Evaluation of Genetic Variability between Local White Chicken and Commercial Lines by RAPD-PCR and Sequencing of 18s rRNA Gene

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Abstract

The present study investigated and identified genetic diversity between native White chicken lines and two commercial Broiler (Rose) and Layer (Isa Brown) chicken breeds using RAPD markers and a sequencing technique. All primers applied produced 151 scorable bands with percentage polymorphic loci of 54.93% within chicken populations, as per the results of the RAPD marker. The maximum amplified fragment by primer OPC-11 was 22 and the fewest by primer OPAA-03 was 7. For all loci analyzed, the effective number of alleles (ne), means the observed number of alleles (na), Shannon's information index (I), and gene diversity (h) was 1.4394, 1.5493, 0.3496, and 0.2441, respectively. The presence of a high number of polymorphisms and targeted (71) loci across all chicken populations indicates that RAPD-PCR techniques provide sufficient genetic distance and higher genetic variation among chicken populations. The highest identity of the blasted sequences of the 18srRNA gene of local white chicken is 90.41% and 84.23%. Likewise, a total of 46 and 27 nucleotides are altered with 27 and 10 gaps in both sequences for the first and second regions, respectively. According to both phylogenetic trees, the local white chicken had a stronger sense of individuality and was slightly closer to the commercial broiler breeds than the layer chicken breeds. As a result, it suggests that enhancing the local chicken line requires a broiler breeding program, as well as cross-breeding with other native chicken lines to obtain hereditarily significant new strains.

1. Introduction:

Gallus sonneratii is the source of modern chicken breeds based on molecular data, as described by [1]. The domestic chicken is the most common domesticated animal species. However, a rising number of indigenous breeds are facing extinction, and essential genotypes and phenotypes are at risk of being lost [2],[3]. The loss of genetic polymorphism in particular traits that are currently insignificant in economic breeding strategies could result from the genetic erosion of these indigenous breeds [4]. Iraq's indigenous chickens (I.I.C.) have been purified and maintained, since 1986 and different genetic lines of I.I.C. were established from the local foundation population that was samples taken from throughout the country, based on feather colour and nakedness. Brown, white naked, white, barred, and black are the five genetic lines of I.I.C. that have been characterized. I.I.C. has a better taste and flavour of eggs and meat when compared to commercial breeds [5],[6]. Heating stress is the main cause of commercial broiler mortality during growing and transporting, resulting in significant financial losses and poor meat qualities [7]. But

I.I.C. is highly adaptable to climate change conditions such as high and low temperatures, high resistance to diseases, poor management, and dietary shortages, as well as farms for both egg and meat production throughout the country. Local chicken strains are recognized for being efficient mothers, good foragers, and requiring minimal caring for growth; they are assumed indigenous genetic resources and play an important role in the improvement of renewable agricultural production. Chickens play an important ecological and economic role in agribusiness as well as being the major source of livestock protein in many family members of developing countries [8],[9]. Several techniques, such as morphological characters, biochemical identification, and molecular marker analysis, have been frequently used to determine genetic diversity [10].The advancement of molecular biological tools has opened new opportunities for livestock genetic improvement and selection procedures. The discovery of the polymerase chain reaction (PCR) has a significant impact on multicellular organisms' genome research, as well as the development and use of numerous DNA markers [11]. This technology is now being used to identify genetic resources from economic essential animals like poultry and other livestock. Molecular DNA markers provide important information on genetic diversity, allow for the description of breed identity among chicken populations, and enable population relatedness to be measured [12]. Some of the DNA molecular markers discovered due to the fast development of present biotechnology include RAPD, SNP, AFLP, RFLP, and microsatellites [13],[14]. The Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) assays, first described by Welsh and McClelland (1990) and Williams et al. (1990), have been used in poultry for determining genetic diversity and correlation in numerous species, as well as for determining potential markers to evaluate phylogenetic relationships of many various poultry species [15],[16],[17]. On the other hand, gene/genome sequencing, one of the most powerful current techniques, has been utilized to characterize biodiversity analyses in poultry and domesticated animals. It has been the most uncomplicated technology and provides more thorough information on the genetic diversity among various chicken varieties because it can identify various variations inside the genomes [18],[19]. From nucleotide sequences, DNA sequencing analysis was utilized to detect the mutation and compare the presence of genetic variations in the qacE1 gene of bacteria with their relatives [20].The majority of biodiversity research has focused on regions of phylogenetically valuable genes (taxonomic "markers"). Due to sequence length restrictions, the method does not readily allow for complete gene characterization. The small subunit of the ribosomal RNA gene is a common marker (SSU rRNA). The genetic diversity of pathogenic organisms in the human intestine has been studied using subunit ribosomal RNA [21],[22]. In poultry, the subunit Ribosomal Ribonucleic Acid (18s rRNA) gene being used in biodiversity investigations is prevalent. The 18s rRNA gene sequence can also be used as referencing phylogenetic tree for comparing to all other 18s rRNA genes sequenced from various chicken strains in different locations that are stored in the National Center for Biotechnology Information (NCBI) database (GenBank). Individual chicken 18S rRNA and 28S rRNA gene sequences are accessible in the GenBank database [22],[23],[24]. Before beginning any breeding or preservation program, phenotypic and molecular characterization is required [25]. As a result, the current study's goal is to compare the genetic parameters of a local white chicken line with those of commercial layers and broilers to evaluate and identify genetic variation as the first step toward genetic resource preservation and the planning and development of a breeding program aimed at improving egg and meat production.

