POLIMORPHIC CHARACTERIZATION OF ESR1 AND FOXL2 GENES AND THE ASSOCIATION OF THEIR INTERACTION WITH PRODUCTIVE TRAITS OF BROWN LOCAL IRAQI CHICKENS

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ABSTRACT

The estrogen receptor 1(ESR1) and fork-head box L2 (FOX L2) genes play a pivotal role in regulation of egg formation in poultry. this study aimed to investigate interaction between ESR1 and FOXL2 in the productive performance of brown local Iraqi chickens (BLIC). A total of 104 BLIC represented from the F4th generation of local chicken selected for high egg productions were used. Two novel single nucleotide polymorphism (SNPs), one of them in ESR1 (T57198C) intron 3, and another of FOX L2 (C919T) gene within encoding region were identified through PCR-DNA sequencing. Six haplotypes (H1=TTCC, H2=TTCT, H3=TCCC, H4=TCCT, H5=CCCC, H6=CCCT) were obtained due to interaction between these two SNPs. Chi-square analysis showed no significant in genotypic and allelic frequencies for each SNP which revealed that both genes were agreement with Hardy-Weinberg equilibrium. Association analysis of haplotypes with production traits revealed that individuals have H4 genotype achieved higher body weight at sexual maturity, at 60 weeks of age and egg weight at 45 and 60 weeks of age, whereas, the higher number of eggs were exhibited in individual with H6 from onset egg till 60 weeks of age. The two haplotypes; H4(TCCT) and H6(CCCT) showed better combination than others with respect to production performance. In conclusion, our findings provided new evidence that the two genes (ESR1 and FOXLl2) with special interaction may have potential effects on productive traits of chickens and beneficial effects in laying breeding programs.

Keywords: DNA Sequence, haplotypes, egg production, local Iraqi chickens, interaction

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توصيف تعدد المظاهر في جينات ESR1 و FOXL2 و علاقة التداخل بينهما مع الصفات الانتاجية في الدجاج العراقي المحلي

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المستخلص

تلعب جينات مستقبل الاستروجين الاول (ESR1) و عامل النسخ Forkhead boxL2(FOX L2) دورا مركزيا في تنظيم عملية تكوين البيض في الدجاج. كان الهدف من هذه الدراسة معرفة تاثير التداخل بين هذه الجينات و الاداء الانتاجي للدجاج العراقي المحلي البني. تم استخدام 104 من اناث الجيل الرابع للدجاج العراقي المحلي المنتخب على اساس انتاج البيض. تم الكشف عن اثنين من تعدد المظاهر الجديد (SNPs), احداهما كانت في الانترون الثالث من جين ESR1 في الموقع,(T57198C), و الثانية وجدت في المنطقة المشفرة من جين FOX L2 في الموقع (C919T) باستخدام تقانة تفاعل البلمرة التسلسلي و معرفة تتابع الحامض النووي الدنا(PCR–DNA sequencing). تم الحصول على ستة من الطرز الوراثية المشتركة من خلال التداخل تعدد المظاهر لكلا الجينين (H1=TTCC, H2=TTCT, H3=TCCC, H4=TCCT, H5=CCCC, H6=CCCT). لم تُظهر الطرز الوراثية و التكرارات الاليلية لكل SNP أي فرق معنوي في تحليل مربع كاي ، و كان ذلك متفق مع قانون اتزان هاردي–واينبرغ.، اظهر تحليل التباين وجود علاقة معنوية للطرز الوراثية مع الصفات الانتاجية، اذ حققت الافراد الحاملة للطراز H4 وزن جسم عالي في عمر النضج الجني و عمر 60 اسبوعا، اضافة الى وزن البيض بأعمار 45 و 60 اسبوع, بينما , تميزت الافراد الحاملة للطراز H6 بارتفاع عدد البيض المنتج منذ بداية وضع البيض و لغاية 60 اسبوعا من العمر. أظهرت الطرز الوراثية المشتركة TCCT و CCCT أفضلية بالمقارنة مع الطرز الاخرى فيما يتعلق بالأداء الإنتاجي. في الختام، قدمت النتائج التي توصلنا إليها دليلًا جديدًا على أن التداخل بين اثنين من الجينات (ESR1 و FOXLI2) قد يكون لهما تأثيرات في الصفات الإنتاجية و اهمية في برامج التربية و التحسين للدجاج البياض.

الكلمات المفتاحية: تتابع الحامض النووي, الطرز الوراثية المشتركة, انتاج البيض, الدجاج العراقي المحلي, التداخل.

