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PCR-RFLP Analysis of Single Nucleotide Polymorphism (SNP) C-2402T at the Promoter Region of *Prolactin* Gene and its Association with Positively Correlated Production Traits in White Leghorn Chicken

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ABSTRACT

Background: The avian *prolactin* gene is highly conserved, located on chromosome number 2 and most sequence polymorphisms occurs in the 5' flanking region, 3' flanking region, and the coding region of signal peptide. The present study was aimed at the identification of SNP C-2402T of *prolactin* gene and its association with production traits in White Leghorn chicken.

Methods: A total of 200 birds of White Leghorn were selected from All India Co-ordinated Research Project on Poultry improvement (AICRP) farm, Mannuthy. Genomic DNA was isolated from venous blood. Polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis was done to identify the SNP C-2402T of *prolactin* gene.

Result: All the birds were observed with the same genotype CC and the frequency of the C allele was one.

Key words: Genotype, PCR, Prolactin, RFLP, SNP, White Leghorn.

INTRODUCTION

In Indian agriculture, the poultry industry is one of the fastest-growing sectors among all livestock sectors. At present, the total poultry population in our country is about 851.81 million numbers as per the 20th livestock census (Government of India, 2020) and the egg production of around 103.318 billion numbers (BAHS, 2019). Persistent efforts and collaborative research on poultry breeding have been carried out for the evolution of high producing layer stock which is suitable for various environment and management practices (Tomar *et al*., 2015). White Leghorn is a non-broody bird and produces more than 300 eggs per year. On one hand, the non-broody behaviour of White Leghorn is due to the presence of a major autosomal recessive gene on the Z chromosome (Romanov, 2001). On another hand, a 24bp insertion polymorphism at the promoter region of the *prolactin* gene suppresses the expression of the *prolactin* gene, and broodiness is prevented (Jiang *et al.*, 2005). Also, White Leghorn is used as a major line in the cross-breeding programmes to develop different layer strains for high egg production in the commercial poultry industry. Poultry breeding is done mainly to increase the level of egg-laying capacity, egg quality and meat quality (Kulibaba *et al*. 2020). Over the past few years, newer DNA technologies, marker-assisted selection and genomic selection have been efficiently employed in poultry selection and breeding (Salisu *et al*., 2018). The foremost objective of commercial poultry breeding is to achieve genetic improvement in age at sexual maturity, egg weight, egg number and body weight. Restriction fragment length polymorphism (RFLP) enables to detection of the large insertion and deletion within the genome sequence and to identify of the single base changes called single nucleotide polymorphism (SNP) (Fulton, 2012). The chicken *prolactin* gene plays a significant role in egg production (Wang *et al*., 2011). Since the avian *prolactin* gene was cloned and sequenced, most of the research has focused on detecting new polymorphic sites in this gene. The results of several studies had shown a significant association of SNPs of *prolactin* gene with economically important traits in different species of poultry (Wilkanowska *et al*., 2014). Specifically, a significant association was found between the SNP of C-2402T genotypes with egg number and laying rate in Fars native chicken (Bagheri Sarvestani *et al*., 2013). Thus, this study aimed to study was to identify the SNP C-2402T at the promoter region of the *prolactin* gene using the PCR- RFLP technique and find out the

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association of this SNP with production traits in White Leghorn chicken.

MATERIALS AND METHODS

Experimental birds

A total of 200 birds of White Leghorn were randomly selected during September 2016 to October 2017 from the All India Research Co-ordinated Project (AICRP) on poultry improvement, Mannuthy, Thrissur, Kerala, India.

Workplace

The present study was carried out at Avian Biotechnology Laboratory, in the Department of Poultry Science, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala.

Collection of blood samples

Approx. 0.5-1 ml of blood was collected from the wing vein under aseptic conditions. The samples were brought to the laboratory at 4ºC in an ice pack.

