GENETICS AND GENOMICS

Genome-wide association study of 8 carcass traits in Jinghai Yellow chickens using specific-locus amplified fragment sequencing technology

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ABSTRACT Carcass traits are important to the commercial chicken industry, and understanding the genetics of these traits will be useful in the development of commercially viable varieties of chickens. We conducted a genome-wide association study based on 8 carcass trait phenotypes in a population of 400 43-week-old Jinghai Yellow chickens. Specific-locus amplified fragment sequencing technology was used to identify 90,961 single nucleotide polymorphisms (SNP) distributed among 29 chromosomes and the mitochondrial genome. SNP that were significantly associated with phenotypic traits were identified by a simple general linear model. Fifteen SNP attained genome-wide significance (P < 1.87E−6) and were associated with 5 of the 8 carcass traits; only one SNP was significantly associated with 2 traits (foot weight and wing weight). Twelve genes were associated with these 15 SNP. A region of chromosome 4 between 75.5 and 76.1 Mb was associated with carcass weight, foot weight, and wing weight. An 84-kb region on chromosome 3 (51.2 Mb) was associated with eviscerated weight and semi-eviscerated weight.

Key words: Carcass trait, chicken, GWAS, SLAF-seq

INTRODUCTION

The increasing worldwide human population has resulted in a growing demand for meat products. To meet this demand, the poultry industry has improved its productivity rate, mainly through genetic improvement. China has many indigenous chicken breeds that together form a large proportion of the poultry consumption in this country (billions of birds).

Carcass traits are among the most important factors in the poultry industry, and many relevant associated genes and quantitative trait loci (QTL) have been identified (Abasht and Lamont, 2007; Atzmon et al., 2008; Fang et al., 2010; Tang et al., 2010). In a previous study, Ambo et al. (2009) mapped a QTL for chicken BW at 35 and 42 d using microsatellite markers in chromosome 4. In another study with the same population, Baron et al. (2011) mapped a QTL for the percentage of thighs and drumsticks in the same region of chromosome 4. However, the application of these QTL results in broiler breeding remains impractical because of low mapping precision. Moreover, the techniques are labor intensive and time consuming.

Genome-wide association studies (GWAS) are currently used to search for single nucleotide polymorphisms (SNP) and functional genes that affect quantitative traits. SNP identified by GWAS can be used in genomic selection, so the identification of specific genes is not required. Moreover, they also can be used to examine traits and genetic markers (Liu et al., 2008; McCarthy et al., 2008; Cho et al., 2009), although their accuracy is greatly affected by population size.

High-throughput sequencing technologies can provide new strategies for sequence-based SNP genotyping. Whole-genome re-sequencing strategies can be used to genotype a large number of SNP among samples (Xia et al., 2009; Lam et al., 2010; Rubin et al., 2010), but it remains cost prohibitive in large populations. Specific-locus amplified fragment sequencing (SLAF-seq) is a new, reduced representation sequencing technology that uses bioinformatics methods to design a tag...
development plan and a screen-specific fragment length to achieve mass labeling using high-throughput technologies. SLAF-seq technology has several distinguishing characteristics: 1) deep sequencing to ensure genotyping accuracy; 2) reduced representation strategy to reduce sequencing costs; 3) a pre-designed reduced representation scheme to optimize marker efficiency; and 4) a double-barcode system for large populations (Sun et al., 2013). This technology has been reported for haplotype mapping, genetic mapping, linkage mapping, and polymorphism mapping. It also can provide an important basis for molecular breeding and germplasm resource identification (Chen et al., 2013).

This study aimed to identify potential loci and candidate genes affecting carcass traits in 43-week-old Jinghai Yellow chickens using SLAF-seq (Sun et al., 2013). In this strategy, repetitive sequences can be avoided using predesignated schemes, and the selected fragment number can maintain the balance between marker density and population size. We conducted a GWAS of 400 chickens from a conservation population of a Chinese local breed (Jinghai Yellow chicken) to measure a total of 8 carcass traits.