2. Materials and Methods:

2.1 Selection of chickens and blood sampling:

This study was conducted at the College of Agricultural Engineering Sciences, Salahaddin University- Erbil, Iraq, in collaboration with the Departments of Animal Science Plant Protection/Directorate of Agricultural Research- Erbil, Ministry of Agriculture and Water Resources, Kurdistan Region, Iraq. For this study, individual blood samples were collected from 52 chickens; Local White (LW) [15 chickens (6 males and 9 females/line)] breeds that have been maintained at the Department of Animal Science, Directorate of Agricultural Research- Erbil and one egg-type layer breed Isa Brown (IB) [17 chickens (7 males and 10 females/breed)] from Shimal Company and one meat-type breed white Broiler Rose, (BR) [20 chickens (10 males and 10 females/breed)] from Eyfan Feed Company. For every individual of all these varieties, One millilitre of venous entire blood from the wing vein was obtained into 2.0-millilitre tubes containing 5 mM of EDTA, transferred on ice, and stored at °C.

2.2 DNA Extraction:

Genomic DNA was isolated from the whole blood using a DNA extracting kit (QIAGEN) according to the manufacturer's directions, except for having a slight change in the sample volume. A (Nanodrop 1000 Thermo Scientific/Labtech) was used to determine the quality and quantity of extracted DNA. Individual acquired DNA was pooled based on their strains.

2.3 Conditions and programs of PCR:

According to the previous analysis, 11 ten-nucleotide decamer primers Table (1) were investigated for RAPD-PCR amplification. Software Primer3 (version: 2.5.0) [26], was used to design particular primers for the 18s rRNA gene-PCR amplification. For parts 1 and 2, the original self-designed primers (F: CGGCTACCTGGTTGATCCTG and R: CGCCGGTCCAAGAATTTCAC, F: CGTATTGTGCCGCTAGAGGT, and

R: TGATCCTTCGGCAGGTTCAC) were used in all populations and all of the primers were synthesized from (Macrogen, Korea). PCRs were carried out Table (2) according to the manufacturer's containing reaction materials of Prime Taq premix (2X), (GeNet Bio, Korea). PCR amplification was carried out in a thermos-cycler (PCR max Alpha, UK), with an initial denaturation step at 95°C for 300 s followed by 35 cycles of 35 s at 95°C, 35 s at (35°C and 60°C), (120 s and 80 s) at 72°C and a final extension step at 72°C for 600 s, for the RAPD-PCR and the 18s rRNA gene-PCR amplification, respectively according to the manufacturer's perform PCR program of Prime Taq premix (2X) with the minor modification (GeNet Bio, Korea).

2.4 Gel Electrophoresis:

Extracted DNA samples were run on a 1% agarose gel containing Red safe dye (GeNet Bio, Korea) and the stained gels were visualized and photographed using a UV-transilluminator. On the other hand, the RAPD-PCR and 18s rRNA gene PCR products were run on a 1.5% and a 2% agarose gel, respectively. The OneMARK 100 DNA Ladder (GeNet Bio (GeneDirex), Korea) was used.