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INTRODUCTION

Animal production is one of the important and diverse economic agricultural elements widespread around the world and has been given more attention today to productive animals in general and poultry in particular due to their high and continuous production of eggs and meat which have significant nutritional importance. (17,18). Egg production is a vital process regulated by a wide range of endocrine system pathways through a set of complex genes that control egg production traits, therefore, it is very difficult to obtain rapid progress using the classical methods of genetic improvement within the breed (5,8,9,10,15,21). Estrogen receptor 1(ESR1) and forkhead box L2 (FOXL2) genes are the most important among these candidate genes as selection indication in local chicken improvement programs. (22,23). The ESR1 gene is located on chromosome 3 in the chicken. Its size 103,705 kilobase (kb) and contains eight exons encoding a 589-amino acid protein called (ERα). The differential expression of ESR1 in chickens is contributed to asymmetric ovarian development (11). The DNA sequence of the ESR1 gene has an important role in the development of ovaries and egg production (6,27). FOXL2 gene is expressed in granulosa membrane cells of growing follicles and in maturing oocytes of hen ovaries (12,19). So it has a role in increasing the number of ovarian follicles and their differentiation in chickens (4). The chicken FOXL2 gene, located on chromosome 9. The size of the gene 1,130 (kb); it contains one exon, supplies instructions for making a protein that links to specific regions of DNA and assists to control the action of particular genes (12). In chickens, the FOXL2 gene contains a single exon encoding a 305–amino acid protein (12). Based on this job, the FOXL2 protein is called a transcription factor. The protein contains one place where a protein building block (amino acid) called alanine is repeated multiple times. This stretch of alanines is known as a polyalanine or poly(A) tract. The function of this poly(A) tract is unknown. (3,13,16). Since there is no study about the possibility of employing these genes in the selection and improvement process of local Iraqi chicken and its close relationship with characteristics of egg production, So, this is the unique study in Iraq which intended to pinpoint the interaction relationship of the polymorphisms in the DNA sequence of these genes with some productive traits and their use as a genetic indicator for improving the egg production traits in the brown local Iraqi chickens.

MATERIALS AND METHODS

Hens and character measurements

This research was conducted at the poultry station in the Animal Resources Department of the Office of Agricultural Research – Ministry of Agriculture. As for laboratory work, it was in the Office of WAHJ ALDNA for chemical analysis, Baghdad –Karrada. The fieldwork continued from 1/3/2018 to 25/4/2019 (420 Days). The laboratory work began on 16/2/2019 until 16/8/2019. A total of 104 hens of the F4th generation of brown local Iraqi chickens were used. The hens were reared inside individual cages equipped with longitudinal feeders and water nipples. The preventive and hygienic programs that recommended by the Office of Agricultural Research was applied. Five types of rations were used, according to age. The lighting program used was depended by the Office of Agricultural Research with a lighting power of 60 watts, with the lighting hours number 15 h from 15 weeks of age to the end of production period(1). The mean of body weight, hen days egg production (HDEP) and the weight of eggs produced was calculated for haplotypes.

DNA Extraction: At 320 days of age. three ml of blood were collected from individuals of the 104 local Iraqi hens under the study via the wing vein. Genomic DNA was extracted using G- spin DNA extraction kit by intron company/ Korea. Prior DNA extraction, blood volume was modification to 10 microliters (2). To ensure genomic DNA purity, according to (24), the agarose gel has been made in 1% condensation by melting 1 g of agarose in 100 ml of previously made TBE buffer (22). Three µl of the processor loading dye (Intron / Korea) has been mixed with 5 µl of the supposed DNA to be electrophoresis, after the mixing process, the mixture is loaded at the gel holds. An electric current of 7 v\c2 has been exposed for 1.5 h till the tincture has reached to the other side of the gel. The gel

has been tested by ultraviolet spectrophotometer source with 336 nm after putting the gel in the pool which contains 30µl Red safe nucleic acid staining solution and 500 ml of distilled water.

Primers designation: To find the genetic variation in the estrogen receptor 1 (ESR1)and forkhead box L2 (FOXL2) genes, primers were designed for the target gene fragment and shown in tables (1). The primers had been supplied by an integrated DNA technologies company(IDT)/Canada. Note: the FOXL2 gene primer is designed depending on the insensitive DNA strand.