**MATERIALS AND METHODS**

**Experimental Animals**

The animals used in this study were obtained from the Jinghai Yellow Chicken Breeding Station in Nan tong City, Jiangsu Province, China. Four hundred 43-week-old female chickens of the same hatch from the same generation were randomly chosen. All had complete genealogical records and were reared in stair-step cages under the same recommended nutritional and environmental conditions. Blood samples were collected from 400 female chickens at 60 d of age. A total of 8 carcass quality traits were measured for the GWAS: carcass weight (CW), foot weight (FW), single wing weight (WW), single breast muscle weight (BMW), single leg muscle weight (LMW), abdominal fat weight (AW), eviscerated weight (EW), and semi-eviscerated weight (SEW). After a 12-h fast, chickens were weighed and slaughtered on d 300 using standard commercial procedures, and CW, FW, and WW values were recorded. Adipose tissue surrounding the proventriculus and gizzard, together with those located around the cloaca, were weighed as AW (Ain et al., 1996; Zhao et al., 2007). The EW and SEW were also determined. The carcasses were then dissected into deboned, skinless thighs and breasts for the assessment of BMW and LMW.

**SLAF-seq Technology Design**

SLAF-seq was used to genotype all 400 chickens, as previously described (Qi et al., 2014), with a few modifications. First, the chicken reference genome was analyzed using restriction enzyme prediction software based on the GC content, repeating sequences, and gene characteristics. Marker selection, digestion conditions, the gel cutting range, and total sequencing amount were determined to ensure uniformity of marker coverage throughout the genome.

Then genomic DNA (≥600 ng) from Jinghai Yellow chickens was extracted from blood samples using Dzup (Blood) Genomic DNA Isolation Reagent (Sangon Biotech, Shanghai, China) and diluted to between 50 and 100 µg/µL. DNA was incubated at 37°C with T4 DNA ligase (New England Biolabs, Hitchin, UK), 0.6 U MseI (New England Biolabs), ATP (New England Biolabs), and MseI adapters. Restriction–ligation reactions were heat-inactivated at 65°C and then digested in an additional reaction with the pre-determined restriction enzyme HaeIII at 37°C. PCR reactions contained the diluted restriction–ligation samples, dNTP, Taq DNA polymerase (New England Biolabs), and an MseI primer containing a barcode. PCR products were purified using a DNA Fragment Purification Kit Ver.2.0 (Takara Bio INC, Otsu, Japan), pooled, and then incubated at 37°C with MseI, T4 DNA ligase, ATP, and Solexa adapters. Samples were purified using a Quick Spin column (Qiagen, Hilden, Germany) and then separated on a 2% agarose gel to isolate 500 to 800 bp fragments using a Gel Extraction Kit (Qiagen, Hilden, Germany). These fragments were PCR amplified with the Phusion Master Mix (New England Biolabs) and Solexa sequencing technology (Illumina, San Diego, CA) using Phusion PCR settings that followed the Illumina sample preparation guide. Samples were gel-purified and products of appropriate sizes (300 to 500 bp) were excised and diluted for sequencing using an IlluminaHiSeq2000. Sequencing produced paired-end reads that were evaluated and mapped using SOAP 2.20 software (Li et al., 2009) to assemble newly referenced genomes (http://ftp.ensembl.org/pub/release-75/fasta/gallus_gallus/dna/).

We compared paired-end sequences with the reference genome sequence and defined the SLAF label using the group with an average depth of sequencing ≥ 4 in line with the comparison error correction result.

