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Polymorphism at 5′UTR region of *ACACB* gene and its association with body weight and HDL concentration in layer chickens

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Understanding genetic regulation of adipose tissue developmental genes would help in designing healthy chicken for human consumption. In the present study, we tried to identify the polymorphism at the 5′UTR region of the Acetyl-CoA Carboxylase Beta (*ACACB*) gene and its association with body weights and High-Density Lipoprotein (HDL) concentration in IWI and IWK lines of White Leghorn layer chicken breed. A total of 500 birds comprising of 250 IWI line and 250 IWK line were included in Single Stranded Conformation Polymorphism (SSCP) data analysis. Results revealed that in IWI lines within group there is a significant ($P \leq 0.05$) effect on body weights at 8th week and 16th week of age. Whereas, in IWK lines within group there is a significant ($P \leq 0.05$) effect on body weights at day old and 20th week of age was observed. In general, the h8h8 haplogroup showed the highest body weights in both the lines. The association analysis of serum concentration of HDL in IWI line revealed that h12h12 haplogroup birds was the highest (61.64±2.99) and in the h1h2 haplogroup it was found to be the lowest (42.72±7.23). In case of IWK, the h8h8 haplogroup birds were the highest (62.47±6.06) serum concentration of HDL and in the h7h7 haplogroup, it was found to be the lowest (44.85±2.64) serum concentration of HDL. In IWI lines within group, there is a non-significant ($P \leq 0.05$) effect on serum concentration of HDL and in IWK lines within group there is a significant ($P \leq 0.05$) effect on serum concentration of HDL was observed. It is concluded that promotor of the *ACACB* gene is highly polymorphic and have a significant effect on body weights and serum concentration of HDL in White Leghorn layer chicken.

Keywords: Genotyping, Haplogroup, High-Density Lipoprotein (HDL), Obesity, Single Stranded Conformation Polymorphism (SSCP), White Leghorn

The prevalence of accumulation of fat affects body composition and quality of meat and egg. In chicken, most fat is deposited under the skin, around the inner organs and in the abdominal part around the gonads. Although high mass of white adipose tissue reduces the nutritional and economic value of a chicken, excess fat may contribute to the development of detrimental traits arising from genetic selection for rapid growth, such as reduced fertility and immunocompetence, both of which are seen in obese humans[1]. High-fat content in poultry can be a risk to human health[2]. Obesity is a serious public health problem around the world that increases the risk of some common diseases, such as type 2 diabetes, cardiovascular disease, metabolic syndrome, hypertension, and a few types of cancer[3,4]. The excessive fat in modern poultry strains has been one of the major problems facing the poultry industry[5]. Hence, the chicken industry requires new strategies to reduce the fatness in chicken meat and egg, which would economically benefit producers and likely improve health and welfare of chicken and human being.

Laying hens also exhibit excessive fat accumulation, which negatively affects their reproductive performance[6]. The genes or molecules that regulate abdominal fat deposition or abdominal adipose tissue development can be identified by many different genomic approaches[7-12]. The *ACACB* gene was first discovered in rat heart[13], localized in chromosomes 15 (NCBI). *ACACB* (M_r ~280 kDa) is found primarily in non-lipogenic tissues such as

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skeletal and heart muscle and to a lesser extent in liver[14-17]. The *ACACB* generated malonyl-CoA functions as 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[18-20]. The net result is reduced fatty acid oxidation and increased fatty acid and triglyceride synthesis, at the expense of glucose utilization. Mice lacking *ACC2* show increased oxidation of fatty acids and decreased fat storage[21].

In the present study, we explored the poly-morphism in the 5'UTR region of the *ACACB* gene and its association with body weights and High-Density Lipoprotein (HDL) concentration in IWI and IWK lines of White Leghorn layer chicken**.**

Materials and Methods

Location

The present study was carried out at ICAR-Directorate of Poultry Research (DPR), Hyderabad, Telangana, India. Hyderabad is situated in central Telangana region and is spread over an area of 260 sq. km. The city lies at 17.366° N latitude and 78.476° E longitude. Hyderabad has a unique combination of tropical wet and dry climates. The mean daily temperature varies from 30°C to 36°C from April to June and from 20°C to 24°C in the months of December and January.

