

A genome-wide single nucleotide polymorphism scan reveals genetic markers associated with fertility rate in Chinese Jing Hong chicken

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ABSTRACT The function of the sperm storage tubules is directly correlated with the fertility of laying hens. However, little is known about the molecular mechanisms regulating the fertility traits in chicken. To identify genetic markers associated with reproductive traits, we calculated fertility rate at 61 to 69 wk (51 D) of Jing Hong chickens parent generation as the phenotype and the genotype were detected by the chicken 600K Affymetrix Axiom High Density single nucleotide polymorphisms (SNP)-array. The genome-wide association study using 190 Jing Hong hens showed that the 20 SNP in chromosomes 3 and 13 were significantly associated with fertility rate. To verify these results, a total of 1900 Jing Hong laying hens from 2 populations (P1 and P2) were further genotyped by polymerase chain reaction–restriction fragments length polymorphisms method. The association analysis results revealed that 12 polymorphisms (AX-75769978, AX-76582632, AX-75730546, AX-75730496, AX-75730588, AX-76530282,

AX-76530329, AX-76529310, AX-75769906, AX-75755394, AX-80813697 and AX-76582809) out of 20 showed highly significant effects ($P < 0.0001$) on fertility rate in P1, P2 and P1+P2. Six haplotypes (TTAA, TTGG, TTAG, CTAA, CTGG, and CTAG) were inferred based on significant loci (AX-75730546 and AX-76530282) also showed significant association with fertility rate, where haplotype CTAG was shown to be markedly associated with the significantly highest ($P < 0.0001$) fertility rate (in P1, 86.42 ± 0.59 ; P2, 85.98 ± 0.59 and P1+P2, 86.16 ± 0.42) followed by other haplotypes for the irrespective of population studied. Collectively, we report for the first time that 12 SNP in the chromosomes 3 and 13 were significantly associated with fertility rate during the later stage of egg production, which could be used as the potential genetic markers that would be able to facilitate in the selection and improvement of fertility rate through chicken breeding.

Key words: Jing Hong hens, fertility rate, GWAS, SNP, association

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INTRODUCTION

After a single mating or artificial insemination (AI) in their breeding season, the female domestic birds appear to be capable of sustained fertility for days or weeks

(depending on the species), which is a widespread phenomenon among numerous avian, reptilian, and mammalian species (Bakst, et al., 1994). This period of sustained fertility is generally identified as the duration of fertility (DF) (Bakst and Vinyard, 2002). The sperm storage function of the sperm storage tubules (SST) is directly correlated with the fertility of laying hens (Han, et al., 2019). Improving in the DF could increase the interval between successive AI, thus to cut down the number of breeder males and labor costs associated with AI (Beaumont, et al., 1992). In previous studies, the possibility of

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increasing the AI intervals by improving DF via the selection of these 2 traits has been proven (Beaumont, 1992; Yu, et al., 2002; Cheng, et al., 2009; Brun, et al., 2012). Recently, with advances in technologies of next generation sequencing, genome-wide association studies (GWAS) have been utilized to identify the associations between genomic loci and reproductive traits with moderate density single nucleotide polymorphism (SNP) arrays in chickens (Liu, et al., 2011; Wolc, et al., 2014; Zhang, et al., 2015). The success of the poultry industry at all scales of production depends on a regular supply of day-old chicks, which is influenced by 2 major parameters such as the fertility and hatchability of the eggs (M King 'ori, 2011). In the modern egg production industry, AI has been widely used to reduce production costs and improved quality of the progeny (Ottinger and Mench, 1989).