Isolation of genomic DNA

Isolation of Genomic DNA was done from the whole blood using ODP304 Origin Genomic DNA isolation kit according to the standard procedure given by manufacturers. The yield and quality of the DNA obtained were checked by Nano-drop spectrophotometer and by 0.8% agarose gel electrophoresis, respectively. The DNA samples showing the OD260/OD280 ratio between 1.7 and 1.9 were used for further investigation.

PCR assay

The polymerase chain reaction was carried out using a specific set of forward (F-5' AGAGGCAGCCCAGGCAT TTTAC3') and reverse primer (R-5'CCTGGGTCTGGTTTGG AAATTG3') to amplify the 439bp fragment of *prolactin* gene containing SNP C-2402T at the promoter region (Cui *et al*., 2006; Bagheri Sarvestani *et al*., 2013). Each diluted primer (10 pM/µl) was added to the template DNA (100 ng/µl) and 2X PCR Smart Mix (origin) in a PCR tube and made up to the final volume of 20 µl using ultra-filtered Millipore water. PCR was done in Bio-Rad thermal cycler and standardization was done with the following program: initial denaturation of 5 min at 94°C; 35 cycles of 94°C for 30 s, annealing at 67.7°C for 30 s, and 72°C for 30 s with a final elongation of 5 min at 72°C. PCR amplicons were checked on 2% agarose gel.

Agarose gel electrophoresis

The genomic DNA and PCR products were checked in agarose gels of 0.8 per cent and 2%, respectively prepared using 1X TBE buffer. The PCR products were loaded along with a molecular weight marker (50bp ladder) for relative sizing. Electrophoresis was carried out at 5V/cm and the gel was photographed in a Gel Doc System (Bio-Rad, USA).

Restriction fragment length polymorphism (RFLP) analysis

For restriction digestion five microlitres of the amplified PCR product (439bp) of promoter C-2402T was added with 5 units of *AluI* enzyme and incubated at 37°C for 1 hr. The composition of the reaction mixture was made with the final volume of 12µl (PCR product-5µl, 10X buffer -1.2µl, *AluI* (10U/µl) -0.5 µl and Distilled water -5.3µl). After restriction digestion, the digested PCR products were separated by electrophoresis in 3% agarose gel in 1X buffer with 50bp DNA size marker. The restriction pattern was visualized under a UV trans-illuminator and documented in a gel documentation system.

The amplicons of the promoter region of *prolactin* gene 439bp and the digested PCR products were sequenced using respective forward and reverse primers in an automated sequencer using Sanger's dideoxy chain termination method at Agri Genome Labs Pvt. Ltd., Cochin, Kerala. Based on the results of RFLP analysis, all the birds were designated with the same genotype and allele frequency was calculated, accordingly. Bodyweight at 16th, 40th and 64th weeks of age, and egg weight at 28th, 40th and 64th weeks of age, respectively were recorded.

Statistical analysis

The association of SNP site C-2402T of *prolactin* with body weight and egg weight were analyzed by one-way ANOVA by using the software SPSS (Version 21.0).

RESULTS AND DISCUSSION

The quality of extracted DNA was checked on 0.8% agarose gel (Fig 1) PCR amplification of 439bp amplicon of *prolactin* gene is represented on 2% agarose gel (Fig 2). On restriction analysis, 439bp PCR products of *prolactin* with *AluI* restriction enzyme revealed the presence of only one genotype CC with different sizes of restriction fragments (160, 144, 81 and 54bp) on 3% agarose gel (Fig 3). The same RFLP pattern was observed in all 200 digested products of PCR samples. Simultaneously, this *AluI* restriction enzyme has been widely used in most of the studies to reveal the restriction sites for the SNP position C-2402T in the chicken *prolactin* gene (Cui *et al*., 2006; Kulibaba *et al*., 2012; Bagheri Sarvestani *et al*., 2013; Kulibaba, 2015). Also, we applied the NEBcutter V2.0 tool to identify the restriction sites within our DNA sequence and found *AluI* restriction enzyme was suitable for the SNP site C-2402T of the *prolactin* gene. Therefore, we have planned to proceed with our research work further but we have not gone through any pilot study for the present work using the *AluI* restriction enzyme. According to the restriction digestion results, it had been confirmed that all the 200 birds of White Leghorn chicken were monomorphic with the same genotype CC for the SNP site C-2402T of *prolactin* gene. Thus, no polymorphism were found in this White Leghorn chicken population which had undergone 29 generations of continuous selection. In contrary to our findings, the *AluI* restriction enzyme produced different sizes of fragments with three genotypes *viz*. CC (four fragments- 160, 144, 81 and 54bp), CT (five fragments- 304, 160, 144, 81 and 54bp) and TT (three fragments - 304, 81 and 54bp), respectively for

