

MODEL FOR TYPE SETTING BEFORE SUBMISSION

Validation of SNPs Identified by Amplicon Sequencing and Investigation of their Association with Egg Production and Egg Weight in Anand Synthetic White Leghorn and Anand Bantamised White Leghorn Chicken

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ABSTRACT

The present study was aimed to validate the previously found SNPs using NGS-based amplicon sequencing and investigate of their association with egg production and egg weight by PCR-RFLP in Anand Synthetic White Leghorn (ASWLH) and Anand Bantamised White Leghorn (ABWLH) chicken. A total of 500 blood samples were collected including, 227 samples of ASWLH and 273 of ABWLH maintained at the Poultry Research Station, Anand Agricultural University, Anand, Gujarat. The study was conducted for a total of eight SNP markers located across the four genes involved in physiological pathways related to egg production and egg weight, viz., GnRHII (T88170424C), GnRHII (G88170427A), VIPR1 (C1826542A), VIPR1 (C1826555T), OCX32 (C21912308T), OCX32 (T21912084G), OCX32 (C21912423A) and GHR (C13415980T). Out of eight SNPs, C21912423A of the OCX32 gene was significantly associated with egg weight at the 40 weeks of age (EW40) in the whole population. The G88170427A of the GnRHII gene was significantly associated with egg weight at the 28 weeks of age (EW28) and C21912308T of the OCX-32 gene was significantly associated with total egg number at the age of 64 weeks (TWN64) in the whole population.

Keywords: Chicken, Egg Production, Egg Weight, PCR-RFLP, SNP validation, Real-Time PCR.

INTRODUCTION

Poultry is one of the fastest-growing segments contributing an important part in India's agricultural sector. In India, the poultry industry has undergone a significant transformation in terms of structure and operation in rural as well as urban regions during the last thirty years (Mitra *et al.*, 2021). India ranks 3rd in the production of eggs and 8th in meat production across the world as per the economic survey 2021-22. Egg production in the country has increased from 78,480 million in 2014-15 to 122,110 million in 2020-21 making up the per capita availability of 91 eggs per annum in 2020-21 among Indian populations. Because of the ongoing increase in

demand for eggs and meat chicken, improved growth and production performance is essential to meeting demand. This can be compensated using genomics and the use of molecular or DNA markers. A molecular marker is used for the selection of superior animals with high productivity. PCR-RFLP is a popular, reliable and cost-effective technique for identifying targeted single nucleotide polymorphisms (SNPs) (Narayanan, 1991). The recent advancement in next-generation sequencing and genotyping technologies is used for the identification of SNPs related to production (Vinh *et al.*, 2021).

The avian egg-production process is mainly in control of the hypothalamic-pituitary-gonadal axis (Kuo *et al.*, 2005). A neuropeptide Y (NPY) is investigated routinely to decode its role in the hypothalamus regulation of reproductive function via controlling GnRH secretion. Besides the GnRH-gonadotrophin system, the vasoactive intestinal peptide (VIP) and prolactin (PRL) neuroendocrine route also control avian seasonal reproduction (Leska and Dusza., 2007). The growth hormone receptor (GHR) and insulin-like growth factor-I (IGF-I) genes have a notable collision on the bio-physiological processes that regulate differentiation and growth production, reproduction, and follicular generation in animals during the rapid growth phase (Bachelot *et al.*, 2002).

The line “Anand Synthetic White Leghorn (ASWLH)” was produced at Anand Agricultural University's Poultry Research Station and has a better egg weight (54 g) and a higher egg production capability (280 eggs/year). This synthetic white leghorn was created by crossing four commercial strain crossings, and it became a major gene pool for egg-type chickens. Feed accounts for roughly 80% of all recurring expenses in poultry production. To enhance feed efficiency, another line was developed at Anand Agricultural University's Poultry Research Station inserting the Bantam gene into WLH. The resultant line "Anand Bantamised White Leghorn (ABWLH)" has a 6.25 % Bantam inheritance and 93.75 % White Leghorn inheritance.