**Genotyping and Statistical Analysis**

The SNP discovery was conducted using the methods described by Sun et al. (2013) with some modification. Briefly, identical ‘useful fragments’ were merged together after base correction (named ‘tag’) in each library, and sequence similarity between tags was detected using one-to-one alignment by BLAT (Kent, 2002). Sequences with more than 90% identity were grouped in one specific-locus amplified fragments (SLAF) locus. Alleles were defined in each SLAF using the MAF evaluation. True genotypes had markedly higher MAF values than genotypes containing sequence errors. Tags with sequence errors were corrected to the most similar genotype to improve data efficiency. In mapping populations of diploid species, one locus can
contain at most 4 genotypes, so the groups containing more than 4 tags were filtered out as repetitive SLAF. SLAF with sequence depth less than 200 were defined as low-depth SLAF and were filtered out of the following analysis. Only groups with suitable depth and fewer than 4 seed tags were identified as high-quality SLAF, and SLAF with 2 to 4 tags were identified as polymorphic SLAF. We evaluated the accuracy of our genotyping using a Bayesian approach described by Sun et al. (2013). Plink software (v1.07) (Purcell et al., 2007) was used for data quality control. SNP with low call frequencies (<85%) and low minor allele frequencies (<5%) were rejected. Finally, 400 samples and 90,030 SNP distributed more than 3 autosomes and the Z chromosome remained for GWAS analysis.

Based on the SNP, we used ADMIXTURE 1.22 software (Alexander et al., 2009) to calculate the sample’s group structure. We assumed that the group number (Q value) of the 400 samples was between 1 and 15 for the cluster analysis, and ensured the number of subgroups by peak ΔQ value positions.

SNP significantly associated with phenotypic traits were identified using a TASSEL 3.0 generalized linear model (GLM) (Zhang et al., 2010)

\[ Y = \mu + X\alpha + Q\beta + e \]

where Y is the phenotypic value, \( \mu \) is the fixed effect value vector, X is the genotype, Q is the population structure matrix calculated by the ADMIXTURE program, with the proportion of each of the different groups fitted as a covariate, \( \beta \) is the weight vector of each group, and K is the relative kinship matrix (K). X is considered the genotype matrix, \( \alpha \) is the weight vector of each marker, and e is the random error. In the present study, we calculated the number of independent SNP and linkage disequilibrium (LD) blocks to construct K and obtain a P value. Independent SNP and LD blocks were calculated using the equation \( r^2 > 0.4 \) by Plink v1.07 through all autosomal SNP and pruned using the indep-pairwise option with a window size of 25 SNP, a step of 5 SNP, and an \( r^2 \) threshold of 0.4. Finally, 15,719 independent SNP and 11,048 LD blocks were obtained. K was constructed from the 15,719 independent SNP using SPAGeDi 1.3a software (Ou et al., 2009). The threshold Bonferroni P value was obtained from the estimated number of independent SNP markers and LD blocks. Because there was a total of 26,767 independent SNP and LD blocks, the threshold Bonferroni P value of suggestive significance was 3.73E−5 (1/26,767), and the threshold Bonferroni P value of genome-wide significance was 1.87E−6 (0.05/26,767). P values were corrected by Bonferroni (Nicodemus et al., 2005). Quantile-quantile plots for each trait and Manhattan plots of genome-wide association analyses were produced using TASSEL 3.0 software (http://www.r-project.org/).

RESULTS AND DISCUSSION

Analysis of SLAF-seq Data and SLAF Markers

After SLAF library construction and sequencing, a total of 52.70 Gb of raw data consisting of paired-end reads each ~80 bp in length was obtained after preprocessing. Of these, 86.1% bases were of a high quality, with quality scores \( \geq 20 \) (i.e., a quality score of 20, indicating a 1% chance of an error, and thus 99% confidence). In total, 236.07 M reads were mapped to the chicken reference genome, resulting in a paired-ends mapping ratio of 71.66%. A total of 103,680 SLAF were identified with an average read depth of 5.46 in the study population. Of these, 88,135 were found to be polymorphic (85%). The number of SLAF markers per chromosome ranged from one to 19,722, and the distribution was even throughout the genome (Figure S1). We then detected SNP among the defined SLAF fragments, identifying 90,961 after quality control measures, distributed among 29 chromosomes (including the Z chromosome) and the mitochondrial genome (Table S1). The average physical distance between 2 neighboring SNP was approximately 10 kb. The mean, maximum, and minimum distance between SNP on each chromosome is shown in Table S2.

