Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

ISSN 1516-635X 2022 / v.24 / n.3 / 001-008

http://dx.doi.org/10.1590/1806-9061-2021-1587

Original Article

Genome-Wide Association Study on Two Immune-Related Traits in Jinghai Yellow Chicken

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■Keywords

Avian influenza; disease resistance breeding; INF-γ; SLAF-seq.

Submitted: 25/October/2021
Approved: 01/March/2022

ABSTRACT

This study was designed to discover molecular marker associated with the interferon INF-γ and avian influenza (AI) antibody titer traits in Jinghai Yellow chicken (*Gallus gallus*). Serum samples were taken from 400 female chickens and the INF-γ concentrations and AI antibody titer levels were measured. A genome-wide association study was carried out using specific-locus amplified fragment (SLAF) sequencing. Bioinformatics analysis was applied to detect single-nucleotide polymorphisms (SNPs) associated with the two traits. After sequencing and quality control, 103,680 SLAFs and 90,961 SNPs were obtained. The 400 samples were divided into 10 subgroups to reduce the effects of group stratification. The Bonferroni adjusted P-value of genome-wide significance was set at 1.87E–06 according to the number of independent SNP markers and linkage disequilibrium blocks. A SNP that was significantly associated with INF-γ concentration was detected in the myomesin 1 (*MYOM1*) gene on chromosome 2, and another SNPthat was significantly associated with the AI antibody titer level was detected in an RNA methyltransferase gene (*Nsun7*), which was found to have an important biological function. We propose that *MYOM1* and *Nsun7* are valuable candidate genes that influence the disease resistance characters of chicken. However, in-depth investigations are needed to determine the essential roles of these genes in poultry disease resistance and their possible application in breeding disease resistant poultry.

INTRODUCTION

Interferons (INFs) are highly bioactive glycoprotein that are produced in cells under the effect of specific inducers. When interferons are introduced into cells, the cells acquire antiviral and anticancer immunity through the activation of natural killer cells, which kill infected cells, and the induction of major histocompatibility complex (MHC), class I genes[1]. In chicken (*Gallus gallus*), there are two types of interferons, type I and II. INF-γ is a type II interferon (also called immune interferon) that can induce the expression of MHCs and immunomodulatory effects. INF-γ is an important cytokine in animals and is the main macrophage activation factor [2-3]. INF-γ has efficient antivirus and antitumor activity as well as strong immunomodulatory effects, and has been widely used in the clinical treatment of human diseases and in the prevention and control of animal diseases. In chicken, INF-γ has antiviral and anticoccidial roles, and is involved in immune regulation, increase in macrophage lethality, cell proliferation, and apoptosis, as well as disease control and prevention[4].

Genetic correlation is the correlation between the genotype influences on two different traits associated with a hybrid population

phenotype. Genetic correlation has been used to investigate phenotypic traits at the molecular level[5]. In chicken, only a few studies have focused on avian influenza (AI) disease resistance traits. Hu et al. used a 60 K SNP chip to detect single-nucleotide polymorphisms (SNPs) that were significantly associated with AI disease resistance in two different kinds of Chinese native chickens[6], but no SNPs were detected at the genome-wide level.

A few molecular marker studies have investigated INF-γ and AI antibody titer in chickens. Traditional methods of detecting quantitative trait loci (QTLs) and SNPs include single-strand conformational polymorphism and restricted fragment length polymorphism analysis; however, these methods tend to have low detection rates and are easily influenced by the environment, leading to low accuracy of the results. Genome-wide association studies (GWAS) have been widely applied in chicken to discover QTLs for important economic traits and SNPs have been detected in candidate genes associated with growth traits, reproduction traits, body composition traits, meat quality traits, and resistance to Newcastle disease and avian infectious bronchitis traits[7-13]. GWAS avoid unnecessary assumptions and can find SNPs that are associated with target traits directly in the genome, and produce reliable results[14].