2.5 Molecular Genetic Data Analysis:

2.5.1 Data Analysis of RAPD-PCR:

For RAPD-PCR amplified, the gel image depending on PCR product bands was scored by PyElph (version. 2.5) program [30] based on its presence (1) or absence (0) with the molecular weight of the bands based on the markers. The total score was then pooled to create only one data matrix that was used to estimate the Genic Variation Statistics Nei's (1987) and Nei's Analysis of Gene Diversity; The effective number of alleles (ne), gene diversity (h), the mean of the observed number of alleles (na), Shannon's Information Index (I), Coefficient of gene differentiation (GST), Total genotype diversity among populations (Ht), and the percentage of polymorphic loci. Nei's (1978) genetic distance and genetic identity, dendrogram construction, and cluster analysis algorithm based on the unweighted Pair Group Method of Arithmetic Means (UPGMA) across populations. POPGENE32 (version 1.32) was used to perform all statistical analyses [31].

2.5.2 Data Analysis of 18s rRNA gene:

All three populations' 18s rRNA gene PCR amplifications were sequenced from (Macrogen, Korea). To determine pairwise similarities and divergences between the 18s rRNA gene of the local white chicken line and other breeds, the nucleotide sequences of the native white chicken line were blasted to all other sequences in the NCBI using the Basic Local Alignment Search Tool (BLAST) tool [32]. Multiple Sequence Alignment-MUSCLE Version (3.8) Tools [33], was used to align sequences and construct dendrograms using the 18s rRNA gene sequences of the three chicken populations and *Gallus gallus* (Sequence ID: KT445934.2, [23]) available in NCBI [34]. In addition, the local white chicken line's 18s rRNA gene sequencing data were submitted to GenBank f or an accession number.

3. Result and Discussion:

3.1 Purity of DNA Extraction:

In this investigation, the quantity and quality of isolated DNA from fifty-two chicken samples of both sexes were correspondingly (93.37 to 114.26) ng/l and (1.89 to 1.97) A260/280. Based on the successful modification of a reasonable sample amount, a sufficient amount and high purity of genomic DNA are extracted.

3.2 Results of RAPD-PCR:

As shown in Fig.(1 and 2), each of the eleven primers tested in this investigation produced various fragments with a different number of scorable bands. The properties of the fragments obtained by uploading the gel picture of the RAPD-PCR results to the PyElph program across population groups by the primers were summarised Table (3). Throughout all population groups, all primers produced a total of 151 cleared bands. The numbers of bands ranged from two to eight, with molecular weights ranging from 340 to 3840 bp. The much more produced fragments by primer OPC-11 were 22 and the fewest by primer OPAA-03 was 7. The overall number of produced fragments for each chicken flock; Local White (LW), Broiler Rose (BR), and Isa Brown (IB), by all primers, was found to be 53, 50, and 48 bands, respectively. The variations in the size and number of produced fragments indicated that the local white chicken population has highly biodiversity genomic DNA.

3.3 Results of Genetic Variation Statistics and Nei's Gene Diversity:

In this investigation, by all the primers across all the chicken populations; based on the Summary of Genic Variation Statistics Nei's (1987); a total of (71) loci targeted and the 39 loci (54.93%) are polymorphic, with 23 (32.39%) representing unique, and 32 (45.07%) are monomorphic. The percent polymorphism loci result is higher than the results given by [29], [11], [8], [14], which were 48.94%, 45%, 34.7%, and 34.56%, respectively. But our finding, however, is lower than that of [27], which was 64.10%. Among the 71 loci analyzed; the result of the Effective number of alleles (ne) was 1.439, which is higher than the 1.238 observed by [14], while smaller than the 1.65 and 1.473 described by [25], [34], respectively. The result of Gene diversity (h) is 0.244, which is lower than the result obtained by [25], [35], [32], which were 0.25, 0.286, and 0.675, respectively. The result of the observed number of alleles (na) was 1.549, which is lower than the results provided by [35], which was 2.00, but greater than the results obtained by [14], which was 1.173. Our finding of Shannon's Information Index (I) is 0.349, which was lower than

Table 1. List of Primers for RAPD-PCR Marker.

