Full Length Article

# POLYMORPHISMS IN THE PROMOTER REGION OF THE *ACACB GENE* AND THEIR ASSOCIATION WITH EGG PRODUCTION TRAITS IN WHITE LEGHORN LAYER CHICKENS

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## ABSTRACT

*The present study was carried out to identify the polymorphism in the promoter of ACACB gene vis-à-vis effect of polymorphism on egg production traits in IWI and IWK lines of White Leghorn layer chicken. A total of 500 birds comprising of 250 IWI line and 250 IWK line of White Leghorn chicken included in this study.. Results showed that, the promoter region was polymorphic. With respect to egg production in all age groups showed higher egg production in IWI line, when compared to the IWK line. A total of twelve haplotypes and eighteen haplogroups were formed. The haplogroup h12h12 showed the highest egg production (193.20±7.56) and the h1h2 haplogroup showed the lowest egg production (126.80±3.54) up to 52 weeks of age. In all age groups h12h12 haplogroup showed the highest egg production in IWI line. It is concluded that the promotor of the ACACB gene was highly polymorphic and had significant effect on egg production traits in White Leghorn layer chicken.*

**Keywords:** ACACB gene, promoter, SSCP, polymorphism, egg production

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## INTRODUCTION

The poultry sector has been one of the most promising industries among the different livestock sectors globally. The chicken egg and meat are relatively cheaper and affordable sources of protein for humans. The traditional selection within populations has not been very encouraging when the selection objective involved several characteristics with unfavorable genetic correlations. The inclusion of markers could increase the genetic response to selection for growth rate in chicken populations up to 15% compared with selection based on family selection (Pongpisantham, 1994). The molecular markers possess unique genetic properties and methodological advantages that make them more useful and

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amenable for genetic analysis compared to other genetic markers. Several molecular markers such as RFLP, Microsatellites, single stranded conformation polymorphism (SSCP) etc. were used in past for the analysis of complex genetic traits (Badola *et al.* 2004; Bhattacharya *et al*., 2011; Bhattacharya *et al*. 2015b; Bhattacharya *et al*., 2016). The Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) is a reproducible, rapid and quite simple method for the detection of deletions/ insertions/substitutions in the gene (Kakavas *et al.*, 2008), developed by Orita *et al*. (1989) is widely used for DNA mutation detection. SSCP detects single-base sequence changes by showing difference in electrophoretic migration of one or both single strands in a non-denaturing polyacrylamide gel (Gruszczynska *et al*., 2005). Early reports used radioactivity, which limited widespread use of the technique. To simplify and make SSCP analysis more efficient, alternative staining methods with ethidium bromide (Ya and McGee, 1992) and with silver stain (Ainsworth *et al*., 1991) were described. In chicken meat, high-fat content is a risk to human health (Milićević *et al*., 2014). Excessive fat accumulation in laying hens also negatively affects their reproductive performance (Xing *et al*., 2009). Genetic regulation of egg production and quality particularly, with respect to fat metabolism and quality of fat is established based on genome wide association studies in chicken. The fatty acid breakdown is mainly accomplished via the mitochondrial β-oxidation pathway. The Acetyl-CoA Carboxylase Beta (ACACB or ACC-β or ACC2) gene plays a key role in β-oxidation pathways. Since the chicken ACACB gene is involved in fatty acid metabolism, it can potentially regulate the quality of the egg. The ACACB generated malonyl-CoA that functions as an inhibitor of the carnitine/ palmitoyl-transferase 1 (CPT1) activity and the transfer of the fatty acyl group through the carnitine/palmitoyl shuttle system to inside the mitochondria for β-oxidation (McGarry and Brown, 1977; Ruderman *et al*., 1999; Wakil and Abu-Elheiga, 2009). The ultimate result will be utilization of more glucose, decreased fatty acid oxidation and elevated fatty acid and triglyceride (TG) synthesis. Understanding genetic variation in chicken ACACB would help in identification of potential variants that are associated with production. Further, understanding such variation in the regulatory region of the gene would help in future directions of breeding for chicken with improved egg quality. Therefore, the present study was conducted to identify the polymorphism in the promoter of ACACB gene vis-a-vis effect of polymorphism on egg production traits in IWI and IWK lines of White Leghorn layer chicken.

## MATERIALS AND METHODS

### Experimental birds

The present study was conducted on 500 birds, belonging to IWI (250 birds) and IWK (250 birds) lines of the White Leghorn breed. The IWI has been undergoing index selection for higher egg production up to 64 weeks of age during last six generations. IWK line is being subjected to selection for higher egg production up to 64 weeks of age. The IWI and IWK lines are improved

egg producing lines laying 298 and 276eggs annually (DPR Annual Report 2018-19). Both the lines are maintained at the ICAR-Directorate of Poultry Research (ICAR-DPR), Hyderabad, India.