Until now, most GWAS in chicken have used SNP chip technologies, which can only detect known SNPs, and therefore cannot find new SNPs. In this study, we used specific-locus amplified fragment sequencing (SLAF-seq)[15-16], which, compared with other techniques, has a number of advantages: (1) millions of high density SNPs can be generated; (2) unknown mutations can be detected in genomes; (3) a reference genome sequence is not required; and (4) complete 2× 100-bp double-end sequence reads can be obtained, which are likely to have a high SNP conversion rate. We performed SLAF-seq to find SNPs that were associated with INF-γ concentration and AI antibody titer level traits in the whole genome of chicken, with the aim of providing a reference resource for breeding disease resistant chickens.

MATERIALS AND METHODS

Experimental animals and index measurement

Four hundred 11th generation female Jinghai Yellow chickens were used as the experimental group. All the hens were hatched in the same batch, in the same pheasantry, under the same conditions. Their health was good and no illnesses were recorded. At 1 month of age, the chickens had tags put on the wing and were immunized in accordance with the established immunization schedule. The standard immune program shown in Table 1 was followed for all the chickens. At 60 days of age, blood samples were collected for the vein under the wings and the serum was separated by the conventional method. Briefly, the blood was left for 2–4 h at room temperature to clot, then centrifuged at 4000 rpm for 2 min to extract the serum. Serum INF-γ concentrations and AI antibody titer levels were measured using chicken INF-γ and AI Elisa kits (RD, USA). Then absorbance was obtained by enzyme-linked immunosorbent assay (ELISA) at 450 nm and standard curves were drawn. INF-γ concentrations and AI antibody titer levels were measured using the corresponding standard curve.

Table 1 – Chicken's immunization schedule.

Age(d)	Vaccine name	Methods	Notes
1	MDV	Neck skin injection	Incubation hall
1	Avinew and h120	Eye drop	
3	Coccidia	Drinking water	
10	AIV	Neck skin injection	0.2ml/a chicken
14	IBD	Drinking water	
21	IBD	Drinking water	
21	henpox	Vaccination	
24	NDV	Neck skin injection	
24	NDV and IBV	Eye drop	
49	NDV	Injection	0.25ml/a chicken

Specific-locus amplified fragment (SLAF) sequencing

Genomic DNA was extracted from the blood of Jinghai Yellow chickens using genomic DNA isolation reagent (Aidlab Biotech Co., Ltd, Beijing, China) and diluted to approximately 100 ng/μL.The extracted DNA was processed as follows: genome enzyme digestion, 5'-end repair, 3'-end plus 'A', connect Solexa adapter, electrophoresis gel extraction, PCR amplification, and sequencing.

Genome enzyme digestion to fragment the genomic DNA was conducted using inhouse restriction enzyme prediction software based on the genome GC content, repeat sequences, and other gene characteristics. Marker selection, digestion conditions, gel cutting range, and total sequencing amount were chosen to ensure uniformity of marker coverage throughout the genome. In this step, the water bath temperature was set to 37 °C, and the reagents (water, genomic DNA, NEB buffer, and restriction enzyme *Hae* III) were kept at 37 °C

for 15 h after they were mixed. The products were purified on a Quick Spin column (Qiagen, Hilden, Germany) and redissolved in the elution buffer. Blunt-end repair was performed on the different types of fragment ends produced by enzyme digestion. Then, phosphorylation modification was performed on the 5'-ends of the fragments, and an 'A' was added to the 3'-ends to connect the Solexa adapters, which have a 'T' at their 5'-end, to both improve the efficiency of the connections and prevent self-connection of the Solexa adapters. Ligation products were purified using a DNA fragment purification kit v2.0 (Takara Bio INC, Ostu, Japan), and pooled. The PCR mixture contained the serum samples, DNA ligase, ATP, and Solexa adapters. The PCR products were purified on a Quick Spin column (Qiagen) and isolated on a 2% agarose gel using a Gel Extraction Kit (Qiagen) to separate the 500- to 800-bp fragments. The purified fragments were PCR amplified with dNTPs, Q5® High-Fidelity DNA Polymerase, and PAGE-purified primers, and the PCR products were purified on a 2% agarose gel and pooled. Products that were 300–500-bp long (including indices and adaptors) were selected and sequenced on an Illumina HiSeq 2500 system (Illumina, Inc, San Diego, CA, USA) according to the manufacturer's instructions. Paired-end reads and reads with a single-base sequencing error rate of over 1/100 were discarded. The remaining reads were assessed and mapped to the Ensembl*Gallus gallus* reference assembly (release 75) (http://ftp.ensembl.org/pub/release-75/fasta/gallus gallus/dna/) using SOAP 2.20[17]. Pair-end reads that were uniquely mapped to the genome were retained, and reads that had an average coverage depth of at least 4 were considered as SLAF fragments and were used in the subsequent analysis.