Population Structure Analysis

Descriptive statistics of the phenotypic measurements of carcass traits are shown in Table 1. All non-normal phenotypic data were normalized using the Box–Cox or Johnson transformations.

| Traits                        | Mean  | Stand deviation | Minimum | Maximum | CV (%) |
|-------------------------------|-------|-----------------|---------|---------|--------|
| Carcass weight (CW, g)        | 1780  | 275             | 1070    | 2540    | 15     |
| Foot weight (FW, g)           | 46    | 7.3             | 30      | 70      | 16     |
| Single wing weight (WW, g)    | 63    | 10              | 40      | 119     | 16     |
| Single breast muscle weight (BMW, g) | 107 | 20             | 47      | 158     | 19     |
| Single leg muscle weight (LMW, g) | 139 | 24             | 67      | 222     | 17     |
| Abdominal fat weight (AW, g)  | 63    | 44              | 7.0     | 226     | 70     |
| Eviscerated weight (EW, g)    | 1500  | 237             | 945     | 2250    | 16     |
| Semi-eviscerated weight (SEW, g) | 1270 | 191            | 793     | 1820    | 15     |

1CV is the coefficient of variation of traits.
Using ADMIXTURE, calculations revealed stratification in the experimental population structure. The population was divided into one to 15 subgroups (k) first. Then the value of cross-validation (CV) error of population under a different k number was calculated. The number of k with a minimum value of CV error was most suitable. The results indicated that a k value of 10 works best (Figures S2A, B) and the samples were divided into 10 k. Given that the population stratification might influence the GWAS result, QQ-plots of 8 traits were drawn (Figure S3). The observed values (ordinate) calculated by association analysis fitted to the expected values (abscissa), which indicated that the population stratification was well corrected. The association analysis result was reliable using a GLM.

**Genome-Wide Association Analysis**

GWAS are commonly used to identify SNP associated with economically important production traits in animal studies (Jiang et al., 2010; Fan et al., 2011; Shen et al., 2012). SLAF-seq technology is a cost-effective way to genotype SNP in such analyses, because it can develop many specific markers and achieve a high success rate (Sun et al., 2013). In the present study, GWAS identified potential loci and candidate genes using SLAF-seq in a population of Jinghai Yellow chickens, which is the first new chicken variety to be authorized by the National Commission for the Livestock and Poultry Genetic Resources in China (Gu et al., 2011; Zhao et al., 2012, 2013).

In the present study, the TASSEL-compressed GLM model was used to do association analysis between SNP and carcass traits. The Manhattan plots of all SNP with 8 carcass traits were drawn (Figure 1, Figure S4). One significant SNP was identified to be associated with CW, which was located on chromosome 4, 1.61 kb downstream of the Gallus gallus fibroblast growth factor binding protein 2 gene (FGFBP2). Some SNP associated with BMW and LMW reached the suggestive significance level (Table S3). FGFBP2 gene is located in a QTL region, which corroborates the former reports (Ankra-Badu et al., 2010; Nassar et al., 2012). It plays an important role in embryogenesis, cellular differentiation, and proliferation in chickens. A previously identified SNP, g.651G > A, in FGFBP2 was associated with thawing loss and meat redness (Felicio et al., 2013). The protein encoded by FGFBP2 recognizes DNA promoter regions and induces the transcription of fibroblast growth factor genes, which cause myoblast proliferation and differentiation in chicken skeletal muscle development (Gibby et al., 2009; Felicio et al., 2013). Thus, FGFBP2 can influence the carcass quality and muscle development.

Five SNP of genome-wide significance for FW were identified. These were clustered within a 0.1-Mb region of chromosome 4 (75.54 to 75.67 Mb), indicating that this 0.1 Mb region might affect FW of Jinghai Yellow chickens. These 5 SNPs were located within or 2.08 to 2.38 kb away from 3 genes. rs731242270, rs736026342, and rs733762928 are 2.08, 2.12, and 2.38 kb upstream from the family with sequence similarity 184, member B gene (FAM184B). rs739233957 is within the quinoid dihydropteridine reductase gene (QDPR) and rs316943436 is within the LIM-domain binding factor 2 gene (LDB2). The latter 2 genes have previously been shown to influence shank circumference (Sun et al., 2013) and be associated with BW in Beijing You chickens (Gu et al., 2011). This is of particular interest because FW positively correlates with BW, and the shank circumference has a direct influence on FW, further suggesting that these 2 genes have an important influence on FW in Jinghai Yellow chickens. FAM184B also has been found to influence the daily gain, CW, and ingestion of cattle (Lindholm-Perry et al., 2011), so it may have similar effects on FW in chickens.