**Management**

Day old Chicks of both the lines were vent sexed, wing banded and reared in open-sided house on deep litter up to 16 weeks of age. They were provided with layer chick starter ration (2,600 kcal/kg metabolizable energy (ME) and 18% crude protein (CP)) up to 8 weeks of age and grower ration (2,500 kcal/kg ME and 16% CP) from 9 to 16 weeks of age. After 16 weeks of age, birds were shifted to individual cages so as to record individual production and the birds were fed with layer ration (2,600 kcal/ kg ME and 16% CP) from the onset of egg production till the completion of the experiment. Light for 16 h (including natural day light) was provided during laying period. All the birds were reared on same management with ad-lib watering. On the day of hatch chicks were vaccinated against Marek's disease and subsequently birds were protected against important diseases like Ranikhet Disease, Infectious Bursal Disease and fowl pox using standard vaccination program. Cooling facilities were provided during summer season through water sprinkling on the roof and proper lighting was arranged in the shed, all other management practices including bio-security measures were carried out in and around the sheds so that birds get congenial environment for performing in optimum potential.

**Sample collection**

Blood samples were collected from the wing vein and genomic DNA was isolated from blood samples following the standard protocol (Sambrook and Russell 2001). Eggs were collected daily for estimation of egg production up to 40th week, up to 52nd week and up to 72nd week.

**PCR**

The 5' upstream region of transcription start site of ACACB gene comprising 534 bp length divided into two non-overlapping fragments namely, ACACB-S1 (301 bp) and ACACB-S2 (233 bp). Primers were designed from the chicken ACACB gene sequence (Accession No. NC_006102) for amplification of the fragments (Table 1). The amplification by polymerase chain reaction (PCR) was carried out in 0.2 ml PCR tubes reaction mix consisted of 10X dream Taq buffer (1 µl), 2.5 mM dNTPs mix (0.5 µl), 1 µl forward and reverse primers (20ng/µl), 0.2 µl Taq DNA polymerase (5U/µl), genomic DNA (~100 ng) as template and remaining nuclease free water to make the volume up to 10 µl. The cycling conditions are 95$^{o}$C for 10 min of initial denaturation followed by 35 cycles of 95$^{o}$C for 30 sec of cycle denaturation, 64$^{o}$C for S1 fragment and 66$^{o}$C for S2 fragment for 30 sec of annealing, 72$^{o}$C for 30 sec of extension and finally 72$^{o}$C for 10 min of final extension. The thermal cycling conditions followed were mentioned in Table 2.

**Single strand conformation polymorphism (SSCP)**

The amplicons of both fragments were denatured at 95°C for 5 min followed by snapped cooling on ice for 15 min followed by separation on 12% polyacrylamide gel comprising of 9.6 ml Acrylamide: Bisacrylamide (49:1), 28.3 ml 1X Tris-borate-EDTA (TBE) buffer, 1.5 ml Glycerol, 100 µl 10% Ammonium per sulphate (APS) and 75 µl Tetramethyl ethylenediamine (TEMED) to identify the variants. The product was loaded in the gel and electrophoresis was performed at 48ºC for 12 h at 200 V. After electrophoresis, the gel was stained with silver nitrate to visualize banding patterns of the fragments (Vohra *et al.*, 2006) to determine the genotypes. The different sized fragment combinations were scored as different genotypes.

**Haplotypes**

Each variant of the SSCP in the fragments of ACACB-S1 and ACACB-S2 regions was recorded. The ACACB-S1 showed three patterns and ACACB-S2 fragment sowed six different patterns. The patterns from both the fragments in a given individual bird were used to deduce haplotype of the individual. In total 12 haplotypes, labelled from h1 to h12, were deduced from combinations of these patterns. Frequencies of haplotypes and haplogroups were calculated by direct counting method (Bhattacharya *et al*., 2013). Haplotypes in diploid state of the individual represent the haplogroup.

