

POU class 1 homeobox 1 gene polymorphisms associated with growth traits in Korean native chicken

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Objective: POU class 1 homeobox 1 (*POU1F1*) mediates growth hormone expression and activity by altering transcription, eventually resulting in growth rate variations. Therefore, we aimed to identify chicken *POU1F1* polymorphisms and evaluate the association between single nucleotide polymorphisms (SNPs) and growth-related traits, and logistic growth curve parameter traits (α , β , and γ).

Methods: Three SNPs (M_1 to M_3) were used to genotype 585 F_1 and 88 F_0 birds from five Korean native chicken lines using a polymerase chain reaction–restriction fragment length polymorphism method.

Results: Single marker analyses and traits association analyses showed that M_2 was significantly associated with body weight at two weeks, weight gain from hatch to 2 weeks, and weight gain from 16 to 18 weeks ($p < 0.05$). M_3 was significantly associated with weight gain from 14 to 16 weeks and from 16 to 18 weeks, and asymptotic body weight (α) ($p < 0.05$). No traits were associated with M_1 . The *POU1F1* haplogroups were significantly associated with weight gain from 14 to 16 weeks ($p = 0.020$). Linkage disequilibrium test and Haploview analysis shown one main haploblock between M_2 and M_3 SNP.

Conclusion: Thus, *POU1F1* significantly affects the growth of Korean native chickens and their growth curve traits.

Keywords: Growth Traits; Korean Native Chicken; *POU1F1* Gene; Association

INTRODUCTION

Recently, rare and heritage animal breeds and their genetic resources have become more important as they offer economically viable and distinct meat quality characteristics. One such example is the Korean Native Chicken (KNC) that is growing in popularity with consumers because of its unique meat qualities. However, native chicken lines tend to have lower growth rates and production performances than commercial breeds. Improving growth rate and production performance in KNC breeds is an increasingly important issue for the Korean chicken industry.

Traditional quantitative genetics approaches cannot discriminate among the effects of sets of genes associated with variations in quantitative traits. However, the recent developments in molecular biology technologies, such as quantitative trait locus analysis and candidate gene approaches have provided better tools for identifying functional genes, and for applying the obtained knowledge in breeding programs to improve economically important traits in animals [1]. Information on functional genes helps the selection of animals with favourable breeding traits and increases the efficiency of selecting for improvements in growth rates, body weights, and carcass traits [2].

POU class 1 homeobox 1 (*POU1F1*) gene is considered as a strong candidate among genes

affecting variation in animal growth rates. *POU1F1* has a POU-domain binding domain and can trans-activate the promotor region of growth hormone (*GH*), prolactin (*PRL*), thyroid stimulating hormone chain (*TBSH*) encoding genes and *POU1F1* itself in the anterior pituitary [3].

POU1F1 is a tissue specific transcription factor that regulates gene expression, particularly in somatotrophs, lactotrophs, and thyrotrophs, which are responsible for growth related hormone secretion [4]. The cDNA sequence of chicken *POU1F1* has been identified [5]. According to the chicken genome assembly *Gallus_gallus-5.0* (Gene ID: 374215), *POU1F1* is located on chromosome 1 and is 14 kb long. Single nucleotide polymorphisms (SNPs) in *POU1F1* have been identified in swine, bovine and chickens and their associations with growth, meat quality, and fatty acid profile have been analyzed [1,6-8]. A non-synonyms SNP (A>T, Asn229Ile) was identified in the POU domain and showed significant association with body weight in 8-week-old chickens [6]. Similarly, Nie et al [9] reported four SNPs and a 57 bp indel polymorphism that were significantly associated with early growth and body weight of a Chinese breed of chicken. Mutation of chicken *POU1F1* may control the expression and activity of growth hormones by altering transcription rates; these alterations eventually result in growth variations. Given the pivotal role of these hormones in growth, *POU1F1* can be considered as a vital candidate gene for production traits in chicken. In this light, the objective of this study was to identify SNPs in *POU1F1* and evaluate their associations with growth related traits (body weight [BW], weight gain [GR], and growth curve parameter traits) in the KNC.