Table 1. The specific primer of ESR1 and FOXL2 genes

Genes	Primer	Sequence	Tm (°C)	GC (%)
ESR1	Forward	**5'- TCC AAG GTT TAG GGT CGT TCA G- 3'**	**56.7**	**50**
ESR1	Reverse	**5'- TTC AAC CAA AAT GGT CTC CCA GT- 3'**	**56.8**	**43.5**
FOX L2	Forward	**5'-GCC GTT GTA GGA GTT CAC CA - 3'**	**57.2**	**55**
FOX L2	Reverse	**5'-GGG GCA AGG AGG AAC TGA G - 3'**	**57.9**	**63.2**

Polymerase chain reaction(PCR)

The total size of the reaction is 25 µl, PCR PreMix 5 µl, DNA 1.5, forward primer 1 µl, reveres primer 1µl, and DdH2O 16.5µl.The temperature has identified (Initial denaturation and annealing) after several experiments (Gradient PCR). The optimum denaturation temperatures 95°C and, annealing temperatures 64°C used to detect target regions of ESR1, and the optimum denaturation temperatures 95°C and, annealing temperatures 62°C used to detect target regions of FOXL2.

PCR product electrophoresis

To preparation of gel, the percentage 2% of agarose was used, by melting 2 g of agarose in100 ml of previously made TBE buffer. five µl of the PCR product was loaded into the holes of the gel and 5 µl of the ladder was loaded into the first hole of the gel. An electric current of 7 v\c2 has been exposed for 1.5h till the tincture has reached the other side of the gel for ESR1 and 1h for FOXL2 gene as shown in figures(1,5) respectively. The gel has been tested by a source of the UV with 336 nm after putting the gel in the pool which contained 30µl red safe nucleic acid staining solution and 500 ml from distilled water. Note: Loading dye will not be added for the PCR product electrophoresis because the master mix contains a dye.

DNA sequencing: PCR product samples were sent to the biogen company in South Korea to determine the target regions sequence for each gene, by sequencer device, then detect the genotypes.

Sequence results analysis: For genotyping, the results of sequence were analyzed by(geneious prime program); it is the world's leading bioinformatics software platform for molecular biology and sequence analysis. Note: an analysis of the valid samples had been adopted, which have high quality, according to the program. Note: the FOXL2 gene sequence has been transferred to the sensitive strand, then genotyping. The genotypes were determined by comparing nucleotides type and number of alleles that appeared in DNA sequence for each individual with the DNA of *Gallus gallus domesticus* the recorded by NCBI.

Statistical analysis: Data were statistically analyzed using the Statistical Analysis System program (25) to study the interaction relationship between the genotypes of ESR1 and FOX L2 genes with body weight and egg production traits for the F4th generation of brown local Iraqi chickens according to the mathematical model shown below and calculated the significant differences between the averages using the polynomial Duncan test (7).

$Yij = \mu + A_i + C_j + AC_{ij} + e_{ijk}$

Yij: the value of observation j that belongs to the genotype i

µ: the average of the measured trait

Ai: the effect of the ESR1 gene polymorphism of the site (T57198C)

Cj: the effect of the FOXL2 gene polymorphism of the site (C919T)

ACij: the interaction between ESR1 and FOXL2 genes

eijk: random error which is distributed normally an average equal to zero and variance of $\sigma^2 e$

Polymorphism evaluations: Frequencies of genotypes and alleles at each SNP site were calculated, with each polymorphism evaluated for Hardy–Weinberg equilibrium using (Chi-square $\chi 2$) test to compare the percentages of genotypes for each gene (degree of freedom=1).

P + q = 1

$$PA = \frac{2^{*}\text{ No. of Homozygous} + 1^{*}\text{ No. of Heterozygous}}{2^{*}\text{ Total number of sample}}$$

RESULTS AND DISCUSSION

PCR of estrogen hormone receptor 1 gene (ESR1): using the primers, which showed a molecular weight of 1500 bp (Figure 1**)** A specific sequence of the estrogen hormone receptor 1 gene was amplified

