A genome-wide association study of growth trait-related single nucleotide polymorphisms in Chinese Yancheng chickens

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ABSTRACT. Chicken (*Gallus gallus*) growth traits are important economic traits, and many studies have been conducted on genetic selection for body weight. However, most of these studies have detected functional chromosome mutations or regions by conventional molecular markers or gene chips. In this study, we performed a new genome-wide association study using specific-locus amplified fragment sequencing (SLAF-seq) technology in purebred Yancheng chickens. Single nucleotide polymorphisms (SNPs) that were significantly associated with phenotypic traits were identified by GAPIT-compressed mixed linear models. Eighteen SNP markers reached 5% Bonferroni genome-wide significance. A region spanning 72.3 to 82.1 Mb on *GGA4* had a strong influence on growth traits. Four genes (*FAM184B*, *KCNIP4*, *MIR15A*, and *GLI3*) were closely associated with body weight. Some SNPs were coincident with previously reported quantitative trait locus regions. Our results would promote the researches of Chinese chickens and accelerate the utilization of Chinese chicken, specially Yancheng chicken.

Key words: Yancheng chicken; SLAF-seq; Genome-wide association study; GWAS

INTRODUCTION

Growth traits are among the most important economic traits in the poultry industry. Remarkable advances in the study of growth traits have been achieved, and many relative genes and quantitative trait loci (QTLs) have been discovered (Sewalem et al., 2002; Carlborg et al., 2004; Jacobsson et al., 2005; Tsudzuki et al., 2007; Moura et al., 2009; Wahlberg et al., 2009). More than 1500 QTLs, covering most of the chicken genome, are associated with growth traits (Hu et al., 2010). Previous genomic studies have generally employed low-density microsatellites as markers; however, this approach can no longer provide novel information. Recently, the genome-wide association study (GWAS) technique was developed and used to search for single nucleotide polymorphisms (SNPs) and functional genes that affect quantitative traits. A GWAS does not assume that certain genes or QTLs are associated with specific traits (Hardy and Singleton, 2009), but is rather a genome-wide study of the association between given traits and genetic markers (Liu et al., 2008; McCarthy et al., 2008; Cho et al., 2009). Most previous studies have used gene chips, but a new and efficient solution for large-scale genotyping called specific-locus amplified fragment sequencing (SLAF-seq) has been developed. SLAF-seq technology has several distinguishing characteristics: i) deep sequencing to ensure genotyping accuracy; ii) reduced representation strategy to reduce sequencing costs; iii) a pre-designed reduced representation scheme to optimize marker efficiency; and iv) a double-barcode system for large populations (Sun et al., 2013). In the present study, a GWAS was conducted using SLAF-seq technology in order to identify SNPs that are significantly associated with the growth traits of a Chinese chicken breed.

MATERIAL AND METHODS

Ethics statement

All of the experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and the protocols were approved by the Institutional Animal Care and Use Committee of the Ethics Institute, Jiangsu Academy of Agricultural Sciences, China.

Experimental animals

The animals used in this study were obtained from the Jinghai Yellow Chicken Breeding Station. The Yancheng chicken is a combination of broiler and layer chickens. It has a thin skin and tender, tasty meat. Yancheng chickens are responsive, active, and lively, and their color is yellow; they exhibit coarse-feeding resistance, disease resistance, social and field foraging ability, high performance, and are suitable for coastal climates and conditions. Two hundred purebred hens of the same batch from the same generation were randomly selected. All of the chickens had complete genealogical records and were reared in stair-step caging under the same recommended nutritional and environmental conditions. The body weights of the 200 chickens were measured biweekly from birth to 16 weeks of age. The statistics of nine growth measurements are presented in Table 1.

Genotyping

DNA was extracted from blood samples using the phenol/chloroform method and diluted to 100 μg/μL. Genomic DNA concentration and quality were assayed using a NanoDrop™ 2000

spectrophotometer to ensure that they met genotyping requirements. SLAF-seq is an efficient method of large-scale genotyping, which is based on a reduced representation library and high-throughput sequencing. The procedure is shown in Figure 1. The *Gallus gallus* sequences were analyzed using SLAF_Predict (Biomarker, Beijing, China), based on the GC content, repeat sequences, and gene characteristics. The plan for marker development was designed by defining the enzyme digestion scheme, the gel cutting ranges, and the sequencing quantity, which were used to verify the density and homogeneity of the markers under development and ensure the successful preparation of the expected target.

Table 1. Statistics of nine growth measurements.

