Research Note: Fine mapping of sequence variants associated with body weight of Lueyang black-boned chicken in the *CCKAR* gene

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ABSTRACT Cholecystokinin A receptor (**CCKAR**) is a key receptor mediating satiety. Previous studies found that decreased expression of *CCKAR* attenuated satiety, and thus contributed to the high-growth of broiler chickens. The objective of this study is to map sequence variants associated with the growth of chickens in the *CCKAR*. The *CCKAR* and upstream 1.4 kb genomic sequences were resequenced to find out all sequence variants using 35 Lueyang black-boned chickens (**LBC**). Haplotypes were reconstructed using the PHASE program. Linkage disequilibrium between variants was analyzed using the Haploview software. Associations of 33 tag SNPs that captured 89% of all variants with body weight of LBC (n = 675) at 16 (BW16), 20 (BW20) weeks of age and the onset (**BWOEP**) of egg production were tested using linear mixed models. A total of 126 SNPs were found and formed 41 haplotypes in 35 resequenced samples. Average length of haplotype blocks is 129 bp, indicating that LBC maintains low linkage disequilibrium at the *CCKAR* locus. Eleven of 33 tag SNPs were significantly associated with BW16, but not with BW20 and BWOEP. These significantly associated variants were most (8/11) distributed in a 2 kb region (chr4:73206169-73208244) around the Exon3. They together with 33 captured variants potentially disrupted binding sites of 471 transcription factors. Twelve variants can disrupt appetite (FOXO1) or lipid metabolism-related TF (AR and C/EBP) motifs. This study recognized chr4:73206169-73208244 as a key region harboring functional variants affecting the growth of chickens.

Key words: Lueyang black-boned chicken, *CCKAR*, satiety, body weight

INTRODUCTION

Cholecystokinin (**CCK**) is a peptide hormone that is mainly synthesized in small intestinal endocrine cells and nervous fibers (Martinez et al., 1993). CCK induces satiety and thus is a key signaling molecule modulating food intake and energy balance of animals. Cholecystokinin A receptor (**CCKAR**) and cholecystokinin B receptor (**CCKBR**) are 2 key receptors mediating CCK actions. CCKBR binds CCK and gastrin with almost equal affinities. Its activation in the stomach stimulates gastric acid secretion, and in the brain is associated with anxiety and pain perception (Guilloteau et al., 2006). In contrast, CCKAR that exhibits a 500-fold higher affinity for CCK than for gastrin is believed to be the primary receptor mediating satiety (Guilloteau et al., 2006). The absence of CCKAR attenuated the satiety signal, which contributed to increasing food intake and obesity in human and rat (Miller et al., 1995; Moran and Bi, 2006). This link between abundant of CCK receptor and appetite was also found in chicken. Decreased expression of *CCKAR* facilitated the growth of chicken by changing satiety set point (Dunn et al., 2013). Sequence variants that affect *CCKAR* expression were associated with body weight of chickens, suggesting that *cis*-regulatory variants in the *CCKAR* form molecular basis controlling the growth of chickens (Rikimaru et al., 2013). Previous studies reported some sequence variants that were significantly associated with diverse growth traits of chickens (Dunn et al., 2013; Rikimaru et al., 2013; Yi et al., 2018). A fine mapping remains necessary to narrow down the region containing functional variants at the gene level. This study resequenced the *CCKAR* locus to find out all sequence variants, and mapped functional variants using 33 tag SNPs.

MATERIALS AND METHODS

Genetic Features of Lueyang Black-Boned Chickens and Measurement of Body Weight

Lueyang black-boned chicken (**LBC**) is an indigenous breed from Lueyang city of Shaanxi province,

China. The breed has a dermal hyperpigmentation phenotype like the Silkie chicken. But its adult BW is 2 folds as large as the Silkie chicken. As LBC was not subjected to selection for production traits, it shows large variations in egg production and growth traits, implying that some QTL could not possibly be fixed in this population (Dang et al., 2020). In addition, our previous studies found that this breed sustained low genomic linkage disequilibrium (Wang et al., 2016). These genetic features make it being a suitable population for fine mapping of some functional variants. In this study, 675 pullets that were produced by mating 47 cocks with 296 hens were used in the association analysis. These pullets were simultaneously hatched and raised in the same hen house by Lueyang LongJia Agro-Tech Ltd. Co. (Lueyang city, Shaanxi, China) from March 2018 to January 2019. Body weights at the 16 (BW16), 20 (BW20) weeks of age and the onset of egg production (**BWOEP**) were measured for each bird after fasting for 12 h. Animal care, slaughter and experimental procedures were approved by Institutional Animal Care and Institutional Ethic Committee of Northwest A&F University (DK-2021020).