Liquid storage of semen at refrigerated temperatures for up to 6 h in turkeys and 24 h in chicken can result in fertility levels comparable to freshly inseminated semen (Donoghuea and Wishart, 2000). In contrast, female chicken can maintain viable and fertile sperm in the oviduct for 3 to 4 wk following AI or natural mating (Pierson, et al., 1988). The anatomical structures associated with prolonged sperm storage are the SST located in the utero-vaginal junction (UVJ) and infundibulum, and UVJ is the primary SST (Froman, et al., 2011). The biological basis of DF has been reported to be associated with the capacity of hens to store a population of sperms in their oviduct for days or weeks after insemination (Birkhead and Jean-Pierre, 2007; Bakst, et al., 2010; Bakst, 2011). To store sperms, hens possess specialized simple tubular invaginations referred to as SST located in the UVJ mucosal folds (Bakst, 1987). Here, the spermatozoa are stored and ultimately released for upward transport toward the infundibulum for ova fertilization (Bakst, 1987). The utero-vaginal SST function as a sperm reservoir in hen's oviduct (Blesbois and Brillard, 2007). In previous studies, the population of immune cells in the UVJ tissues of infertile hens significantly increased in hens receiving AI (Higaki K Y Y, 1995; Zheng, et al., 2001), and infiltration of lymphocytes into the SST of hens with low fertility was observed (Das, et al., 2005). Along these lines, Bakst reported that successful sperm storage in the SST depends on the immune privilege of the sperms which is thought to relate to an allergen residing in SST (Bakst, 1993).

Reproduction traits are controlled by quantitative trait loci, and a genome-wide scan is an effective approach that can be used to gain an understanding of these complex traits. As a statistical tool, GWAS is one of the most effective methods for identifying important SNP and functional genes that affect quantitative traits (Jin, et al., 2015). The technique is more efficient at identifying genetic characteristics for economic traits than the candidate gene approach. In addition, the development of the Affymetrix 600K Chicken SNP array allows further efficient screening for causal loci and genes with relevance to target traits (Kranis, et al., 2013). As multibreed GWAS can improve precision because of

less linkage disequilibrium (LD) across breeds (Raven et al., 2014), in the present study, a GWAS was performed on 190 Jing Hong chickens of parent generation for a period of 51 D at 61 to 69 wk of age to observe fertility rate as the phenotype. Further, the genotype was detected by the chicken 600K Affymetrix Axiom HD SNP-array to identify molecular markers associated with fertility rate. However, GWAS usually focuses on common variants only, because genetic markers (SNP) that show minor allele frequencies, lower than 0.05, are generally excluded from the analysis. Therefore, this study was planned to investigate SNP in the chromosomes 3 and 13 and their associations with fertility rate to evaluate the effect of this SNP on fertility rate in chicken that can be used to advance poultry breeding by molecular marker assisted selection programs.

MATERIALS AND METHODS

Ethics Statement

This research was performed in strict accordance with the guidelines for experimental animals established by the Ministry of Science and Technology. All the experimental procedures and research on animals were conducted in strict conformity with the recommendations in the Guide for the Care and Use of Laboratory Animals according to the regulations of proclaim of the Standing Committee of Hubei People's Congress (No. 5) and approved by the Biological Studies Animal Care Committee of Hubei Province, P. R. China and the ethics committee of Huazhong Agricultural University, Wuhan, P. R. China (Permission number: HZAUCH-2016-008).

Experimental Birds

The 190 Jing Hong parent chickens were picked up from first population but not use in SNP identification study (association study analysis), which is divided into case and control group according to the nonfertility rate trait to perform GWAS as the first exploration step. A total of 1900 healthy Chinese Jing Hong laying chicken from 2 populations were used for genotyping and marker-trait association analysis under this study as the second validation step. First population (P1), comprising 858 Chinese Jing Hong laying chickens (61-69 wk old), were obtained from the poultry farm of Jingzhou Yukou Poultry Industry Co. Ltd., Jingzhou-434020, Hubei, P. R., China. The second population (P2), comprising a total of 1042 Chinese Jing Hong laying chickens (also 61-69 wk), were reared at Huadu Yukou Poultry Industry Co. Ltd., Beijing-100000, P. R., China. The fertility rate of hens was measured in 3 age periods (61 ± 63 , 64 ± 66 , and 67 ± 69 wk). The birds were fed iso-caloric and an iso-nitrogenous corn-soybean-based diet containing 2,850 kcal ME/kg dry matter and 16.83% crude protein and same environmental management.