Measurement	Mean	Standard error	Minimum	Maximum
BW0[1]	32.5	0.58	20	45
BW2	95.4	1.28	73	136
BW4	192.5	4.90	145	302
BW6	378.7	41.30	260	520
BW8	479.9	11.50	355	675
BW10	689.4	45.40	550	862
BW12	859.3	27.60	640	1060
BW14	979.7	45.50	795	1355
BW16	1106.9	23.40	970	1520

[1]The unit of body weight (BW) is the gram. 0, 2, 4, etc., refer to the week in which the BW measurement was taken.

An SLAF library was constructed in accordance with the pre-designed scheme. *Hae*III (New England Biolabs) was used to digest the genomic DNA. A single nucleotide A overhang was then added to the digested fragments using the Klenow fragment (3'→5' exo-) (New England Biolabs) and dATP at 37°C. Duplex Tag-labeled sequencing adapters [polyacrylamide gel electrophoresis (PAGE) purified, Life Technologies] were then ligated to the A-tailed fragments using T4 DNA ligase. A polymerase chain reaction (PCR) was performed using diluted restriction-ligation DNA samples, dNTP, Q5® High-Fidelity DNA Polymerase, and the PCR primers 5'-AATGATACGGCGACCACCGA-3' and 5'-CAAGCAGAAGACGGCATACG-3' (PAGE purified). The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK) and then pooled. The pooled sample was separated by electrophoresis on a 2% agarose gel. Fragments that were between 500 and 800 bp in size (with indexes and adaptors) were excised and purified using a QIAquick Gel Extraction Kit (Qiagen). The gel-purified products were sequenced on an Illumina HiSeq 2500 system (Illumina Inc., San Diego, CA, USA) according to the manufacturer protocol.

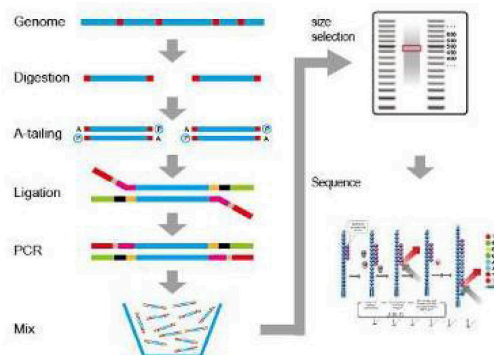

Figure 1. Flowchart of the specific-locus amplified fragment sequencing (SLAF-seq) method.

Raw reads were sorted to individuals according to the barcode sequences, and low-quality reads (quality score < 20) were filtered out. We evaluated and mapped the eligible reads using the SOAP 2.20 software (Li et al., 2009) to assemble newly referenced genomes (http://ftp.ensembl.org/pub/release-75/fasta/gallus_gallus/), to ensure that the original sequencing data were effectively obtained. We deemed that the reads with double ends were reliable compared to the only locus of the genome, and could do SLAF label development. We chose the group whose average depth of sequencing was not less than 4 to define the SLAF label, in line with the comparison error correction result. PLINK (1.07) (Purcell et al., 2007) was used to perform data quality control. SNPs with a low call frequency (<85%) and a low minor allele frequency (<5%) were rejected.

Statistical analysis

We used the ADMIXTURE 1.22 software (Alexander et al., 2009) to calculate the samples' group structure, based on the SNPs. We assumed that the 200 samples' subgroup number (Q value) was 1-10 for the cluster analysis, and confirmed the number of subgroups by their peak ΔQ value positions. The subgroups with a minimum ΔQ peak value were deemed the best.

The SNPs that were significantly associated with phenotypic traits were identified using a GAPIT compressed mixed linear model (MLM, II), which was as follows:

$$y = X\alpha + Q\beta + K\mu + e$$

where Y is the phenotypic value, X is the genotype, Q is the population structure matrix calculated by the ADMIXTURE program, β is the weight vector of each group, K is the relative kinship matrix, α is the weight vector of each marker, and e is the random error. K was constructed from all of the SNPs using the SPAGeDi 1.3a software (Hardy and Vekemans, 2002). The threshold P value of the 5% Bonferroni genome-wide significance was based on the estimated number of independent markers and linkage disequilibrium (LD) blocks for autosome markers (Nicodemus et al., 2005). We assessed the population structure using ADMIXTURE, and all of the autosomal SNPs were pruned using the indep-pairwise option, with a window size of 25 SNPs, a step of five SNPs, and an r^2 threshold of 0.2 (Wang et al., 2009). An LD block was defined as a set of contiguous SNPs that had pairwise r^2 values exceeding 0.4. We estimated the number of independent SNP markers using this approach. Given that the different genetic backgrounds of the population might lead to population stratification, we evaluated the population stratification status using ADMIXTURE. A quantile-quantile (Q-Q) plot was drawn to detect the population stratification for each trait.