the SNP site C-2402T of *prolactin* gene in Fars native chicken of Iran (Bagheri Sarvestani *et al*., 2013). Similarly, all three genotypes CC, CT and TT were observed in five different chicken populations (Yangshan, Taihe Silkies 1, White Rock, Nongdahe and Taihe Silkies 2) with six SNPs (Cui *et al*., 2006). Also, Chau, (2016) reported similar findings in the Noi native chicken of Vietnam. In comparison, Kulibaba *et al*. (2015) conducted research on polymorphism of *prolactin* gene in connection with egg production in Poltava clay chicken of Ukraine and found SNP at the position of C-2402T with all three possible genotypes in this population. Allele C had three restriction sites for *AluI* (two monomorphic and one polymorphic) and allele T had two restriction sites. Similar polymorphism (C-2402T) was reported by Kulibaba *et al*. (2012) in chicken lines of Ukrainian selection.

Our findings revealed that all 200 birds of White Leghorn chicken carry CC genotype. As a result, all White Leghorn

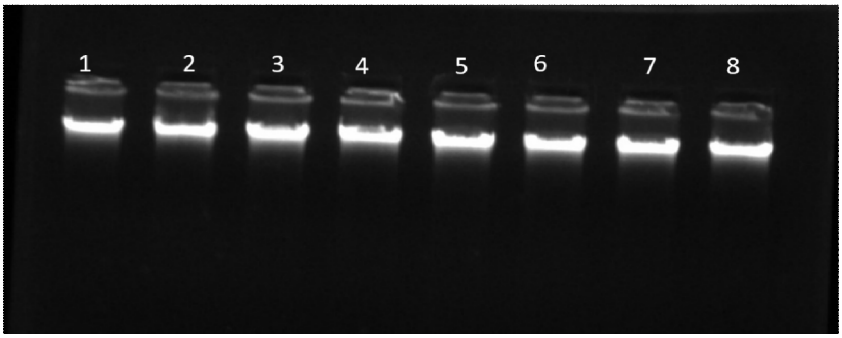

Fig 1: Testing the quality of extracted Genomic DNA on 0.8% agarose gel
Lane 1-8: Genomic DNA isolated from venous blood.

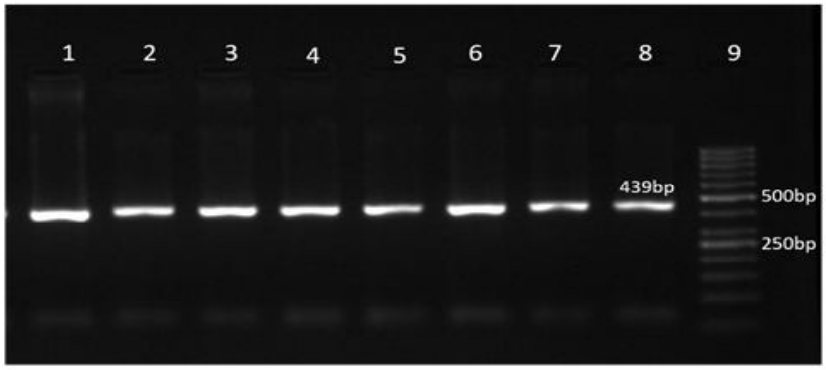

Fig 2: PCR amplification of 439bp fragment of *prolactin* gene on 2% agarose gel
Lane 1- 8 : 439bp PCR product.
Lane 9: 50bp ladder.