Data Collection and Measured Traits

All hens were artificially inseminated once with 2.00×10^8 viable spermatozoa per mL issued from pooled ejaculates collected from 60 sexually mature, proven fertile Jing Hong rooster stocks by natural abdominal palpation to confirm their semen quality before experimental AI. Artificial insemination was carried out with 25 μ L extended semen (1.00×10^8 spermatozoa) within 30 min of semen collection. To prevent any undesirable effects of the interval between inseminations and oviposition on subsequent fertility, insemination was done identically in the afternoon from (15:00–16:00) on the first day of 61, 64, and 67 wk. Eggs of each hen were collected and recorded for each hen from day 2 to the last fertile egg before 2 consecutive clear eggs to up to and/or within the day 18 after AI and later set for incubation every week. All eggs were candled on the day 10 of incubation, and those which did not contain an apparent live embryo were removed and opened for visual confirmations as infertile or early dead. Eggs classified as early embryonic death were counted as fertile. The fertile eggs, dead embryonic eggs, and clear eggs (assumed as infertile) were recorded. The parameter of fertility rate was expressed as the averages of 3 cycles of measurements where each cycle contained 17 D. The number of eggs produced from the individual experimental bird was recorded daily for 51 D duration (61–69 wk).

Fertility is defined as the proportion of the total number of eggs set in each laying period that is fertile (as determined by candling). Finally, the fertility of each hen is determined by a number of eggs set related only to the total number of settable eggs laid by the individual hen within the period. The fertility rate was calculated and/or expressed from the data concerning the number of egg production of each hen using the following equation:

$$\text{Fertility rate (FR)\%} = \frac{\text{Number of fertile eggs}}{\text{Total number eggs}} \times 100$$

Blood Sample Collection and Genomic DNA Extraction

Blood samples were collected from the wing vein in a tube containing EDTA as an anticoagulant. All the samples were collected in an ice box and subsequently preserved at -20°C until further use. The genomic DNA was extracted according to the phenol–chloroform method as described by SAMBROOK and RUSSEL, 2006 (Sambrook and Russell, 2006), with some minor modifications. The concentration and quality of the extracted DNA were quantified using the ND-2000 spectrophotometer (NanoDrop Co., Ltd., Thermo Fisher Scientific, Madison, WI) and agarose gel electrophoreses, respectively. All of the genomic DNA preparations from a total of 1,900 (P1, $n = 858$ and P2, $n = 1,042$) samples were within the ratios of 1.6 - 1.8 (A_{260}/A_{280}) and equilibrated to 50 ng/ μ L and were used for genotyping.

Discovery of Candidate SNP Markers Associated with Fertility Rate using GWAS

Jing Hong parent hens with extreme performance differences were used to detect SNP markers associated with fertility rate using GWAS. A high-density SNP array was employed herein to identify associated variants underlying fertility rate using the chicken 600K Affymetrix Axiom HD SNP-array (Aviagen Ltd., Midlothian, UK) to investigate whether the effects of these quantitative trait loci is associated with fertility traits (Kranis et al., 2013). Genome-wide association studies were performed using a total of 190 Jing Hong parent chicken from among a total of 858 hens of the first population to identify the association between the SNP and fertility rate using MVP software (VanRaden, 2008). The general linear model (Price, et al., 2006), mixed linear model (Price et al., 2006; Yu, et al., 2006), and FarmCPU (Liu, et al., 2016) were used to estimate the association effects of SNP on each of the phenotypes. Jing Hong chicken is resistant to rough feeding and has strong adaptability. It is suitable for China's extensive breeding environment. The survival rate is high, 98% of the chickens are raised, and 93% of the laying hens are 3 or 2 percentage points higher than the foreign varieties. The peak of egg production is long, and the egg production rate of more than 90% can reach more than 180 D. In parental generation, there have been high qualified rate of eggs, high fertilization rate, high rate of healthy mothers, and only 4 to 5 births in the year of the chicken. The qualified rate of eggs was 90%, the fertilization rate was 92.3%, and the number of healthy mothers was 108. In commodity generation, near about 311 eggs is produced at 72 wk of age, with a total egg weight of 19.5 kg and an egg-to-egg ratio of 2.2:1. In GWAS, principal content analysis was done first. The top 3 principal contents were added in the models as a fixed effect. The significant and suggestive significant thresholds were set at $P = 1\text{E-}06$ and $P = 1\text{E-}04$, respectively. The 20 SNP of P -value were used to further analysis. In GWAS, all individuals were divided into 2 groups according to the nonfertility rate. The chickens whose nonfertility rate was lower and higher than 0.01 were considered as the case group and control group, respectively.