RESULTS

SLAF-seq results

The distribution of all of the SLAFs in the genomes of the 200 samples was determined by the number of SLAFs per 100 kb in the genomes (Figure 2). SLAFs were relatively evenly distributed throughout the genomes, which indicated that the SLAF data were reliable. We then detected the SNPs among the defined SLAF fragments. The SLAF marker information for each chromosome is summarized in Table 2.

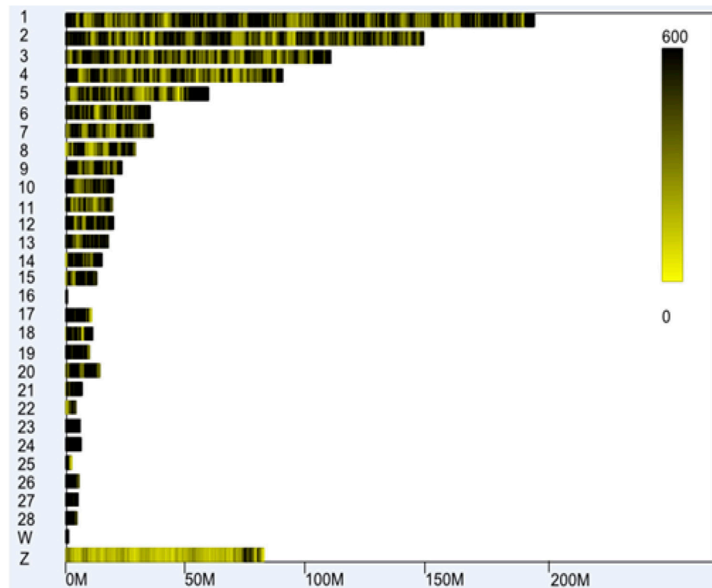

Figure 2. Specific-locus amplified fragment (SLAF) distribution on chicken chromosomes. Horizontal lines represent chromosomes, with the chromosomal length on the x-axis. The scale in the top-right corner indicates the number of SLAFs, with black indicating more than 50 SLAFs.

Table 2. Characteristics of single nucleotide polymorphism (SNP) markers on the chicken physical map.

Chromosome	Physical map (Mb)	No. of SNP markers	Marker density (kb/SNP)
1	199.4	76,296	95.6
2	154.4	53,596	85.9
3	113.6	40,068	88.2
4	94.0	31,996	85.1
5	62.0	22,608	91.1
6	37.4	14,472	96.7
7	38.4	13,904	90.5
8	30.5	11,076	90.6
9	25.4	9,308	91.6
10	22.4	8,288	92.5
11	21.9	6,312	72.1
12	20.4	8,876	108.8
13	18.4	7,384	100.0
14	15.8	6,620	104.8
15	13.0	5,340	102.7
16	0.43	300	174.4
17	11.2	6,116	136.6
18	10.9	4,912	112.7
19	9.8	4,488	114.5
20	13.9	6,204	111.6
21	6.7	3,436	128.2
22	3.8	984	64.7
23	6.0	2,964	123.5
24	6.4	3,288	128.4
25	2.0	664	84.0
26	5.1	2,456	120.4
27	4.6	2,816	153.0
28	4.4	2,040	116.0
W	0.939	580	154.4
Z	74.6	6,168	20.7

Group structure and cluster analysis

The result of calculating the samples' group structure indicated that a Q value of 1 was the lowest peak value (Figure 3), meaning that samples could be classified into one group. Regarding the Bonferroni P value, the total number of independent markers and LD blocks was 28,976. Therefore, at a threshold P value of 5%, the Bonferroni genome-wide significance was 1.72E-06 (0.05/28,976). Inter-individual genetic relationships may have influenced the population stratification; therefore, Q-Q plots of body weight at eight time points were drawn (Figure 4). The observed values (ordinates) were calculated by association analysis and fitted to the expected values (abscissae), which indicated that there was no population stratification. Therefore, the association analysis result was reliable using the GAPIT mixed linear model.