The present study was carried out with the objectives to validate already identified SNPs in previous study (by NGS based amplicon sequencing) using PCR-RFLP technique in coding regions of GnRHI, GnRHII, VIPR1 and OCX32 genes and to assess an association of identified SNPs with egg production in Anand Synthetic White Leghorn and Anand Bantamised White Leghorn chicken.

MATERIALS AND METHODS

Collection and Processing of Samples

A total of 500 blood samples were collected including, 227 samples of ASWLH and 273 of ABWLH maintained at Poultry Research Station, Anand Agricultural University, Anand, Gujarat (India) under the aseptic condition in a sterile 4 mL EDTA vacutainer. The DNA was isolated from blood as per John's method using the standard phenol-chloroform extraction method (John *et al.*, 1991). The evaluation of the quality of DNA was done by agarose gel

electrophoresis and the quantity of DNA was estimated by UV spectrophotometer.

Real-Time PCR for SNPs Validation

Primers were designed using Primer 3 with web-based tools to amplify the flanking areas of the targeted SNPs (Table 1). Real-Time melt curve shows different curves according to the melting temperature of a particular sequence and identify alleles based on amplicon length and was used to distinguish them. This study first analyzed 500 samples in 96 sample batches and after the result data showed that around 100 samples were having different T_m , those samples were subjected to the second run of Real-Time PCR. The Real-Time PCR was carried out with primers of genes-specific targeted SNP. Real-Time PCR-based melt curve analysis was performed initially to discriminate the amplicons having different nucleotide compositions. The final PCR reaction volume of 10 μ L which consisted of 0.5 μ L template DNA, 1 μ L forward primer, 1 μ L reverse primer, 5 μ L 2x QuantiNova SYBR Green qPCR master mix, 0.5 μ L QN ROX reference dye and 2 μ L nuclease free water. The entire reaction was conducted in the Quant Studio 5 thermal cycler system.

PCR-RFLP for SNPs Validation

Approximately 20 samples needed to be analyzed for every targeted gene through PCR-RFLP after the screening of 500 samples using melt-curve analysis. At first, the PCR reaction was carried out in a total of 25 μ L system and the PCR amplification was conducted in an automated thermal cycler as per the protocol of particular SNPs. At first 12.5 μ L PCR master mix, 1 μ L forward primer, 1 μ L reverse primer, and 9.5 μ L nuclease-free water, 1 μ L of genomic DNA were taken in 0.2 mL labeled thin-walled PCR tubes. The PCR product was analyzed by agarose gel electrophoresis in 1.5% agarose gel. The products were examined under UV light and images were documented using a gel documentation system. The restriction sites and the corresponding enzymes were selected using the online tool NEB cutter V3.0 at <http://nc2.neb.com/NEBcutter2/> (Table 2). A total of 30 μ L reaction mixture consisted of 10 μ L PCR product, 3 μ L 10X RE buffer, 1 μ L RE enzyme (10 units/ μ L), 16 μ L nuclease free water. The whole reaction was conducted at 37°C for 1 h in a thermal cycler with the restriction digestion mix.

Table 1: Primer sequences, SNP markers and PCR product size of various candidate genes

Gene	SNP marker	Forward/ Reverse Sequence	Amplicon size
GnRHII	T88170424C	F:5'CCAACACTCTCCCCTTTCCT3' R:5'TACCTCTGCAACAGCAGCAC3'	293 bp
GnRHII	G88170427A	F:5'GAGGCTCGGATTGAAGAGTG3' R:5' ACTGCAGGAcTGAGGCAGAG3'	298 bp
VIPR1	C1826542A	F:5'GCTATTACCCCTATGTACTCTCTCC3' R:5' AGGGATCCTTCCCCTCTCTTG3'	299 bp
VIPR1	C1826555T	F:5'TGCTGCAGTGTCTCTCTCC3' R:5' AGGGATCCTTCCCCTCTCTTG3'	367 bp