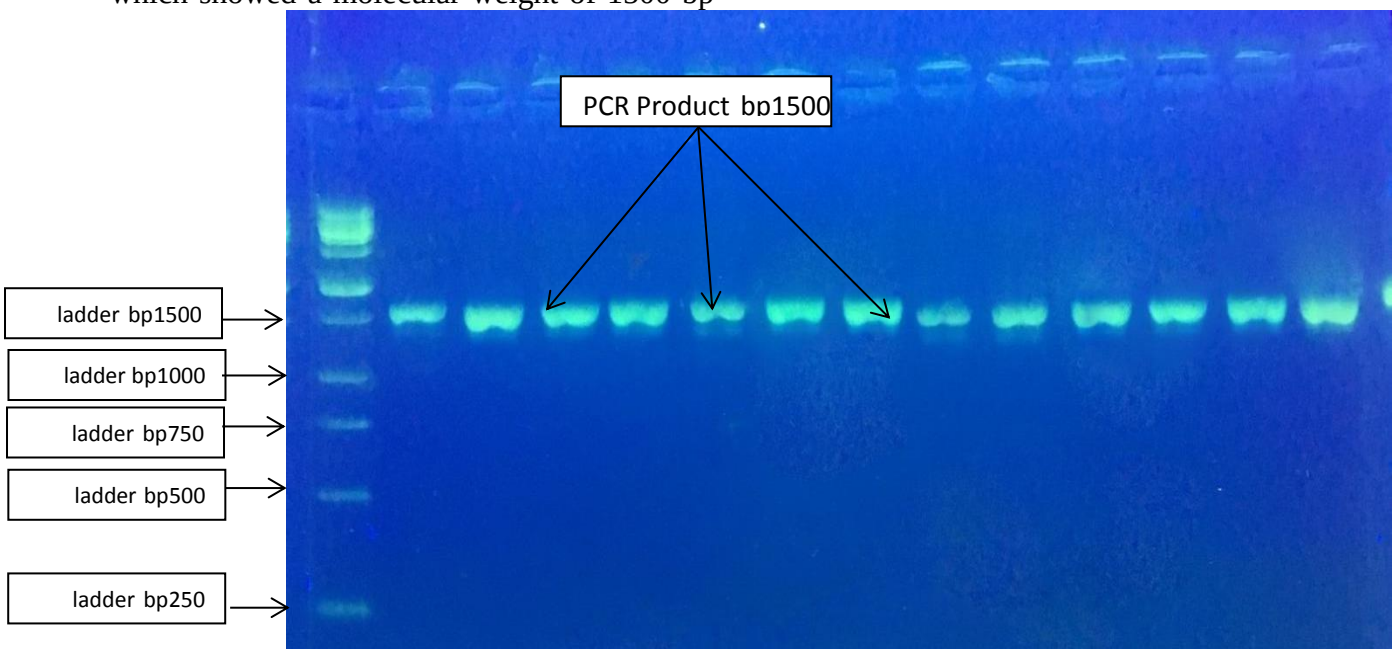
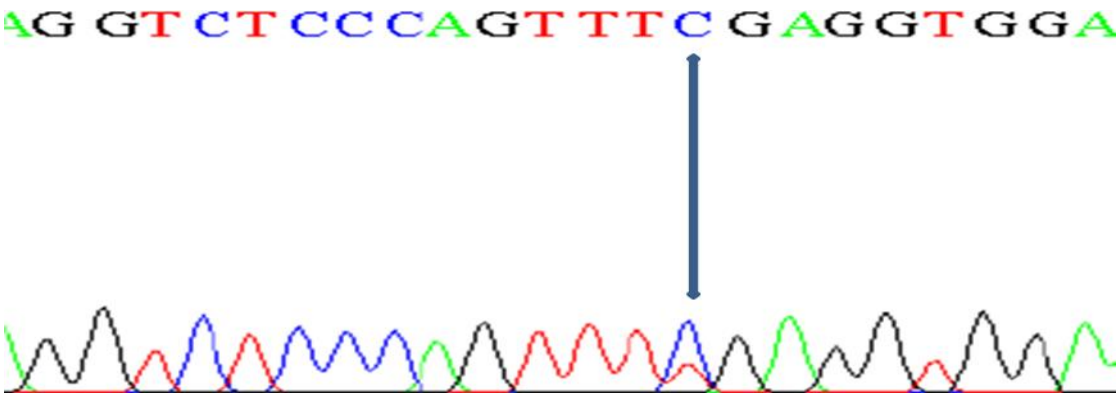


Figure 1. Electrophoresis of PCR product for ESR1 gene, the product was electrophoresis on 2% agarose gel at 7 volt/cm2 for 1.5 hour. The molecular size 1500 bp. Ladder of DNA (250-10000) bp

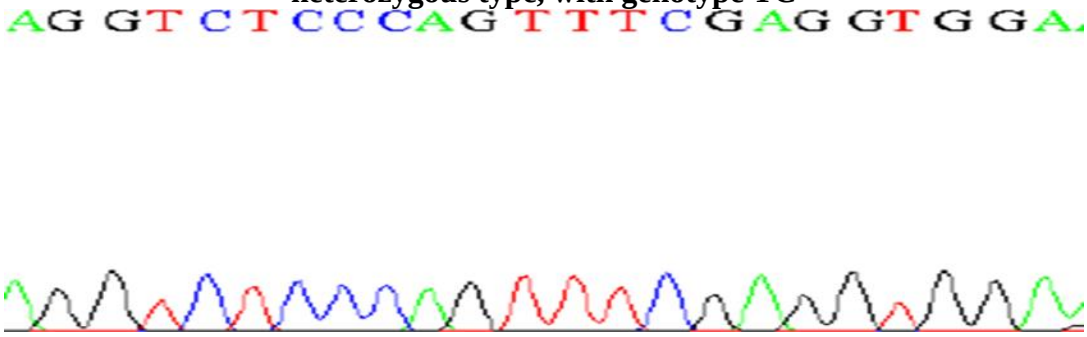
Detection of polymorphism and genotypes in ESR1 gene