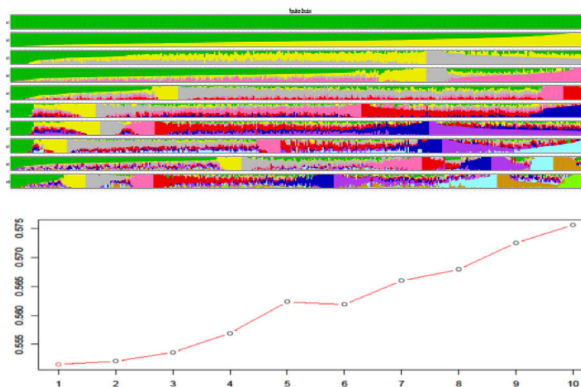

Figure 3. Group structure clustering. In the upper panel, each color represents a group and each line represents a cluster. The lower panel displays the clustering, where the cluster number ranges from 1 to 10.

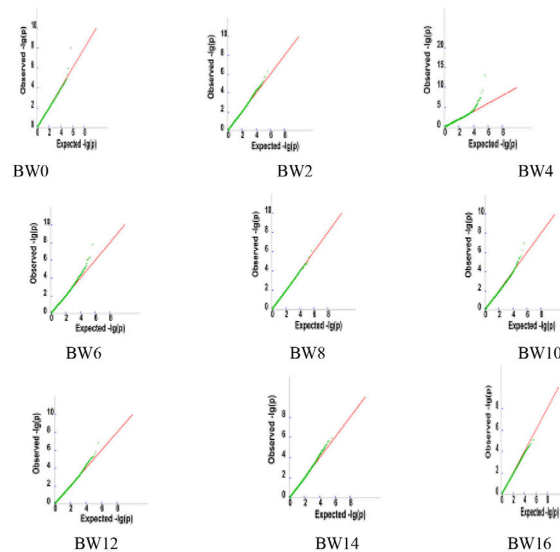

Figure 4. Quantile-quantile plots of body weight at eight time points. The ordinates are higher than the abscissae, which indicated that there is no population stratification.

Genome-wide association analysis

According to the quality control criteria, 200 chickens and 89,560 SNPs that were distributed on 29 autosomes and the Z chromosome were eligible for genome-wide analysis. Based on the GAPIT mixed linear model and a Bonferroni correction, 18 SNPs exhibited genome-wide associations with growth traits in Yancheng chickens (P < 1.72E-06). We searched for these SNPs in the National Center for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/snp/) according to their position in the genome, in order to obtain their SNP ID and nearest genes (Table 3).

Table 3. Significant genome-wide single nucleotide polymorphisms (SNPs).

Trait	SNP ID	Chr	Pos. (bp)[1]	MAF	P value	Nearest gene
BW0	rs13840709	1	25,098,872	0.375	4.25E-08	Novel gene
BW2	rs15990496	2	51,360,125	0.3548	8.71E-07	*GLI3*
BW2	rs15229556	26	1,958,988	0.4785	1.71E-06	*RBBP5*
BW4	rs16444875	4	82,169,307	0.375	4.54E-14	*CFAP99*
BW4	rs15368284	1	103,569,548	0.3548	7.98E-09	*GRIK1*
BW4	rs16023603	4	75,511,560	0.2247	6.91E-08	*FAM184B*
BW4	rs15990496	2	51,360,125	0.4126	8.71E-07	*GLI3*
BW4	rs13701151	3	96,483,790	0.2967	2.60E-07	*ATP6V1C2*
BW4	rs14144201	2	16,380,387	0.2063	4.69E-07	*GPR158*
BW6	rs16023603	4	75,511,560	0.2071	2.14E-07	*FAM184B*
BW6	rs15498187	1	168,713,010	0.4329	3.71E-07	*8 D MIR 15A*
BW10	GGaluGA265806	4	74,347,058	0.3548	1.16E-07	*KCNIP4*
BW10	rs16023603	4	75,511,560	0.375	1.36E-06	*FAM184B*
BW10	rs15498187	1	168,713,010	0.2336	1.63E-06	*8 D MIR 15A*
BW12	rs14085822	3	11,691,800	0.2987	4.44E-07	*CDC42BPA*
BW12	rs16023603	4	75,511,560	0.1899	1.59E-06	*FAM184B*
BW14	GGaluGA265806	4	74,347,058	0.0404	1.25E-06	*KCNIP4*
BW14	rs15938574	2	31,538,341	0.375	1.39E-06	*20 D STK31*

[1]SNP positions are based on WADHUC2 and SNP ID codes from the dbSNP database. MAF = minor allele frequency.

DISCUSSION

SLAF-seq technology was developed based on high-throughput sequencing. SLAF-seq is more appropriate for GWAS than using SNP chips, can obtain millions of SNPs at once, and detect novel SNPs on the genome (Xu et al., 2015). SLAF-seq can be applied to species with a reference genome, as well as to species without a reference genome (Sun et al., 2013).

