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≪Research Note≫

Effect of a Single-Nucleotide Polymorphism in the *Cholecystokinin Type A Receptor* Gene on Growth Traits in the Hinai-dori Chicken Breed

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We have previously reported the association between chicken *cholecystokinin type A receptor* gene (*CCKAR*) haplotypes and growth traits in an F_2 resource population produced by crossing low- and high-growth lines of the Hinai-dori breed. The high-growth line was developed from a low-growth founder from the Preservation Society of the Hinai-dori breed by long-term selection for growth performance at the Akita Prefectural Livestock Experiment Station. In the present study, we determined the effect of a single-nucleotide polymorphism (SNP, AB604331: g.420 C>A) in the 5′-untranslated region of *CCKAR* on the growth traits of the F_2 population. A mismatch amplification mutation polymerase chain reaction assay was developed to distinguish between the 3 genotypes (A/A, A/C, and C/C) in the F_2 population, and the effect of the SNP on growth traits was estimated. The data showed that body weight at 10 and 14 weeks of age, and average daily gain between 4 and 10 weeks, 10 and 14 weeks, and 0 and 14 weeks of age in individuals with the A allele was superior to that in those with the C allele. The electrophoretic mobility shift assay was performed to clarify the contribution of the g. 420 C>A SNP in the predicted YY1 binding site. As a result, the YY1 protein showed a stronger binding affinity for g.420 A probe, suggesting the possibility that the SNP affects transcriptional efficiency of the *CCKAR*. The A allele frequencies in the high- and low-growth lines maintained in fiscal year 2010 were 0.889 and 0.124, respectively. The difference in the allele frequencies of these lines is thought to be caused by long-term selection for growth performance since the probability was too significantly ($P<0.01$) low to be caused by random genetic drift.

Key words: chicken, cholecystokinin type A receptor, growth traits, Hinai-dori breed, single nucleotide polymorphism

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Introduction

The Hinai-dori is a slow-growing breed of chicken native to Akita Prefecture in northern Honshu Island, Japan. Although Hinai-dori meat has a characteristic taste and the breed has been used for a long time, it has decreased in numbers in recent times due to the introduction of exotic breeds and for a while was at risk of extinction. The Hinai-dori breed has been conserved by hobbyists who belong to the Preservation Society (PS) of the Hinai-dori breed and has mainly been used for exhibition purposes. To effectively use the breed, selection experiments have been performed at the Akita Prefectural Livestock Experiment Station (LES) since 1973 when fertilized eggs were introduced at the LES from the PS with a view toward increasing growth performance. To date, the average body weight (BW) of LES males at 300 days of age is approximately 1 kg heavier than that of PS males (Rikimaru *et al*., 2011). F_1 chickens produced by crossing improved LES Hinai-dori sires with Rhode Island Red dams have been commercialized as the Hinai-jidori chicken, which is one of the most popular high-quality chickens on the Japanese market (Rikimaru and Takahashi, 2007).

In the breeding programs for meat-type chickens, breeding for BW and larger gains is a major consideration. Mapping quantitative trait loci (QTLs) and the identification of genes that affect growth traits will greatly enhance progress toward this goal (Goddard and Hayes, 2009; Georges, 2007). We have previously identified QTLs for BW and average daily gain (ADG) in a common region between *MCW0240* (G32009; chr 4: 67.7 Mb) and *ABR0622* (AB186718; chr 4:

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82.9 Mb) on chicken chromosome 4 in an F_2 resource population produced by crossing low- and high-growth lines of the Hinai-dori breed (Rikimaru *et al.*, 2011). We assumed that *cholecystokinin type A receptor* (*CCKAR*, chr 4: 72.8 Mb) is a candidate gene for growth traits because it was located just under the QTL peak on chromosome 4 and has also been identified as a candidate gene for human obesity (Arya *et al.*, 2004). Accordingly, we analyzed polymorphism in the *CCKAR* gene and tested its association with growth traits in the F_2 population. As a result, 5 *CCKAR* haplotypes were identified, and significant associations were observed between *CCKAR* haplotypes and growth traits. We found a single-nucleotide polymorphism (SNP) (A/C) in the predicted YY1 binding site (Shrivastava and Calame, 1994) in the 5′-untranslated region (5′-UTR) of the *CCKAR* gene, and the haplotype data implied that this SNP might be associated with growth traits.