MATERIALS AND METHODS

Animal care

All practices and procedures in this study strictly followed “The Guide for Care and Use of Laboratory Animals” published by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (NIAS) (2012-C-037) in Korea.

Chicken population

The chicken population described in previous studies was used [10-12]. It consisted of five F_1 lines of KNCs: Black line (88 chickens), Grey line (111 chickens), Red line (135 chickens), White line (121 chickens), and Yellow line (130 chickens), and the parents (F_0 , 88 chickens). All the birds were nurtured under the standard feeding and environmental conditions of the National Institute of Animal Science (NIAS) Korea. Phenotypes were measured during the experiment period as part of the breeding program. The body weight of each individual was measured at two-week intervals from hatching to 20-week-old and used to calculate the weight gain in two-week intervals.

The longitudinal body weight data were fitted to a logistic growth model ($W_{(t)} = \alpha/[1+\beta e^{-\gamma t}]$) to obtain three growth curve parameters: α = asymptotic body weight; β = the log-function for the proportion of the asymptotic mature weight to be gain after birth (week); γ = scale proportional to the overall growth rate. For the molecular analysis, blood samples were collected in ethylene diamine tetra acetic acid containing tubes and were provided by NIAS Korea. Genomic DNAs were isolated according to the published method [13]. The purity and concentration of the DNAs were assessed by spectrophotometry method (NANODrop 2000, Thermo Scientific, Wilmington, DE, USA). Stock DNAs were diluted with ultrapure water to produce a working concentration of 25 ng/ μ L and stored at -20°C until use.

Genotyping of SNPs in *POU1F1*

Three SNP polymorphisms of chicken *POU1F1* gene were genotype by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method. SNPs were named as M_1 to M_3 . The M_1 SNP is a mutation at g. 6758 T>C in exon 5 of *POU1F1* and not reported previously. The SNP M_2 (g. 9432T>C) and M_3 (g.11041T>C) were referred to gene bank accession number: rs13687127, rs13687128, respectively and were previously reported in chicken [9] (Table 1). All the Three SNPs were first screened to confirm by using the next generation sequence data of KNCs parents’ population. Three pairs of PCR primers were designed based upon the chicken *POU1F1* gene sequences using Primer 3.0 software (Table 1). PCR was performed to amplify sequence fragments of the markers (Thermal cycler T100, Bio-Rad, Hercules, CA, USA) in exons 5, 6, and intron 5 of *POU1F1* gene. The PCR mixture (20 μ L) contained 50 ng/ μ L of chicken genomic DNA, Prime PCR buffer (Genetbio Inc., Daejeon, Korea), 1.6 μ L dNTPs, 0.2 U *Taq* DNA polymerase (Genetbio Inc., Korea), 0.8 pmol of each forward and reverse primer (M_1 to M_3), and distilled water. PCR was performed using the following PCR thermal cycler conditions. Initial denaturation at 94°C for 10 min; 34 to 35 cycles of 94°C for 30 s, specific annealing temperature for each marker (60°C to 67°C) for 30 s, and 72°C for 30 s; and final extension at 72°C for 10 min. The PCR products were digested at 37°C for 6 to 12 h with *HhaI*, *EcoRI*, or *BspHI* (Table 1). The digestion mixture (20 μ L) contained 15 μ L PCR product, 1 \times digestion buffer, two units of each enzyme and distilled water. Genotypes of M_1 to M_3 were determined using an ultraviolet trans illuminator after 3.0% agarose gel electrophoresis of the digestion mixture at 120 V for 30 minutes.