OCX32	C21912308T	F:5'GATAACCATGGGCTCGAAGA3' R:5'CCAACTTCCACTGTGTATAATGGT3'	497bp
OCX32	T21912084G	F:5'GCTCCACGAAATTCCAACAC3' R:5'TGCTCACCTGTGTGTGTATGC3'	485bp
OCX32	C21912423A	F:5'CACAGGTGAGCATAACACAGG3' R:5'CCAACTTCCACTGTGTATAATGGT3'	214bp
GHR	C13415980T	F:5'AGCAAGGCAGGCTACAAGAA3' R:5'GCACAGGAGGAAAAATCAGC3'	245bp

Table 2: Details of gene, SNP position, Amplicon size, Restriction enzyme with their cutting site, and optimal reaction temperature of REs

Gene	SNP position	Sizes of amplicon (bp)	REs	Sizes of RE digests (bp)	optimal reaction temp
GnRHII	T88170424C	292	AhdI	129, 163	37°C
	G88170427A	298	HinP1I	78, 220	37°C
VIPR1	C1826542A	299	NlaIV	5, 62, 94, 138	37°C
	C1826555T	367	BfaI	30, 128, 209	37°C
GHR	C13415980T	245	EarI	88,157	37°C
OCX32	C21912308T	497	AleI	211, 286	37°C
	T21912084G	485	HpyF10VI	240, 245	37°C
	C21912423A	214	Tsp45I	111, 103	37°C

RESULTS AND DISCUSSION

Real-Time PCR is rapidly becoming a standard method in many diagnostic and research laboratories. The PCR was carried out in a 0.2 mL nuclease free PCR tube in a final volume of 25 µL in a thermal cycler for a total of eight SNP markers located across the four genes involved in physiological pathways related to egg production and egg weight, viz. GnRHII (T88170424C), GnRHII (G88170427A), VIPR1 (C1826542A), VIPR1 (C1826555), OCX32 (C21912308T), OCX32 (T21912084G), OCX32 (C21912423A) and GHR (C13415980T) with amplicons size 293bp, 298bp, 299bp, 367bp, 497bp, 485bp, 214bp and 245bp, respectively.

In PCR-RFLP different SNP marker shows different band pattern on agarose, i.e (i) T88170424C/AhdI resulted two fragment pattern 292bp and 129bp, 163bp, 292bp those referred as TT and TC genotype (Fig. 1) (ii) G88170427A/HinP1I resulted into three pattern 298bp/GG, (78bp, 220bp/GA), (78bp, 220bp, 298bp/AA) (Fig. 2) (iii) C1826542A/NlaIV polymorphism showing single digestion pattern 5bp, 62bp, 94bp, 138bp that referred as AA genotype (Fig. 3) (iv) C1826555T/BfaI shows absence of SNPs (Fig. 4) (v) C13415980T/EarI resulted in two digestion pattern 245bp/CC and 88bp, 157bp, 245bp/CT (Fig. 5) (vi) C21912308T/ AleI revealed that 211bp, 286bp/TT and 211bp, 286bp, 497bp/CT (Fig. 6) (vii) T21912084G/ HpyF10VI not shows any SNPs (Fig. 7) (viii) C21912423A/ Tsp45I resulted two digestion pattern 111bp,

103bp/AA and 111bp, 103bp, 214bp/CA (Fig. 8) .

In the association study polymorphism was associated with egg production and egg weight in ABWLH and ASWLH. First, we have calculated the gene and allele frequency of all genotypes to find a significant association via Hardy Weinberg equilibrium. According to Hardy Weinberg equilibrium, GnRHII (G88170427A) and GHR (C13415980T) are significantly associated with egg production traits. The association of candidate gene genotype with egg production and egg weight was assessed by performing a T-test. The C21912423A of OCX32 gene was significantly ($p<0.05$) associated with EW40 in the whole population. The G88170427A of GnRHII gene were significantly ($p<0.05$) associated with EW28 and C21912308T of OCX-32 gene were significantly ($p<0.05$) associated with TWN64 in the whole population.