Genotyping and statistical analysis

Identical fragments were merged after base correction in library and sequences that shared >90% similarity by one-to-one BLAST alignments[18] were classified as being in the same SLAF locus. Only groups with suitable depth were considered to be reliable SLAFs. Plink (v1.07) was used for data quality control. SNPs with low minor allele frequencies (<5%) and low call rates (<85%) were rejected [19]. Finally, 90,030 SNPs distributed in 30 autosomes and the Z chromosome were retained for later GWAS analysis.

To avoid the negative impact of population stratification, ADMIXTURE 1.22 software was used to analyse the chicken group structure based on the detected SNPs[20]. Group numbers (Q value) were assumed to be between 1 and 15 for the cluster analysis and best subgroup numbers were ensured by the peak ΔQ value positions. The generalized liner model of TASSEL 3.0 (http://www.r-project.org/) was used to identify SNPs that were associated with the target traits[21].

$$Y = \mu + X\alpha + Q\beta + e,$$

where Y is the phenotypic value, X is the genotype, and Q is the population structure matrix that was calculated by ADMIXTURE, with the proportion of each of the different groups fitted as a covariate, where μ is the fixed effect value vector, α is the weight vector of each marker, β is the weight vector of each group; and *e* is the random error.

A Bonferroni adjusted *p* value was used as the threshold by analyzing the estimated number of independent SNP markers and linkage disequilibrium (LD) blocks. Independent SNPs were determined using r^2 >0.2 for all the autosomal SNPs, and chosen using an in-depth pairwise option with a window size of 25 SNPs with steps of 5 SNPs, and r^2 threshold of 0.2. The number of LD blocks was calculated with r^2 >0.4[22-23]. Finally, 11,048 LD blocks and 15,719 independent SNPs were obtained. The Bonferroni adjusted P value threshold of genome-wide significance was 1.87E–06 (0.05/26,767) and the suggestive significance was 3.73E–05 (1/26,767)[24]. Quantile-quantile (QQ) plots were constructed for the target traits to detect the effect of group structure, and Manhattan plots of the GWAS results were generated by TASSEL 3.0 (http://www.r-project.org/).

RESULTS AND DISCUSSION

SLAF-seq data analysis and identification of SNPs

The SLAF-seq generated 52.70 Gb of raw data that consisted of pair-end reads, and 86.1% of the bases had quality scores >20 (indicating 99% confidence and <1% possibility of an error). A total of 71.66% of the reads were successfully mapped to the *Gallus gallus* reference assembly. We defined a SLAF as having an average read depth >5, and identified 103,680 SLAFs from the mapped reads; 88,135 of them were polymorphic (85%). The distribution of SLAFs on each chromosome is shown in Figure 1. A total of 90,961 eligible SNPs were detected in the SLAFs after quality control, and their distribution on the autosomes, and Z and mitochondrial chromosomes is shown in Table S1.

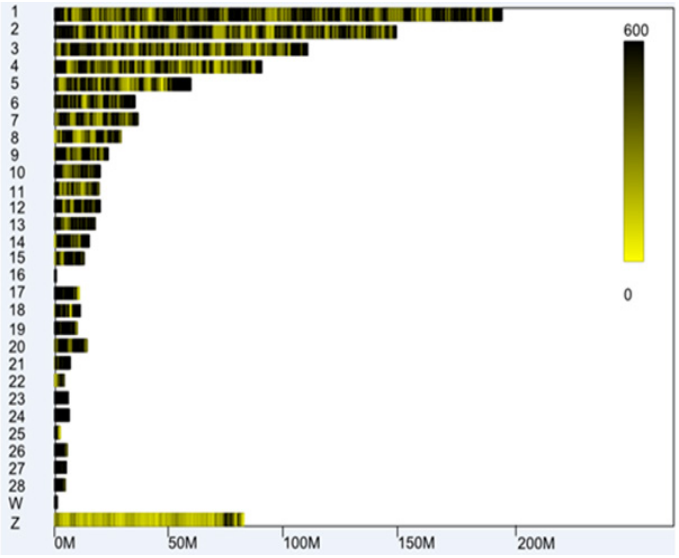


Figure 1 – SLAF distribution on chromosome.