Statistical analysis

Genotype and allele frequencies were calculated by the direct counting method [14]. Hardy–Weinberg equilibriums for KNC population was analysed using Chi Square (χ^2) tests with a significance level of $p < 0.05$, using the ‘Hardy–Weinberg’

Table 1. PCR-RFLP primers and restriction enzymes used to identify *POU1F1* gene polymorphisms

Marker name	Location ¹⁾	Region	Primer (Forward/Reverse) (5'-3')	Restriction enzyme	Amplicon (bp)
<i>M_1</i>	g.6758T > C	Exon 5	AGTATAGCTCTGTGGTGAC TATGCCCTCAGATGCCAG	<i>HhaI</i>	626
<i>M_2</i>	g.9432T > C	Intron 5	GGGGATTTGCCACTTAGGG TGGTAAGGCTCTGGCACTGT	<i>EcoRI</i>	442
<i>M_3</i>	g.11041T > C	Exon 6	GGGGTACCACTCAACTCAG TAGGGTACCTGCAATGGGG	<i>BspHI</i>	750

PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; *POU1F1*, POU class 1 homeobox 1.

¹⁾ Nucleotide positions are numbered according to the first base of gene as it appears in GenBank. *M_2* and *M_3* were refer to NCBI accession number of rs13687127, and rs13687128, respectively. *M_1* marker identified in Korean Native Chickens.

package in R version 3.2.5 [15]. Based on the allele distribution results, all the lines were pooled. Single SNP marker association analyses were performed with the general linear model procedure of analysis of variance in Minitab version 15 [16]. Haplotype inferences and haplotype frequencies were obtained using Phase 2.1 software [17] and the Haploview program [18]. Associations between haplogroups and traits were calculated using same statistical model by replacing Genotype_i to Haplogroup_i term in model.

Linear mixed model analysis was performed using following mathematical model;

$$Y_{ijklmno} = \mu + Genotype_i + Sex_j + Batch_k + Line_l + Sire_{m(line)} + Dam_{n(lineSire)} + e_{ijklmno}$$

Where, $Y_{ijklmno}$ is the response of each growth trait, μ is the overall mean, Sex_j is the fixed effect of the i^{th} sex (i = male, female), $Batch_k$ is the fixed effect of the k_{th} batch (k = 1, 2), $Line_l$ is the fixed effect of the l^{th} line (l = 1, 2, 3, 4, 5), $Sire_{m(line)}$ is the effect of the m^{th} sire nested in the l^{th} line, $Dam_{n(lineSire)}$ is the effect of the n^{th} dam nested in the l^{th} line and m^{th} sire, and $e_{ijklmno}$ is the random residual effect. The level of significance for an association was taken and pairwise comparisons between genotypes were obtained using Tukey’s test at p value < 0.05. Regression analyses of traits and SNPs were performed to obtain regression coefficients for dominance and additive effects [14]. To calculate the dominance and additive effects of each SNP, “genotype effect” in the model was replaced by dominance effect (X_{dom}), (genotypes were coded as 0-1-0 for CC, CT, TT, respectively) and additive effect (X_{add}) (coded as 0, 1, for two homozygous and 2 for heterozygous based on the minor allele frequency) [19].

RESULTS

Genotypes and haplotype inference

In this study, we describe three SNPs (*M_1* to *M_3*) in the KNC (Figure 1); two of these SNPs have previously been identified in the reciprocal crosses of white recessive rock male and Chinese Xinghua female chicken breed [9]. Digestion of PCR

fragment of the *M_1* SNP in exon 5 with *HhaI*, produced fragments lengths of 626 bp for the TT genotype, and 417 bp and 209 bp for the CC genotype and 626,417,209 for CT genotype. The CC, CT, and TT genotype frequencies of *M_1* were 0.86, 0.10, and 0.04, respectively. The corresponding C and T allele frequencies were 0.91 and 0.09, respectively. The *M_2* SNP in intron 5 (genebank accession number rs13687127) PCR product was digested with *EcoRI*, produced a 442 bp fragment for the CC homozygote, 246 and 196 bp fragments were in the TT homozygote and 442, 246, and 196 bp in heterozygous CT genotype. The frequency of the CC genotype was 0.87 while the frequency of the CT and TT genotypes were 0.11 and 0.022, respectively. PCR product of *M_3* SNP (genebank accession number rs13687128) produced fragments of 283, 251, and 216 bp for CC the genotype and 467 bp and 283 bp for the TT genotype and 467,283,251, and 216 bp for heterozygous CT genotype after digest with *BspHI*. In *M_3* SNP, CC genotype was most frequent (0.83) and the TT genotype was least frequent (0.03); the CT genotype also occurred at a low frequency value of 0.14.