Identification of Candidate SNP Associated With Fertility Rate Using Polymerase Chain Reaction–Restriction Fragments Length Polymorphisms and Reconstruction of Haplotypes

All experimental birds (P1 and P2) were then genotyped considering identified SNP location following the polymerase chain reaction–restriction fragments length polymorphisms (PCR-RFLP) technique. A total of 20 primers were designed for genotyping of 20 tag SNP in chicken chromosomes 3 and 13. Tabular description of 20 identified database SNP in chicken chromosomes 3

and 13 is shown in [Supplementary Table 1](#). The name of genes which possess the identified database SNP in the chromosomes 3 and 13 in chicken is shown in [Supplementary Table 2](#). The nucleotide sequences of chicken chromosomes 3 and 13 from reference sequence of chicken Galgal4 (GCA_000002315.2) were used to design primers to amplify DNA fragments which contain those SNP by Oligo6 software. Primer synthesis was completed using oligonucleotide synthesis technology of Sangon Biotech Co., Shanghai, China. A detailed description of primer sets and their corresponding products size with location is that were used to genotype 20 tag SNP is shown in [Supplementary Table 3](#). The 10.0 μL reaction mixture volume included 1.3 μL of DNA template, 0.15 μL of each primer ($100 \text{ nmol } \mu\text{L}^{-1}$), $10 \times$ buffer 1.0 μL , dNTP 0.1 μL , rTaq 0.1 μL , and 7.2 μL sterile distilled water were used for PCR. The amplification program consisted of an Eppendorf Thermal Cycler was programmed for an initial incubation temperature at 95°C for 5 min, followed by 35 cycles each with denaturing at 95°C for 20 s, annealing at 46.6°C to 56.4°C ([Supplementary Table 3](#)) for 20 s, and extension at 72°C for 20 s. A final extension at 72°C for 7 min and then at 25°C for 1m were maintained. After the reaction process was completed, the PCR products were separated on 1.5% (w/v) agarose gel electrophoresis and stained with ethidium bromide and visualized in a BIO-RAD Image Lab gel documentation system (BIO-RAD, Berkeley, SC). The amplified PCR products were digested by 0.03 μL of 250 specific restriction enzymes (**RE**) (New England BioLabs Inc., Ipswich, MA). The digestion mixture contains PCR products: 5 μL , cut smart buffer, 1 μL , DNase free ddH₂O-4 μL , and 3.0 U of each enzyme. After that, it was incubated at the temperature as per manufacture's instruction for specific RE requirements for overnight. The genotype patterns were visualized from the digested products that were separated on 5% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The genotype pattern was recorded by the gel documentation system (BIO-RAD Image Lab) to check the genotype category of each chicken considering each SNP position. The ingredients of SNP in chromosomes 3 and 13 PCR-RFLP optimized RE digestion mixture is shown in [Supplementary Table 4](#). The RE used in the

AX-75755394, SNP-19: AX-80813697, and SNP-20: AX-76582809, for each of the population. Two association analysis of haplotypes were also performed; that is, one is based on 2 significant SNP, SNP-04: AX-75730546 and SNP-07: AX-76530282 for fertility rate, and another one is based on SNP-13: AX-75769906 and SNP-20: AX-76582809 for DF traits according to the genotyping data obtained from all experimental population of P1 and P2 used in the present study applying the PHASE 2.0 program ([Stephens, et al., 2001](#)). The minimum haplotype frequency was set at 2%.

Polymorphism Evaluation

The genotype and allele frequencies at each SNP site were calculated for all populations according to electrophoresis results of the genotype categorization. Genotypic frequencies of different PCR-RFLP patterns were estimated from the combination of various RFLP alleles generated based on the presence or absence of one or more restriction sites. Different genotypes were identified based on different patterns. The genotypic and allelic frequencies among all birds were calculated by the standard procedure ([Frankham, 1996](#)). The test for the Hardy–Weinberg equilibrium (**HWE**) at each SNP site was conducted separately for the P1, P2, and P1+P2 population, using the Haploview version 4.2 software (<http://www.broad.mit.edu/mpg/haploview/>) ([Barrett, et al., 2005](#)). Genotypic and allelic frequencies at each SNP site were calculated, with each polymorphism evaluated for the HWE using a Pearson's goodness-of-fit chi-square test (degree of freedom = 1). We performed LD analysis to characterize 10 causal SNP in a strong LD region where 7 significant SNP were identified by the solid spine algorithm in Haploview version 4.2 (Cambridge, MA) as being clustered ([Barrett et al., 2005](#)). The indexes of genetic variability in population genetics including gene homozygosity (*Ho*), gene heterozygosity (*He*), the effective number of alleles, and the polymorphism information content were calculated according to the Botstein's methods ([Botstein, et al., 1980](#)) and Nei's methods ([Nei and Roychoudhury, 1974](#)).