**Statistical analysis**

The allelic and genotypic frequencies for each fragment were calculated using POPGENE software (Yeh *et al*., 1997). A Univariate General Linear Model was used to analyze the association with egg production traits between the two lines, as implemented in Statistical Package for Social Sciences (SPSS) v17.0. While performing the association analysis, the haplogroups which were represented for less than 3 were excluded. Thus, the model used for the analysis was as follows:

$$Yijk = \mu + Li + Gj + Sk + eijk$$

Where, $Yijk$ = dependent variable;
$\mu$ = overall population mean
$Li$ = fixed effect of lines
$Gj$ = fixed effect of genotype or diplotype
$Sk$ = fixed effect of sex
$eijk$ = random error with NID (0, $\sigma$2)

The significant differences were calculated between least-squares means of different genotypes by Duncan's method at 5% level of significance.

## RESULTS AND DISCUSSION

**Polymorphism in ACACB gene promoter**

The ACACB-S1 (301bp) fragment revealed six different banding patterns in both IWI and IWK lines, whereas the ACACB-S2 (233bp) fragment revealed three different banding patterns in both the populations. By combining these patterns from two fragments, twelve haplotypes (h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11

and h12) (Fig.1), and eighteen haplogroups (h1h1, h1h2, h2h2, h3h3, h3h4, h4h4, h5h5, h5h6, h6h6, h7h7, h7h8, h8h8, h9h9, h9h10, h10h10, h11h11, h11h12 and h12h12) (Fig.2), were deduced (Table 3 and Table 4).

**Association of haplogroups with egg production**

In IWI lines within group there is a significant (P≤0.05) effect on egg production up to 52 weeks of age. Whereas In IWK lines within group there is a non-significant (P≤0.05) effect on egg production at all ages. The haplogroups of ACACB gene promoter had non-significant (P≤0.05) effect egg production at all ages between the group of IWI and IWK lines with the exception of h12h12 up to 52 and 72 weeks of age. With respect to egg production in all age groups showed higher egg production in IWI line, when compared to the IWK line. The haplogroup h12h12 showed the highest egg production (193.20±7.56) and the h1h2 haplogroup showed the lowest egg production (126.80±3.54) up to 52 weeks of age. In all age groups h12h12 haplogroup showed the highest egg production in IWI line (Table 5).

The ACACB gene is known for its association with nephropathy, obesity, diabetes, and end stage renal diseases (Tang *et al*., 2010; Riancho *et al*., 2011; Zain *et al.*, 2017). Genetic polymorphism in the promoter region would alter the function of the gene and ultimately leading to differential association of the gene with many traits in chicken (Cui *et al.*, 2006; Zhu and Jiang, 2014). For the polymorphism analysis, two genetic lines of white leg horn layer chicken – IWI and IWK were chosen. The IWI line is selected for egg production and IWK line is selected for both egg production and egg weight.

Both the fragments in the present study revealed polymorphism based on SSCP analysis. The 301 bp fragment revealed higher polymorphism compared to 233 bp fragment. Based on analysis of both the fragments, 12 haplotypes were identified. These 12 haplotypes formed 18 haplogroups in both the populations studied. Promoter polymorphism is also observed in matrix metalloproteinase 9 gene (Zhu and Jiang 2014), prolactin gene (Cui *et al*., 2006; Bhattacharya *et al*., 2015a) etc. in chicken. Generally the variation is associated with difference in function.

No association of haplogroups with egg production up to 40 weeks of age is observed. Haplogroup h12h12 is significantly associated with higher egg production in IWI line up to 52 weeks of age. Between the two lines, the haplogroup displayed more egg production in IWI line. However, in general the IWI line has higher egg production than IWK line due to the difference in type of selection practiced in both the lines. The selection for high egg production in IWI line could be the reason for the association of h12 h12 haplotype with higher egg production in this line.

From the results obtained in this study, it can be concluded that the promoter region of ACACB gene is polymorphic and certain haplogroups are associated with egg production. It would be interesting to further

validate the specific mutations to understand their precise role in the egg production traits in the two lines, which would help in marker assisted selection.

## DECLARATION OF INTERESTS

All the authors declare that they have no competing interests. All authors read and approved the final manuscript.

**Table.1. Details of primers used for amplification of *ACACB* gene promoter.**

| Primer name | Primer Sequences (5'-3') | Amplicon Size (bp) |
|---|---|---|
| ACACB-S1 | F: CAGTCCTGCTTTGCTTCCGTG<br>R: TTGTGGGACAAAGGTGCCATG | 301 |
| ACACB-S2 | F: AGGTGAGCTCCATGGCACTG<br>R: GACAGCCAGACCTCTCACAT | 233 |

