RESEARCH ARTICLE

Identification of Single Nucleotide Polymorphisms in GnRH-I, GnRH-II/MRPS26, GnRHR exons and their Association with Egg Production in Anand Synthetic White Leghorn and Anand Bantamised White Leghorn Chicken

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Abstract

The aim of study was to identify Single Nucleotide Polymorphisms (SNPs) in the exon regions of GnRH-I, GnRH-II/MRPS26 and GnRHR genes and to assess their association with egg production up to 64 weeks of age (EN64) in chicken. Blood samples were collected from 48 Anand Synthetic White Leghorn (ASWLH) and 48 Anand Bantamised White Leghorn (ABWLH) chicken. Custom panel was designed for these genes' exon regions and amplicon sequencing was performed on IlluminaMiseq platform. Total 41 SNPs (19 previously reported and 22 novel SNPs) were identified from 96 samples. Association analysis revealed that rs314996211 ($p<0.05$), rs313100310 ($p<0.01$), g.17308550T>C ($p<0.01$) of GnRHR were significantly associated with EN64. Conclusively, rs314996211, rs313100310, g.17308550T>C of GnRHR can be implemented as selection markers associated with EN64 in chicken.

Key words: Amplicon sequencing, Chicken, Egg production, GnRH, SNP.

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Introduction

Present-day commercial layers exhibit excellent laying capacity and have shown consistent improvement in egg production mainly due to phenotypic selection. Continued increase in egg production indicates that genetic variability is still present. Production can be improved further by taping the genetic variability present in the genes involved in egg production pathways. Several candidate genes have been studied to improve reproductive traits and reduce broody days and frequency in poultry (Vinh *et al.,* 2021).

Egg production is a polygenic inheritance trait having a low to moderate heritability which depends on the period involved (AL-Jaryan *et al.*, 2021). The avian egg-production process is strictly controlled by the hypothalamic-pituitary-gonadal axis (Kataki and Khanikar, 2022). The gonadotropin releasing hormone (GnRH*)*, a neuro-endocrine decapeptide, plays an essential role in regulating the gonadal development and sexual maturation in vertebrate species (Gharaei *et al.*, 2011). The hypothalamus regulates gonadotropin secretion via pulsatile secretion of the GnRH into the pituitary's portal circulation which is essential for proper gonadal function. GnRH, binding with its receptor GnRHR, stimulates gonadotropin secretion from the pituitary gland and evokes steroidogenesis in the gonads, resulting in egg production in hens (Sonez *et al.,* 2010). The majority of evidence suggests that in chickens, GnRH-I is the primary regulator of gonadotropin release and GnRH-II might be independent of GnRH-I. Ikemoto and Park (2006) identified that mitochondrial ribosomal protein S26 (MRPS26) gene was partly overlapped with the downstream part of the GnRH-II in the chicken and the GnRH-II/MRPS26 locus generated at least five distinct types of transcripts with different expression patterns and three of them might produce functional GnRH-II decapeptide.

In genomic DNA, a base position with sequence alternatives is considered as an SNP only when the least frequent allele has at least 1% frequency.Previously, haplotype combinations were used to associate biological traits to their causal genes but with the advent of next-generation sequencing (NGS) technology, it is now possible to effectively classify SNPs linked to the genes involved in the traits of interest. Amplicon sequencing is an approach

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of targeted resequencing that involves amplification and purification of regions of interest using primer-mediated highly multiplexed oligonucleotide probes. Although a number of research works and association studies have been done on these genes using conventional techniques like PCR-RFLP, however, we have performed amplicon sequencing of these genes' exon regions to enable deep sequencing at much higher coverage levels, allowing greater confidence for calling variants of low-frequency alleles, and their association with egg production in Anand Synthetic White Leghorn (ASWLH) and Anand Bantamised White Leghorn (ABWLH) Chicken.

Materials and Methods

Experimental Birds

All the experimental birds were subjected to the rules and regulations provided by Institutional Animal Ethics Committee (IAEC). The Anand Synthetic White Leghorn (ASWLH) line was developed by crossing four different commercial strain-crosses, *viz*., BV-300, Shaver Star cross-280, Hisex white and B.H.78 chicken. The present experimental ASWLH birds were developed through selection for egg number and egg weight at 40 and 56 weeks, respectively, for 12 generations and after that selected based on egg number and egg weight at 64 weeks of age. The Anand Bantamised White Leghorn (ABWLH) line was developed by crossing Bantam chicken and two strains of White Leghorn, *viz*., IWP and IWN and possesses 6.25% Bantam inheritance. All the selected birds were maintained in individual cages in a three-tier cage system under standard feeding, management and health care practices. For present study 24 highest egg producing birds and 24 lowest egg producing birds of ASWLH and ABWLH chicken whole populations developed by Poultry Research Station, Anand Agricultural University, Anand were selected to maximize the genetic variation.