The allele and genotype frequencies were calculated by standard formula as follows:

$$\text{Genotype frequency} = \frac{\text{Number of individuals of a particular genotype}}{\text{Total number of animals of all genotypes}}$$

present study and their respective restriction sites are presented in the [Supplementary Table 5](#).

Haplotypes were reconstructed among 12 significant SNP, that is SNP-01: AX-75769978, SNP-03: AX-76582632, SNP-04: AX-75730546, SNP-05: AX-75730496, SNP-06: AX-75730588, SNP-07: AX-76530282, SNP-08: AX-76530329, SNP-11: AX-76529310, SNP-13: AX-75769906, SNP-14:

$$\text{Gene frequency} = \frac{2D+H}{2N}$$

where, D = number of animals homozygous for a particular allele.

H = number of heterozygous animals.

N = total number of animals.

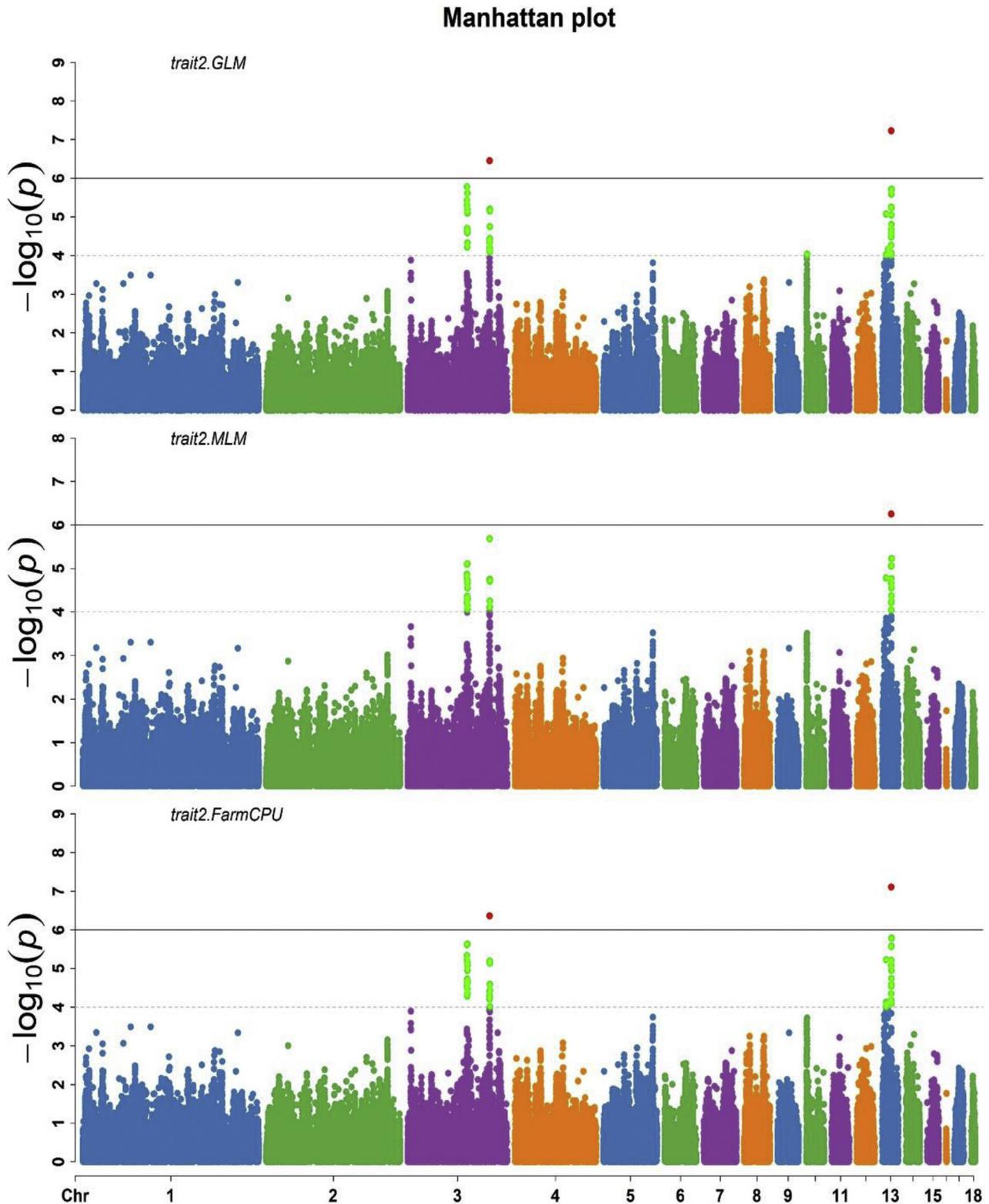


Figure 1. Rectangular Manhattan plot of the P-values in the genome-wide the association study analysis showing association of single nucleotide polymorphisms (SNP) with fertility rate in Chinese Jing Hong Chicken. Figure 1 illustrating 3 methods showing upper figure is general linear model, middle one is multiple linear model, and lower one is fixed and random model circulating probability unification (FarmCPU). Each dot represents an SNP. The figure illustrated the level of statistical significance (y-axis), as measured by the negative log of the corresponding P-value, for each SNP. Each typed SNP is indicated by dots of different colors are arranged by chromosomal location (x-axis). Imputation was performed on chromosomes 3 and 13 only using 190 Genomes data. The SNP above the solid and dotted line were significantly and suggestive significantly associated with chicken nonfertility rate trait.