Cholecystokinin (CCK) was the first gut peptide to be implicated in the control of appetite (Gibbs *et al.*, 1973). In mammals, CCK promotes gallbladder contraction, increases the secretion of pancreatic enzymes and bicarbonate, inhibits gastric acid secretion, slows gastric emptying, and reduces food intake (Gibbs *et al.*, 1973; Kissileff *et al.*, 1981). Two G-protein-coupled receptors for CCK have also been identified: CCKAR (Sankaran *et al.*, 1980) and CCK type B receptor (CCKBR) (Innis and Snyder, 1980). Although both receptor subtypes are distributed throughout the central nervous system and gut, CCKAR is predominant in the alimentary tract and CCKBR is predominant in the brain. The functions of CCK have been extensively studied in birds and mammals. For example, intravenous injection of CCK suppresses food intake in chickens (Savory and Gentle, 1980). CCK also modulates intestinal motility (Martín *et al.*, 1995; Martínez *et al.*, 1995; Rodríguez-Sinovas *et al.*, 1997) and biliary flow in the gallbladder of chickens (Duke *et al.*, 1987). Furthermore, CCK stimulates amylase secretion from the pancreas in chickens and ducks (Satoh *et al.*, 1994; Xiao and Cui, 2004). These reports suggest that CCK plays a key role in the regulation of appetite in birds and mammals; however, limited information is available on the functions of the CCK receptors in birds. A binding study using CCK agonists showed that CCKAR is more prevalent than CCKBR in chicken pancreas (Vigna *et al.*, 1986; Rodríguez-Sinovas *et al.*, 1995). Ohkubo *et al.* (2007) reported that the mRNA of CCKAR is mainly located in the alimentary tract of chickens (except for the proventriculus and gizzard), whereas CCKBR mRNA is located predominantly in the brain.

The purpose of the present study was to confirm the association between the SNP in the predicted YY1 binding site of the *CCKAR* gene and growth traits in a Hinai-dori F_2 population, and to test whether the SNP allele frequencies differ significantly between PS and LES populations.

Materials and Methods

Experimental Birds

F_2 Resource Population

F_1 chickens were produced by crossing 3 cocks from a low-growth line (obtained from PS) with 9 hens from a high-growth line (obtained from LES). In this cross, 1 to 3 hens were randomly selected to mate with each male. The F_2 individuals were produced by full-sib mating of 17 F_1 cocks and 60 F_1 hens. We obtained 418 F_2 individuals that included 206 cocks and 212 hens. The F_2 chicks were hatched on the same day, raised in the same chicken house, and fed the same diet *ad libitum* for the duration of the experiment. The chicks were raised in a battery cage until 4 wk of age and were raised in a cage system poultry house until 14 wk of age. The chicks were fed starter diet (ME, 3,000 kcal/kg; CP, 24% (wt/wt)) from 0 to 4 wk, first grower diet (ME, 2,850 kcal/kg; CP, 18%) from 5 to 10 weeks, and second grower diet (ME, 2,900 kcal/kg; CP, 16%) from 11 to 14 wk. Water was provided *ad libitum* for the duration of the experiment. BW was measured at 4 wk (BW-4 wk), 10 wk (BW-10 wk), and 14 wk (BW-14 wk) of age. ADG between 0 and 4 wk of age (ADG 0–4 wk), 4 and 10 wk of age (ADG 4–10 wk), 10 and 14 wk of age (ADG 10–14 wk), and 0 and 14 wk of age (ADG 0–14 wk) was calculated from the BW at each wk of age.

Divergent Selection Lines

A total of 507 individuals in the LES line, including 124 males and 383 females, and 178 individuals in the PS line, including 85 males and 93 females, that were maintained at LES in fiscal year 2010 were used for *CCKAR* genotyping. Feeding and management of the chickens were performed as described above.

CCKAR Genotyping

In the previous study (Rikimaru *et al.*, 2012), 5 *CCKAR* haplotypes (haplotypes 1–5) were identified in the parents of the F_2 population; however, no individuals with haplotype 2 were detected in the F_2 population since, by chance, F_1 individuals with a heterozygous haplotype 2 were not selected for construction of the F_2 population. In addition, since only one 1/5 individual was detected in the F_2 population, this individual was not included in the comparative analyses of *CCKAR* haplotypes. Accordingly, we observed that haplotype 1 was superior to haplotype 3 and 4 in growth traits. Since haplotype 1 has an "A," while haplotype 3 and 4 have a "C" at the g.420 C>A site, this SNP may affect growth. Therefore, we recalculated the SNP effects in the F_2 population.