No.	Primer's Name	Sequences (5' - 3')	*GC%*	References
1.	OPA-10	GTGATCGCAG	60	
2.	OPB-07	GGTGACGCAG	70	[8], Saudi Arabia
3.	OPB-08	GTCCACACGG	70	
4.	OPA-20	GTTGCGATCC	60	[27], Bangladesh
5.	OPAA-03	TTAGCGCCCC	70	
6.	OPAA-07	CTACGCTCAC	60	[28], India
7.	OPC-01	TTCGAGCCAG	60	[11], Iran
8.	OPC-11	AAAGCTGCGG	60	
9.	OPU-09	CCACATCGGT	60	[29], China
10.	OPA-05	AGGGGTCTTG	60	
11.	OPZ-11	CTCAGTCGCA	60	[25], Jordan

Table 2. Components of polymerization reaction materials.

Components	18s rRNA gene- PCR	RAPD-PCR
2X Prime Taq premix (master mix)	12 µl	10 µl
Forward primer (10 p.mol/µl)	2 µl	—-
Reverse primer (10 p.mol/µl)	2 µl	—-
Primers (10 p.mol/µl)	—-	2 µl
template DNA (80 & 45 ng/µl)	2 µl	2 µl
Sterilized D.W.	7 µl	6 µl
final volume	25 µl	20 µl

the results given by [25], [35], which were 0.58 and 0.44, respectively. The coefficient of gene differentiation (GST) was (1.000). The total genotype diversity among populations (Ht) equalled 0.244, which is lower than the finding reported by [35], which was 0.286. When compared to commercial layer and broiler breeds. The presence of a significant degree of polymorphisms and targeted (71) loci across all chicken populations in this investigation by all primers may show a significant genetic distance and increased genetic heterogeneity of local chicken populations.

3.4 Results of Genetic Identity Matrix based on RAPD marker and 18s rRNA gene:

According to the results obtained from RAPD marker Nei's unbiased genetic identity and genetic distance and percent identity matrix obtained by aligning the sequences of the first section of the 18s rRNA gene sequential alignments, the indigenous white chicken line has the maximum genetic similarity of 0.676 and 92.73 and the minimum genetic distance 0.391 with the commercial Broiler Rose, while it was with Isa Brown has 0.647 and 92.58 and 0.434, respectively Table 4 and 5. The RAPD marker result is similar to that described by [36], who found that Chinese local chickens and fast-broiler chickens are quite similar. The outcomes of the RAPD marker and 18s rRNA gene sequencing showed that the genetic divergence of the local white chicken line has a more genetic identity and is closer to Broiler Rose than the Isa Brown breed.

3.5 Phylogenetic tree construction based on RAPD marker and of 18s rRNA Gene:

Depending on a comparative analysis of the total loci identified with the eleven RAPD markers across populations, the dendrogram based on Nei's (1978) genetic distance using UPGMA methods was constructed. Fig.3 illustrates the phylogenetic tree, which shows the three chicken populations clustered into two major groups. The lowest genetic distance between Broiler Rose and local white, which are clustering together, was observed to be 0.19 in the first main group. The genetic distance of the second major group consists of Isa Brown chicken, which is shown to be 0.24. Also, the Phylo-

Table 3. Properties of the fragments produced across populations by the RAPD-PCR technique.

Chicken Populations					
Name of primers	**Local White (LW)**	**Broiler Rose (BR)**	**Isa Brown (IB)**	**Overall/**	**primers**
	No. of bands	**No. of bands**	**No. of bands**	**Size ranged (bp)**	**No. of bands**
OPAA-03	3	2	2	2130-3340	7
OPB-07	5	5	4	500-2760	14
OPB-08	6	5	4	570-3840	15
OPA-05	3	3	3	780-1480	9
OPA-10	6	4	6	340-1520	16
OPA-20	6	6	5	390-2380	17
OPAA-07	5	6	5	500-2730	16
OPC-01	3	4	4	730-1960	11
OPC-11	7	7	8	420-2620	22
OPU-09	4	4	4	640-3080	12
OPZ-11	5	4	3	730-2860	12
Overall/ populations	53	50	48	340-3840	151

Table 4. Nei's Unbiased Measures of Genetic Identity (above diagonal) and Genetic distance (below diagonal).