Sample Collection and DNA Isolation

2 mL blood samples were collected from the wing vein of 96 birds under an aseptic condition in a sterile 4 mL EDTA vacutainer. DNA was extracted from 200 µL blood and the isolated DNA samples were stored at -20°C until further use. The concentration of genomic DNA was evaluated on NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., USA). The quality of genomic DNA was checked by 0.8% agarose gel electrophoresis. For library preparation, DNA concentration was measured using Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, MA) using DNA HS (High Sensitivity) kit as per protocol provided by the manufacturer.

Custom Amplicon Design and Ampliseq Library Preparation

The Illumina custom amplicon panel for exon regions of GnRH-I, GnRH-II/MRPS26, GnRHR genes was designed using Illumina's Design Studio (San Diego, CA, USA) from the reference genome Gallus_gallus-4.0 (galGal4). The library size distribution was checked on Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States) with the Agilent DNA 1000 kit. Amplicon sequencing was performed on IlluminaMiseq platform. Details of amplicons and primer sequences are shown in Table 1.

Bioinformatics Analysis

After sequencing run, quality of data in Fastq format was checked using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data having minimum quality mean of 35 were filtered using PRINSEQ stand alone lite v0.20.4 (https://sourceforge.net/projects/prinseq/files/). Filtered Fastq files were used for mapping against candidate genes derived from Galgal4 using Burrows-Wheeler Alignment (BWA v0.7.17) (https://sourceforge.net/projects/bio-bwa/files/). Alignment metrics of reads stored in the BAM files were generated using Picard tool (v2.25.6) (https://broadinstitute.github.io/picard/). Variant calling was performed using SAM tools (v1.12) (https://www.htslib.org/doc/samtools.html). Mapping characteristics and variants of each sample were viewed in Integrative Genomics Viewer (IGV v2.10.0) (https://software.broadinstitute.org/software/igv/igvtools). All identified variants were annotated and filtered using SnpEff (https://snpeff.sourceforge.net/SnpEff.html) and SnpSift (v5.0e) (https://snpeff.sourceforge.net/SnpSift.html). Each SNP position was verified using UCSC genome browser (https://genome-asia.ucsc.edu/cgi-bin/hgGateway) to confirm proper annotation. Association of identified variants with EN64 was performed using PLINK (v1.07) (https://zzz.bwh.harvard.edu/plink/). To generate significance level, permutation within cluster was employed that enabled desirable properties, *viz*., relaxing assumptions about normality of continuous phenotypes and Hardy-Weinberg equilibrium, dealing with rare alleles and small sample sizes.

Results and Discussion

Comparative performances of ASWLH and ABWLH chicken populations (24 highest egg producing birds and 24 lowest egg producing birds) are presented in Table 2.

As for larger exons, it needed more than one amplicon to cover entire exon, the custom amplicon panel included a total of 23 amplicons with a maximum amplicon length of 375 bp with two primer pools. Sequencing run on IlluminaMiseq platform generated raw data with 96% clusters passing filter and 90.6% raw data had Q-Score 30 (*i.e.,* minimum 99.9% accuracy in base calling) or higher. All reads achieved minimum 99% alignment with reference genome.