Note: The distribution of all the SLAFs in the genomes of 400 samples was determined by calculating the number of SNPs per every 100kb in the genome. Horizontal lines represent chromosomes, with 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.

Analysis of population structure

The INF-γ concentrations and AI titer levels in the serum samples are shown in Table 2. Johnson or Box-Cox transformations were applied to normalize the non-normal traits data.

Table 2 – The descriptive statistics of two disease resistance characters.

Traits	Maximum	Minimum	Mean	Stand deviation	CV[1] (%)
AI	15	1	5.1	1.47	28.8
IFN-γ (ng.l)	289	91	176	19.8	11.3

[1]CV is coefficient of variation of traits.

For the population structure analysis, ADMIXTURE was applied and the samples were divided into 1 to 15 subgroups (k value) for the cluster analysis. The cross-validation (CV) error for the population was calculated under different k numbers. We considered the most suitable subgroup was the one that had the lowest CV error. The results showed that dividing the samples into 10 subgroups produced the lowest CV error (Figure 3). QQ plots of the target traits were drawn to verify the revision effect of the population stratification (Figure 2). The plots show that the population stratification was well corrected because of the close fitting between the observed values, calculated by association analysis, and the expected values, which indicated the results of population structure analysis were reliable and could be used in the subsequent analysis.

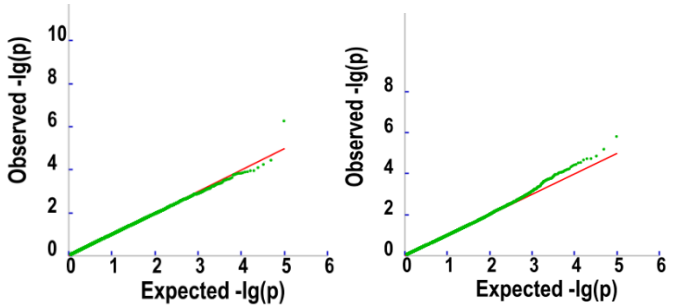
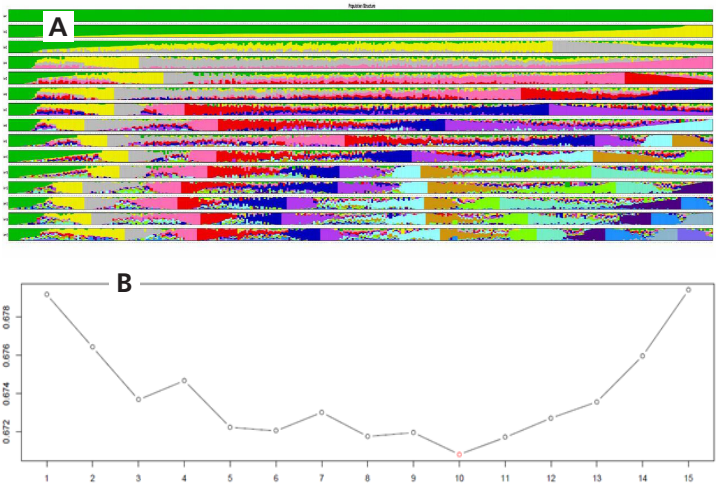


Figure 2 – The QQ-plot of two disease resistance characters. IFN-γAI



Note: We assumed that the 400-samples' subgroup number (Q value) was 1-15 for the cluster analysis and ensured the number of subgroups by the peak ΔQ value positions. The subgroups with a minimum ΔQ peak value were the best. The rsults indicated that a Q value of 10 is the lowest peak value.

Figure 3 – The structure of the samples.