Genomic DNA was extracted from blood samples using an FTA card (WB120205; GE Healthcare, Buckinghamshire, UK). The blood was dropped on the card and dried at room temperature. Using a Harris Micro Punch with a diameter of 1.2 mm (WB100028; GE Healthcare, UK), a single disk was transferred to each well of a 96-well plate. One hundred microliters of FTA Purification Reagent (WB120204; GE Healthcare, UK) were added, and the sample was incubated for 1 h at room temperature. After the reagent was removed, 100 μL of DNAzol BD reagent (10974-020; Invitrogen, Carlsbad, CA, USA) were added, and the sample was incubated for 30 min. After the reagent was removed, the discs were washed 3 times with 100 μL of sterile deionized water. Then, 100 μL of sterile deionized water were added, the

Table 1. **The primers and target position in the 5′-UTR of *cholecystokinin type A receptor* gene for the mismatch amplification mutation assay**

Primer sets	Primers (5′→3′)	Product (bp)	Target position (AB604331; Takahashi, 2011)	PCR amplification genotype A	PCR amplification genotype C
CCKAR-Hinai1	GAATGTGTGTCTGCGTGCTT GGATCCACAGGTTAGCTGCgAt	225	g.420 C＞A	＋	−
CCKAR-Hinai2	GAATGTGTGTCTGCGTGCTT GGATCCACAGGTTAGCTGCgAg	225	g.420 C＞A	−	＋

The underlined bases shown in lower case represent induced mismatches. The bases shown in lowercase only at the 3′ end represent the target single nucleotide polymorphisms (SNPs). The genotypes that could and could not be amplified by PCR are shown as "＋" and "−", respectively.

sample was heated to 90℃ for 10 min, and the water containing DNA was collected.

To detect an SNP (AB604331: g.420 C＞A) in the YY1 binding site in the 5′-UTR of the *CCKAR* gene, 2 sets of polymerase chain reaction (PCR) primers (Table 1) were designed for a mismatch amplification mutation assay (Cha *et al*., 1992). PCR amplification was performed in an 8-μL reaction volume, which included 2 pmol of each primer, and 4 μL of 2× PCR mix (EmeraldAmp; Takara, Otsu, Japan), and 1 μL of DNA template. Reactions were performed in a 96-well plate in a thermal cycler (GeneAmp System 9700; Perkin-Elmer, Foster City, CA, USA) using the following conditions: 30 cycles of 98℃ for 10 s, 65℃ for 30 s, and 72℃ for 30 s. PCR products (2 μL each) were then electrophoresed on a 2.0% agarose gel with 1× Tris-acetate EDTA (TAE) buffer at 150 V for 30 min in a horizontal gel electrophoresis apparatus (BE-548B; BIO CRAFT, Tokyo, Japan) and stained with ethidium bromide for 30 min.

Electrophoretic Mobility Shift Assay (EMSA)

To test the hypothesis that the g.420 C＞A SNP disrupts YY1 binding *in vitro*, we performed an EMSA. Pairs of unmodified and 5′-biotinylated 30-bp oligonucleotides were designed for the 2 g. 420 C＞A alleles (g.420 A: 5′-AAACCAGCCTTCTTCATAGCAGCTAACCTG-3′ and AB604331: g.420 C: 5′-AAACCAGCCTTCTTCCTAGCAGCTAACCTG-3′). The complementary oligonucleotides were annealed in 10 mM Tris-HCl (pH 7.5), 50 mm NaCl, and 1 mm EDTA to form double stranded probes for the EMSA. The biotinylated double stranded probes (20 fmol) were incubated with 1 μg of YY1 overexpression lysate (LY401166; OriGene, Rockville, MD, USA) using an EMSA kit from Signosis (Sunnyvale, CA, USA) according to the manufacturer's instructions. Competition experiments were performed using a 10- and 100-fold molar excess of unlabelled probe. Negative control reactions did not contain the YY1 overexpression lysate. The reaction mixtures were analyzed by 6% polyacrylamide gel electrophoresis using a vertical gel apparatus in 0.5× Tris-borate-EDTA (TBE) buffer at 120 V for 90 min (AE-6530; ATTO, Tokyo, Japan), and transferred to NB membranes (Signosis) using a tank transfer apparatus (BE-351; BIO CRAFT) according to the manufacturer's instructions. Biotinylated oligonucleotides were detected on the membranes with an EMSA kit and a FluorChem imaging system (IS-8900; Alpha Innotech, San Leandro, CA, USA). All samples were tested in duplicate.