Genotypes and allele frequencies for all markers (*M_1* to *M_3*) and Chi Square (χ^2) tests for Hardy-Weinberg equilibrium were calculated and are summarized in Table 2. The CC genotype was more frequently observed in KNC than in other genotypes, and the C allele frequency was higher than that of the T allele in all SNPs. None of the SNPs was in Hardy-Weinberg equilibrium in KNC population.

Four haplotypes were reported in this study. Among these, CCC (H1) was the major haplotype (88.01%, 1,029/1168), three minor haplotypes were also found at frequencies of lower than 1%: CTT, TCC, TTT (H4 (32/1,168), (H5 49/1,168), and H6 (58/1,168), respectively).

Log of the likelihood odds ratio scores (LOD), a pairwise measure of linkage disequilibrium $D' = |D|/D_{max}$ [20] between the three SNP loci were estimated. Higher D' indicates a low recombination rate between the loci. The D' , LOD, and regression coefficient (r^2) values between *M_1* and *M_2* were 0.596, 36.83%, and 0.294, respectively; between *M_1* and *M_3*, they were 0.563, 32.63%, and 0.314, respectively. The highest D' and r^2 values were found between *M_2* and *M_3* and were

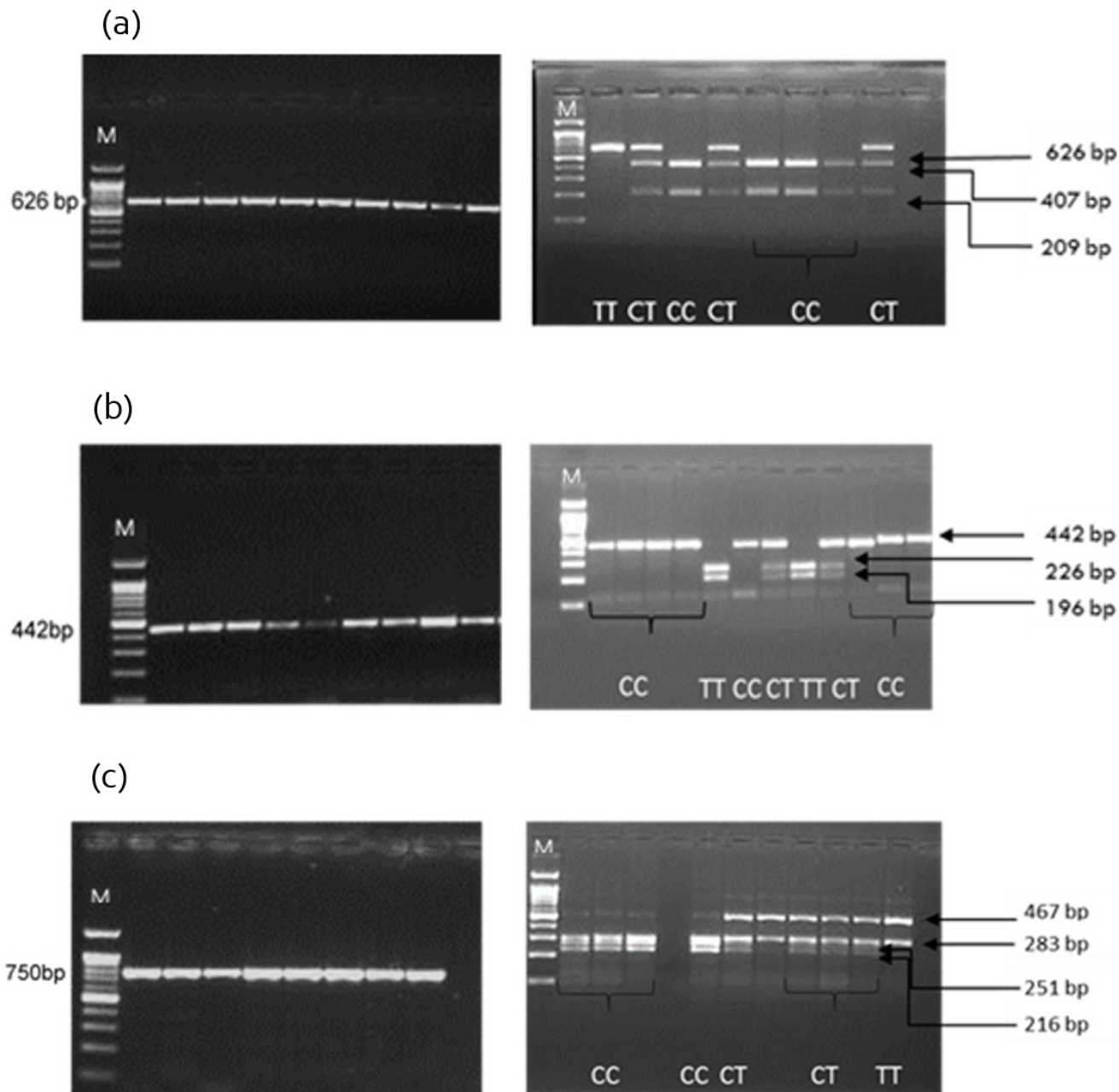