Populations	**Local White (LW)**	**Broiler Rose (BR)**	**Isa Brown (IB)**
Local White (LW)	****	0.6761	b0.6479
Broiler Rose (BR)	0.3915	****	0.5775
Isa Brown (IB)	0.4340	0.5491	****

gram tree is obtained by aligning the sequences of the first section of the 18s rRNA gene. Fig.4 shows a dendrogram that has been clustered into three major groups. The closest genetic relationship between Isa Brown and *Gallus gallus*, which were clustered together, was discovered to be 0.17 in the first main group. Broiler Rose chickens have a phylogenetic relationship of 0.23 in the second major group, and local white chickens have a genetic divergence of 0.37 in the third major group. The outputs of both computed dendrograms disagree with the result given by [37], [38], wherein the commercial broiler was positioned in a separate cluster. Based on both phylogenetic tree constructed results, local chicken lines have a stronger sense of individuality and it shares greater genetic similarities with the Broiler Rose than the commercially Isa Brown.

3.6 Blasted Sequence Results of 18s rRNA Gene:

In the current investigation, both sequence's information of the 18s rRNA gene of the local white chicken line was submitted to GenBank and published in Nucleotide-NCBI under the accession numbers MT889761 and MT889762 for the first and second parts of the gene sequences, respectively. The first part blasted sequences of the 18s ribosomal RNA gene of the local white chicken line against *Gallus gallus* (Sequence ID: KT445934.2, [23]), showing the highest query coverage of 98% and percent identity of 90.41% from 1868 to 2704 nucleotides, and it is covered 837 base pairs with the identity 773/855 (90.41%), gaps 27/855 (3%) and 0.0 of E-value, which is shown in Fig.5. Furthermore, both the local white chicken and *Gallus gallus* have 46 nucleotides altered, with 27 gaps in their sequences, which are 9 gaps in the query sequence and 18 gaps in the subject sequence. Likewise, blasted sequence of the second part from 2765 to 3114 nucleotides, the highest query coverage of 63% and percent identity 84.23% were found, as well as 350 nucleotides, were covered with the identity 299/355(84.23%), gaps 10/355(2%), and 0.0 of E-value (the figure is not present) with the sum of 27 nucleotide bases changed across both sequences. The

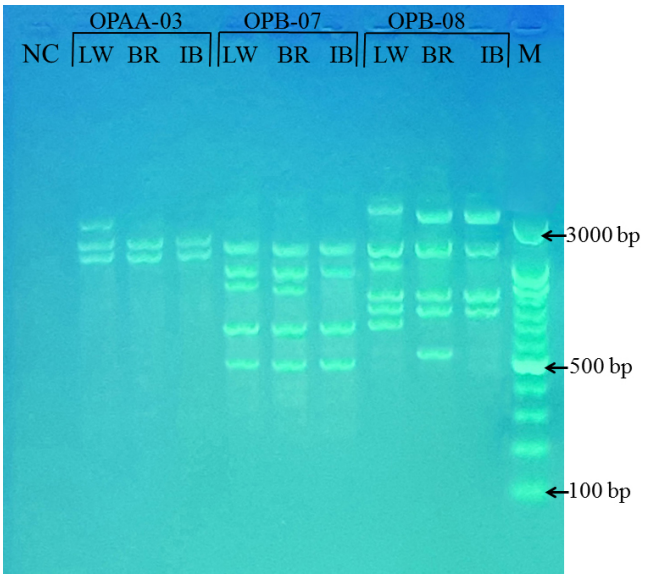

Figure 1. RAPD-PCR products of different chicken populations using OPAA3, OPB-07, and OPB-08 primers (NC; negative control, LW; Local White, BR; Broiler Rose and IB; Isa Brown Samples, M; 100 bp (OneMARK) DNA Ladder).

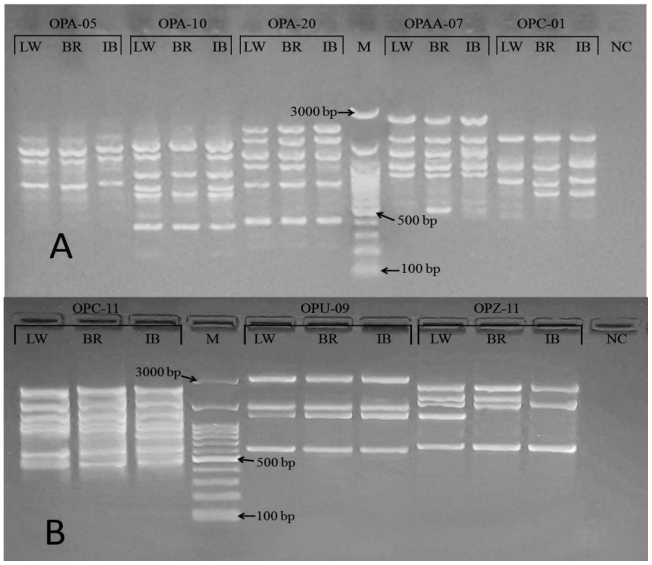

Figure 2. RAPD-PCR products of different chicken populations using (A: OPA-05, OPA-10, OPA-20, OPAA-07, and OPC-01), (B: OPC-11, OPU-09, and OPZ-11) primers (LW; Local White, BR; Broiler Rose and IB; Isa Brown Samples, M; 100 bp (OneMARK) DNA Ladder, NC; negative control).