To detect polymorphism in the ESR1 gene, all samples of the PCR products were performed using the DNA sequence, as previously described. One novel polymorphism was found within the target fragment of estrogen hormone receptor1 gene, it was a single nucleotide polymorphism (SNP), T57198C transition located within ESR1 gene (intron 3), two alleles (T and C) and three genotypes were found: Wild(TT), Heterozygous(TC)and Homozygous(CC) as shown in the figures 2, 3 and 4 respectively.

Figure 2. ESR1 gene, wild genotype (TT), showed one peak, belongs to both natural alleles with nucleotide (T) marked with an arrow, which is the site of genetic variation assumed in the third intron of the ESR1, one peak for nucleotide (T) indicates to there is no genetic variation in this site of the DNA, and the birds of this group are considered wild type, with genotype TT



Figures 3.ESR1 gene, heterozygous genotype (TC), It was visible from the figure (3) two peaks, represent two alleles, in the site of genetic variation of the third intron of the ESR1, one for the natural nucleotide (T), another for the variance nucleotide(C) where had been a transition (T > C), and marked with an arrow, and the birds of this group are considered heterozygous type, with genotype TC



Figures 4. ESR1 gene, homozygous genotype (CC), a one peak with nucleotide (C), marked too, there had been a transition (T/ C) transition in a site of genetic variation for the third intron of the ESR1, to both alleles, were marked too, and the birds of this group are considered homozygous type, with CC genotype

From the foregoing, there were allelic variation in the untranslated region of ESR1 gene, which is a single nucleotide polymorphism(SNP) resulting in three genotypes TT, TC and CC. The results of study agree with previous study (27) which indicated a polymorphism in untranslated regions of the ESR1 gene in blue egg-shell chicken.

Distribution of genotypes and allele frequency in ESR1 gene(SNP T57198C)

Table (2) showed non-significant differences between the distribution of genotypes TT, TC, and CC of ESRI in the F4th generation of brown local Iraqi chickens, and the percentages were TT (57.4%), TC (33.7%) and CC (8.9%), and the birds' numbers of different genotypes were 58, 34 and 9, respectively, adding there was no-significant dominance for allele T over allele C according to Hardy–Weinberg law, reached 0.743 and 0.257 respectively. The wild genotype showed the numerically superior and with regard to the relative percentage compared to birds of other genotypes, this is consistent to research (27) which refers to the SNPs of untranslated region. Our assured scientific explanation that the F4th generation of brown local Iraqi chickens previously will not be sufficiently subject to an intense selection program, so it naturally contains a lot of polymorphism in several of DNA locus.

Table 2. Distribution of genotypes and allele frequency in ESR1 gene(SNP T57198C) of the F4th generation of brown local Iraqi chicken

The polymorphism	Genotype	No of hens	Genotype frequency	Allele	Allele frequency	χ^2
	TT	58	57.4%	T	0.743	
	TC	34	33.7%	C	0.257	
SNP T57198C	CC	9	8.9%	_	_	1.44
Total		101	100%	_	1	
	3					
P-value			0.49			

PCR of forkhead box l2 gene (FOXL2).
A specific sequence of the FOXL2 gene was amplified using the primers shown in the chapter of materials and methods ,the size of amplified PCR product was found to be -625 bp as shown in figure (5).