Figure 1. PCR-RFLP patterns for the SNPs in *POU1F1*. (a) Genotypes of *M*₁ (g.6758T>C) digested with *HhaI*, (b) genotypes of *M*₂ (g.9432T>C) digested with *EcoRI*, (c) genotypes of *M*₃ (g.11041T>C) digested with *BspH1*. PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; SNP, single nucleotide polymorphism; *POU1F1*, POU class 1 homeobox 1.

Table 2. Genotype and gene frequency of *POU1F1* SNP polymorphisms in Korean native chickens¹⁾

Item	Genotype frequency			Allele frequency		χ^2 test ²⁾	
	CC	CT	TT	C	T	χ^2	p<0.005
<i>M</i> ₁	0.858	0.101	0.041	0.908	0.092	85.92	0.000
<i>M</i> ₂	0.868	0.110	0.022	0.923	0.077	27.84	1.316e-7
<i>M</i> ₃	0.844	0.130	0.026	0.909	0.091	23.82	1.059e-06

POU1F1, POU class 1 homeobox 1; SNP, single nucleotide polymorphism.

¹⁾ SNP position in *POU1F1* gene, based on gene counting method genotypes and allele frequency for SNPs were shown in each row.

²⁾ Chi Square test for Hardy-Weinberg equilibrium for each SNP in the sampled population.

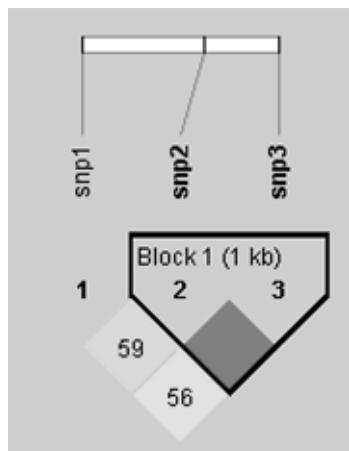


Figure 2. Linkage disequilibrium (LD) in the *POU1F1* SNP haplotype block. Haplotype frequency and pairwise measures of LD (D'), which represent the degree of LD between two blocks, are shown for Korean native chickens. Observation of D' values indicate one main haplotype block covering the SNP2 to SNP3 region in *POU1F1* gene (SNP1, g.6,758T>C; SNP2, g.9,432C>T; SNP3, g.11,041C>T). *POU1F1*, POU class 1 homeobox 1; SNP, single nucleotide polymorphism.

0.999, and 0.836, respectively (Figure 2).