**Table.2. PCR amplification conditions followed for *ACACB* gene promoter fragment.**

| Fragment | Thermal cycling conditions | | | | |
|---|---|---|---|---|---|
| | Initial denaturation at 95ºC for 10 minutes | Cycles (35) | | | Final Extension at 72ºC for 10 minutes |
| | | Denaturation | Annealing | Final Extension | |
| ACACB-S1 | | 95ºC for 30 seconds | 62ºC for 40 seconds | 72ºC for 40 seconds | |
| ACACB-S2 | | 95ºC for 40 seconds | 62ºC for 40 seconds | 72ºC for 1 minute 30 sec | |

**Table.3. Haplotype frequencies of *ACACB* gene in IWI and IWK lines.**

| Haplotype | Haplotype frequencies | |
|---|---|---|
| | IWI | IWK |
| h1 | 0.038 | 0.048 |
| h2 | 0.182 | 0.048 |
| h3 | 0.090 | 0.028 |
| h4 | 0.014 | 0.240 |

| | | |
|---|---|---|
| h5 | 0.012 | 0.014 |
| h6 | 0.084 | 0.038 |
| h7 | 0.094 | 0.020 |
| h8 | 0.034 | 0.036 |
| h9 | 0.086 | 0.234 |
| h10 | 0.114 | 0.146 |
| h11 | 0.202 | 0.234 |
| h12 | 0.050 | 0.146 |

**Table.4. Haplogroup frequencies of *ACACB* gene in IWI and IWK lines.**

| Haplotype | IWI | | IWK | |
|---|---|---|---|---|
| | Number of birds | Frequencies | Number of birds | Frequencies |
| h1h1 | 6 | 0.024 | 8 | 0.032 |
| h1h2 | 7 | 0.028 | 8 | 0.032 |
| h2h2 | 42 | 0.168 | 8 | 0.032 |
| h3h3 | 21 | 0.084 | 3 | 0.012 |
| h3h4 | 3 | 0.012 | 8 | 0.032 |
| h4h4 | 2 | 0.008 | 2 | 0.008 |
| h5h5 | 2 | 0.008 | 1 | 0.004 |
| h5h6 | 2 | 0.008 | 5 | 0.020 |
| h6h6 | 20 | 0.080 | 7 | 0.028 |
| h7h7 | 23 | 0.092 | 4 | 0.016 |
| h7h8 | 1 | 0.004 | 2 | 0.008 |
| h8h8 | 8 | 0.032 | 4 | 0.016 |
| h9h9 | 15 | 0.060 | 52 | 0.208 |
| h9h10 | 13 | 0.052 | 13 | 0.052 |
| h10h10 | 22 | 0.088 | 30 | 0.120 |
| h11h11 | 48 | 0.192 | 44 | 0.176 |
| h11h12 | 5 | 0.020 | 29 | 0.116 |
| h12h12 | 10 | 0.040 | 22 | 0.088 |

**Table.5. Association of haplogroups with egg production up to 40 wk, 52 wk and 72 wk in IWI and IWK line**

| Haplo group | Egg Production | | | | | |
|---|---|---|---|---|---|---|
| | Up to 40 weeks | | Up to 52 weeks | | Up to 72 weeks | |
| | IWI | IWK | IWI | IWK | IWI | IWK |
| h1h1 | 91.00±8.73 | 84.57±8.42 | 175.25±5.70$^{ab}$ | 142.57±2.24 | 251.25±8.15 | 230.29±8.14 |
| h1h2 | 71.80±8.92 | 92.25±9.37 | 126.80±3.54$^{a}$ | 155.75±3.89 | 207.50±7.68 | 261.63±4.36 |
| h2h2 | 91.42±6.69 | 87.25±7.82 | 169.95±7.75$^{ab}$ | 151.71±4.03 | 250.71±2.44 | 260.17±2.00 |
| h3h3 | 89.62±8.23 | 86.00±0.03 | 149.00±2.11$^{ab}$ | 138.00±5.72 | 201.71±7.70 | 246.33±2.77 |
| h3h4 | 97.00±1.00 | 77.00±7.15 | 141.50±8.50$^{ab}$ | 135.88±3.45 | 218.50±2.50 | 246.33±7.00 |
| h5h6 | - | 92.80±1.06 | - | 155.20±3.37 | - | 286.20±5.54 |
| h6h6 | 93.15±7.33 | 70.29±1.92 | 156.54±1.06$^{ab}$ | 129.00±7.51 | 243.17±9.32 | 222.71±4.45 |
| h7h7 | 90.00±6.73 | 77.25±6.84 | 165.00±6.19$^{ab}$ | 144.00±4.53 | 253.88±9.10 | 250.50±5.61 |
| h8h8 | 86.00±5.00 | 91.50±6.71 | 151.50±9.50$^{ab}$ | 145.75±7.66 | 230.00±8.00 | 247.75±2.05 |
| h9h9 | 80.00±8.02 | 87.13±3.18 | 145.27±9.25$^{ab}$ | 140.06±5.81 | 230.64±6.80 | 245.92±7.65 |
| h9h10 | 76.38±1.58 | 94.08±4.39 | 147.57±2.90$^{ab}$ | 159.25±5.28 | 215.29±5.04 | 250.64±1.72 |
| h10h10 | 78.63±8.98 | 86.62±3.50 | 149.24±1.20$^{ab}$ | 149.12±5.62 | 234.35±5.23 | 247.36±6.16 |
| h11h11 | 82.69±5.98 | 83.80±3.85 | 145.62±8.40$^{ab}$ | 142.49±4.99 | 233.71±10.78 | 232.35±6.60 |
| h11h12 | 93.00±4.54 | 89.64±5.08 | 154.00±5.82$^{ab}$ | 150.64±6.43 | 221.40±0.60 | 220.93±3.17 |
| h12h12 | 98.17±8.31 | 89.75±4.02 | 193.20±7.56$^{bx}$ | 151.92±5.11$^{y}$ | 287.40±2.95$^{x}$ | 245.09±1.16$^{y}$ |

