Validation of SNPs Identified by Amplicon Sequencing and 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.

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

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Introduction

Poultry is one of the fastest-growing segments contributing an important role 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 meet the 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

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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 Tm, 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 µL which consisted of 0.5 µL template DNA, 1 µL forward primer, 1 µL reverse primer, 5 µL 2x QuantiNova SYBR Green qPCR master mix (Cat# 208056), 0.5 µL QN ROX reference dye and 2 µL 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 µL system and the PCR amplification was conducted in an automated thermal cycler as per the protocol of particular SNPs. At first 12.5 µL PCR master mix (Cat# RR310B), 1 µL forward primer, 1 µL reverse primer, and 9.5 µL nuclease-free water, 1 µL of genomic DNA were taken in 200 µL 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 µL reaction mixture consisted of 10 µL PCR product, 3 µL 10X RE buffer, 1 µL RE enzyme (10 units/ µL), 16 µL nuclease free water. The whole reaction was conducted at 37°C for 1 h in a thermal cycler with the restriction digestion mix.

RESULTS AND DISCUSSION

Real-Time PCR is rapidly becoming a standard method in many diagnostic and research laboratories. The melt curve analysis shows different melting temperature of a particular sequence and identify alleles based on amplicon length and was used to distinguish them, so in this study based on Tm samples were divided into different categories, by performing Real-time PCR samples size was reduced from 500 to 96 samples. The PCR was carried out in a 200 µL 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 292 bp, 298 bp, 299 bp, 367 bp, 497 bp, 485 bp, 214 bp and 245 bp, respectively.

In PCR-RFLP different SNP marker shows different band pattern on agarose, *i.e* (i) T88170424C/AhdI resulted two fragment pattern 292 bp and 129 bp, 163 bp, 292 bp those referred as TT and TC genotype (Fig. 1) (ii) G88170427A/HinP1I resulted into three pattern 298 bp/GG, (78 bp, 220 bp/GA), (78 bp, 220 bp, 298 bp/AA) (Fig. 2) (iii) C1826542A/NlaIV polymorphism showing single digestion pattern 5

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' AGGGATCCTTCCCTCTCTTG3'	299 bp
VIPR1	C1826555T	F:5'TGCTGCAGTGTTCTCTCTCC3' R:5' AGGGATCCTTCCCTCTCTTG3'	367 bp
OCX32	C21912308T	F:5'GATAACCATGGGCTCGAAGA3' R:5'CCAACTTCCACTGTGTATAATGGT3'	497bp
OCX32	T21912084G	F:5'GCTCCACGAAATTCCAACAC3' R:5'TGCTCACCTGTGTGTGTATGC3'	485bp
OCX32	C21912423A	F:5'CACAGGTGAGCATACACAGG3' 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