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

Gene	Ch.	Exon No.	Start Coordinate	End Coordinate	Primer Sequence (5′→3′)
GnRH-I	22	Exon 1	827692	828015	F: GAAAATCCCTGCTGATATTTGACAG R: AGTAAAGCACATAATTGGAGCAGCT
		Exon 2	828228	828550	F: TGTTGAGAGAGAGAGACTCCTGGTA R: GACTTTCAGCTCTGACCTCCTGCAC
		Exon 3	829008	829335	F: GCTGGGAGTGCCACATGTGAACTAA R: CATGTGCTCTAACAGCTGTAGAATG
		Exon 4	830604	830935	F: TTCCCCACCATTGGACTACGTGTTC R: AAATCTGACCCGACGTGATCTCTCT
GnRH-II/ MRPS26	4	Exon 1	88165773	88166019	F: GGAGGATGGAGATGCTGCAGAGGAT R: TTGCTGAGATGGAGCCCACCCCAGA
		Exon 2	88166637	88166968	F: GAGGGGGTGGTGGTTATAGCACTGA R: AAGGGATGAGCATCTGTGGATGCTG
		Exon 3	88167571	88167897	F: GAGTCGCAGACCAATGAAAGGAGAG R: TGAGG-TAAAAATTCTGACTGTGAGG
		Exon 3	88167884	88168137	F: TACCGCTCGGTGACCACATAGAGCT R: ACGATGGGAATTGTAGTGCGGCGCG
		Exon 3	88168079	88168320	F: GCCAGCCCGACTTCTATTCTTCTGC R: ATCGTACCGCGATTTTCGGCCCCGA
		Exon 4	88169020	88169327	F: AACCACTCAGGTTTATTTTTACAAA R: AGAAGCCTCAGCCCAATGTAACCCC
		Exon 5	88169678	88170009	F: CAGGAAGGAGCCAACATTTGCCTTC R: TCATCCCTTTAACCTCTGCCTGCAT
		Exon 6	88170260	88170539	F: ACAGGAAGCAGGCTGTTTGTTCATC R: CAAAGGGTTGCTAGGATCTGCCTTT
		Exon 6	88170528	88170846	F: AAGACAGAGAGTGACCAGAGATCGA R: TGATTTGTGCATTAAAAGGAAGAAC
		Exon 6	88170835	88170982	F: GTACAAGACCAGTCAAGATACAATC R: TTAGTGTAGGAGACAAAGGGATGAG
		Exon 6	88170971	88171280	F: CACTCTGCCTCCTGTTTATAAACTC R: TGTTACAAAGGCCCAGGTACAGTCT
GnRHR	10	Exon 1	17307065	17307395	F: AGTCCTTTCACTGCATCCATCCCTT R: TTTAGTCCTAATGAGCCAGCCGTGG
		Exon 2	17307886	17308156	F: TGGGGCGGTTCAGCTTCGATTAAAG R: TTTAGCTCTTTTCAGGGCAGCAGAG
		Exon 2	17308155	17308456	F: CAGGAAAGGTCTCCACCATACCACT R: AGCTGGTAATTAGAGAGAGATCACA
		Exon 2	17308480	17308712	F: ATGCTATGATGGATTCATTGCGGAA R: AGGAAAGGTCTCCACCATACCACTG
		Exon 3	17308732	17309011	F: TATAGAGGGTGGTGAAGGTGAACAT R: ATGATGGATTCATTGCGGAAGCCCT
		Exon 3	17309000	17309164	F: GGTGTGAGAGAACAAGGAGAACGTA R: TTTCTTCCCAGTGTGCTCGGAAGCT
		Exon 4	17309070	17309399	F: CATGTGTGCAGCAAACCAAAGAGAA R: ACGTAGCAAACAATCATGATGCTCA
		Exon 4	17309388	17309679	F: TTGTCTCCAAAATCATCCTCTCAGA TTGATGTACTCGGGCATCCTCTGGA

Table 2: Comparative analysis of egg production performance between high and low egg producing groups within ASWLH and ABWLH populations

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	

Table 3: Details of identified SNPs

Gene	Region	Reference SNP Identifier	Gene	Region	Reference SNP Identifier
GnRH-I	Exon 3	g.829159G>A	GnRH-II	Exon 3	g.88168008G>A
GnRH-II	Exon 2	rs731196463	GnRH-II	Exon 3	g.88168009C>T
GnRH-II	Exon 3	g.88167792C>T	GnRH-II	Exon 3	g.88168048T>A
GnRH-II	Exon 3	g.88167805G>A	GnRH-II	Exon 3	g.88168132C>T
GnRH-II	Exon 3	rs731943627	GnRH-II	Exon 3	g.88168134G>A
GnRH-II	Exon 3	rs735432780	GnRH-II	Exon 3	g.88168135C>T
GnRH-II	Exon 3	g.88167814T>C	GnRH-II	Exon 3	g.88168171A>G
GnRH-II	Exon 3	rs735398423	GnRH-II	Exon 6	rs80597663
GnRH-II	Exon 3	g.88167868A>G	GnRH-II	Exon 6	rs741121319
GnRH-II	Exon 3	g.88167870T>C	GnRHR	Exon 1	rs317908788
GnRH-II	Exon 3	g.88167888T>C	GnRHR	Exon 2	rs315958476
GnRH-II	Exon 3	g.88167939A>G	GnRHR	Exon 2	rs315111882
GnRH-II	Exon 3	g.88167945G>A	GnRHR	Exon 2	rs735377169
GnRH-II	Exon 3	g.88167957G>A	GnRHR	Exon 2	rs313334462
GnRH-II	Exon 3	g.88167962C>A	GnRHR	Exon 2	rs317273381
GnRH-II	Exon 3	rs736598919	GnRHR	Exon 2	rs314996211
GnRH-II	Exon 3	g.88167997C>T	GnRHR	Exon 2	rs733217146
GnRH-II	Exon 3	g.88167998C>T	GnRHR	Exon 2	g.17308550T>C
GnRH-II	Exon 3	g.88168005G>A	GnRHR	Exon 3	rs313100310
GnRH-II	Exon 6	rs736424967	GnRHR	Exon 4	rs316736677
GnRH-II	Exon 6	rs734923321	--	--	--