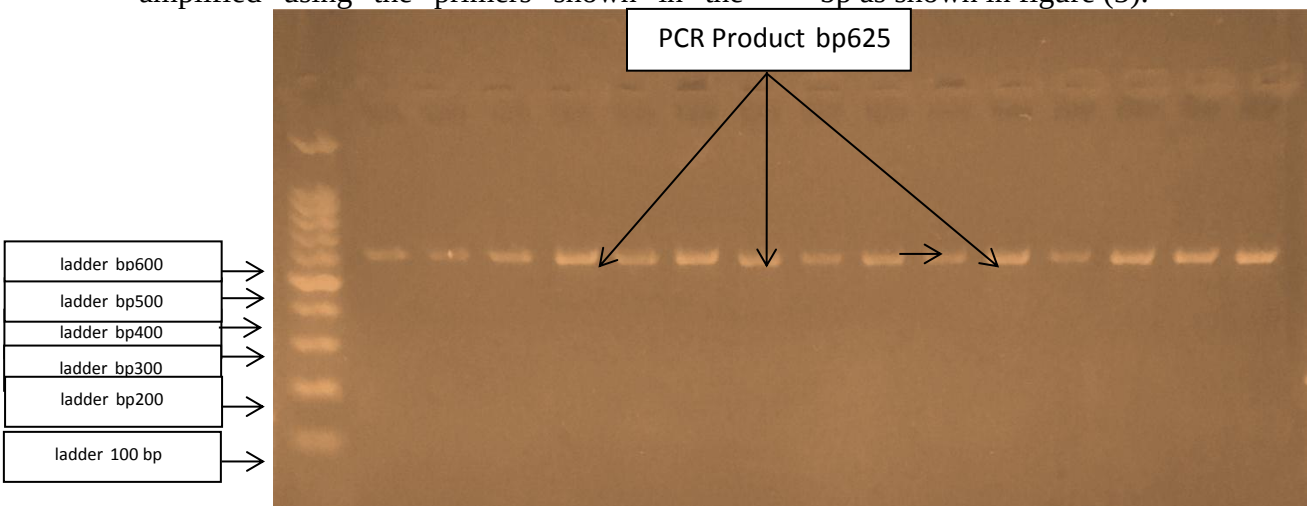
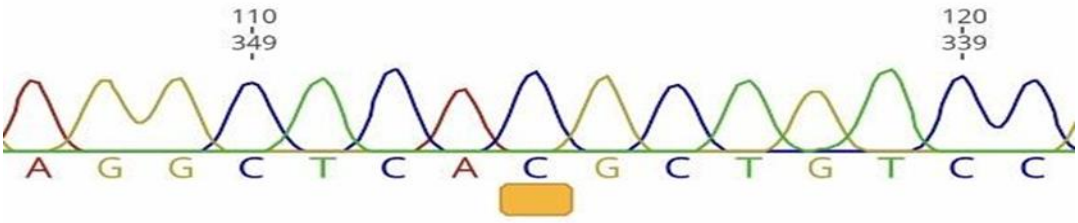
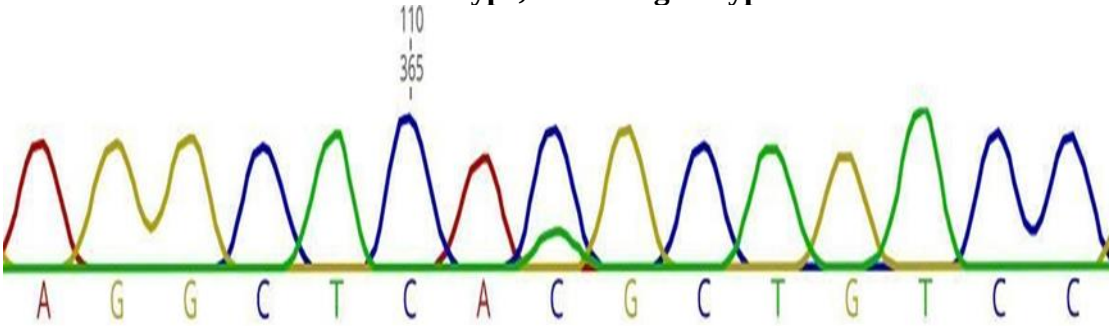


Figure 5. Electrophoresis of PCR product for FOXL2 gene, the product was electrophoresis on 2% agarose gel at 7 volt/cm2 for 1 hour. The molecular size 625 bp. Ladder of DNA (100-1500) bp

Detection of polymorphism in FOXL2 gene: To investigate polymorphism in the FOX L2 gene, all samples of the PCR products were analyzed using the DNA sequence, as formerly described. One polymorphism within the target fragment FOX L2 gene was identified, The (SNP), C919T transition in the gene, was located exclusively at encoding region, two alleles (C and T) and two genotypes were found: Wild(CC) and heterozygous(CT) as shown in the figures (6,7) respectively. The SNP C919T(FOXL2) leads to a non-synonymous substitution (threonine ACG 69- to-methionine ATG).