Experimental birds

The present study was conducted on IWI and IWK lines of White Leghorn chicken breed. Birds of the two selected pure lines were bred by pedigree mating using artificial insemination. One day old Chicks 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 body weight and provided 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 hatching, chicks were vaccinated against Marek's disease and subsequently birds were protected against important diseases like RD (Ranikhet Disease), IBD (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 were 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.

Collection of samples

Blood samples

A total of 250 birds of IWI line and 250 birds of IWK line were included in the present experiment to study polymorphism in the promoter of Acetyl Co-A carboxylase B (*ACACB*) gene. About 0.5-1.0 mL of blood was collected from the wing vein of each bird in a sterile polypropylene 2 mL centrifuge tubes containing 2.7% ethylene diamine tetra acetic acid (EDTA) (60-70 µL/1 mL of blood) as an anti-coagulant under sterile conditions. After collection of blood, the tubes were tightly capped and mixed up and down for proper mixing of blood with the anti-coagulant. The vials were then kept immediately in ice box containing ice and gel cool packs and were transported to the laboratory immediately. After reaching the laboratory, samples were stored at –20°C till DNA isolation.

Serum samples

The blood (1-2 mL) was collected from wing veins of birds of IWI (250) and IWK (250) lines into a sterile polypropylene 2 mL centrifuge tubes aseptically. This serum was used for association study.

PCR-SSCP

The 5′ upstream region of transcription start site of *ACACB* gene 534 bp divided into two non-overlapping fragments namely, ACACB-S1 and ACACB-S2. These two small fragments were screened through single stranded conformation polymorphism technique. Their sensitivity is inversely proportional to the fragment length *i.e.*, one single base pair difference can be resolved 99% of the time for 100-300 bp fragments and more than 80% for 400 bp according to[22]. 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 tube's reaction mix consisted of 10X dream Taq buffer (1 µL),

2.5 mM dTNPs mix (0.5 µL), 1 µL gene specific forward and reverse primers (20 ng/µL), 0.2 µL Taq DNA polymerase (5U/µL), genomic DNA (1 µL) as template and remaining nuclease free water to make the volume up to 10 µL. The thermal cycling conditions followed were mentioned in Table 2.

Genotyping

Genotyping by a single strand chain polymorphism was done in an attempt to screen the chicken populations for polymorphism in *ACACB* gene promoter using a 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 ethylene diamine (TEMED). Samples were denatured at 95°C for 5 min followed by snapped cooling on ice for 15 min. Then, the product was loaded in the gel and electrophoresis was performed at 48°C for 12 h at 200 V. After electrophoresis was over, the gel was stained with silver nitrate to visualize banding patterns of the fragments[23].

Measurement of traits

Body weights

The body weights were measured in total of 500 birds comprising of 250 lines of IWI and 250 lines of IWK (each 200 female and 50 male) of White Leghorn chicken at day old, 8th week, 16th week and 20th week.

Estimation of high-density lipoprotein (HDL) concentration in serum

About 1-2 mL of blood samples without anticoagulant were collected from 250 birds of IWI lines and 250 birds of IWK layer chicken line aseptically collected and kept in 2 mL tubes. This blood samples were kept in slanting position at room temperature (24°C) for 2 h, then the tubes were centrifuged at 1000 RPM for 5 min and upper serum samples were collected and kept in 1.5 mL tubes. These serum samples were used for estimation of high-density lipoprotein (HDL) level in serum. The HDL content were estimated using the high-density lipoprotein test kit (Ref-10112; lot No-28478: Identi) and Thermo Chem 100 automatic blood analyzer.

Statistical analysis

The allelic and genotypic frequencies for each fragment were calculated using POPGENE software[24]. Univariate General Linear Model of Statistical Package for Social Sciences (SPSS) 17.0 was performed to analyze the association studies with different traits of birds.