bp, 62 bp, 94 bp, 138 bp that referred as AA genotype (Fig. 3) (iv) C1826555T/BfaI shows absence of SNPs (Fig. 4) (v) C13415980T/EarI resulted in two digestion pattern 245 bp/CC and 88 bp, 157 bp, 245 bp/CT (Fig. 5) (vi) C21912308T/AleI revealed that 211 bp, 286 bp/TT and 211 bp, 286 bp, 497 bp/CT (Fig. 6) (vii) T21912084G/ HpyF10VI not shows any SNPs (Fig. 7) (viii) C21912423A/ Tsp45I resulted two digestion pattern 111 bp, 103 bp/AA and 111 bp, 103 bp, 214 bp/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 the other 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.
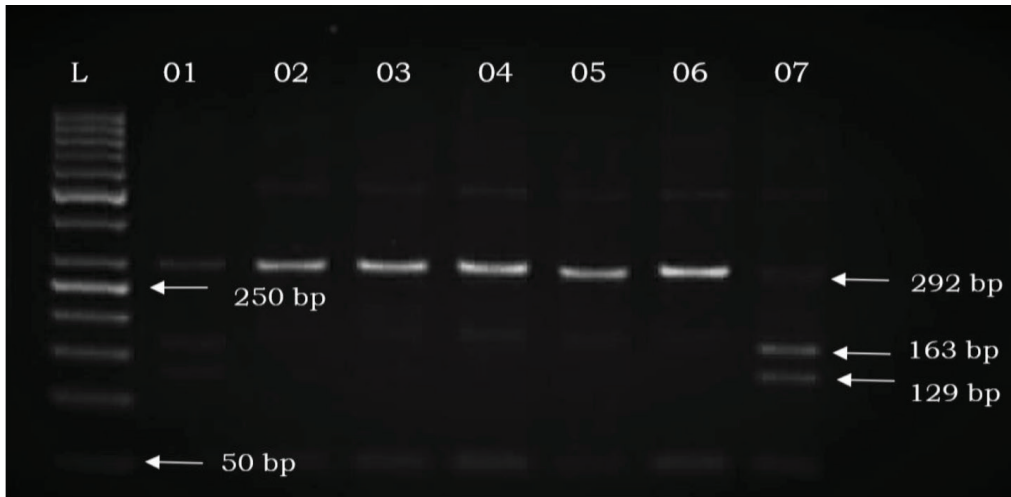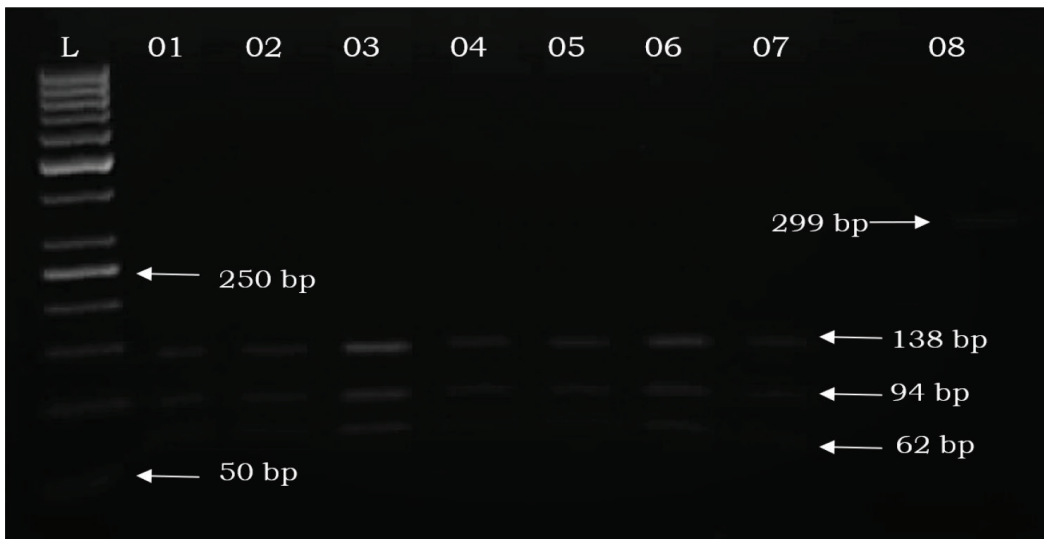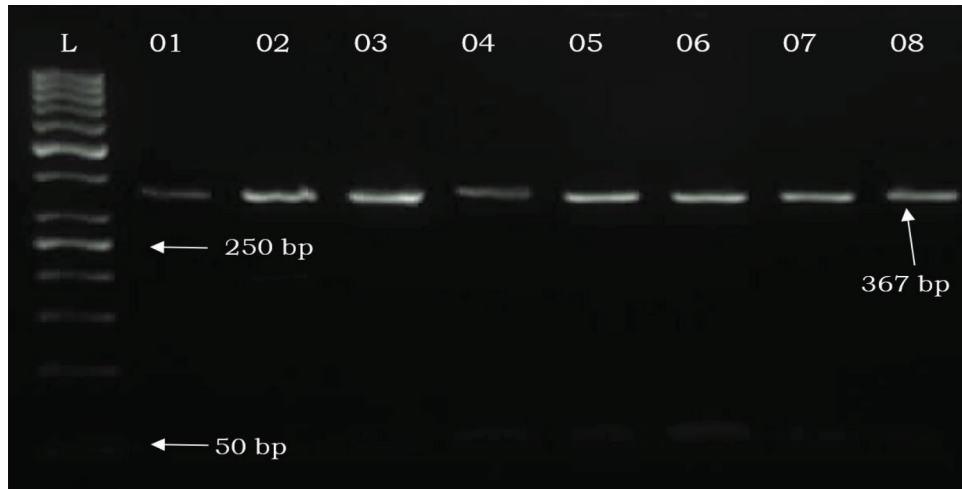
Fig. 1: Representative agarose gel electrophoresis image for PCR-RFLP of GnRHII (T88170424C) SNP marker
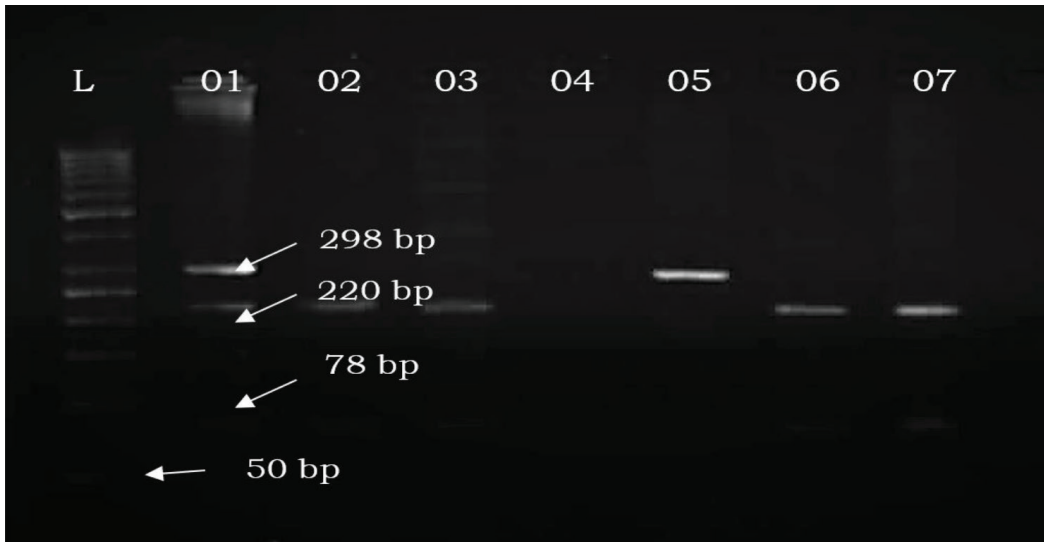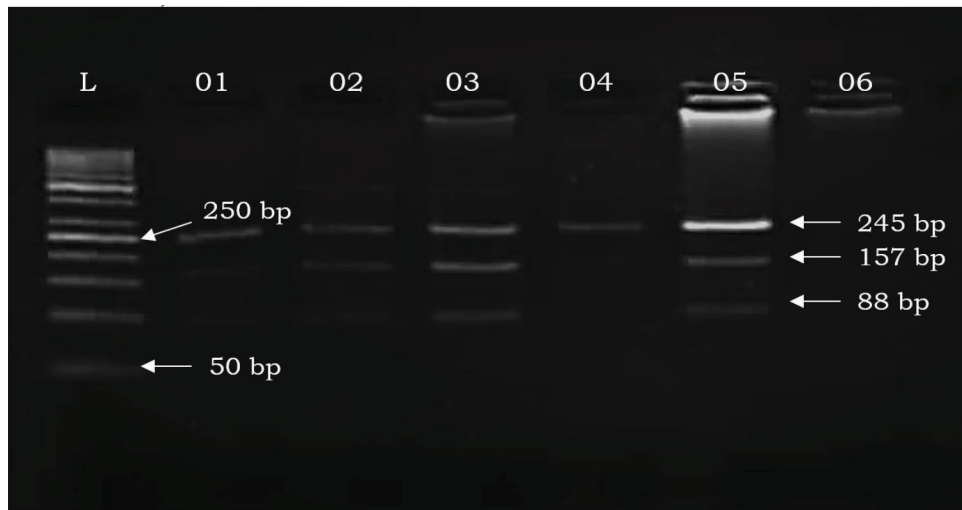