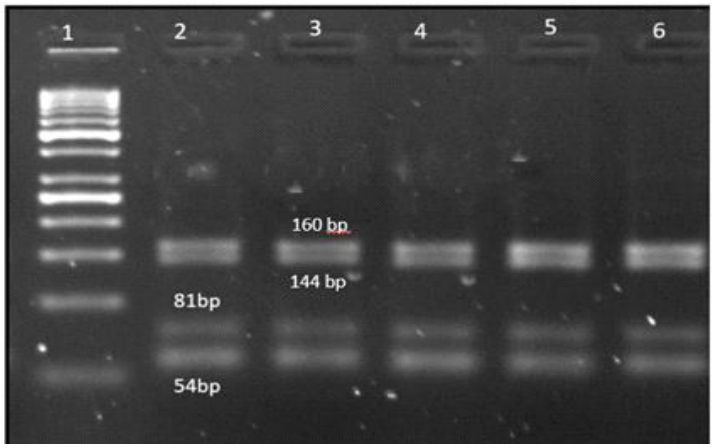

Fig 3: RFLP analysis of 439bp fragment of *prolactin* gene digested with *AluI* on 3% agarose gel.
Lane 1 : 50bp Ladder.
Lane 2-6 : 160, 144, 81 and 54bp restriction fragments of CC genotype.

chicken selected from the AICRP farm are monomorphic for the allele C and the allele frequency of C was 1.0 in this population. Similar findings were reported by Cui *et al*. (2006) in the White Leghorn chicken population for the SNP site C-2402T. In contrary to these findings, Bagheri Sarvestani *et al*. (2013) reported the allele frequency as 0.66 (C) and 0.34 (T) in Fars native chicken of Iran. In comparison, Chau. (2016) found the allele frequency as C (0.17) and T (0.83) in the Noi native chicken of Vietnam. Furthermore, Kulibaba *et al.* (2015) reported the allele frequency as C (0.372) and T (0.628) in the Poltava clay chicken population of Ukraine. In addition, Kulibaba *et al*. (2012) found the allelic frequency as C (0.745) and T (0.255) for the chicken line A in Ukrainian selection. According to these research findings, it has been observed that frequency of the C allele varies in different breeds of the chicken population. Allele frequency is a measure of the relative frequency of an allele on a genetic locus in a population and usually, it is expressed as a proportion or a percentage. Hence, the variation in allele frequency is possibly due to gene flow, genetic drift, natural selection, and mutation. These are referred to as the four fundamental forces of evolution. Besides, the mutation can create new genetic variation and the other three forces simply rearrange this variation within and among populations.

Association of SNP C-2402T at the promoter of *prolactin* gene with body weight and egg weight in White Leghorn chicken

Bodyweight at 16th, 40th and 64th weeks of age and egg weight at 28th, 40th and 64th weeks of age, respectively were recorded for all 200 birds of White Leghorn which were randomly selected from AICRP on poultry improvement farm, Mannuthy, Thrissur. By the acquired statistical results, the mean body weight at 16th, 40th and 64th weeks of age were 1036.75±2.62, 1561.00 ±6.07 and 1544.00±6.31 g and mean egg weight at 28th, 40th and 64th weeks of age were 48.00±0.01, 52.16±0.10 and 54.16±0.37 g, accordingly for the genotype CC of SNP site C-2402T. Since only CC genotype was found in this White Leghorn chicken population, association study with positively correlated traits was not possible.

CONCLUSION

Our research findings revealed the presence of only one genotype CC for the SNP site C-2402T of *prolactin* gene in all the 200 birds of White Leghorn chicken using PCR- RFLP analysis. It indicates that the *prolactin* gene is monomorphic for this chicken population and the frequency of the C allele was one. Hence, further research could be done in other breeds of the chicken population to confirm the association of SNP C-2402T of *prolactin* gene with production traits.

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