Results

Polymorphism in *ACACB* gene promoter and its association with different traits

The PCR products corresponding to 301 bp and 233 bp promoter fragments of the *ACACB* gene were subjected to single strand conformation polymorphism, followed by silver staining displayed polymorphism was detected in both the fragments of IWI and IWK lines. The *ACACB*-S1 (301 bp) fragment revealed six different banding patterns were observed in both IWI and IWK lines (Fig. 1a), whereas the *ACACB*-S2 (233 bp) fragment revealed three different banding patterns in both the populations (Fig. 1b). By combining these patterns from two fragments, 12 haplotypes (h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11 and h12) and 18 haplogroups (h1h1, h1h2, h2h2, h3h3, h3h4, h4h4, h5h5, h5h6, h6h6, h7h7, h7h8, h8h8, h9h9, h9h10, h10h10, h11h11, h11h12 and h12h12) were formed.

Association of haplogroups with body weight and serum HDL concentration

The association of haplogroups with the body weight and serum HDL concentration in IWI and IWK lines of white leghorn chicken was analyzed using Univariate general linear model (GLM) technique and the results are presented in the Tables 3 and 4 with their Least square means (LSM).

Body weight

IWI lines, within the group, showed a significant ($P \leq 0.05$) effect on body weight at 8th week and 16th week of age. Similarly, the IWK lines, within the group, showed a significant ($P \leq 0.05$) effect on body

Table 1 — Details of primers used for cloning of *ACACB* gene promoter

Primer name	Primer sequences (5'-3')	Amplicon size (bp)
ACACB-P1	F: AGATCTAGGTGAGCTCCATGGCACTG R: GAATTCTTGTGGGACAAAGGTGCCATG	534
ACACB-P2	F: AGATCTTCTGCTGCTGTCAACTTGATG R: GAATTCTTGTGGGACAAAGGTGCCATG	1518

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

Fragment	Thermal cycling conditions (35 Cycles)				
	Initial denaturation	Denaturation	***Annealing***	***Final Extension***	Final extension
ACACB-P1	at 95°C for 10 min	95°C for 30 s	62°C for 40 s	72°C for 40 s	at 72°C for 10 min
ACACB-P2		95°C for 40 s	64°C for 40 s	72°C for 1 min 30 s	

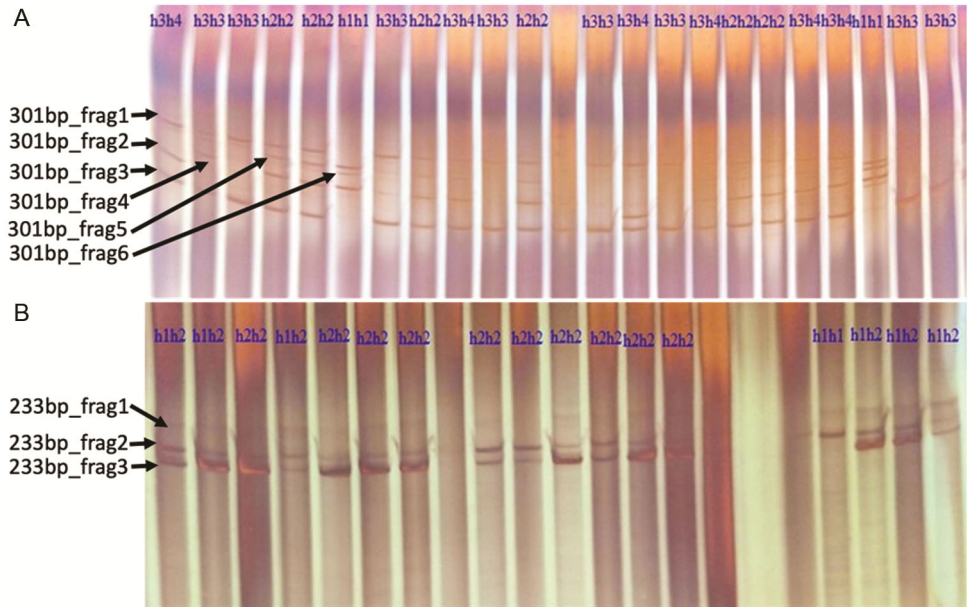

Fig. 1 — Polyacrylamide gel electrophoresis of (A) 301bp; (B) 233bp fragment showing representative SSCP patterns in IWI and IWK lines of *ACACB* gene promoter