Statistical Analysis

F_2 Resource Population

A mixed-inheritance animal model was used to evaluate the effects of genotypes on traits in the F_2 population. The snp_ad option of the Qxpak software (Pérez-Enciso and Misztal, 2004) was used for the g.420 C＞A SNP. The assumed model for the phenotypic data of each trait was as follows:

$$y_i = sex_{ij} + C_a a + C_d d + u_i + e_i$$

where y_i is the phenotype of the i-th animal; sex_{ij} is the fixed effect of sex j (2 classes) for animal i; u_i is the infinitesimal genetic effect of animal i, which is distributed as $N(0, \mathbf{A}\sigma_u^2)$ (A is the numerator relationship matrix); and e_i is the residual effect for animal i. The additive effect (a) is a covariate coefficient with C_a being values −1, 0, and 1, and the dominance effect (d) is a covariate coefficient with C_d being values 0, 1, and 0, for genotypes A/A, A/C, and C/C, respectively. Pedigrees of base population animals were traced back to the parental population to create the numerator relationship matrix, and a total of 520 animals were included in this study. Likelihood ratio tests were performed by not including the additive and dominance effects in the model, and nominal P-values were obtained by assuming a χ^2 distribution of the likelihood ratio test.

The proportion of additive genetic variance accounted for by the genotypic effect was calculated as:

$$variance\ percentage = [2pq\,(a + d(q - p))^2]/V_A$$

where p and q are the frequencies of alleles A and C, respectively, and V_A is the additive genetic variance of the trait obtained from animal model analysis by ignoring the g.420 C＞A genotype effects (Falconer and Mackay, 1996).

Divergent Selection Lines

The 14-week body weight in the LES line was corrected for the effect of sex, and the 14-week body weight in the PS line was corrected for the effect of sex and hatch. The sex and hatch effects were estimated using the Best Linear Unbiased Estimator (BLUE). The assumed model for BLUE in each line was as follows:

LES: y = sex + additive + dominance + polygene + e

Table 2. **Phenotypic values for growth traits and the effects of the g.420 C>A genotype in the YY1 binding site in the 5′-UTR of *cholecystokinin type A receptor* gene on the traits of the F_2 population**

Trait	n	Phenotypic values	LRT	*P* value	Additive effects	Donimance effects	Va	Vsnp	h^2	Vsnp /Va (%)	SNP effect (%)
		Mean±SD			Mean±SE	Mean±SE					
BW-4 week (g)	417*	231.1±38.3	1.5	n.s.	—	—	—	—	—	—	—
BW-10 week (g)	418	960.6±163.5	36.7	1.1×10^{-8}	49.1±8.1	−3.4±10.8	9231.0	1209.6	0.56	13.1	7.3
BW-14 week (g)	418	1,466.9±260.7	55.2	1.1×10^{-12}	83.0±11.0	−2.6±14.6	18137.9	3427.0	0.57	18.9	10.8
ADG 0-4 week (g/day)	417*	5.2±1.0	1.3	n.s.	—	—	—	—	—	—	—
ADG 4-10 week (g/day)	417*	17.4±3.3	46.2	9.3×10^{-11}	1.1±0.2	0±0.2	3.7	0.6	0.54	17.2	9.3
ADG 10-14 week (g/day)	418	18.1±4.4	45.0	1.7×10^{-10}	1.2±0.2	0±0.2	1.8	0.7	0.25	42.0	10.4
ADG 0-14 week (g/day)	418	14.6±2.7	55.0	1.1×10^{-12}	0.8±0.1	0±0.1	1.9	0.4	0.57	18.9	10.7