Association of SNPs with growth traits

A single marker trait analysis showed that M_1 SNP was not significantly associated with any of the growth traits tested in KNC. Whereas, M_2 SNP was significantly associated with body weight at two weeks (BW02), weight gain from hatch to two weeks (GR0-2), weight gain from 16 to 18 weeks (GR16-18) ($p < 0.05$), and with asymptotic body weight (α) ($p < 0.1$) (Table 3). The highest BW02 and GR0-2 were found in the TT type animals, while the lowest was observed in the CT type. However, for weight gain, GR16-18, the CC animals had a

higher weight gain than the TT animals, while the CT type showed moderate weight gain. The M_3 SNP was significantly associated with weight gain from 14 to 16 weeks (GR14-16), weight gain from 16 to 18 weeks (GR16-18), and with asymptotic body weight (α) ($p < 0.05$). The CC type had the highest weight gain and asymptotic body weight (α), while TT type had the lowest values. Overall, GR14-16, GR16-18, and α trait in the CC animals were significantly different from those in the TT animals by 19%, 25%, and 1.1%, respectively (Table 3).

Association between haplotypes and growth traits

We screened the haplotypes of the 584 individuals in this study and found four haplotypes and eight diploids: H1H1 (482), H1H2 (14), H1H3 (14), H1H4 (37), H2H2 (5), H2H4 (8), H3H3 (11), H3H4 (13). The haplogroup H1H3 was associated with GR14-16 and had higher weight gain than those of the H1H4 and H2H2 (Supplementary Table S3).

DISCUSSION

This study was initiated to identify polymorphisms in the chicken *POU1F1* gene that were related to growth, growth curve parameters, and weight gain traits in KNC. Previous studies have examined *POU1F1* of domestic animals and identified its polymorphisms associated with growth traits [8,21]. Among the SNPs reported to be associated with growth related traits in chickens, most had significant effects [6,9,22].

Variability in allele frequencies among populations indicates genetic differences in their base population [23]. The genotype frequencies for all three SNPs in our study did not conform to the Hardy-Weinberg equilibrium. Especially, in line G, W, and Y only observed homozygous CC allele type for all SNP. While R and L lines were consisted all the three gen-

Table 3. Association of *POU1F1* polymorphisms and growth traits (least square means)

Trait ¹⁾	Genotype of M_2			p-value	Effect	
	CC(507)	CT(64)	TT(13)		Additive	Dominance
BW02 (g)	142.41 ± 1.13 ^a	134.70 ± 2.27 ^b	143.68 ± 4.42 ^a	0.032	7.89	-8.345
GR0-2 (g)	103.66 ± 1.07 ^a	96.59 ± 2.64 ^b	106.37 ± 3.60 ^a	0.008	2.73	-8.425
GR16-18 (g)	218.04 ± 5.10 ^a	178.95 ± 24.0 ^b	148.56 ± 22.3 ^b	0.016	-28.80	-0.042
α (g)	7.679 ± 0.018	7.598 ± 0.077	7.598 ± 0.077	0.062	-	-
Genotype of M_3						
	CC(493)	CT(76)	TT(15)			
GR0-2 (g)	103.48 ± 1.07	98.52 ± 2.67	105.21 ± 4.30	0.081	-	-
GR14-16 (g)	217.93 ± 3.53 ^a	182.78 ± 8.51 ^{ab}	174.57 ± 17.8 ^b	0.043	-11.95	-13.47
GR16-18 (g)	217.93 ± 5.10 ^b	185.32 ± 224.70 ^b	162.97 ± 20.50 ^a	0.040	-24.95	-5.13
γ (wk)	0.228 ± 0.001	0.239 ± 0.003	0.228 ± 0.006	0.066	-	-
α (g)	7.684 ± 0.018 ^{ab}	7.599 ± 0.36 ^b	7.598 ± 0.077 ^b	0.030	-0.095	-0.04

POU1F1, POU class 1 homeobox 1.

¹⁾ BW, body weight at 2 weeks; GR0-2, weight gain from 0 to 2 weeks; GR14-16, weight gain from 14 to 16 weeks; GR16-18, weight gain from 16 to 18 weeks; α , asymptotic final body weight (g); γ , constant scale that is proportional to the overall growth rate (Numbers in bracket refer to sample size for each genotype).

