RESEARCH ARTICLE

Association of SNPs in ESR1 and ESR2 Genes with Egg Production in Anand Synthetic White Leghorn and Anand Bantamised White Leghorn Chicken

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Abstract

The objective of the present study was to find Single Nucleotide Polymorphisms (SNPs) in ESR1 and ESR2 genes and examine their association with egg production in chickens up to 64 weeks of age (EN64). Blood samples were taken from 48 Anand Synthetic White Leghorn (ASWLH) and 48 Anand Bantamised White Leghorn (ABWLH) chickens. Amplicon sequencing was carried out using the IlluminaMiseq platform, and a custom panel was developed to cover the exon regions of these genes. Total 91 SNPs, 59 previously reported and 32 novel SNPs were identified from 96 samples. In conclusion, the genetic variants g.52944897 T>C and rs3181386 inside the ESR2 gene exhibit potential as selection markers linked to EN64 in chickens.

Key words: Amplicon sequencing, Anand Bantamised White Leghorn, Anand Synthetic White Leghorn, ESR1, ESR2, Single Nucleotide Polymorphisms.

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Introduction

The production of eggs is a significant economic characteristic within the poultry industry. Based on the findings of Kolluri *et al.* (2021), the chicken industry in India makes a major contribution of approximately USD 17.31 billion to the country's overall gross domestic value. Furthermore, this sector serves as a significant source of employment, providing livelihoods for approximately 50 million individuals. Egg production has steadily increased in contemporary commercial layers primarily because of phenotypic selection, which has contributed to their exceptional laying capacity. The persistence of genetic variability is signified by the ongoing rise in egg production. The genetic variability inherent in the genes involved in egg production pathways can be used to boost production even more. Several potential genes have been explored in poultry to increase reproductive features and reduce broody days and frequency (Vinh *et al*., 2021). Many current chicken breeds have been developed using traditional breeding methods (such as family selection supported by self-selection). Fortunately, recent advancements in molecular biology technologies have made it possible to expedite the breeding process for desired characteristics. Implementing such a methodology facilitates enhanced breeding for traits associated with egg production in poultry.

The genes for estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2) are known to play a role in ovarian follicular development and ovulation in both chickens and mice (Gonzalez-Moran *et al.,* 2013; Gonzalez-Moran, 2014), which may influence characteristics of laying hen's egg production. Estrogen is a crucial intrafollicular modulator that promotes the proliferation of granulosa cells and facilitates the differential effects of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) on these cells (Lu *et al*., 2004; Moriarty *et al*., 2006). The ESR1 gene, which is found on chromosome 3, comprises eight exons that encode a 589-amino acid protein (ER alpha) (Lucinda *et al*., 2004). A study provided evidence that genetic variations within the ESR1 gene, identified using SNP analysis, exhibited a significant

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correlation with laying characteristics in quails (Tremblay *et al*., 1998).

The chicken ESR2 gene, on chromosome 5, has been cloned and is made up of eight exons that code for a 472-amino acid protein (ER Beta). The ovarian granulosa cells mostly express ERβ, which is needed for the formation of the antrum, the maturation of preovulatory follicles, and ovulation during follicle growth (Emmen *et al*., 2005). However, ERα and ERβ have different roles in folliculogenesis (Drummond and Fuller, 2012). Therefore, the results of this study suggest that sequence mutations in ESR1 and ESR2 could significantly impact the development of chicken ovary function and egg-laying performance. The present study was focused on association of SNPs in ESR1 and ESR2 genes with egg production in Anand Synthetic White Leghorn and Anand Bantamised White Leghorn Chicken developed at KU, Anand.

Materials and Methods

This study was conducted on 96 chicken with approval of Institutional Animal Ethics Committee of the institute. Four commercial strain crosses: the BV-300, the Shaver Star cross-280, the Hisex white, and the B.H.78 chicken were used to create the Anand Synthetic White Leghorn (ASWLH) line. The current experimental ASWLH birds were produced by selecting for egg number and egg weight at 40 and 56 weeks, respectively, for 12 generations, and then selecting for egg number and egg weight at 64 weeks of age. The Anand Bantamised White Leghorn (ABWLH) line was made by crossing Bantam chicken with two types of White Leghorn, IWP and IWN. It has 6.25 % Bantam DNA. All the designated birds were housed in individual cages within a three-tiered cage system using standard feeding, management, and health care procedures. For this study, the genetic variation was maximised by selecting the 24 birds with the maximum egg production and the 24 birds with the lowest egg production from the ASWLH and ABWLH chicken populations developed by the Poultry Research Station at Kamdhenu University, Anand.