BW-4 week, BW-10 week, and BW-14 week: body weight measured at 4 weeks, 10 weeks, and 14 weeks of age. ADG 0–4 week, ADG 4–10 week, ADG 10–14 week, and ADG 0–14 week: average daily gain between 0 and 4 weeks, between 4 and 10 weeks, between 10 and 14 weeks, and between 0 and 14 weeks of age. SD: standard deviation. SE: standard error. LRT: loglikelihood ratio test statistics, n.s. not significant. Va: genetic variance. Vsnp: SNP variance. h^2: heritability. Vsnp/Va: the percentage of SNP variance to genetic variance. SNP effect: the percentage of SNP variance to phenotypic variance. Additive and dominance effects are genotypic value of (AA−CC)/2 and AC−(AA+CC)/2 at g.420 C>A, respectively.
* Since BW-4 weeks has a missing datum, ADG 0–4 week and ADG 4–10 week also have a missing datum.

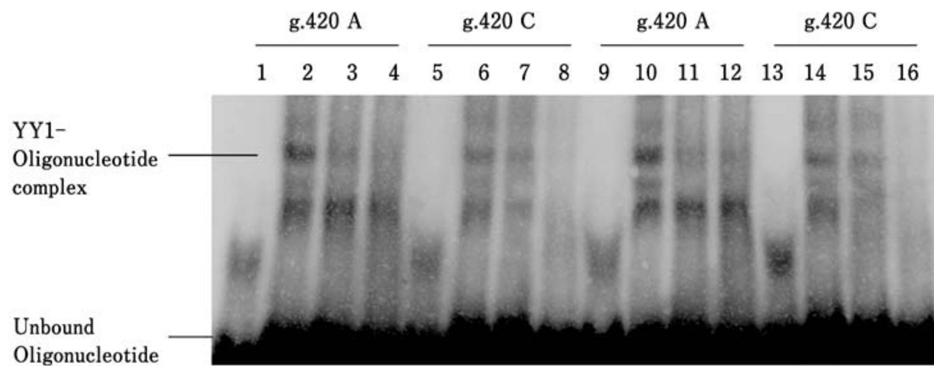

Fig. 1. **Electrophoretic mobility shift assay for YY1 binding to DNA containing the g. 420 C>A alleles.** Lanes 1–4 contain: (1) biotinylated g.420 A probe alone; (2) biotinylated g.420 A probe and YY1; (3) biotinylated g.420 A probe, YY1, and a 10-fold excess of unlabeled competitor probe; (4) biotinylated g.420 A probe, YY1, and a 100-fold excess of unlabeled competitor probe. Lanes 5–8 contain: (5) biotinylated g.420 C probe alone; (6) biotinylated g.420 C probe and YY1; (7) biotinylated g.420 C probe, YY1, and a 10-fold excess of unlabeled competitor probe; (8) biotinylated g.420 C probe, YY1, and a 100-fold excess of unlabeled competitor probe. Lanes 9–16 show the results from an independent duplicate experiment, with lanes 9–16 corresponding to lanes 1–8, respectively.

PS: y=sex+hatch+additive+dominance+e

The genotype and allele frequencies of the g.420 C>A SNP were calculated from observed genotypes. The effective population size (Ne) of the LES and PS line was calculated according to Falconer and Mackay (1996).

Results and Discussion

The effects of the g.420 C>A SNP on the growth traits of the F_2 population are shown in Table 2. This SNP was significantly associated with BW-10 wk, BW-14 wk, ADG 4–10 wk, ADG 10–14 wk, and ADG 0–14 wk in the F_2 population, and the A allele was significantly superior to the C allele in these traits. The SNP effects on the genetic and phenotypic variance in these traits accounted for 13.1% to 42.0% and 7.3 % to 10.8%, respectively. These data suggest that *CCKAR* gene is associated with growth traits, and the allele A is superior to the allele C in the Hinai-dori breed, although we do not deny that the associations detected in the present study may be produced by linkage disequilibrium between the SNP and another linked DNA polymorphism directly involved in

Table 3. **Genotype and allele frequency of the g.420 C＞A genotype in the YY1 binding site in the 5′-UTR of *cholecystokinin type A receptor* gene**

Line	*n*	14-wk body weight, g* Mean±SD	g.420 A＞C genotype			Allele	
			AA	AC	CC	A	C
LES	507	2023.9±284.5	0.781	0.215	0.004	0.889	0.111
PS	178	1199.4±211.4	0.017	0.213	0.770	0.124	0.876