Resequencing of CCKAR and SNP Calling

The *CCKAR* gene and upstream 1.4 kb genomic sequence (chr4:73201947-73210290) of 35 chickens were resequenced by the Sanger sequencing. The resequenced samples were randomly selected from 675 pullets. Blood samples were collected using wing venipuncture. Genomic DNA was extracted using FlexGen Blood DNA Kit (CWBIO, Beijing, China) according to the manufacture's instruction. Seven fragments were amplified using 5'-CTACCAAATCAATGCCTGTC-3' and 5'- TACATC-TATCTCATTTAGCG-3' for fragment1, 5'- AGCAG CTCAAGTTTACAAGT-3' and 5'- TCAGATCACA-TAACTCCAAT-3' for fragment2, 5'- GGCAGGAAA-GAGTTCAGTAT-3' and 5'-CAAAGTTCTCAAG GGCGTAA-3' for fragment3, 5'-CGCCCTTGAGAA CTTTGATG-3' and 5'-GCAAATGGCACTGTACCG CT-3' for fragment4, 5'-AGCACGGAGACCAA-GACCCA-3' and 5'-CAGAAGGACAGGAGCGGTTG-3' for fragment5, 5'-CAACCCTCCAGTAGGTGCCA-3' and 5'-TCACAGGCGGCTTCTGCTTA-3' for fragment6, and 5'-TCCCATCTCCTTTATCCACC-3' and 5'-AGCTCACATCTGCCCTTTAC-3' for fragment7. PCR was performed using Dye-added $2 \times$ Es Taq Master-Mix (CWBIO, Beijing, China) in the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Thermal cycle program was 95°C 3 min, 33 cycles of 95°C 30s, 60°C 30 s and 72°C 60 s followed by final elongation at 72°C for 5 min. PCR products were purified using Universal DNA purification kit (TIANGEN, Beijing, China). Purified PCR products were sequenced in 2 directions by GENERAL BIOL Co (Chuzhou, China). Sequence polymorphisms were found by multiple sequence alignment using ChromasPro 1.33.

Linkage Disequilibrium Analysis and Haplotype Reconstruction

SNPs with Hardy-Weinberg equilibrium *P*-value >0.001 and minor allele freq >0.05 were included in the linkage disequilibrium (**LD**) study. LD was analyzed using the Haploview 4.2 software. Haplotype block was defined using the confidence interval algorithm. Twenty-six tag SNPs were picked out in pairwise mode with $r^2 \geq 0.7$ and LOD ≥ 3.0 using the Tagger program in the Haploview 4.2 software. Additional 7 tag SNPs that belong to non-synonymous mutations or were reported to be associated with body weight of chickens were forced into the tag list. *CCKAR* haplotypes were reconstructed using the PHASE 2.1.1 program with the default parameters.

Genotyping Tag SNPs

Thirty-three tag SNPs were genotyped in 675 chickens using Sequenom MassARRAY platform. Multiplex PCR was performed in 384-well plates using PCR mix consisting 10 ng of gDNA, 0.5 μM PCR primer mix, 8 mM $MgCl_2$, 2.5 mM dNTP, and 1 U of hot-start enzyme (Qiagen, Hilden, Germany), 0.625 μL of $10 \times$ PCR buffer and 1.75 μL of ddH$_2$O per reaction. Non-incorporated dNTPs were dephosphorylated by adding 2 μL mixture consisting of 0.3 μL (1.7 U/μL) shrimp alkaline phosphatase (NEB, Ipswich, MA), 0.17 μL SAP buffer (10 $\times$) and 1.53 μL H$_2$O. The dephosphorylated products were then subjected to the single base extension reaction according to the iPLEX Assay protocol (Sequenom, San Diego,CA). The resulting products were dispensed onto a 384-element SpectroCHIP Bioassay with a nanodispenser (Sequenom). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry MassARRAY was performed on a MassARRAY Analyzer Compact. MassARRAY results were analyzed with the SpectroTYPER 4.0 (Sequenom).