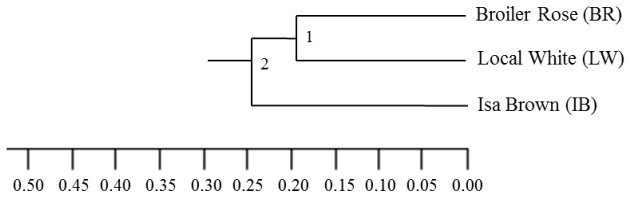

Figure 3. UPGMA Dendrogram Based Nei's (1978) Genetic distance.

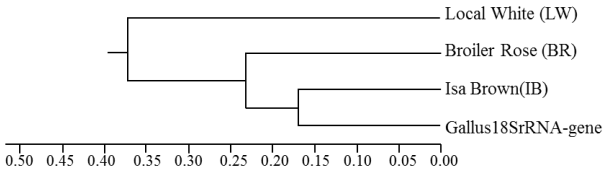

Figure 4. UPGMB Phylogram tree Based on the 18s rRNA gene of chicken by Multiple Sequence Alignment-MUSCLE tools.

result of percent identity and covered base pairs less but nucleotides altered more for both sections of the 18s rRNA gene sequencing. By using whole sequences from the 18S rRNA gene as a reference tree one can compare sequences originating from different regions. However, the bias from the lack of complete sequences in the reference tree will still affect the results [21]. Also, the majority of T and A nucleotides in the local white chicken were changed to C and G, indicating that our local chicken line has an independent and also more stable 18s rRNA gene sequence. On the other hand, the finding demonstrated great genetic resources for determining the genetic biodiversity of the local white chicken and how far it is from other chicken breeds. Additionally, our results demonstrated sufficient expertise and precision in the setting of the PCR programs, as well as the designed and selected primers, and also a sufficiently large beneficial size of the population. The results suggest that the indigenous white chicken has a lot of evolutionary divergences. The overall outcomes of 18s rRNA gene sequence analysis demonstrated that the local white chicken had a stronger sense of individuality and was more similar to Broiler Rose than Isa Brown.

4. Conclusions:

Each RAPD marker and 18s rRNA gene sequencing approach observed enough genetic variations in the local white chicken line, and it has a stronger sense of individuality, slightly closer to commercial broiler breeds, and the results presented here could be very good information of highly valuable hereditary resources about the biodiversity of native white chicken. Our findings will support the establishment of conservation projects, as well as the long-term use and official recognition of this line. As a result, we believe that accomplishing

Table 5. Percent Identity Matrix of the 18s rRNA gene nucleotide sequences.

Populations	*Gallus gallus*	Broiler Rose (BR)	Isa Brown (IB)
Local White (LW)	92.59	92.73	92.58
Gallus gallus		99.87	99.87
Broiler Rose (BR)			99.87