Table 3 — Haplogroup-wise body weights in IWI and IWK lines of White Leghorn chicken breed

Haplo-group	Body weights (g)							
	Day old		8th week		16th week		20th week	
	IWI	IWK	IWI	IWK	IWI	IWK	IWI	IWK
h1h1	34.34±1.34^{x}	38.34±1.15^{aby}	509.80±33.13^{cx}	461.29±0.94^{x}	884.80±8.82^{ax}	999.43±6.56^{x}	1199.80±0.10^{abcx}	1097.86±7.20^{ax}
h1h2	35.58±1.75^{x}	38.91±1.80^{abx}	439.33±5.49^{bcx}	457.86±0.89^{x}	978.50±7.84^{ax}	901.71±6.26^{x}	1186.67±1.55^{abcx}	1080.86±6.42^{ax}
h2h2	35.81±1.46^{x}	39.34±1.57^{abx}	458.88±4.12^{bcx}	499.57±1.85^{x}	998.22±5.57^{abx}	1050.43±4.61^{x}	1318.25±4.93^{bx}	1113.57±7.61^{abx}
h3h3	34.71±1.04^{x}	38.57±2.54^{abx}	416.76±5.29^{abx}	455.00±9.70^{x}	1000.18±2.21^{abx}	967.00±6.66^{x}	1307.24±9.49^{bcx}	1175.67±7.94^{abx}
h3h4	35.75±1.15^{x}	37.45±1.74^{abx}	340.50±9.50^{ax}	459.00±2.50^{y}	949.50±5.50^{ax}	974.17±0.29^{x}	1081.00±10.00^{ax}	1131.17±7.80^{abx}
h6h6	36.35±0.82^{x}	41.13±1.78^{by}	448.84±5.50^{bcx}	519.00±3.69^{x}	916.58±1.87^{ax}	985.00±5.22^{x}	1222.16±8.29^{abcx}	1094.75±4.17^{ax}
h7h7	35.43±0.97^{x}	35.65±3.05^{ax}	436.75±9.95^{bcx}	414.00±8.00^{x}	920.56±0.10^{ax}	1004.50±5.50^{x}	1222.94±0.33^{abcx}	1041.00±3.00^{ax}
h8h8	36.24±1.41^{x}	38.10±3.90^{abx}	520.50±6.78^{cx}	500.00±2.00^{x}	1142.13±5.75^{bx}	1065.00±4.00^{x}	1529.13±5.74^{cx}	1287.50±1.50^{bx}
h9h9	37.40±0.87^{x}	38.06±0.45^{abx}	440.29±0.39^{bcx}	496.21±1.82^{y}	906.59±5.29^{ax}	943.26±6.89^{x}	1229.41±3.09^{abcx}	1207.61±4.25^{abx}
h9h10	35.83±0.70^{x}	37.29±0.93^{abx}	414.27±1.88^{abx}	484.78±7.60^{x}	847.91±6.00^{ax}	976.22±8.20^{y}	1217.64±5.18^{abcx}	1054.89±2.64^{ay}
h10h10	36.93±0.61^{x}	37.36±0.97^{abx}	463.48±2.80^{bcx}	487.13±5.61^{x}	880.16±7.52^{ax}	926.38±9.40^{x}	1187.71±9.55^{abcx}	1151.25±4.30^{abx}
h11h11	36.19±0.49^{x}	37.76±0.76^{abx}	429.17±1.11^{bcx}	480.75±1.87^{x}	908.70±9.34^{ax}	962.42±9.21^{x}	1162.19±3.23^{abcx}	1192.46±7.82^{abx}
h11h12	37.58±0.87^{x}	39.69±0.71^{abx}	458.67±3.87^{bcx}	508.44±6.10^{x}	857.25±2.48^{ax}	889.38±9.57^{x}	1157.00±3.60^{abx}	1215.88±3.70^{abx}
h12h12	36.57±0.75^{x}	40.05±1.28^{abx}	432.56±1.94^{bcx}	472.22±1.11^{x}	948.33±0.38^{ax}	952.56±5.33^{x}	1265.47±3.98^{abcx}	1204.00±3.20^{abx}