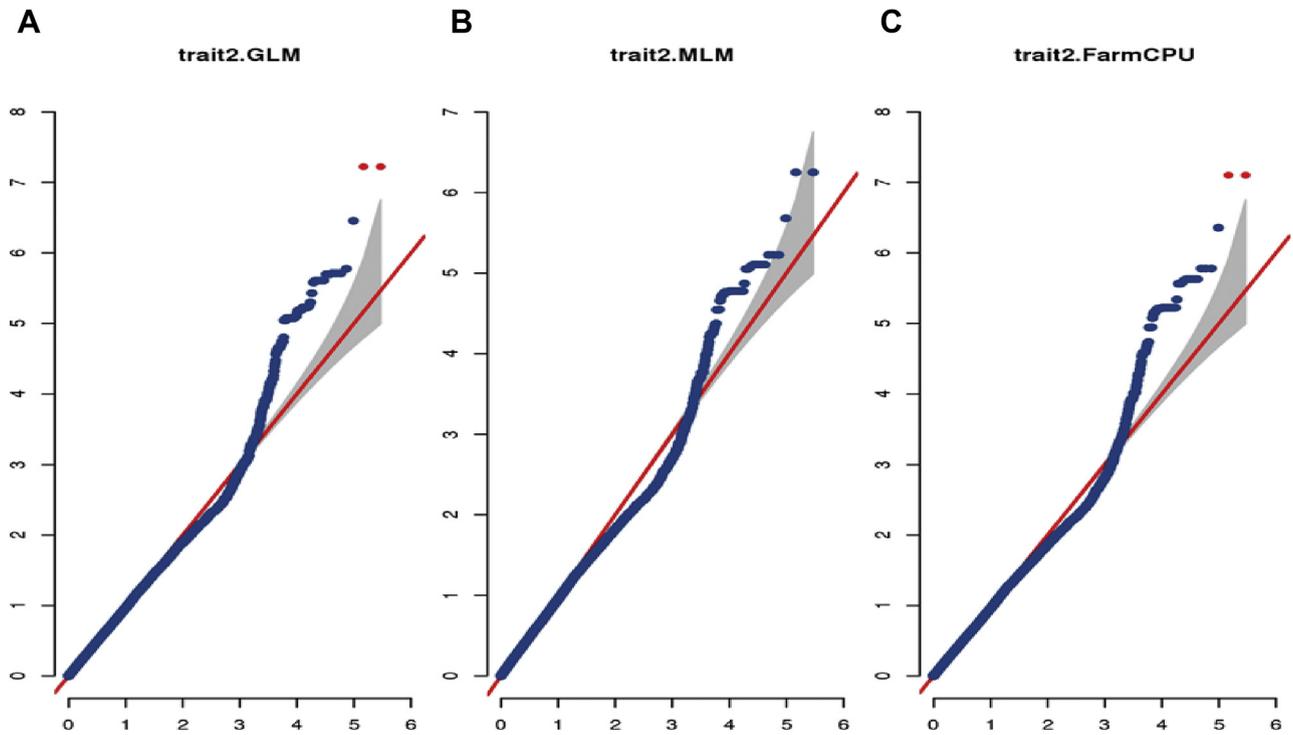


Figure 2. Q-Q (Quantile–Quantile) plots of P-values adjusted after Bonferroni correction in GWAS analysis for quality control check and visualizing crude association. (A), (B), and (C) represented the result of general liner model, mixed liner model and FarmCPU, respectively. GWAS, genome-wide the association study.

Marker-Trait Association Analysis

The association analyses of the 20 SNP genotypes or haplotypes with phenotypic data of fertility rate trait were analyzed in the Chinese Jing Hong layer chicken population using the general linear model procedure for least square means (LSM) with Statistical Package for the Social Sciences (SPSS) statistics analytical software package (version 20.0; IBM Corp., Armonk, NY). Data were processed in excel, and the genetic effects were analyzed by a linear mixed effect model procedure.

$$Y_{ijk} = \mu + L_i + G_j + F_k + e_{ijk}$$

where.

Y_{ijk} = Phenotypic value of the target trait, such as fertility rate trait.

μ = population mean of fertility rate trait.

L_i = Fixed effect of the line.

G_j = fixed effect of the SNP genotype or haplotype.

F_k = Random effect of the family.

e_{ijk} = the overall error term.

Type III sum of squares was used in each test. The threshold for significance was set at $P < 0.05$ and for high significance at $P < 0.01$. All values are presented as least square means with standard errors of the mean (LSM \pm SEM). Means were compared for significant differences using Duncan's Multiple Range Test (Duncan, 1955).

RESULTS

Discovery of Most Significant SNP in Chromosomes 3 and 13 by GWAS