Process of Acquiring and Extracting DNA Samples

A sterile 4 mL EDTA vacutainer was used to collect 2 mL of blood from the wing vein of 96 poultry chickens in an aseptic manner. From 200 µL blood sample, the DNA was separated and kept at -20°C. Genomic DNA concentration was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The quality assessment of genomic DNA was conducted using 0.8% agarose gel electrophoresis. In the process of library preparation, the concentration of DNA was determined with the help of the Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, MA). The DNA HS (High Sensitivity) kit was utilised in accordance with the manufacturer's instructions.

Design of Individualised Amplicons and Preparation of an Amplicon-Seq Library

From the reference genome Gallus_gallus-4.0 (galGal4), the Illumina custom amplicon panel for exon regions of the ESR1 and ESR2 genes was designed using Illumina's Design Studio (San Diego, CA, USA). The library size distribution was assessed using the Bioanalyzer 2100 (Agilent Technologies in Santa Clara, CA, United States). The Agilent DNA 1000 kit was utilized for this purpose. The Illumina Miseq platform was utilized for amplicon sequencing. The amplicons and primer sequences are provided in Table 1.

Data Analysis

The quality of the data in Fastq format was assessed after the sequencing run using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The data were filtered using PRINSEQ stand-alone lite v0.20.4, which has a minimal quality mean of 35 (source: https://sourceforge.net/projects/prinseq/files/). Using Burrows Wheeler Alignment (BWA v0.7.17) (https://sourceforge.net/projects/bio-bwa/files/), filtered Fastq files were utilised to map against candidate genes derived from Galgal4. Picard tool (v2.25.6) (https://broadinstitute.github.io/picard/) was utilised to generate alignment metrics for reads contained in BAM files. SAMTools (v1.12) were used for variant calling (https://www.htslib.org/doc/samtools.html). Integrative Genomics Viewer (IGV v2.10.0) was used to view the mapping characteristics and variations of each sample (https://software.broadinstitute.org/software/igv/igvtools). SnpEff (https://snpeff.sourceforge.net/ SnpEff.html) and SnpSift(v5.0e) (https://snpeff.sourceforge.net/SnpSift.html) were used to annotate and filter all discovered variants. The UCSC genome browser (https://genome-asia.ucsc.edu/cgi-bin/hgGateway) was utilised to validate the annotation of each SNP position. A correlation analysis was conducted between the identified variants and EN64 utilising PLINK (v1.07) (https://zzz.bwh.harvard.edu/plink/). We used permutation within the cluster to find the significance level. This had good qualities, like letting go of assumptions about the normality of continuous phenotypes and Hardy–Weinberg equilibrium, which helped us deal with rare alleles and small sample numbers.

Results and Discussion

The present study examined the comparative performances of two chicken populations, namely ASWLH and ABWLH. The populations consist of 24 birds each, with the highest egg producing birds and the lowest egg producing birds being included for analysis (Table 2). The custom amplicon panel contained a total of 43 amplicons with a maximum amplicon length of 375 bp and two primer pools; however, for bigger exons, several amplicons were required to cover the whole exon.

Raw data was obtained from sequencing runs on the IlluminaMiseq platform, with 96% of clusters passing filter and 90% of raw data having a Q-Score of 30 (*i.e.,* at least 99.9% accuracy in base calling). All the reads obtained in this study showed a minimum alignment of 99% with the reference genome.

Variant Calling

After filtering variants based on a minimum read depth of 20 and QUAL score of 30 (each individual base has been sequenced at least 20 times), 91 SNPs were discovered. Out of the 91 SNPs, 59 were previously reported in the dbSNPbuild145 database and 32 were novel. The SNPs are described in detail in Table 3.

Transition and transversion both can lead to change in amino acid sequence which ultimately leads to change in protein. However, transversions have a more significant impact on regulatory DNA as emphasized in the study conducted by Guo *et al*. (2017). In the present study, transitions accounted for approximately 70.9% of all the identified SNPs, with transversions making up the remaining 29.1%. The most frequent substitutions in the event of transitions were T-to-C (22%), A-to-G (19%), followed by C-to-T (16%) and G-to-A (12.9%). The present finding is fully in agreement with the findings reported by Kachchhi *et al*. (2024[b]), who also noted that transitions were more common than transversions and that G-to-A and C-to-T substitutions were the most common types of transitions.