Figures 6. FOXL2 gene, SNP (C919T), wild genotype (CC), appeared one peak, belongs to both natural alleles with nucleotide (C) at the site of genetic variation supposed of the FOXL2 gene (Exon), that confirms there is no variation in this site of the DNA, and these birds were deemed wild type, with the genotype CC



Figures 7. FOXL2 gene, heterozygous genotype (CT), A transition (C- T) in the second group of birds was found, where two peaks appeared, belong two alleles, in the site of polymorphism of FOXL2 gene, one for the natural allele (C), another to the differ allele (T). This group is considered a heterozygous type, with the genotype CT

Distribution of genotypes and allele frequency for SNP C919T of the FOX L2 gene : Two alleles (C and T) and two genotypes (CC and CT) of the FOXL2 C919T in the F4th generation of brown local Iraqi chicken were observed, the frequency of allele C 0.911 was higher than allele T with 0.089. This result revealed that this population are agreement with Hardy– Weinberg equilibrium. The percentage of genotype CC (82.29%) by 79 birds higher than genotype CT with percentage (17.71%) and 17 birds. Non-significant differences showed between the genotypes percentage according to the Chi-square test as shown in table (3).

Table 3. Distribution of genotypes and allele frequency for SNP C919T of the FOX L2 gene in the F4th generation of brown local Iraqi chickens

The polymorphism	Genotype	No of hens	Genotype frequency	Allele	Allele frequency	χ^2
SNP C919T	CC	79	82.29%	C	0.911	0.03
	CT	17	17.71%	T	0.089	
Total	2	96	100%		1	
p-value		0.8534				

The interaction between the genotypes

The genetic interaction between genotypes was performed to explore the greatest variation in productive traits to enable selection. An interaction between the ESR1 and FOXL2 genes has been proposed, which included the least number of haplotypes, which allowed the opportunity to provide an appropriate number of individuals for every haplotype to statistical analysis. Six haplotypes were observed: H1=TT(ESR1)&CC(FOXL2), H2=TT(ESR1)&CT(FOXL2),H3=TC(ESR)& CC(FOXL2),H4=TC(ESR1)&CT(FOXL),H5= CC(ESR1)&CC(FOXL2),H6=CC(ESR1)&CT (FOXL2),with percentages: H1:46%, H2:0.095%, H3:0.30%, H4:0.042%, H5:0.042%, and H6:0.053%.

Effect of interaction the ESR1 and FOXL2 genes in body weight: It is evident from table 4. Non-significant differences between combined genotypes (Haplotypes) in the mean of body weight for ages 17 and 40 weeks despite the presence of arithmetic variance, while both body weight at age of sexual maturity and 60 weeks recorded significant differences, as the combined genotype (haplotype)H4 was significantly associated ($P<0.05$) with higher body weight compared to H6 reached 1722.8±108.33 g, and 1425.8± 13.9g at the age of sexual maturity, and 2350±174.9, and 1900±104.4 at 60 weeks of age for H4 and H6 respectively. It is noticed that the genetic interaction was showed the high-productivity haplotypes and others with low-productivity when the variance became largest between the combined genetic groups (Haplotypes) comparing to single genotypes in the body weight especially at 60 weeks, it is estimated at 450g among H4 and H6 haplotypes. There is no previous study on the interaction between the ESR1 and FOXL2genes, but previous studies indicated the importance of the association between these genes and other genes and their effect on body weight characteristics as well as the possibility of their use in selection programs(22,23).

Table 4. Effect of interaction the ESR1 and FOXL2 genes in body weight

Body weight	Haplotypes H1 TTCC	H2 TTCT	H3 TCCC	H4 TCCT	H5 CCCC	H6 CCCT	P-Value
At 17 weeks (g)	1052.98±22.32	1000±53.99	1000.69±28.54	1076±37.4	925±66.02	928± 69.07	0.26
Sexual maturity (g)	1600.23±28.4 ^{ab}	1544±57.72 ^{ab}	1565.29±39.8^{ab}	1722.8±108.33 ^{a}	1587.5±144.06 ^{ab}	1425.8±13.9^{b}	0.05
At 40 weeks (g)	1921.05±49.8	1885±98.68	1790.83±47.75	2081.3±141.6	1871.3±162.68	1736.3±111.6	0.3
At 60 weeks (g)	2052.95±69.5 ^{ab}	2035±137.9 ^{ab}	1998.97±57.51 ^{ab}	2350±174.9 ^{a}	1950±209.51$^{a\ b}$	1900±104.4 ^{b}	0.05

Means within a row with the different superscripts per trait are significantly different (P<0.05).
H1=TT(ESR1)&CC(FOXL2):H2=TT(ESR1)&CT(FOXL2):H3=TC(ESR1)&CC(FOXL2): H4=TC(ESR1)&CT(FOXL2):H5=CC(ESR1)&CC(FOXL2):H6=CC(ESR1)&CT(FOXL2