Association Analysis

This study fit a univariate linear mixed model (**LMM**) to test association of tag SNPs with body weight in the following form: $y = W\alpha + x\beta + u + \varepsilon$; $u \sim MVN_n(0, \lambda\tau^{-1}K)$, $\varepsilon \sim MVN_n(0, \tau^{-1}I_n)$ where y is an n-vector of BW; W is an $n \times c$ matrix of covariates consisting of a column of 1s and c-1 column of covariates; α is a c-vector of coefficents; x is an n-vector of marker genotypes; β is the effect size of the marker; u is an n-vector of random effects; ε is an n-vector of errors; τ^{-1} is the variance of the residual errors; λ is the ratio of random effect and residual error variances; K is a $n \times n$ relatedness matrix and I_n is a $n \times n$ identity matrix. MVN_n denotes the n-dimensional multivariate normal distribution. The W is a 675-vector of 1s in the LMM for BW16 and BW20, and a 675×2 matrix consisting of a column of 1 s and a column of ages at the onset of egg production in the LMM for BWOEP. Missing genotypes were

imputed based on LD information in 35 resequenced and 675 MassARRAY genotyped samples using the BIM-BAM software prior to association analysis. Missing BW data was predicted using outputs from Bayesian sparse linear mixed model. Relatedness matrix was calculated using the kinship2 library in R program based on the pedigree of 675 chickens. LMMs were fit using the GEMMA software (Zhou and Stephens, 2012). GEMMA performed Wald test to get the effect size estimate for each tag SNPs and the corresponding p value. *P*-values were adjusted using the Benjamini and Hochberg approach to overcome the multiple testing problems. A Benjamini and Hochberg adjusted *P* -value (q-value) <0.05 is considered significant (Benjamini and Hochberg, 1995).

RESULTS AND DISCUSSION

A total of 126 SNP were found in the *CCKAR* and upstream 1.4 kb region, and formed 41 haplotype in the 35 resequenced samples (Figure 1). There were no structural variants in the region. Twenty-four SNP were located in exons, and the others were distributed in promoter and introns (Figure 1). These variants sustained low LD as only 15 short haplotype blocks with an average length of 129 bp were present in the region

(Figure 1). Low LD enables us to map functional variants within the *CCKAR* locus.

Thirty-three SNP that captured 89% of 126 SNP at r^2 ≥0.7 were selected as tag SNPs to map functional variants (Figure 1). Eleven of 33 tag SNPs were significantly associated with BW16, but not with BW20 and BWOEP (Table 1). Most (8/11) of significantly associated variants were distributed in a 2 kb region (chr4:73206169-73208244) around the Exon3 (Table 1). Rs314291631 is a missense mutation in the Exon2. A previous study found that this variant was significantly associated with feed intake of Tianlu Black chicken (Yi et al., 2018). But rs314291631 and another two missense mutations were not associated with BW of LBC, implying that the changes of protein function are not a key factor affecting the growth of LBC (Figure 1; Table 1).