^{a,b} Least square mean within a row with different superscript differ significantly (p-value).

otypes. Since the Hardy-Weinberg principle cannot apply at single genotype situation. Alleles in each line were pooled and considered as one population to calculate the Hardy-Weinberg equilibrium test. Moreover, in pooled population also all the SNPs genotype frequencies were deviate from Hardy-Weinberg equilibrium. The deviation of Hardy-Weinberg equilibrium may be due to the high selection pressure resulting from a 15-year program to restore and maintain the five distinct lines of KNC. To date, the chicken population is being maintained as five pure lines.

Since 1994, the Korean government has implemented a project for restoration of KNC. As result, NIAS has established aforementioned five lines of native chicken based economic traits and their plumage colour [11], consisting Black (Heuk-saek Jaerae-jong), Grey (Hoegalsaeck Jaerae-jong), Red (Jeokgalsaeck Jaerae-jong), Yellow (Hwanggalsaeck Jaerae-jong), and White (Baeksaeck Jaerae-jong) [DAD-IS; <http://dad.fao.org/>]. Further, this population were proposed as candidates for selection due to their meat quality and growth differences observed among the lines [12]. These results further supported with high heritability value for body weight traits, moderate heritability values of carcass weight of five lines of KNC [10]. The M_2 and M_3 SNPs described in our study were previously reported by Nie et al [9]. In his study, these two markers showed significant associations with body weight at 85 days and body weight at 28, 42 days and average daily gain from 0 to 4 weeks, respectively. By comparison, in KNC, M_2 was associated with body weight at 2 weeks, weight gain from hatch to 2 weeks, and weight gain from 16 to 18 weeks. Additionally, the M_3 SNP marker was significantly associated with weight gain from 14 to 16 weeks and weight gain from 16 to 18 weeks, and with asymptotic body weight. Whereas, no statistical difference at M_1 genotypes was observed for growth traits. Furthermore, not all the other growth traits reported in our study were associated with the SNPs identified here; when compared to the results have been reported by Nie et al [9] and Bhattacharya et al [22] (Supplementary Table S1, S2).

Our results also indicate that the T allele of M_2 is important for high body weights at 2 weeks and for the weight gain from 0 to 2 weeks in this population (TT > CC/CT). These results were similar to those reported by Nie et al [9]. The C allele in M_3 was associated with the highest weight gain from 14 to 16 weeks and 16 to 18 weeks and with asymptotic body weight. Further, Jiang et al [6] reported that a *POUIF1* polymorphism was associated with chicken growth at 8 weeks of age, and suggested that the SNP (c.299A>T) was effective in early life but that effect was decreased with age. However, in the KNC, the M_2 and M_3 polymorphisms showed a significant association with weight gain at later growth stages and with the asymptotic body weight (α). These findings support the previously described correlation between α and weight gain

from 16 to 18 weeks [24]. Our findings indicate that *POUIF1* may be one of the genes in group that control weight gain from 16 to 18 weeks and also asymptotic body weight in KNC.

The linkage disequilibrium map of the haplotypes identified in our study shows a higher pairwise correlation value (D') between M_2 and M_3 SNPs (Figure 2). This relationship indicates strong linkage disequilibrium between these two SNPs and, thus, that one of them can easily act as a proxy for the other. However, the correlation between M_1 and M_2 , and between M_1 and M_3 was low and, therefore, neither of the other two haplotypes can be used as a proxy for M_1 . One main haploid block was identified between M_2 and M_3 , therefore, both variants inherent together. The results of the analyses of D' between M_2 and M_3 were further supported by the haplotype and single marker association results.

Overall, our analyses indicate that *POUIF1* gene polymorphisms have an effect on the growth of KNCs. M_2 and M_3 in *POUIF1* gene were significantly associated with weight gain at late growth period of KNC. T allele of M_2 was responsible for higher body weight at early growth and whereas, C allele of M_3 was responsible for higher body weight at later growth in KNC. This information will be of value for the improvement of native chicken breeds.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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