Effect of interaction the ESR1 and FOXL2 genes in the mean of egg production and egg weight: The interaction between genes resulted in many significant differences between haplotypes (Table 5). The haplotypes H3, and H4 outperformed H6 (P<0.05) in egg weight (EW) at 30 weeks (45.74, 47.01 and 41.9g) for H3, H4, and H6 respectively, while the other haplotypes did not show significant differences in EW at 30 weeks, in addition to the superiority of haplotype H4 (P<0.05) over haplotypes H2, H5, H6 in EW at 45 weeks (47.03, 52.3, 48.36and 44.82g) for H2, H4, H5,and H6 respectively, as well the outperforms of haplotypes H1 and H3 (P<0.05) over the H6 haplotype in EW at 45 weeks (48.84, 48.72 and 44.82g) for H1, H3 and H6 respectively. The haplotype H4 was recorded a significant increase (P<0.05) compared to haplotypes H2 and H6 in EW at 60 weeks (48.37, 54.04 and 46.43g) for H2, H4, and H6 respectively. The haplotype H6 was characterized by a significant increase (P<0.05) in egg production at 60 weeks of age compared to the H5 haplotype (148.75 and 182.25eggs) for H5 and H6 respectively, in addition to its mathematical superiority over other haplotypes. The previous studies were point out the estrogen receptors role in egg production as well as egg-laying series in chickens (6,27) also, a recent study showed that mutations in the ESR1 gene, which were detected using SNP analysis, were associated with the egg production traits in quail(26). The FOXL2 gene plays an important role regulating the development of follicles and ovulation in chickens (14,20). A separated prior studies were showed a significant effect of the polymorphism in the FOXL2 and ESR1genes of egg production traits, these included the number of eggs produced and the eggs weight, in addition to noting the importance studying the relationship of interaction between genes and its effectiveness to increasing the variation among haplotype in productive traits in chicken, this was consistent with the study results (22,23). So the present study support ESR1 and FOX2 roles of hen egg production when doing a gene interaction process, which confirm the additive action for these genes to improvement the quantitative traits like egg production, egg weight and body weight, wherever the greatest yield was obtained via the study of polymorphism effect of two genes, and this maybe lead to conclude that two SNPs in ESR1 and FOXLl2 genes may supply importance genetically markers of chicken breeding, via prediction and early selection for beneficial phenotypes of the body weight, and egg weight by choosing the haplotype H4 (TCCT), and the number of eggs produced by H6 (CCCT).

Table 5. Effect of interaction ESR1 and FOXL2 genes in the mean of egg production and egg weight

Traits	Haplotypes						P-Value
	H1 TTCC	H2 TTCT	H3 TCCC	H4 TCCT	H5 CCCC	H6 CCCT	
HDEP at 30 weeks (No)	38.69±1.91	36.75±4.81	39.63±2.84	27.25±11.77	25.66±11.55	38±5.5	0.4
EW at 30 weeks (g)	45.32±0.4^{ab}	43.92±1.14^{ab}	45.74±0.6^{a}	47.01±1.52^{a}	43.67±1.9^{ab}	41.9±1.07^{b}	0.05
HDEP at 45 weeks (No)	111.5±3.38	115.63±5.76	113.78±4	104.5±10.26	94±9.2	116±2.94	0.54
EW at 45 weeks (g)	48.84±0.4^{ab}	47.03±1.15^{bc}	48.72±0.64^{ab}	52.3±2.27^{a}	48.36±2.14^{bc}	44.82±0.51^{c}	0.02
HDEP at 60 weeks (No)	166.29±5.52^{ab}	172.13±5.78^{ab}	170.18±6.8^{ab}	165.75±9.2^{ab}	148.75±10.37^{b}	182.25±3.68^{a}	0.05
EW at 60 weeks (g)	50.29±0.48^{ab}	48.37±1.3^{b}	50.09±0.68^{ab}	54.04±2.19^{a}	50.34±1.5^{ab}	46.43±0.68^{b}	0.03

Means within a row with the different superscripts per trait are significantly different (P<0.05)
H1=TT(ESR1)&CC(FOXL2):H2=TT(ESR1)&CT(FOXL2):H3=TC(ESR1)&CC(FOXL2):
H4=TC(ESR1)&CT(FOXL2):H5=CC(ESR1)&CC(FOXL2):H6=CC(ESR1)&CT(FOXL2
HDEP= Hen days egg production: EW= Egg weigh

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