Association of Identified SNPs with Egg Production

Two SNPs located in the exons of the ESR2 gene were found to have a significant association with EN64 in the entire experimental population, with a p-value less than 0.05. Nonetheless, single nucleotide polymorphisms (SNPs) located on the ESR1 gene did not demonstrate a statistically significant correlation ($p<0.05$) with EN64, whereas those within the ESR2 genes did exhibit such association. The details about SNPs that exhibit a significant association with EN64 are shown in Table 4.

Missense variants are relatively more concerning because they alter the amino acid sequence of the translated protein, which may lead to a change in phenotype, specifically egg production (Kachchhi *et al.,* 2024[a]). In the present study, 52 SNPs were discovered in ESR2 gene, out of which 11 SNPs were found to be novel. The g.52944897 T>C variant and rs3181386 variant of the ESR2 gene are associated with an Intronic region as determined by annotation. In a prior work, researchers investigated the relationship between ESR2 genotypes and egg-laying characteristics in Leizhou black ducks. Researchers discovered a strong correlation ($p<0.05$) between egg weight and the SNP g.56805646 T>C. In the present study, 39 SNPs were discovered in ESR2 gene, out of

Table 1: Details of amplicon targets and primer sequences used to amplify target regions

Ch.	Exon No	Start Coordinate	End Coordinate	Primer Sequence
3	Exon 1	48327426	48327749	F: CTCAGCAGAAATCATGTTAAAGCAG R: GCTGAAATCAGACCAGGAAGACTAG
	Exon 1	48327738	48328053	F: ACTGGAGCGGTAGTACCAAAATCGT R: TATTCCTCTGATCTCGGCAAGCAGG
	Exon 1	48328042	48328359	F: TGAAAGTGTAGGGCAGACATATTTC R: TCTGGGTAGTTAAAAACTCCTGTCT
	Exon 2	48354999	48355311	F: CCTGGGCTCAGGCTGTAATCTCAAG R: TCCTAAGAGCTAAGAAAGAAGAGCA
	Exon 3	48367725	48368042	F: CAGCTCAGAAAATAAAAATTCCTGC R: CTAACATCGCAGGCAAAGGGTAGTT
	Exon 4	48385293	48385580	F: AGAGCCGGACTGTTCTTCTTGTTAT R: TCTTACATGAGTAGTTGGAGAGGAA
	Exon 4	48385569	48385890	F: ATGCTTTCTATTCCCAAAACTGTAT R: AGTGGACTTGTCCAAAGGGTTGGAG
	Exon 5	48410289	48410559	F: CATCAAATTGTAGCATCAATGAGAT R: ACAACAGCATCCAGAGAACCAGATA
	Exon 6	48424191	48424494	F: GAAATGGAAACACTACCAGGGACAA R: ACGATAGAGCACAAGCTAGCATGTG
	Exon 7	48410289	48410559	F: ACAACACACACACACAGTGAAGGAA R: GCCTTATAGTGGCCTCTTTGGCTGT
	Exon 8	48430928	48431255	F: GGACTGCAGGGAATGAGATGAAGCT R: AATTCAGGATGGCCATGAATGATTG
	Exon 8	48431244	48431491	F: GAAACGTCTTTCACTTACCTAGAAT R: CAGTTGGTTTCGGTTCTCCTCTTCC