The 190 Jing Hong parent chickens were divided to the case and control group according to the nonfertility rate trait, and the results were as in [Supplementary Table 6](#). In results, 19 and 171 chickens were found in case group and control group, respectively. In addition, significant difference was identified between the case and control group. The result of the GWAS was shown in [Figure 1](#). There was no dramatic deviation between observed and expected $-\log_{10}(P)$ in the (Quantile–Quantile) plot ([Figure 2](#)), suggesting that there was little or no evidence of residual population structure effects in test statistic inflation. In result, the top 20 SNP of P-value was on chromosomes 3 and 13. The GWAS results of 190 laying birds show that 20 identified SNP in chromosomes 3 and 13 were associated with fertility rate traits. This SNP could serve as a new candidate genetic marker for the fertility rate trait at late laying stage of production, yet their roles need to be verified in further studies. The positions of the SNP and the information were obtained based on the ICGSC annotation of *Gallus gallus* genome version 4.0. The results found that 20 SNP in chromosomes 3 and 13 were significantly associated with fertility rate. Tabular description of 20 identified database SNP in chromosomes 3 and 13 is shown in

Supplementary Table 1. For the validation, these selected significantly associated SNP by GWAS were further genotyped using PCR-RFLP approach, and association studies were performed with fertility rate trait in 2 expanded populations.

