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Genotypic confirmation of non-broody behaviour of white leghorn by 24bp indel(s) polymorphism at the promoter region of prolactin gene

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Abstract
Broodiness is a sex-linked observable trait in most of the domestic hens but in case of White Leghorn breed this trait was found to be seldom. Prolactin hormone plays a major role in broodiness or incubation behaviour in birds. The main aim of the present study was genotypic confirmation of non-broody behaviour of White Leghorn by identifying a 24bp insertion-deletion (indel)s at the promoter region of *prolactin* gene. A total of 200 birds of White Leghorn were randomly selected from All India Co-ordinated Research Project (AICRP) on Poultry Improvement, Mannuthy, Thrissur, Kerala. Blood samples were collected from the selected birds and isolation of genomic DNA was done. Polymerase chain reaction (PCR) assay was carried out to identify a 24bp indel(s). Based on the polymorphic patterns, birds were categorized to three different genotype groups namely, II, ID and DD. Association of non-broody behaviour and egg production with 24bp insertion polymorphism was analyzed in White Leghorn. Our findings support the view that loss of maternal incubation behaviour in the White Leghorn breed is the result of 24bp insertion polymorphism and intensive selection which improves the egg laying persistency.

Keywords: Broodiness, prolactin, white leghorn, PCR, polymorphism, genotype

Introduction
Broodiness is a normal physiological (or) natural behaviour of hens that allows them to incubate and hatch out the chicks from eggs. Broodiness is also known as incubation and it's a feature of maternal behavior with complex trait. Brooding is defined as act of sitting on a nest of eggs to incubate them and hatch a clutch of eggs (Jiang *et al.*, 2010) [6]. Prolactin is a polypeptide hormone secreted from an anterior pituitary gland which is the major responsible for broodiness (Cui *et al*., 2006) [3]. The secretion of this prolactin is controlled by vasoactive intestinal peptide (VIP) hormone. VIP is released from hypothalamus and it has high affinity to lactotroph cells in the anterior pituitary. On domestication of chickens, broody behaviour has been lost in some breeds of chicken especially the White Leghorn (WLH) breed while other breeds of poultry have retained this behaviour (Basheer *et al*., 2015) [1]. Broodiness is commonly observed where the birds are reared under deep litter system rather than the cage system. Genetically, non-broody behavior of White Leghorn due to the major autosomal recessive gene which is located on 'Z' chromosome (M.N. Ramanov, 2001) [13]. The WLH breed is categorized under the Mediterranean class of chicken breeds. It has higher egg production rate and known for the best layer breed in the world. In this breed, incubation behavior is practically absent, that is because of the intensive artificial selection commercially practiced in WLH. It has been reported that a 24bp insertion polymorphism at the promoter region of *prolactin* gene in WLH is associated with lower expression of *prolactin* gene and decreased broodiness (Jiang *et al*., 2005) [5]. The present study was aimed at identification 24bp insertion-deletion (indel) polymorphism in the promoter region of *prolactin* gene and its association with non-broody behavior of White Leghorn.

Materials and Methods

Experimental birds

A total of 200 birds of White Leghorn (WLH) which had undergone 28 generations of continuous selection were randomly selected from All India Research Co-ordinated Project (AICRP) farm on poultry improvement, Mannuthy, Thrissur, Kerala.

Collection of blood samples

From each bird, 0.5-1 ml of blood was collected from the wing vein using 2.5 ml disposable syringe in a EDTA vial under aseptic condition. The samples were brought to the laboratory at 4ºC in ice pack.

Isolation of Genomic DNA

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

PCR assay

Polymerase chain reaction was carried out using specific set of forward (F-5' TTTAATATTGGTGGGTGAAGAGACA3') and reverse primer (R-5 ATGCCACTGATCCTCGAAAACTC3') to amplify the 130/154bp fragment of *prolactin* gene containing 24bp indel(s) polymorphism at the promoter region. Each diluted primer (10 pM/µl) was added to the template DNA [working solutions prepared from stock solution by diluting with sterile distilled water (Millipore) to get a final concentration of 100 ng/µL] and 2X PCR Smart Mix (origin) in a PCR tube and made upto the final volume of 20 µL using ultra filtered Millipore water. PCR was done in Bio-Rad thermal cycler and standardization was done for each reaction by mild adjustment of concentration of ingredients and annealing temperature with the following profile: initial denaturation of 5 min at 94 °C; 35 cycles of 94 °C for 30 s, annealing at 58 °C for 30 s, and 72 °C for 30 s with a final elongation of 5 min at 72 °C. PCR amplicon was subjected to 2% agarose gel.