Variants Calling

Total 41 SNPs were identified after filtering variants based on minimum read depth of 10 (*i.e.,* each individual base had been sequenced at least 10 times). Out of the 41 SNPs, 19 SNPs were previously reported in dbSNPbuild145 database and 22 SNPs were novel. Details of the SNPs are shown in Table 3.

The amino acid composition of the resulting protein can be changed by both transition and transversion, which has a stronger impact on regulatory DNA by allele-specific transcription factors binding and transcription factors binding motifs (Guo *et al*., 2017). In the exon regions, a total of 36 transitions and five transversions were identified, resulting in a transition to transversion ratio of 7.2. In case of transitions, G- to -A were the most common substitutions (33.33%), followed by C- to -T (27.78%), T- to -C (25.00%) and A- to -G (13.89%). These findings confirmed with those of Weng *et al*. (2020), who also observed that transitions were more frequent than transversions and the most frequent transitions were G-to-A and C-to-T substitutions.

Association between Identified SNPs and Egg Production

Total three SNPs on GnRHR gene's exons were significantly ($p<0.05$) associated with EN64 in the whole experimental population. However, no SNP on GnRH-I and GnRH-II/MRPS26 were significantly ($p<0.05$) associated with EN64. Details of SNPs significantly associated with EN64 are presented in Table 4.

Table 4: Details of significantly associated SNPs

Gene	Region	SNP	P Value
GnRHR	Exon 2	rs314996211	0.04
	Exon 2	g.17308550T>C	0.005
	Exon 3	rs313100310	0.001

Missense variants are of comparatively greater concern since these variants change amino acid sequence in the translated protein and thereby, may result in modification of phenotype, *viz.,* egg production. Following annotation, we found that the rs314996211 of GnRHR gene leads to alanine-to-valine shift, whereas the g.17308550T>C of GnRHR results in leucine-to-proline shift. Synonymous SNPs are well recognized to alter mRNA stability, protein or nucleic acid binding sites, translation rate, protein folding, protein solubility and fitness (Durosaro *et al*., 2021). The rs313100310 of GnRHR was identified as synonymous SNP after annotation.

The present findings are consistent with the observations by Fatemi *et al*. (2012) and Xu *et al.* (2007), who also reported significant association of GnRHR gene polymorphism with egg production, although the variants were different from present study. However, Xu *et al.* (2011) reported no significant association between GnRHR polymorphism and egg production. The present findings are also in contrast to those of Bhattacharya *et al.* (2019) who reported that different haplo group combinations of GnRH-I and GnRH-II genes had a significant association ($p<0.05$) with egg production. This disagreement may be attributed to the limited sample size used in our study.

Conclusions

This is perhaps the first comprehensive study employing amplicon sequencing to identify polymorphisms in the GnRH-I, GnRH-II/MRPS26, and GnRHR genes and their association with egg production in Anand Synthetic White Leghorn (ASWLH) and Anand Bantamised White Leghorn (ABWLH) layer lines. We have identified three SNPs, *viz.,* rs314996211, rs313100310 and g.17308550T>C of GnRHR gene's exon region which were significantly associated with egg production in 64 weeks of age (EN64). We conclude that these SNPs can be used in marker assisted selection studies for chicken egg production. However, since the sample size in our study was small, there is a need to explore the association of these SNPs with the egg production on a large phenotyped chicken population.

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