High-growth line obtained from the Akita Prefectural Livestock Experiment Station (LES).
Low-growth line obtained from the Preservation Society (PS) of the Hinai-dori Breed.
* The 14-wk body weight in the LES line was corrected for the effect of sex, while 14-wk body weight in the PS line was corrected for the effect of sex and hatch. The sex and hatch effects were estimated using the Best Linear Unbiased Estimator (BLUE). The assumed model for BLUE of each line was as follows: LES: y＝sex＋additive＋dominance＋polygine＋e, PS: y＝sex＋hatch＋additive＋dominance ＋e.

the regulation of these traits.

The results from the EMSA showed that YY1 protein clearly bound to oligonucleotides A and C and that the protein had a stronger binding affinity for oligonucleotide A (Fig. 1). By calculating the average pixel values of the bands associated with oligonucleotides A and C, the binding of YY1 protein to A was estimated to be 78% higher than binding to C.

The g. 420 C＞A SNP in the predicted YY1 binding site might be associated with functional effects. One hypothesis that might explain the observed association between *CCKAR* genotypes and growth traits is that g. 420 A represses the transcriptional activity of the *CCKAR* gene, leading to a reduced satiety effect and increased appetite and growth. In pigs, Houston *et al*. (2006) reported that a low-affinity YY1 binding site in the 5′-UTR of the *CCKAR* gene might be related to high growth performance. Meanwhile, our data showed that a high affinity YY1 binding site in the 5′-UTR of the *CCKAR* gene might be related to high growth performance in chickens. Taken together, these data suggest that YY1 plays an important role in regulating *CCKAR* gene expression, and that the action of YY1 varies according to animal species. Further experiments are required to test our hypothesis, including a comparison of mRNA levels between alternative genotypes.

The genotype and allele frequencies at the g.420 C＞A site in the LES and PS lines are shown in Table 3. The frequency of the A/A genotype was highest (0.781) in the LES line, while the frequency of the C/C genotype was highest in the PS line. The A allele frequencies in the LES and PS lines were 0.889 and 0.124, respectively.

The LES line was separated from the PS line in 1973, and since then, each line has been reared in a small closed population. We have to verify whether the marked difference in the A allele frequency between PS and LES lines occurred by random genetic drift. There are no records for the breeding structure of the PS line. However, the number of hatched chicks was approximately 3,000 in 1980s, and has decreased every year, and was 420 in 2012. Therefore, the present number of birds used for breeding, namely, 61 cocks and 145 hens (Ne＝171.7) is thought to be the minimum for the whole period. The number of hatched chicks in the LES line was constant at approximately 1,000 throughout this time period. However, we only have detailed records for the breeding structure over the most recent 5 years. The number of birds used for breeding each year during this period was 19–20 cocks and 99–151 hens. Since selection during this period was stronger than during earlier generations, the size of breeding population must be smaller than that in earlier generations. The minimum population size of the LES line was thought to be 19 cocks and 99 hens (Ne＝63.8).

We calculated the probability that the difference in the A allele frequency in the 2 idealized populations (Falconer, 1981) of Ne＝63 and Ne＝171 became larger than 0.765 (＝ 0.889–0.124) after 37 generations due to random genetic drift. The probability was calculated as 0.0036. This criterion was strict enough; however, to confirm the result, we recalculated the probability due to random genetic drift using a more strict criterion of Ne＝140 (50 cocks and 120 hens for PS) and Ne＝56 (17 cocks and 80 hens for LES), and after 40 generations. As a result, the probability was calculated as 0.0098. This suggests that the A allele difference between the PS and LES lines was not caused by random genetic drift, but by the long-term selection for growth performance in the LES line. As shown above, since the effects of the g.420 C＞A SNP on the genetic and phenotypic variances in growth traits are relatively large, individuals that have an A allele might be chosen without the SNP information.

In conclusion, we confirmed that an A allele at the g.420 C ＞A site in the 5′-UTR of the *CCKAR* gene was superior to a C allele for BW-10 wk, BW-14 wk, ADG 4–10 wk, ADG 10–14 wk, and ADG 0–14 wk in the F_2 population. This suggested that the difference in the g.420 C＞A allele frequency between the LES and PS lines was due to the long-term selection of growth performance.

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