Genetic correlation analysis of the SLAF-seq results with the target traits

Most genetic correlation research in chickens has focused mainly on economic traits such as growth and reproduction, and studies focused on disease resistance traits are few, possibly because the boundaries of such traits were difficult to distinguish and detection of these traits were challenging and expensive[6]. However, immune antibody levels to a particular disease can reflect the strength of the humoral immune response against a disease, and therefore seeking important molecular markers that influence diseases or immune-related traits is important. GWAS have been widely used to detect SNPs associated with important traits in animals. Compared with re-sequencing, SLAF-seq is a highly efficiency and cost-effective method to genotype SNPs because it can 'simplify' the genome and develop many specific fragments, and SLAF-seq has a high success rate[25]. In this study, SLAF-seq was applied to identify potential SNPs and candidate genes associated with the AI and INF-γ disease resistance traits in chickens.

We used the generalized liner model of TASSEL for an association study between SNPs and the AI and INF-γ disease-resistant traits. Details of the identified SNPs and candidate genes are given in Table 3. Manhattan plots of all the detected SNPs associated with the two target traits are shown in Figure 3. For INF-γ, one significant SNP (rs13794539, A→G) was found to be genome-wide associated with this trait. This SNP is located in the myomesin 1 (MYOM1) gene on chromosome 2. MYOM1 plays an important role in regulating the activity of protein kinase and protein dimers, and participates in embryonic morphogenesis of the heart and in muscle contraction[26]. The MYOM1 sequence is highly conserved in rodents, chimpanzees, macaque, humans, and poultry. In humans, the alternative splicing of MYOM1was aberrantly regulated in myotonic dystrophy type[27], and a mutation in the MYOM1 sequence was associated with hypertrophic cardiomyopathy and was found to disrupt its dimerization properties[28]. MYOM1 was also found to be closely related with porokeratosis in humans[29]. In mice, MYOM1 was detected in the nucleus of mature myocardium cells, and its expression led to the differential expression of 42 other genes[30]. In cattle, LD in MYOM1 differed greatly among different breeds, which suggested it could be used as a candidate gene for improving cattle resilience and production performance traits[31]. These findings imply that MYOM1 could possibly be useful in chicken breeding. Until now, there are no reports of the role of MYOM1 in chicken. However, we suggest that MYOM1 can be regarded as a candidate gene that impacts the INF-γ trait, and that it can act as a reference for marker-assisted selection in chickens.

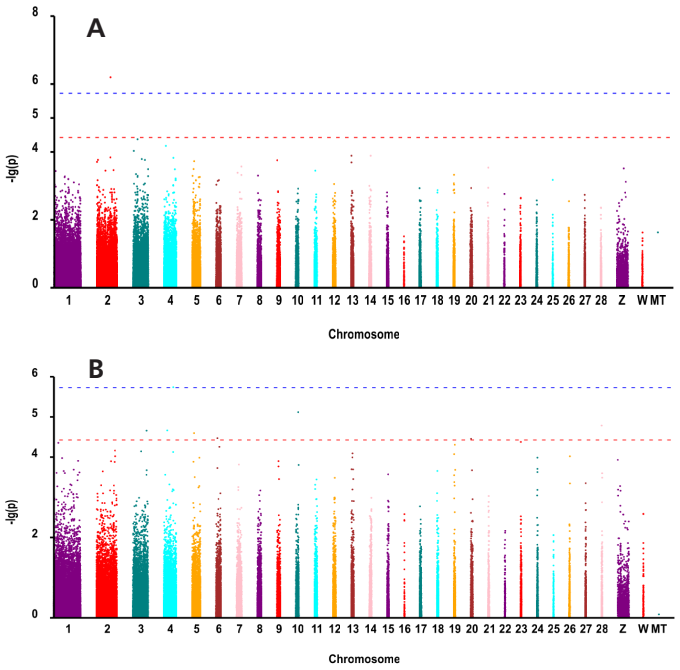


Figure 3 – Manhattan plot for genome-wide association study on INF-γ (A) and AI (B).