SNPs reaching 5% genome-wide significance in this study were located on four different chromosomes. An obvious central region of *GGA4* (72.3-82.1 Mb) was identified. This result agrees with the results of Gu et al. (2011), who used an F2 resource population derived from Silky Fowl and White Plymouth Rock chickens to detect SNPs associated with body weight at 7-14 weeks, and identified a region of *GGA4* (71.6-80.2 Mb). Zhang et al. (2012) performed a GWAS with Beijing fatty chicken, and found no associated SNPs in the central region. Xie et al. (2012) identified SNPs in a region of *GGA1* (173.5-175 Mb) that strongly affected the body weights of F2 chickens, which were based on a cross of Xinghua and White Plymouth Rock chickens. Differences between these results may have been caused by breed differences.

Eighteen SNPs and 12 valuable genes were detected in this study. Among the SNPs in these genes, the largest effects were exerted by rs15990496 in *GLI3* for BW2 and BW4; rs16023603 in *FAM184B* for BW4, BW6, BW10, and BW12; GGaluGA265806 in *KCNIP4* for BW10 and BW14;

and rs15498187 in *8DMIR15A* for BW6 and BW10. *FAM184B* (family with sequence similarity, member B) can influence cattle ingestion, average daily gain, and carcass weight (Lindholm-Perry et al., 2011). In addition, a gene called *NCAPG* (non-SMC condensin I complex, subunit G) that is 1 kb downstream of *FAM184b* encodes a subunit of condensation protein complexes. *NCAPG* is a new candidate gene for cattle growth and carcass traits, and the polymorphism of this gene can influence the growth, bodily form, and carcass traits of different varieties of cattle at different times (Eberlein et al., 2009). Although *FAM184B* has not been reported in chickens, it could be a new candidate gene that influences chicken growth traits because of its important biological function. *KCNIP4* belongs to a family of potassium channel-interacting proteins, and is highly expressed in normal kidney cells (Bonne et al., 2007). Potassium channels have extensive physiological adjustment functions, including neurotransmitter release, contraction of smooth muscle, heart rate adjustment, and insulin secretion. Therefore, we hypothesize that *KCNIP4* has a significant effect on chicken growth. Regarding *GLI3* and *8DMIR15A*, a previous study found that these genes are important in controlling brain development (Laclef, 2014), which partly demonstrates that this gene affects growth traits. *8DMIR15A* encodes a type of noncoding regulatory RNA, and is expressed in several chicken tissues, particularly the lung. *8DMIR15A* influences many kinds of oncogenes, such as *BCL2*, *MCL1*, *CCND1*, and *WNT3A* (Galin et al., 2008; Aqeilan et al., 2010).

Regarding the other genes found in this study, *STK31* is a member of the TDRD protein family. In mice, this protein has a significant effect on the development of middle-late germ cells and fertilization (Bao et al., 2012), and may have a similar function in chickens. *GPIK1* is the main neurotransmitter receptor in mammals, and participates in a variety of neural physiological processes. This gene has also been identified as an important candidate anti-epileptic gene (Mulle et al., 1998). The CDS42BPA protein specifically binds CDC42, a phosphoglycerate kinase enzyme that regulates morphological changes in the cell, cell migration, the swallowing function, and the cell-cycle progression (Gong et al., 1997). Therefore, CDC42BPA may affect chicken growth via CDC42. *GPR158* can control G-protein signaling pathways by regulating the position of the RGS7 complex, and its family member *GPR120* is involved in human fat metabolism (Watson et al., 2012). Two other genes play important roles in biological processes: in Piétrain pigs, *ATP6V1C2* functions in the negative regulation of apoptosis, immune responses, cell-cell signaling, cell growth and migration, and metabolic processes (Ropka-Molik et al., 2014); *RBBP5* controls the efficiency of FOS transcript processing (Teoh and Sharrocks, 2014). We could not find any reports concerning the function of the *CFAP999* gene product.

In summary, we identified 18 SNPs with genome-wide significance. Several SNPs were associated with more than one body weight trait. Most of the SNPs have not previously been reported in chickens, although some overlapped with QTLs for growth traits that have been described in previous studies. Twelve candidate genes that affect growth traits were also detected in our study, and according to previous reports, nearly all of these genes have important biological functions that might regulate the growth of Yancheng chickens. These candidate genes require further study. These results will benefit those involved in the research and utilization of Yancheng chickens, as well as other Chinese chicken breeds.

Conflicts of interest

The authors declare no conflict of interest.

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