In contrast to the current study, Dunn *et al.* (2003) performed PCR-RFLP for the identification of genotypes. They obtained GnRHR/Bpu1102I with three genotypes. They found two significant associations of GnRHR genotype with egg production. In the present study, VIPR1 (C1826542A) was not associated with production traits, on another hand Ngu *et al.* (2015) studied VIPR-1/TaqI and VIPR-1/HhaI polymorphism. They revealed that VIPR-1 genes showed a significant association with the egg productivity of Noi chicken in 20 weeks of laying ($P<0.05$). Li *et al.* (2008) observed that the GHR gene was not associated with egg production and egg weight. They found two restriction fragments pattern 428 bp/290 bp for the A1A1 genotype and 258 bp/170 bp/290 bp for the A2A2 genotype and both were homozygous same as in the present study. Demir *et al.* (2020) performed PCR-RFLP for OCX32-exon2/HpyCH4IV and OCX32-exon4/NcoI and showed that OCX-32 gene polymorphism was correlated with egg production and quality in chicken.

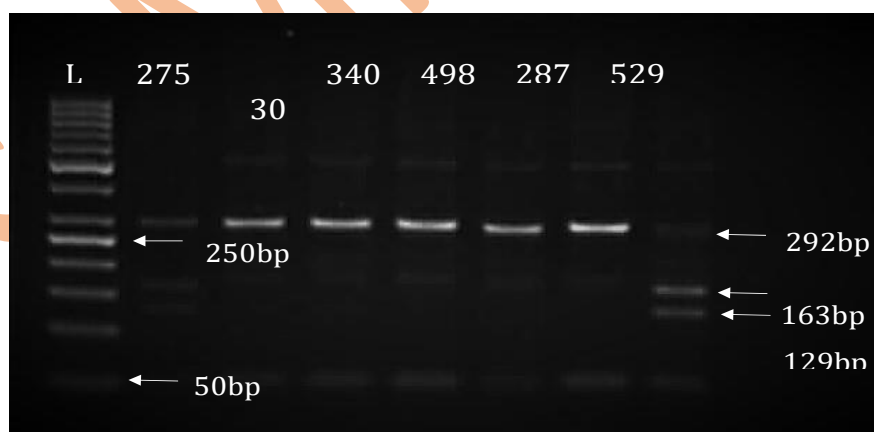


Fig. 1: Representative agarose gel electrophoresis image for PCR-RFLP of GnRHII (T88170424C) SNP marker

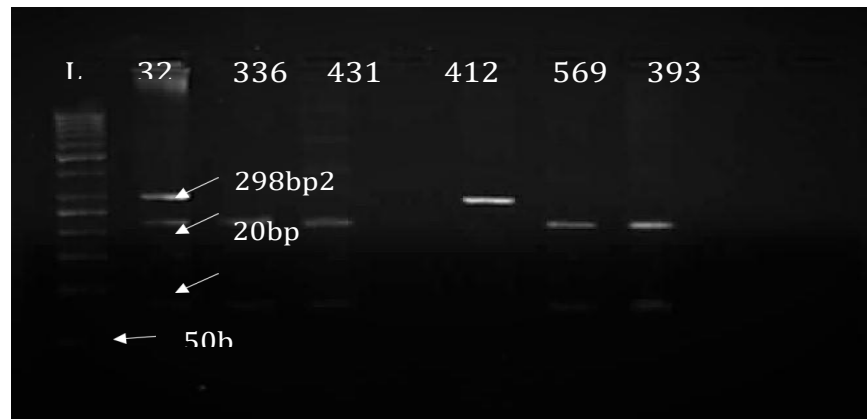


Fig. 2: Representative agarose gel electrophoresis image for PCR-RFLP of GnRHII (G88170427A) SNP marker