[Means with different superscripts (a-c) within each column differ significantly ($P \leq 0.05$) (Duncan's Multiple range test (MRT); and Means with different superscripts (x,y) in each row for each age group differ significantly ($P \leq 0.05$) (Independent sample t-test)]

weight at 1-day old and 20th week birds. The haplogroups of *ACACB* gene promoter had non-significant ($P \leq 0.05$) effect on body weights at all ages between the group of IWI and IWK lines with the exception of h1h1 and h6h6 at day old, h3h4 and h9h9 at 8th week and h9h10 at 16th and 20th week of age.

In general, IWI lines showed lower body weight up to 8 weeks of age, followed by higher body weights was observed up to 20 weeks of age compare to that of IWK lines. The haplogroup h8h8 maintained the highest body weight throughout the period of study with 520.50 ± 6.78 g, 1142.13±5.75 g and 1529.13 ± 5.74 g for 8th, 16th and 20th week, respectively. The h3h4 haplogroup showed the lowest body weight (340.50±9.50g) at 8th week as well as 20th week (1081.00 ± 10.00 g). The h9h10 haplogroup showed the lowest (847.91 ± 6.00 g) body weight at 16th week of age. Contrastingly, in IWK line, 1-day old h6h6 haplogroup displayed the highest body weight (41.13 ± 1.78 g) and the h7h7 haplogroup displayed the lowest body weight (35.65 ± 3.05 g), while the haplogroup h8h8 showed the highest body weight

Table 4 — Haplogroups-wise serum concentration of HDL in IWI and IWK lines of White Leghorn chicken breed

Haplo group	Serum HDL concentration (mg/dL)	
	IWI	IWK
h1h1	45.83±10.36	55.66±5.44^{abc}
h1h2	42.72±7.23	51.65±3.90^{abc}
h2h2	52.55±2.54	60.24±6.43^{bc}
h3h3	51.42±3.11	50.72±7.90^{abc}
h3h4	-	60.53±4.38^{bc}
h5h6	-	57.02±2.08^{abc}
h6h6	53.55±4.13	57.97±4.55^{abc}
h7h7	47.53±2.78	44.85±2.64^{a}
h8h8	58.24±3.25	62.47±6.06^{c}
h9h9	62.64±9.27	54.21±1.70^{abc}
h9h10	49.72±4.04	47.43±2.86^{ab}
h10h10	47.28±6.22	48.68±1.51^{ab}
h11h11	51.68±4.53	50.19±1.74^{abc}
h11h12	-	53.02±2.23^{abc}
h12h12	61.64±2.99	52.20±2.12^{abc}

[Means with different superscripts (a-c) within each column differ significantly ($P \leq 0.05$) (Duncan's MRT)]

(1287.50 ± 1.50 g) and the h7h7 haplogroup showed the lowest body weight (1041.00 ± 3.00 g) at 20th week of age. In general, h8h8 showed the highest body weights in both the lines.

Association of haplogroups with serum concentration of HDL

Different haplogroups were found to be associated with the serum HDL concentration in IWI and IWK lines. While performing the association, the haplogroups which had the less number were excluded. The association analysis of serum concentration of HDL in IWI line revealed that h12h12 haplogroup birds was the highest (61.64 ± 2.99) and in the h1h2 haplogroup it was found to be the lowest (42.72 ± 7.23). In case of IWK, the h8h8 haplogroup birds were with highest (62.47 ± 6.06) serum concentration of HDL and in the h7h7 haplogroup it was found to be the lowest (44.85 ± 2.64) serum concentration of HDL. In IWI lines, within the group, there was a non-significant ($P \leq 0.05$) effect on serum concentration of HDL, whereas, in IWK lines within group it was a significant ($P \leq 0.05$).