	Exon_1	52910473	52910795	F: GAGTTTACTGCTCATAAATCCAGCA R: ACAGAAGTTCCTCCTCCTCCTTTCT
	Exon_2	52920734	52921018	F: CAACTGAGAAAAGTCCTTGTGAGAA R: AGACAACACTTGTTGAGTTCAAACA
	Exon_2	52921007	52921220	F: TGTTGTAATTCAGCATAGCAGGACT R: GGACATTTTGATTCAAGACTGAAGG
	Exon_2	52921209	52921522	F: TCAGATGCGCAGAAATAAAATGTTT R: TTGCAGAATATTCATGCCTGTTTTC
	Exon_3	52928043	52928372	F: AATCAACCCACTACCAGAGCAATTG R: TCACTGGAGATGTGAACAAGCAGCT
	Exon_4	52930723	52931004	F: TTGTTGTGCCTTTTTAGAATTCTGG R: CAACTCATCTACTTCTTATCTAAGC
	Exon_5	52940112	52940393	F: CCCATGCAATCTTCTGAATTACGAT R: GGAAAATCCCACTGACATCAAAAAG
	Exon_5	52940382	52940692	F: TCACACTGCACATTTACAGTGCTAT R: ATTCGATACCCACAGCGTTCTCTTC
	Exon_6	52944802	52945062	F: TTAAGACTTCCATCCAGCAGCTTTC R: TGAAATGATAGAACCACAGAATCCT
5	Exon_6	52945051	52945316	F: TCAACAGCACTATTTACACTCAGCA R: CTTGGTCATAGAGGCTGAGATCAAT
	Exon_8	52948944	52949273	F: CCCTCAGGGCCACTATGGAGTTCTA R: TTCCTGACACATTCACACACCCCAA
	Exon_9	52950273	52950597	F: CAGCCCTGTATTTCTGTTCAGACCT R: TCTACCATCTAGAGAACCAAGATTT
	Exon_9	52950586	52950856	F: CTTGCAGGACTGTTCTGAGGCTTAA R: ACTTGTTCAAACTCAGGATGTGTGA
	Exon_9	52950845	52951163	F: CAGCCACAGCCATTGCATATTTAAG R: GCTTTTAAGAGTCTCACAGAAGGCA
	Exon_9	52951152	52951390	F: ACATCTTCATCTCACAGACAGAACA R: CTCCTTTACACTGTGCACACCACCT
	Exon_9	52951379	52951691	F: GGATCCCGCACAAAATATGGAACAG R: TCAGCACTGACTGAAAGAAGGGGGC
	Exon_9	52951680	52951888	F: CAATATTGGCAGCGACTGGAACAAA R: AATCGCTTGGAGAACGAACAAGCAG
	Exon_9	52951877	52952187	F: GTACAAGATCAAAAACAGGACAAGA R: TGCTCTTTGCTGAGGTGGTCAGATG
	Exon_9	52952176	52952487	F: TAGAAGTTTCTTTGAAAAACTGTAT R: CTCACTGACAATGCTTTATGCCCCT

Table 2: Comparison of egg production performance in ASWLH and ABWLH populations with high and low egg-producing groups

Statistics	ASWLH Low Production	ASWLH High Production	ABWLH Low Production	ABWLH High Production
Observations	24	24	24	24
EN64 Range	191-215	234-248	261-281	282-299
Mean	204.33±1.37	241.83±0.89	269.13±1.58	289.35±0.89
Variance	44.75	60.28	18.92	18.94
P (T<=t) one-tail	1.86E-32		1.29E-36	

which 21 SNPs were found to be novel. No SNPs were found to be associated with EN64. One of the previous studies revealed a correlation between the alteration of ESR1 expression and the corresponding pattern observed in egg production among laying hens (Ni *et al*., 2007[a,b]). In addition, the study of goose has revealed that ESR1 has a significant impact on egg production. The results showed that the expression of ESR1 in goose ovary increased significantly ($p<0.05$) after entering the laying period (Kang *et al*., 2009). In a similar study, three SNPs (rs314996211, rs313100310, and g.17308550T>C) in the GnRHR gene's exon region were found to be strongly linked with egg production at 64 weeks of age (Pal *et al.,* 2023).