**Means with different superscripts (a,b) within each column differ significantly (P≤0.05) (Duncan's MRT)**
**Means with different superscripts (x,y) in each row for each age group differ significantly (P≤0.05) (Independent sample t-test)**

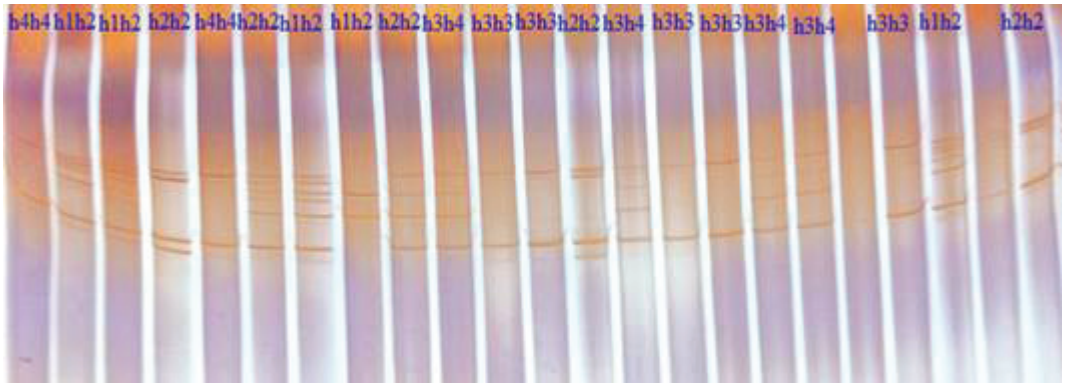


**Fig.1. Polyacrylamide gel electrophoresis of 301bp fragment showing representative SSCP patterns in IWI and IWK lines of ACACB gene promoter.**

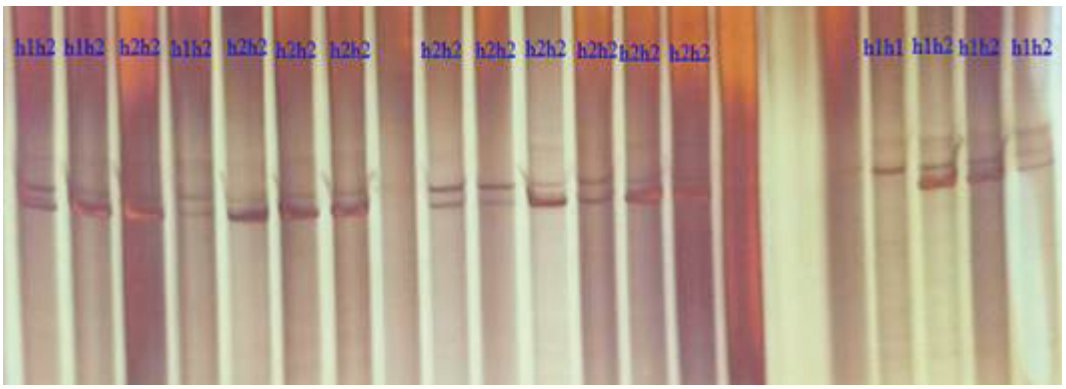


**Fig.2. Polyacrylamide gel electrophoresis of 233bp fragment showing representative SSCP patterns in IWI and IWK lines of ACACB gene promoter.**

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