There are 6 tag SNPs distributed within upstream 1.4 kb region of *CCKAR*, a key region controlling gene expression (Figure 1). But none of them were associated with BW of LBC (Table 1). Rs313822901 were reported to disrupt YY1 binding, and was significantly associated with BW of Japanese indigenous chickens (Rikimaru et al., 2013). But this variant was not associated with BW of the LBC (Table 1). Rs315131298 was another marker that was reported to have a significant additive effect on BW (Dunn et al., 2013). We confirmed the association not only for rs315131298, but for other 8
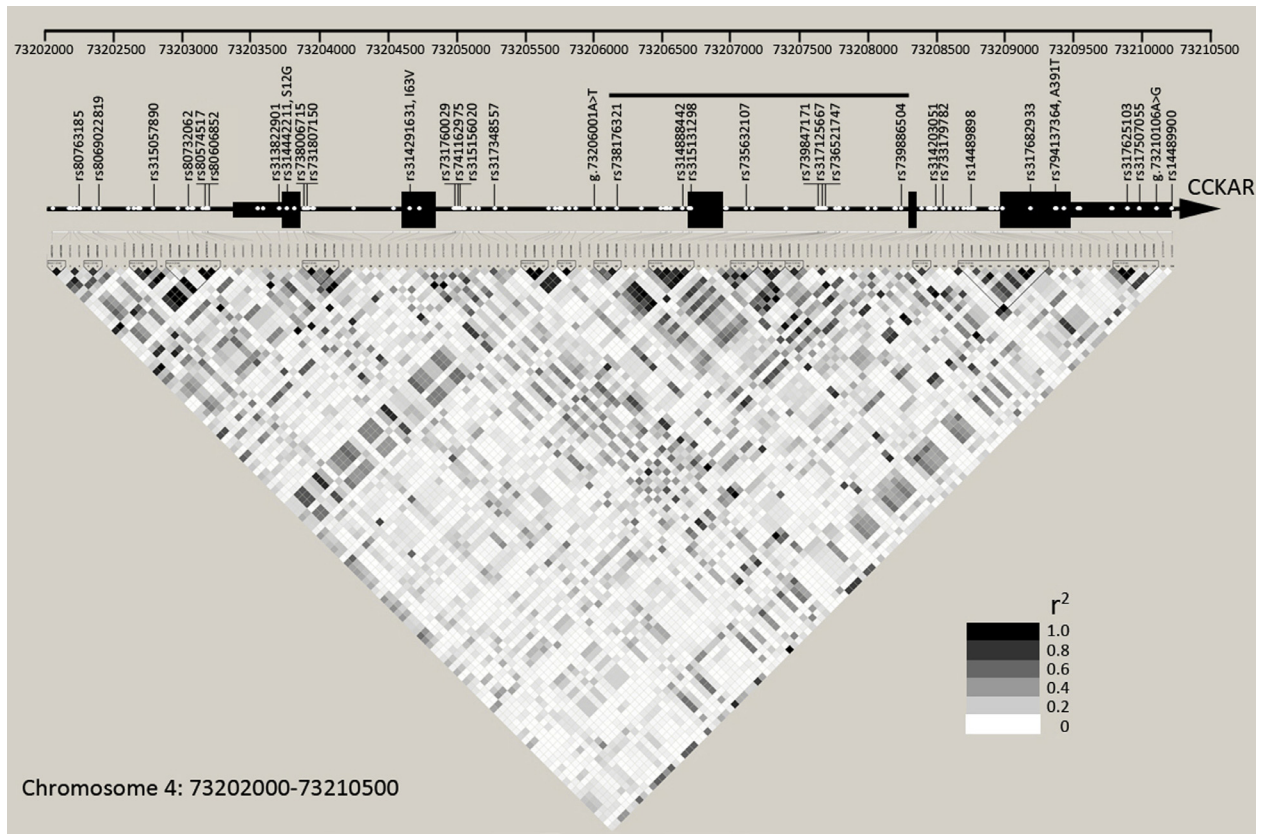

Figure 1. Linkage disequilibrium structure and distribution of sequence variants in the *CCKAR*. This diagram shows the linkage disequilibrium structure of genomic region at Chromosome 4: 73202000-73210500. This region covers the whole *CCKAR* gene and upstream 1.4 kb region from transcription start site. The white points in the gene structure diagram represent 124 variants. The other two variants were excluded from linkage disequilibrium analysis due to minor allele frequencies less than 0.05. Those shown on the gene structure diagram are 33 tag SNPs. The bold black line indicates the region where most (8/11) of significantly associated tag SNPs were distributed. Haplotype blocks are outlined in black triangles on the linkage disequilibrium structure graph.