Table 3: Information about the observed SNPs

Gene	Region	Reference SNP Identifier	Gene	Region	Reference SNP Identifier
ESR1	Exon 1	rs312335415	ESR2	Exon_2	rs731988160
ESR1	Exon 1	rs741031787	ESR2	Exon_2	rs316925667
ESR1	Exon 1	g.48385309A>G	ESR2	Exon_3	rs317377771
ESR1	Exon 2	g.48385317A>G	ESR2	Exon_4	rs313766889
ESR1	Exon 3	g.48385464G>A	ESR2	Exon_5	rs316142043
ESR1	Exon 1	g.48385480A>C	ESR2	Exon_5	rs737627884
ESR1	Exon 1	rs737908350	ESR2	Exon_6	rs314238777
ESR1	Exon 1	g.48385818C>T	ESR2	Exon_1	rs314204640
ESR1	Exon 2	rs16273463	ESR2	Exon_2	rs312486878
ESR1	Exon 3	rs315693030	ESR2	Exon_2	rs15734466
ESR1	Exon 1	rs316489702	ESR2	Exon_2	rs13592397
ESR1	Exon 1	rs316715942	ESR2	Exon_3	rs15734470
ESR1	Exon 1	rs733536058	ESR2	Exon_1	rs318138666
ESR1	Exon 2	g.48355039T>C	ESR2	Exon_2	rs312753241
ESR1	Exon 3	g.48355069G>T	ESR2	Exon_2	g.52944882A>T
ESR1	Exon 1	g.48385465C>T	ESR2	Exon_2	g.52944891T>G
ESR1	Exon 1	rs731028834	ESR2	Exon_3	g.52944897T>C
ESR1	Exon 1	g.48385484A>G	ESR2	Exon_6	g.52944908T>C
ESR1	Exon 2	g.48385529T>C	ESR2	Exon_5	g.52944927T>C
ESR1	Exon 3	rs740591712	ESR2	Exon_5	g.52944930T>C
ESR1	Exon 1	g.48355039T>C	ESR2	Exon_5	g.52944935T>C
ESR1	Exon 1	rs312335415	ESR2	Exon_6	rs16513679
ESR1	Exon 1	g.48355128A>G	ESR2	Exon_6	rs14550608
ESR1	Exon 1	rs741031787	ESR2	Exon_8	rs16513690
ESR1	Exon 1	g.48385465C>T	ESR2	Exon_8	rs14550609
ESR1	Exon 4	g.48385478A>C	ESR2	Exon_8	rs315367925
ESR1	Exon 4	g.48385480A>C	ESR2	Exon_8	rs317835591
ESR1	Exon 5	rs737908350	ESR2	Exon_9	rs312517223
ESR1	Exon 6	rs16273463	ESR2	Exon_9	rs313006366
ESR1	Exon 7	rs315693030	ESR2	Exon_9	rs315271297
ESR1	Exon 8	rs316489702	ESR2	Exon_9	rs312998151
ESR1	Exon 8	rs316715942	ESR2	Exon_9	rs314120558
ESR1	Exon 4	rs733536058	ESR2	Exon_9	rs312497585
ESR1	Exon 4	rs741278680	ESR2	Exon_9	rs14550611
ESR1	Exon 5	g.48385309A>G	ESR2	Exon_9	rs313119515
ESR1	Exon 6	g.48385317A>G	ESR2	Exon_9	rs313923225
ESR1	Exon 7	g.48385460A>G	ESR2	Exon_9	rs316796029
ESR1	Exon 8	g.48385466G>A	ESR2	Exon_9	rs16513694
ESR1	Exon 8	g.48385497A>C	ESR2	Exon_9	rs16513695
ESR1	Exon 4	g.48385500A>C	ESR2	Exon_9	rs16513696
ESR2	Exon_2	g.52921427T>C	ESR2	Exon_2	rs731988160
ESR2	Exon_9	rs312747231	ESR2	Exon_9	rs16513697
ESR2	Exon_9	rs316958312	ESR2	Exon_9	rs312484187
ESR2	Exon_2	g.52920942G>A	ESR2	Exon_9	rs314548180
ESR2	Exon_9	g.52951740T>C	ESR2	Exon_6	rs14550578
ESR2	Exon_2	g.52921295G>A			

Table 4: Details of Novel SNPs that are significantly linked to EN64

Gene	Region	SNP	p value
ESR2 (ABWLH_High/Low)	Exon_6	g.52944897T>C	0.00235
ESR2 (ASWLH_High/Low)	Exon_5	rs3181386	0.04057

Conclusions

The present study utilized amplicon sequencing to discover polymorphisms in the ESR1 and ESR2 genes, and examined their potential association with egg production in two specific layer lines, namely ASWLH and ABWLH. Two SNPs, specifically g.52944897T>C and rs3181386 of the ESR2 gene, were found to be significantly associated with egg production at 64 weeks of age (EN64). Based on present finding, we conclude that these two SNPs can be employed in investigations involving marker-assisted selection for the selection of birds for egg production. However, because our sample size was small, further research into the association of these SNPs with egg production in a large phenotype chicken population is required.

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