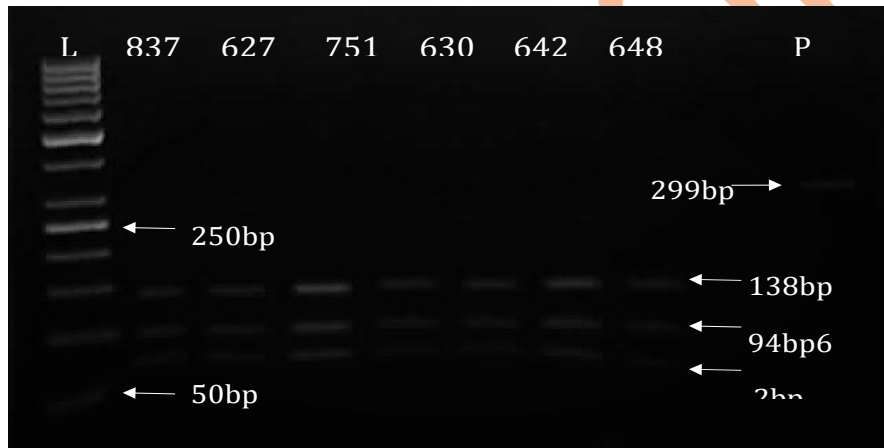


Fig. 3: Representative agarose gel electrophoresis image of PCR-RFLP forVIPR1 (G88170542A) SNP marker validation.

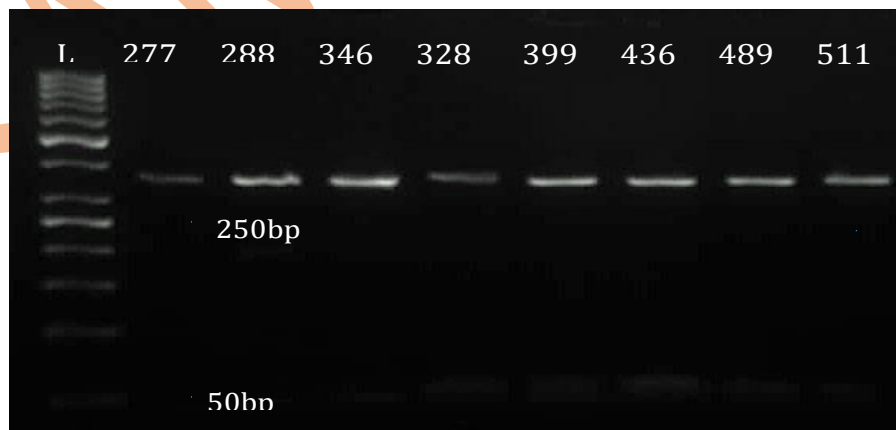


Fig. 4: Representative agarose gel electrophoresis image of PCR-RFLP for VIPR1 (C1826555T) SNP marker validation.

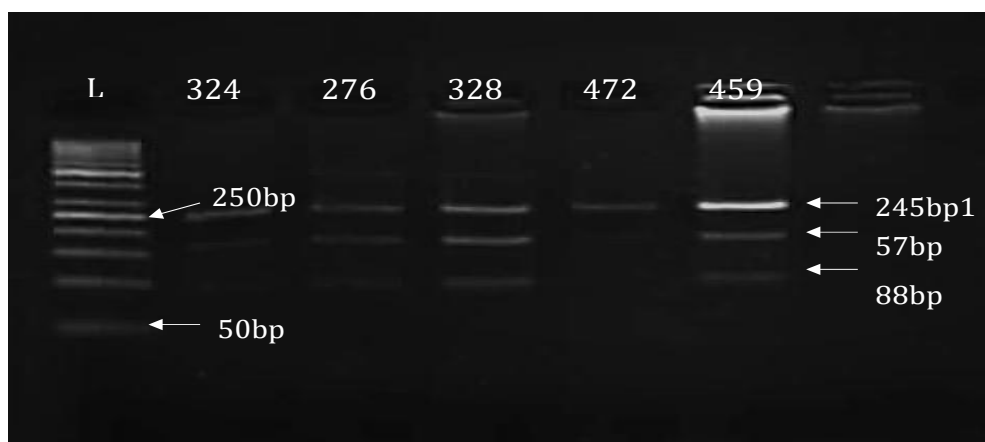


Fig. 5: Representative agarose gel electrophoresis image of PCR-RFLP for GHR (C13415980T) SNP marker validation.



Fig. 6: Representative agarose gel electrophoresis image of PCR-RFLP for OCX32 (C21912308T) SNP marker validation.