Discussion

The acetyl-coenzyme A carboxylase beta (*ACACB*) gene is known to be associated with nephropathy[25], obesity, diabetes[26], end stage renal diseases[27], etc. The enzyme plays a crucial role in fatty acid oxidation by catalyzing the synthesis of malonyl-CoA, which is a substrate for fatty acid synthesis and a regular fatty acid oxidation[21]. Mice lacking *ACACB* gene are reported to be protected against obesity and diabetes[28]. Attempts were made to understand the role of *ACACB* gene in livestock. Mutations in the promoter region in cattle are observed to affect milk production traits in dairy cattle[29]. Though putative mutations of *ACACB* gene or its promoters with functional role are yet to be identified in animals including chicken, due to the functional role of the enzyme in regulation of fatty acid oxidation, the gene is likely to play a role in body weight and serum HDL concentration. Understanding genetic polymorphism and characterization of functional regions in the gene would help in planning future genetic manipulations of the gene towards body fat composition. In the present study, we tried to understand the polymorphism and its association with the body weight and serum HDL concentration in IWI and IWK lines of white leghorn chicken.

Genetic polymorphism of *ACACB* gene promoter region in chicken

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[30,31]. Practically, polymorphism can be detected by direct sequencing, SSCP, PCR-RFLP, etc. However, keeping the economics and potential of the techniques in mind, the genetic polymorphism in the *ACACB* gene was identified using PCR-SSCP analysis in this experiment. The minimal promoter identified in this study is further divided into two non-overlapping fragments of 301 bp (named as *ACACB*-S1) and 233 bp (named as *ACACB*-S2).

Association of haplogroups with body weights, serum concentration of HDL

Perusal of literature revealed lack of genetic polymorphism association studies in either promoter or coding or intronic region of *ACACB* gene with production parameters of chicken. In the present study, it is attempted to verify effect of SSCP variants on body weight and serum HDL concentration in IWI and IWK chicken. The IWI line is selected for egg production and IWK line is selected for both egg production and egg weight. In order to verify association of haplogroups with body weight and serum HDL concentration in these two genetic groups that are selected with different objectives, Univariate General Linear Model analysis is performed in the preset study.

Body weight

In the present study, the association of body weights with different haplogroups for both the

genetic groups at 1-day old, 8th, 16th and 20th week was analyzed. In IWI lime, at day old, no association of body weight with haplogroups was observed. But during 8th, 16th and 20th week, the association of body weight with haplogroups showed significant effect where h8h8 haplogroup observed to be 35, 26 and 29% higher body weight, respectively, than the haplogroup with lowest performer in the group. On the other hand, in IWK line at day old, the association of body weight with haplogroups was significant where the h6h6 haplogroup birds showed 13% higher body weight than the h7h7 haplogroup. At 20th week of age, body weight was significantly associated with haplogroups where h8h8 haplogroup birds showed 19% higher body weight than the h7h7 haplogroup birds. In earlier study also, mutant mice of *ACACB* gene showed an association with lower body weight[32].

Serum HDL concentration

Since *ACACB* gene is involved in fatty acid oxidation, association of different haplotypes with serum HDL concentration was analyzed. The mean serum HDL concentrations ranged between 42.72 ± 7.23 and 61.64 ± 2.99 in IWI line. There was no significant association observed between haplogroups and HDL concentration in IWI line. But, in IWK line, the h8h8 haplogroup was associated with high serum HDL cholesterol concentration of 62.47 ± 6.06 and the h7h7 haplogroup was associated with low serum HDL cholesterol concentration of 44.85 ± 2.64. Genetic association studies on HDL cholesterol level in chicken are scanty. The common variants of *ACACB* gene in human were not associated with HDL cholesterol[33]. But interestingly in the present study, the h8h8 haplogroup in the promotor region is associated with high serum HDL cholesterol level and h7h7 haplogroup is associated with low cholesterol level. The specific mutations at the nucleotide level in these haplogroups would give better understanding of the molecular mechanism underlying such association and understanding such mechanisms would be interesting for future genetic manipulations for regulating serum HDL cholesterol concentrations in chicken.

Conclusion

The present study revealed genetic polymorphism in the 5′UTR region of the chicken *ACACB* gene promotor region based on PCR-SSCP analysis and genetic association of the polymorphism with the HDL cholesterol levels and body weights in chicken.

Conflict of interest

Authors declare no competing interests.

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