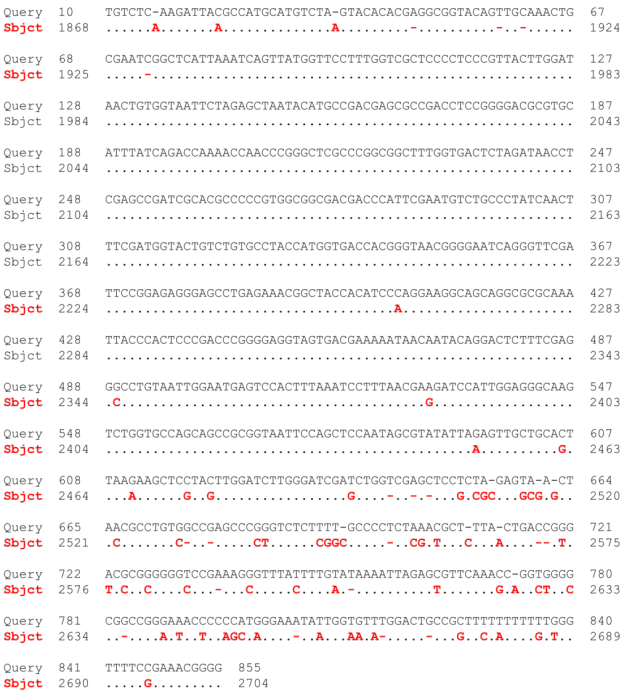

Figure 5. Polymorphic sites of the pairwise similarity observed in the 18s rRNA gene sequences of the local white chicken line (Query) and *Gallus gallus* (subject). The dots (.) indicate identity with the reference sequence (GenBank, Sequence ID: KT445934.2, [23]) by the BLAST tool.

the indigenous white chicken line requires a Broiler development program, as well as cross-breeding with some other local chicken lines, to collect hereditarily important genetic resources and obtain a hereditarily significant new variety of native chicken. We recommend all the local chicken lines that remain to be studied in the future with the same DNA marker and some other productive trait genes for finding the real similarity between or among local chicken lines, then we can decide to choose two close lines of the local chicken for making cross-breeding between them, and obtain a new chicken line.

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Data Availability Statement: All of the data supporting the findings of the presented study are available from corresponding author on request.

Declarations:

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: The manuscript has not been published or submitted to another journal, nor is it under review.

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تقيم لتباين الوراثي بين خط الدجاج الابيض المحلي وخطوط التجارية اعتمادا على *RAPD – PCR* و *18s rRNA* تسلسل الجيني

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الخلاصة

الدراسة الحالية تم فحصها وتحديد التنوع الجيني بين سلالات الدجاج الأبيض المحلي مع سلالتين تجاريتين من الدجاج البياض (*IsaBrown*) والفروج اللحم (*Rose*) باستخدام المؤشر غاعض وتقنية التسلسل. أنتجت جميع البادئات المطبقة 151 حزما قابلاً للتسجيل مع النسبة المئوية للحزم المتباينة 54.93 داخل مجاميع الدجاج، وفقا لنتائج المؤشر .*RAPD* كان وجداً أعلى عدد من الحزم المتباينة في البادئ *OPC – 11* هو 22 وكان أقل عدد بواسطة البادئ *OPAA – 03* هو .7 بالنسبة لجميع المواقع التي تم تحليلها، العدد المؤثرة للأليلات (*ne*) ، متوسط عدد الأليلات المشاهدة (*na*) ، الدليل معلومات شانون (*I*) والتنوع الجيني (*h*) كان 1.4394 و 1.5493 و 0.3496 و 0.2441 على التوالي. اشار عدد كبير من التباين والمواقع المستهدفة (71) في جميع مجاميع الدجاج إلى أن تقنيات *RAPD – PCR* توفر مسافة وراثية كافية وتنوع جيني أعلى بين مجموعات الدجاج. أعلى مطابقة للتسلسل *blasted* لجين *18srRNA* للدجاج الأبيض المحلي هو 90.41 و .84.23 وبالمثل، تم تغيير ما مجموعه 46 و 27 نيوكليوتيد مع 27 و 10 فجوات في كلا التسلسل للمنطقتين الأولى والثانية، على التوالي. وفقا لشجرة القراية، كان دجاج الأبيض المحلي أقوى معنويا بالفردية وكان أقرب قليلاً إلى سلالات الدجاج اللاحم التجارية من سلالات الدجاج البياض. ونتيجة لذلك، يقترح أن يتم تحسين خط الدجاج المحلي من خلال برنامج تربية الدجاج اللاحم، بالإضافة إلى تهجينه مع سلالات أخرى من الدجاج المحلي للحصول على سلالات جديدة وراثية مهمة.

الكلمات الدالة: شجرة، التباين الوراثي، مؤشرات الجزيئية، دجاج محلي، *MT*889761 و *MT*889762 .

التمويل: لايوجد.

بيان توفر البيانات: جميع البيانات الداعمة لنتائج الدراسة المقدمة يمكن طلبها من المؤلف المسؤول.

اقرارات:

تضارب المصالح: يقر المؤلفون أنه ليس لديهم تضارب في المصالح.

الموافقة الأخلاقية: لم يتم نشر المخطوط أو تقديمها لمجلة أخرى، كما أنها ليس قيد المراجعة.