Table 1. Association test of 33 tag SNPs in the *CCKAR* with body weight of Lueyang black-boned chickens.

dbSNP No.	Description[1]	Position	BW16[3]		BW20[3]		BWOEP[3]	
			Effect size (kg)	q value[2]	Effect size (kg)	q value	Effect size (kg)	q value
rs80763185	g.73202243T>A	Promoter	0.06 ± 0.06	0.45	0.12 ± 0.08	0.32	0.03 ± 0.05	0.72
rs8069022819	g.73202391C>T		0.07 ± 0.10	0.57	0.16 ± 0.13	0.39	0.02 ± 0.07	0.84
rs315057890	g.73202789G>A		0.02 ± 0.05	0.77	-0.03 ± 0.06	0.65	0.02 ± 0.03	0.74
rs80732062	g.73203040C>A		0.18 ± 0.18	0.47	0.28 ± 0.25	0.46	0.05 ± 0.14	0.81
rs80574517	g.73203169T>C		0.01 ± 0.04	0.87	-0.04 ± 0.06	0.60	0.01 ± 0.03	0.81
rs80606852	g.73203188G>A		0 ± 0.01	0.87	-0.01 ± 0.02	0.60	0 ± 0.01	0.81
rs313822901	g.73203705A>C	5'UTR	0.05 ± 0.11	0.75	-0.03 ± 0.15	0.89	0.09 ± 0.08	0.52
rs314442211	g.73203766A>G	Exon1	0.04 ± 0.05	0.52	0 ± 0.06	0.99	0.02 ± 0.03	0.71
rs738006715	g.73203887T>G	Intron1	0 ± 0.03	0.95	-0.03 ± 0.04	0.59	0 ± 0.02	0.90
rs731807150	g.73203909T>C		0.12 ± 0.10	0.39	0.09 ± 0.14	0.64	0.05 ± 0.08	0.66
rs314291631	g.73204661G>A	Exon2	0.14 ± 0.06	0.06	0.18 ± 0.08	0.11	0.06 ± 0.05	0.37
rs731760029	g.73204992G>A	Intron2	0.37 ± 0.20	0.13	0.15 ± 0.28	0.65	0.18 ± 0.15	0.47
rs741162975	g.73205010C>T		0.22 ± 0.14	0.21	0.07 ± 0.20	0.76	0.1 ± 0.11	0.54
rs315156020	g.73205026C>T		0.08 ± 0.04	0.12	0.05 ± 0.05	0.53	0.04 ± 0.03	0.37
rs317348557	g.73205278C>A		0.37 ± 0.13	**0.03**	0.44 ± 0.18	0.11	0.16 ± 0.10	0.34
Novel SNP	g.73206001T>A		0.18 ± 0.10	0.13	0.25 ± 0.13	0.15	0.08 ± 0.07	0.48
rs738176321	g.73206169C>T		0.09 ± 0.03	**0.03**	0.10 ± 0.04	0.11	0.04 ± 0.02	0.34
rs314888442	g.73206650G>A		0.04 ± 0.02	**0.03**	0.04 ± 0.02	0.11	0.02 ± 0.01	0.34
rs315131298	g.73206714C>T	Exon3	0.03 ± 0.01	**0.03**	0.04 ± 0.02	0.11	0.02 ± 0.01	0.34
rs735632107	g.73207113A>G	Intron3	0.21 ± 0.08	**0.03**	0.22 ± 0.11	0.12	0.10 ± 0.06	0.34
rs739847171	g.73207633A>G		0.20 ± 0.08	**0.03**	0.23 ± 0.11	0.11	0.10 ± 0.06	0.34
rs317125667	g.73207665T>C		0.06 ± 0.02	**0.03**	0.07 ± 0.03	0.11	0.03 ± 0.02	0.34
rs736521747	g.73207685T>C		0.09 ± 0.03	**0.03**	0.10 ± 0.05	0.11	0.05 ± 0.03	0.34
rs739886504	g.73208244G>C	Exon4	0.34 ± 0.14	**0.04**	0.44 ± 0.19	0.11	0.17 ± 0.10	0.34
rs314203051	g.73208495C>T	Intron4	0.08 ± 0.04	0.12	0.09 ± 0.05	0.21	0.04 ± 0.03	0.37
rs733179782	g.73208549T>C		0.14 ± 0.06	0.07	0.14 ± 0.09	0.24	0.05 ± 0.05	0.47
rs14489898	g.73208752A>T		0.07 ± 0.06	0.40	0.07 ± 0.08	0.53	0.06 ± 0.04	0.37
rs317682933	g.73209189T>C	Exon5	0.02 ± 0.03	0.48	0.04 ± 0.04	0.46	0.02 ± 0.02	0.61
rs794137364	g.73209364G>A		0.13 ± 0.14	0.48	0.27 ± 0.19	0.31	0.08 ± 0.10	0.61
rs317625103	g.73209893T>C	3'UTR	0.18 ± 0.06	**0.03**	0.18 ± 0.09	0.11	0.10 ± 0.05	0.34
rs317507055	g.73209978A>G		0.09 ± 0.11	0.51	0.11 ± 0.15	0.60	0.01 ± 0.08	0.90
Novel SNP	g.73210106A>G		0.36 ± 0.14	**0.03**	0.47 ± 0.19	0.11	0.19 ± 0.11	0.34
rs14489900	g.73210214A>C		0.18 ± 0.10	0.16	0.13 ± 0.14	0.53	0.08 ± 0.08	0.52