Four SNP located on chromosomes 4, 18, 20, and Z were shown to have genome-wide significance with WW (Table 2). The SNP on chromosome 18 is novel and located in the *G. gallus* zinc finger protein 302

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**Figure 1.** Manhattan plots showing association of all SNP with CW from GLM.
Table 2. SNP with genome-wide significance for 8 carcass traits by GLM.

| Traits | SNP ID | Chromosome | Position (bp) | P-value | Nearest gene | Distance |
|--------|--------|------------|--------------|---------|--------------|----------|
| CW     | rs733409553 | 4         | 76149439     | 1.79E-06 | FGFBP2      | 1.61 D   |
| FW     | rs73124270 | 4         | 75548514     | 1.51E-06 | FAM184B     | 2.08D    |
|        | rs736026342| 4         | 75548554     | 1.51E-06 | FAM184B     | 2.12D    |
|        | rs733762928| 4         | 75548610     | 2.43E-07 | FAM184B     | 2.38D    |
|        | rs733943436| 4         | 75614139     | 3.43E-05 | QDPR        | within   |
|        | rs736026340| 4         | 75641139     | 8.62E-10 | QDPR        | within   |
| WW     | rs739233957 | 4         | 75641139     | 2.96E-07 | QDPR        | within   |
|        | rs316943436| 4         | 75679707     | 1.02E-06 | LDB2        | within   |
|        | New      | 18        | 11502599     | 6.80E-10 | PGO2        | 1.28 U   |
|        | Z        | 20        | 26128672     | 1.17E-08 | SMARCA2     | within   |
| AW     | 739097850 | 2         | 18144674     | 1.67E-06 | PGO2        | within   |
|        | New      | 2         | 18594879     | 1.67E-07 | PLXDC2      | within   |
|        | rs317160657| 14        | 12246236     | 1.31E-06 | ITFG3       | within   |
|        | rs317099348| 5         | 56337998     | 3.31E-05 | CGRRF1      | within   |
| EW     | rs737570558| 3         | 51207135     | 1.41E-06 | TULP4       | within   |
|        | rs315486571| 3         | 51291142     | 1.18E-06 | TULP4       | within   |
|        | New      | 3         | 51298965     | 1.02E-06 | TULP4       | within   |

1SNP positions are obtained from ENSEMBL.
2U = upstream, D = downstream. The unit of the distance is kb.

(ZNF302), ZNF302 belonging to the zinc finger protein family. This protein family is responsible for genital malformations (hypospadias) in male humans (Gana et al., 2012). rs317080707 on chromosome 20 is 1.28 kp upstream from an uncharacterized protein encoded by PGO2. The SNP on chromosome Z is novel within the SMARCA2, but rs739233957 on chromosome 4 is within the QDPR, which also demonstrates a genome-wide significant association with FW. QDPR has significant correlations with growth, shank circumference, and FW traits as described above. To our knowledge, no previous reports have investigated the possible function of PGO2. However, the other 3 genes are potential candidates for further WW research.

No SNP was identified to be associated with BMW and LMW traits in Jinghai Yellow chickens. However, 9 and 10 SNP reached a suggestive significance level for BMW and LMW, respectively. Fifteen genes located in or nearby those SNP were identified (Table S1). For SEW, it shares a consistent region with EW that has a P value slightly higher than 3.73E-5. The result may be caused by the correlation between the 2 traits.

We identified 3 genome-wide significant SNP associated with EW, which were all located on chromosome 3 and clustered within an 84-kb region. These were located in TULP4, 29 kb upstream from TMEM181. TULP4 encodes a member of the tubby protein family, members of which act as bipartite bridges through their phosphoinositide binding (Mukhopadhyay and Jackson, 2011). This family has unique amino-terminal functional domains that coordinate multiple signaling pathways, including ciliary G-protein-coupled receptor trafficking and Shh signaling (Mukhopadhyay and Jackson, 2011). A more recent study reported that TULP4 was a new candidate gene for cleft palate (Vieira et al., 2015). No SNP reached genome-wide significance for SEW; however, the 3 SNP significantly associated with EW also reached suggestive significance for SEW. The difference in significance levels is likely to reflect differences between the traits of SEW and EW.