Agarose gel electrophoresis

The genomic DNA and PCR products were checked in agarose gels of 0.8 percent and 2%, respectively prepared using 1X TBE buffer. Agarose was weighed and mixed with required volume of 1XTBE buffer and boiled in a microwave oven till the solution became clear. After cooling to 60 °C (bearable warmth), ethidium bromide (0.5 µg/ml) was added and mixed well. It was then poured into casting tray which was already set with comb in place. After solidification, the comb and sealing were removed and the tray was immersed in a buffer tank containing 1X TBE buffer. DNA and PCR products were mixed with loading dye (6X) and loaded in wells. The PCR products were loaded along with a molecular weight marker (50bp) for relative sizing. Electrophoresis was carried out at 5V/cm until the bromophenol blue dye migrated more than two by third length of the gel and was photographed in a Gel Doc System (Bio-Rad, USA). The amplicons of promoter region of *prolactin* gene 130/154bp were sequenced using respective forward and reverse primers in an automated sequencer using Sanger's dideoxy chain termination method at AgriGenome Labs Pvt. Ltd., Cochin. Allelic and genotypic frequency was calculated based on the polymorphic patterns. The egg production of each bird was recorded for further analysis

Results and Discussion

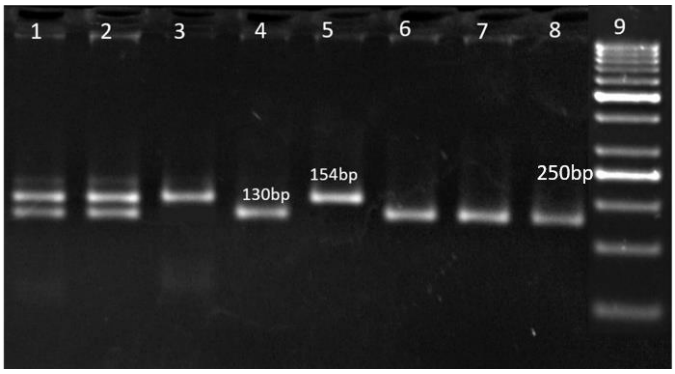

Fig 1: PCR amplification of 130/154bp fragment (24bp indel) of promoter region of *prolactin* gene on 2% agarose gel Lane 1, 2: 130 and 154bp fragments of ID genotype Lane 3, 5: 154bp fragments of IIgenotype Lane 4, 6, 7, 8: 130bp fragments of DD genotype (Native chicken as control) Lane 9 : 50bp ladder

Polymorphism at the promoter region of *prolactin* gene was observed with three different size of fragments namely 154, 130/154 and 130bp representing the II, ID and DD genotypes, respectively. According to the polymorphic patterns, the number of birds were grouped in to different genotypes II, ID and DD, respectively.

Genotype and allele frequency

The allelic frequency of I and D of promoter region of *prolactin* gene (24bp indel) was 0.9825 and 0.0175, respectively in White Leghorn. The genotypic frequency of II, ID and DD were 0.965, 0.035 and 0.000, respectively in White Leghorn. Jiang *et al*. (2005) [5] and Cui *et al*. (2006) [3] found the similar values as 1.000, 0.000 and 0.000, respectively in White Leghorn. Liang *et al*. (2006) [10] also found similar genotypic frequencies as 0.950, 0.050 and 0.000, respectively in White Leghorn. As there was insignificant number of polymorphisms in promoter region of *prolactin* gene of White Leghorn, the frequency of D allele was found to be very less in this population. This results were accordance with that of Jiang *et al*. (2005) [5], Cui *et al*. (2006) [3] and Liang *et al*. (2006) [10].

Association of 24bp insertion-deletion (indel) polymorphism with non-broody trait of White Leghorn

Out of 200 birds of White Leghorn 193 birds observed with II genotype, 7 birds were observed with ID genotype and none of the birds were produced DD genotype. Genotype insertion-insertion (II) and insertion-deletion (ID) is associated with non-broody behavior of White Leghorn. Genotypically a 24bp insertion at the promoter region of *prolactin* gene is the major responsible for non-broody behaviour of White Leghorn. Since, the insertion of a 24bp in the promoter region of *prolactin* gene, a possible ecotropic viral integration site-1 encoded factor (Evi-1) binding site is located in the 5′ flanking region of the chicken *prolactin* gene (Cui *et al*., 2005) [2]. Evi-1 was shown to be involved as a repressor in transcription of many genes (Vinatzer *et al*., 2001 and Izutsu *et al*., 2002) [14, 4]. It has been reported that Evi-1 represses the expression of *prolactin* gene in White Leghorn chickens by binding the Evi-1 binding site and further prevents broodiness

(Jiang *et al*., 2005) [5], that can improve egg production to some extent. Hence, the White Leghorn chicken is producing more than 300 eggs per year. In the present study also, all the 200 birds were observed with higher egg production (an average of 306 eggs/bird/year).

Conclusion

The present study revealed the presence of 24bp nucleotide sequence insertion at the promoter region of *prolactin* gene. Broodiness is seldom because of this 24bp insertion polymorphism which suppress the *prolactin* gene expression in White Leghorn and the egg production is increased subsequently. In our study, the obtained results confirmed that White Leghorn is a non-broody bird with higher egg production. Hence, this 24bp insertion polymorphism could be considered as a molecular marker to assess the non-broodiness and production potential in other breeds of chicken.

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