Fig. 2: Representative agarose gel electrophoresis image for PCR-RFLP of GnRHII (G88170427A) SNP marker

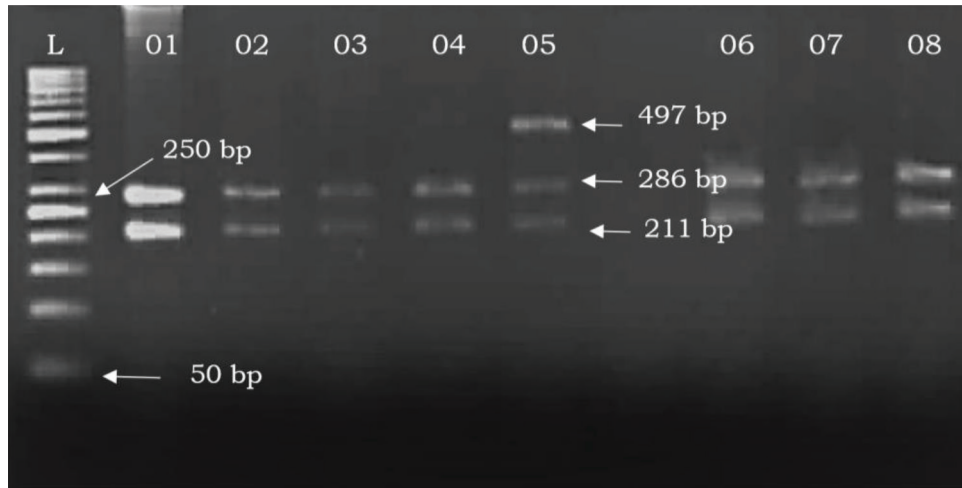

Fig. 3: Representative agarose gel electrophoresis image of PCR-RFLP forVIPR1 (G88170542A) SNP marker validation.

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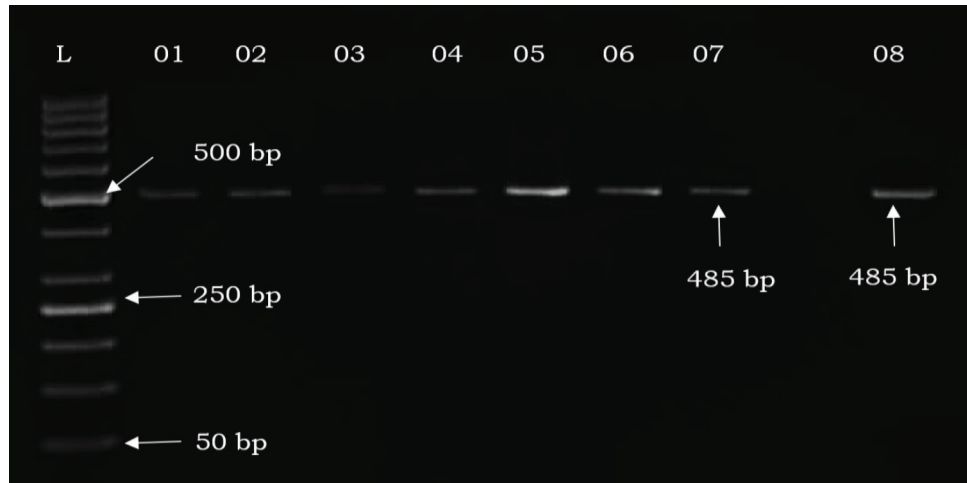

Fig. 5: Representative agarose gel electrophoresis image of PCR-RFLP for GHR (C13415980T) SNP marker validation.
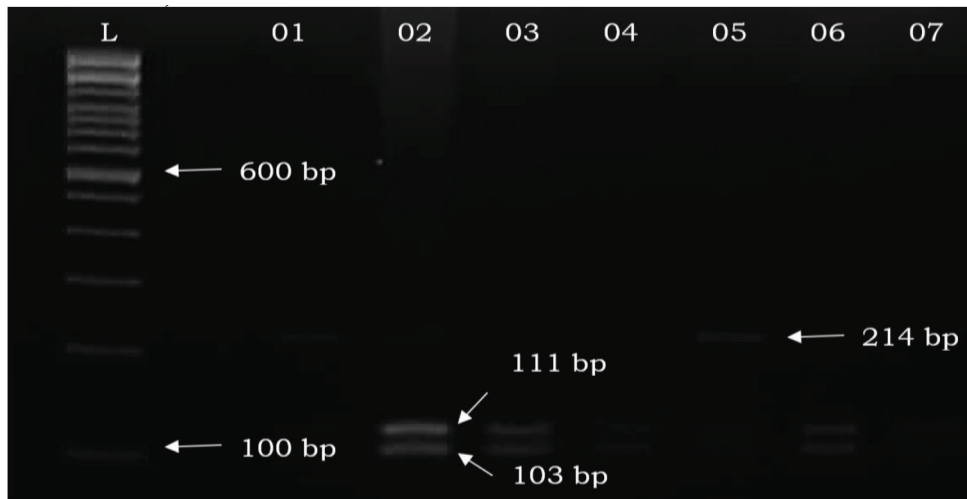

Fig. 6: Representative agarose gel electrophoresis image of PCR-RFLP for OCX32 (C21912308T) SNP marker validation.

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.

CONCLUSIONS

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.

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