For the AI trait, one SNP was detected at the genome-wide association level, and another SNP potentially showed genome-wide association with AI. In an F2 group of Beijing Oil chickens and Cobb chickens, a GWAS showed that no SNPs reached the genome-wide association level, presumably because of the different chicken varieties that were used[6]. The potential SNP for the AI trait was significant at the chromosome level, but no gene were located near this SNP. The genome-wide level SNP (rs15613786, C→T) in the RNA methyltransferase gene Nsun7 is located on chromosome 4. Nsun methyltransferases belong to the family of RNA transmethylases and are involved in the methylation of RNA throughout the transcriptome; However, few studies have investigated the function of RNA transmethylases. Nsun2 and Nsun7 were found to be closely related to cell proliferation and differentiation, protein biosynthesis, and cancer. A mutation in Nsun2 was found to be closely associated with intellectual disability[32], and the transversion of 26,248 bp in exon 7 of Nsun7 was thought to cause a change in protein folding that reduced the energy of the movement of sperm in men[33]. Furthermore, Nsun2 was found to be significantly associated with thymus weight of Beijing Oil chicken, which is a very popular Chinese native chicken[34]. Thymus is the main immune organ, and therefore the Nsun gene family is thought to have an important effect on the immune ability of chicken. Accordingly, *Nsun7* can be considered as a candidate gene for the avian influenza resistance trait in chicken, and deserves further research.

Table 3 – Annotation of candidate genes.

Traits	SNP ID	Chr	Pos (bp)	Alleles	MAF	Nearest Gene	*p*-value	Distance
AI	rs15613786	4	69254247	C/T	0.338	Nsun7	1.82E-06	within
IFN-γ	rs13794539	2	101259916	A/G	0.297	MYOM1	6.32E-07	within

Note: 1. SNP position were based on WASHUC2.

The first allele is minior gene frequency.

Genes' information were based on NCBI and Ensembl.

ACKNOWLEDGMENT

This paper was supported by the University level Project of Jiangsu Agricultural and Animal Husbandry Vocational College (NSF2022CB14), the University level Project of Jiangsu Agricultural and Animal Husbandry Vocational College (NSF2021TF05), and the Double-high Construction Project of Jiangsu Agricultural and Animal Husbandry Vocational College (No. 2019 [14]). We thank Margaret Biswas, PhD, from Liwen Bianji (Edanz) (www.liwenbianji.cn/) for editing the English text of a draft of this This paper was supported by the University level Project of Jiangsu Agri-animal Husbandry Vocational College(NSF2022CB14), the University level Project of Jiangsu Agri-animal Husbandry Vocational College (NSF2021TF05), and the Double-high Construction Project of Jiangsu Agricultural and Animal Husbandry Vocational College (No. 2019 [14]). We thank Margaret Biswas, PhD, from Liwen Bianji (Edanz) (www.liwenbianji.cn/) for editing the English text of a draft of this manuscript.

Table S1 – Basic information of SNP markers on physical map in chicken.

Chr	Length of each chr	SNP number	Density of markers
1	195,276,750	19074	10.24
2	148,809,762	13399	11.11
3	110,447,801	10017	11.03
4	90,216,835	7999	11.28
5	59,580,361	5652	10.54
6	34,951,654	3618	9.67
7	36,245,040	3476	10.43
8	28,767,244	2769	10.39
9	23,441,680	2327	10.07
10	19,911,089	2072	9.61
11	19,401,079	1578	12.29
12	19,897,011	2219	8.97
13	17,760,035	1846	9.62
14	15,161,805	1655	9.16
15	12,656,803	1335	9.48
16	535,270	75	7.14
17	10,454,150	1529	6.84
18	11,219,875	1228	9.14
19	9,983,394	1122	8.90
20	14,302,601	1551	9.22
21	6,802,778	859	7.92
22	4,081,097	246	16.59
23	5,723,239	741	7.72
24	6,323,281	822	7.69
25	2,191,139	166	13.20
26	5,329,985	614	8.68
27	5,209,285	704	7.40
28	4,742,627	510	9.30
MT	16,775	145	0.11
W	1,248,174	163	7.66
Z	82,363,669	1542	53.41

Note: Approximately 10 kb was the average physical distance between 2 neighboring SNPs.

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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