Four SNP were shown to be of genome-wide significance for AW. Two are located on chromosome 2, within the plexin domain containing 2 genes (PLXDC2), Integrin alpha FG-GAP repeat containing 3 (ITFG3), cell growth regulator with ring finger domain containing 1 (CGRRF1), and another with no annotated genes nearby. PLXDC2 is a type I transmembrane protein with some homology to nidogen and to plexins. It is a Mitogen for Neural Progenitors in chicken (Miller et al., 2011). The third SNP rs317160657 on chromosome 14 is within the integrin alpha FG-GAP repeat containing 3 (ITFG3). All significant SNP, suggestive SNP, and the Manhattan plots for all traits with significant SNP are shown in Table 2, Table S3, Figure 1 and Figure S4.

A heatmap (Figure S5) and correlation coefficient matrix (not shown) of the 8 traits in these analyses show the existence of a high correlation among these traits, with the exception of AW. In particular, CW was highly correlated with EW and SEW, with correlation coefficients of 0.94 and 0.96, respectively. EW was also highly correlated with SEW, with a correlation coefficient of 0.97. Meanwhile, SNP rs736007178 and rs733409553 were identified as being significantly associated or having suggestive significance in their association with all 3 traits. This indicates that the 3 traits might be genetically correlated with each other, and that they could be improved by the 2 SNP. rs736007178 is located within SERAC1, whereas rs733409553 is located 1.6 kb downstream of FGFBP2, indicating that both genes might be important candidates for affecting carcass traits in Jinghai Yellow chickens.
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SUPPLEMENTARY DATA

Table S1. Distributions of SLAF-SNP and the average lengths between adjacent SNP on each chromosome

Table S2. Distance between SNP on each chromosome

Table S3. SNP with suggestive genome-wide significance for 8 carcass traits by GLM and MLM

Figure S1. SLAF Distribution on Chromosomes. X-axis is the length of chromosome; Y-axis represents different chromosome of chicken. Scale on the top right corner indicates that the greater the density of SNP, the deeper the color. If the SNP number is more than 600, the color is pure black. The average physical distance between 2 neighboring SNP was approximately 10 kb.

Figure S2A. X-axis is the number of subgroups; Y-axis represents the chicken name. In the figure, a color represents a group.

Figure S2B. X-axis is the cross-validation (CV) errors; Y-axis is the number of subgroups (k). The number of k with a minimum value of CV error was most suitable. The results indicated that a k value of 10 works best and the samples should be divided into 10 k.

Figure S2. Grouping structure picture of the group.

Figure S3. Quantile-quantile (Q-Q) plots for 8 carcass traits. Plotted on the x-axis are the expected p-values under the null hypothesis and on the y-axis are the observed p-values. A: CW, carcass weight; B: FW, foot weight; C: WW, single wing weight; D: BMW, single breast muscle weight; E: LMW, single leg muscle weight; F: EW, eviscerated weight; G: SEW, semi-eviscerated weight; H: AW, abdominal fat weight.

Figure S4. Manhattan plots of all SNP with carcass traits. S3A: FW, foot weight; S3B: WW, single wing weight; S3C: BMW, single breast muscle weight; S3D: LMW, single leg muscle weight; S3E: EW, eviscerated weight; S3F: SEW, semi-eviscerated weight; S3G: AW, abdominal fat weight. The red dotted line is the threshold of 1.0E-7. The blue dotted line is the threshold of 1.0E-5.

Figure S5. Heatmaps of correlation between 8 carcass traits in Jinghai Yellow chickens. This map was created based on the correlation coefficient between 8 carcass traits. The deeper the blue color, the higher the correlation and the deeper the red color, the lower the correlation.

Supplementary data is available at PSA Journal online.

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