016) who reported the naked neck as having the highest number of haplotypes. Arbor Acre and Normal feathered has equal number of haplotypes. BHATTACHARYA *et al*. (2015) discovered twelve (12) haplotypes in *IGF 1* gene sequence of Cornish, control layer and Aseel chickens while ILORI *et al*. (2016) discovered twenty-two (22) haplotypes in promoter and 5' UTR of six populations comprising three Nigerian indigenous chickens (Frizzle Feathered, Normal Feather and Naked Neck), the FUNAAB Alpha 1, the FUNAAB Alpha 2 and the Arbor Acre commercial broiler chicken. Also,

BIAN *et al*. (2008) reported that haplotypes based on three *IGF 1* polymorphisms (5'-flanking, exon 3, and 3'-flanking regions) were associated with body weight (BW) traits.

Naked neck had the highest haplotype diversity, nucleotide diversity, average number of nucleotide differences, number of polymorphic (segregating) sites, parsimony informative site and singleton variable site when the sequences are pulled together. This showed that the Naked neck *IGF 1* gene had the highest rate of mutation and degree of allelic variation compared to the other strains 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 phylogeny of the Nigerian indigenous and Arbor Acre chickens showed that Naked neck chicken was more diverse from others because it had higher rate of mutation which can increase the fitness of this genotype in the environment. The phylogeny tree of the six strains of chicken can be separated into two clusters with first cluster consisting of Rizhao Partridge as a distinct strain on the evolutionary scale while other strains Luxi Game chicken, Frizzle Feathered, Arbor Acre, Naked Neck and frizzled feathered chickens converged together. They second cluster further diverged into two sub-clusters which composed of Luxi Game chicken, on one hand and Frizzle Feathered, Arbor Acre, Naked Neck chicken, and Normal Feathered chicken on the other hands. This showed that the Luxi Game chicken, Nigerian indigenous chicken and Arbor Acre chicken had common ancestor. Also, the inclusion of the Luxi Game chicken in the trend illuminates the tendency of alleles to be shared between strains not minding the geographical barrier. This strongly supports the existence of trans-species polymorphism as reported by YAKUBU *et al.* (2017). The result of the phylogenetic tree showed that Normal feathered was more diverse from others. This was contrary to the findings of ILORI *et al.* (2016). They reported that Naked neck was more diverse from others in the evolutionary relationship.

CONCLUSION

Analysis of the 5'UTR *IGF 1* gene sequence in Nigerian local and Arbor Acre chickens showed the existence of polymorphisms except in frizzle feathered chicken. The Naked neck chicken had the highest number of polymorphisms. The phylogenetic tree showed that small genetic differentiation exists among the chicken populations studied. For the final confirmation of the observed new allelic pattern, the association studies between these alleles and productive and reproductive traits in our native chickens should be investigated. Whether the new variants observed are unique to this population remains to be seen. It would be possible to direct our selection schemes to favour the desired genotypes for improved growth rate and other related parameters in chicken.

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ANALIZA SEKVENCI INSULINSKOG FAKTORA RASTA 1 GENA KOD NIGERIJSKIH AUTOHTONIH I *ARBOR ACRE* PILIĆA

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Izvod

Faktor rasta pileta *Insulin-like growth factor 1* (IGF1) je kandidat za rast, telesnu kompoziciju i metabolizam, karakteristike skeleta i rast masnog tkiva i taloženje masti kod pilića. Mapiran je na 165,95 cM na hromozomu 1 i sastoji se od četiri egzona i tri introna, raspona više od 50 kb. Genomska DNK je ekstrahovana iz uzoraka krvi prikupljenih od eksperimentalnih pilića pomoću Qiagen kita za ekstrakciju DNK. Lančana reakcija polimera (PCR) izvedena je upotrebom utvrđenih prajmera. Sekvencirani su PCR amplikoni koji uključuju 5' region. Sekvence su analizirane kako bi se identifikovali polimorfizmi, njihove genetske raznolikosti i evolutivni odnosi između tri rase nigerijskih autohtonih pilića [Frizzle Feathered (7), Normal Feathered (19) i Goli vrat (19), i pilići Arbor Acre (17)] . Generirane nukleotidne sekvence su uređene i poravnate pomoću *Codon Code Aligner*. Analiza raznolikosti urađena je pomoću DnaSp, dok je softver MEGA6 korišćen za crtanje filogenetskog stabla metodom maksimalne verovatnoće. Ukupno je otkriveno devetnaest polimorfizama pojedinačnih nukleotida (SNP) od 560 bp delova 5'UTR među četiri proučene populacije pilića, a nijedna nije otkrivena kod rase Frizzle. Pilići rase Goli vrat imala je najveći broj SNP-a (13), haplotipova (6), raznolikosti haplotipa (0,778), raznolikosti nukleotida (0,00487), prosečnog broja nukleotidnih razlika (2,725), najviše polimorfnih mesta (13). Rasa pilića Goli vrat je prema tome imala najveću stopu mutacije i stepen alelnih varijacija u poređenju sa ostalim rasama koje su korišćene u ovom radu. Filogenetsko stablo pokazalo je da postoji mala genetska diferencijacija među proučavanim populacijama pilića. Neki od SNP-a su novootkriveni; stoga je povezanost između ovih alela i produktivnih osobina kod nigerijskih autohtonih pilića poželjna u budućim studijama.

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