[1] Alleles in front are effect alleles. Physical positions of SNP at the chromosome 4 are given according to the chicken reference genome *Galgal* 6.0 in the UCSC database.

[2] Q-values represent Benjamini and Hochberg adjusted *P*-values. Those shown in bold indicate significant association between tag SNPs and body weight.

[3] BW16, BW20, and BWOEP represent body weight at 16, 20 weeks of age and the onset of egg production.

variants around the rs315131298 (Table 1). In addition, 2 SNPs located in the 3' UTR also showed significant association with BW (Table 1).

These significantly associated tag SNPs together with adjacent 33 SNPs captured by them became promising candidates affecting *CCKAR* expression and the growth of LBC. We predicted effect of the 44 SNPs on TF motifs using the JASPAR database. These variants can disrupt 471 TF motifs. They were widely involved in differentiation of hematopoietic, lymphoid, endothelial, cardiac muscle and bone resorbing cells, regulation of premature reproductive senescence, energy metabolism, tumorigenesis, and development of vascular calcification, metabolic and cardiovascular diseases, etc. Forkhead Box O1 (**FOXO1**) represents one of promising TFs as it is involved in appetite regulation by integrating leptin and insulin signals in hypothalamic neurons (Peng et al., 2020). Three variants (rs316313304, rs737347140, and rs317125667) have a potential disrupting effect on the FOXO1 motif. Although almost nothing is known about relationship between FOXO1 and CCK system, these FOXO1 motif variants can affect avian appetite by regulating *CCKAR* expression given CCK system may play a more important role in appetite regulation in birds than that in mammals (Honda et al., 2017). Androgen receptor (**AR**) and members of the

CCAAT/enhancer-binding protein (**C/EBP**) are another class of TFs that may regulate *CCKAR* expression. AR and C/EBP members are involved in lipid metabolism by regulating expression of IGF-1, leptin, inulin, PPARγ etc. (Staiger et al., 2009; Rana et al., 2011). Variants in TF itself and motifs were widely associated with obesity and related metabolic diseases in humans and mice (Bennett et al., 2010; Rana et al., 2011; Ren et al., 2014). In this study we found that 9 variants potentially disrupted the AR (rs314054492) and the C/EBP (rs318084470, rs741082602, rs14489892, rs735511990, rs317507055, rs739162066, rs315744114, and rs315844714) motifs. CCK signals play an important role in regulation of energy balance (Richards, 2003). Therefore, it is possible that these AR and C/EBP motif variants modulate the growth of chickens by affecting *CCKAR* expression.

This study identified a 2 kb region (chr4:73206169-73208244) around the Exon3 as a promising region containing functional variants affecting the growth of chicken.

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DISCLOSURES

The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Fine mapping of sequence variants associated with body weight of Lueyang black-boned chicken in the cholecystokinin A receptor gene". The authors have no conflicts of interest.

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