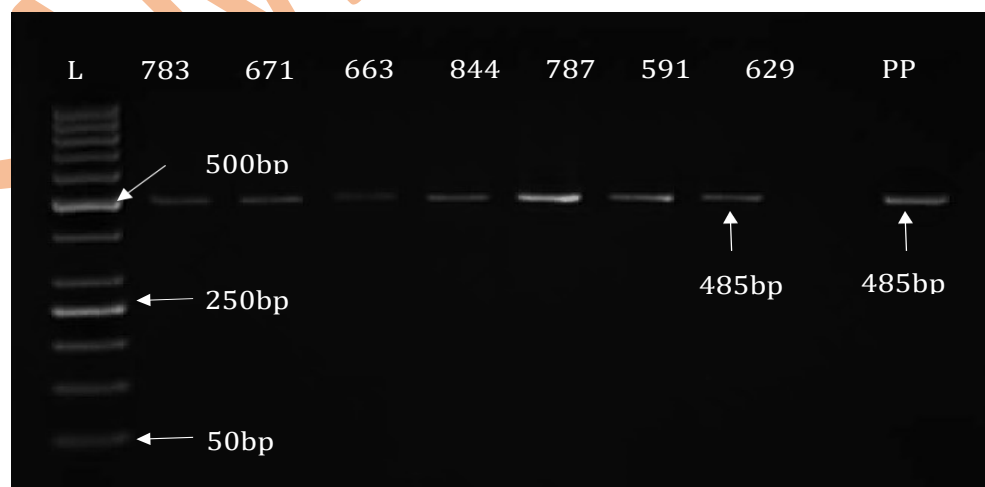


Fig.7: Representative agarose gel electrophoresis image of PCR-RFLP for OCX32 (T21912084G) SNP marker validation.

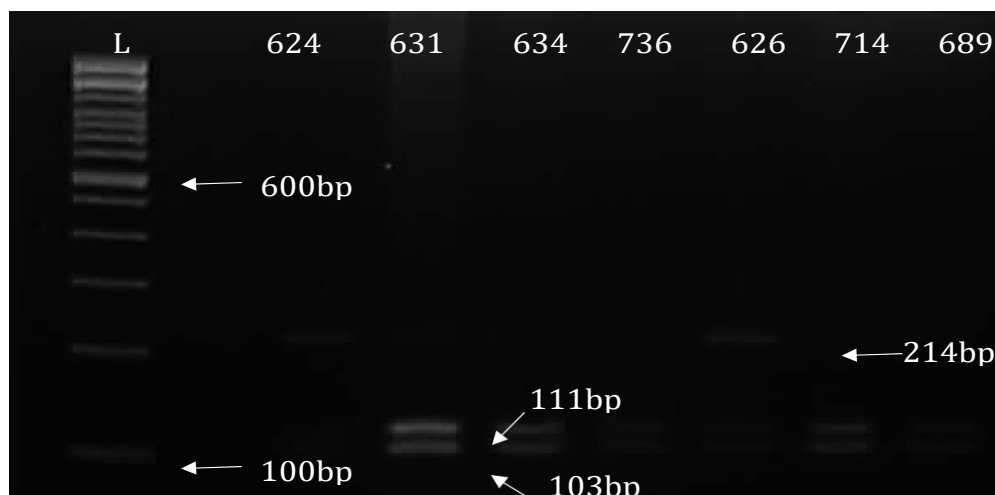


Fig. 8: Representative agarose gel electrophoresis image of PCR-RFLP for OCX32 (C21912423A) SNP marker validation.

CONCLUSION

Based on the variable melting temperature in melt curve analysis in Real-Time PCR, the number of samples to be screened for SNP through PCR-RFLP could be successfully and drastically reduced. This approach saves time, labor resources and is cheaper and faster as compared to PCR-RFLP. Out of 8 SNPs present in 4 genes (GnRHII, VIPR1, OCX32 and GHR), 3 SNPs were found to be associated with egg production and egg weight in ASWLH and ABWLH. Hence, these SNPs could be used as a marker for the selection of these traits in chickens.

ACKNOWLEDGEMENTS

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Anand for providing necessary instrumentations and consumables to carry out the study.

Conflict of Interest: None

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