Genotyping by PCR-RFLP and Reconstruction of Haplotypes

The genotype patterns of the SNP in chromosomes 3 and 13 were checked using the PCR-RFLP technique and found 2 genotype patterns in SNP AX-75769978 (AA and AG), AX-75730546 (CT and TT), AX-75730496 (AG and GG), AX-76530329 (AC and CC), AX-76530331 (CC and CT), AX-76530309 (AA and AG), AX-75730343 (CC and CT), AX-75769906 (CT and TT), AX-75755413 (CC and CT), AX-75755447 (AA and AG), and AX-75755457 (CC and CT) and 3 genotype patterns in SNP AX-75769993 (AA, AG, and GG), AX-76582632 (CC, CT, and TT), AX-75730588 (AA, AG, and GG), AX-76530282 (AA, AG, and GG), AX-76529310 (CC, CT, and TT), AX-75755394 (GG, GT, and GT), AX-75730254 (AA, AG, and GG), AX-80813697 (AA, AT, and TT), and AX-76582809 (AA, AG, and GG) ([Supplementary Figures 1–20](#)). The genotypic and allele frequencies were calculated by observing the presence of various RFLP patterns in chromosomes 3 and 13 and are presented in [Supplementary Table 7](#).

Haplotypes were reconstructed among 12 significant SNP, that is SNP-01: AX-75769978, SNP-03: AX-76582632, SNP-04: AX-75730546, SNP-05: AX-75730496, SNP-06: AX-75730588, SNP-07: AX-76530282, SNP-08: AX-76530329, SNP-11: AX-76529310, SNP-13: AX-75769906, SNP-14: AX-75755394, SNP-19: AX-80813697, and SNP-20: AX-76582809, and their frequencies among all studied individuals are shown in [Supplementary Table 8](#). Among the haplotypes, the most abundant 3 haplotypes were *H1* (ATTGGGCTTTTG), *H2* (ACTGAGCCTGAA), and *H3* (GCCAAAACCGAA) and accounted for 65.19% genetic information in P1 population. In the P2 population, the most abundant 3 haplotypes were *H1* (ATTGGGCTTTTG), *H2* (GCCAAAACCGAA), and *H3* (ACTGAGCCTGAA) and accounted for 79.16% genetic information. Finally, in the P1+P2 population, the most abundant 3 haplotypes were *H1* (ATTGGGCTTTTG), *H2* (GCCAAAACCGAA), and *H3* (ACTGAGCCCGAA) and accounted for 73.06% genetic information. From the analysis results of haplotype frequencies, it was found that haplotype *H1* (ATTGGGCTTTTG) was common and predominant in all groups of the population studied.

Another haplotype reconstruction was performed based on 2 significant SNP, that is SNP-04: AX-75730546 and SNP-07: AX-76530282, with fertility rate according to these genotyping data, and 6 haplotypes (TTAA, TTGG, TTAG, CTAA, CTGG, and CTAG) were identified among the individuals. For the

P1 population, the haplotype present at the highest frequency was the CTAG haplotype (0.35), with the TTGG haplotype is the next most frequent (0.33), followed by TTAG (0.12), CTGG (0.11), and TTAA (0.05), and CTAA haplotype is the lowest frequent (0.04). For the P2 population, the haplotype present at the highest frequency was the CTAG haplotype (0.43), with the TTGG haplotype is the next most frequent (0.36), followed by TTAG (0.08), TTAA (0.06), and CTAA (0.05), and CTGG haplotype is the lowest frequent (0.02). For the total (P1+P2) population, the haplotype present at the highest frequency was the CTAG haplotype (0.39), with the TTGG haplotype is the next most frequent (0.35), followed by TTAG (0.10), CTGG (0.06), and TTAA (0.06), and CTAA haplotype is the lowest frequent (0.04).

Frequencies of Genotypes and Alleles at the SNP Locus

The genotypic and allelic frequencies of each identified SNP in chromosomes 3 and 13 are presented in [Supplementary Table 7](#). All the population was found to exhibit significant genetic disequilibrium ($P \geq 0.05$), except in the SNP site AX-76530282, AX-76530329, AX-76530309, and AX-76530309 in P1 and SNP site AX-76530309 in P2 population among concern alleles in chromosomes 3 and 13. This has shown a low genetic diversity in the population which might result from the cause of selection. As shown in [Supplementary Table 9](#), the gene *Ho* was higher than gene *He* for all of the locus as well as for all of the population, with the effective allele numbers. The value of polymorphism information content was not higher for all of the locus as well as for all of the population. Genotypic and allelic frequencies of 20 SNP were inconsistent (except very few) with HWE. This has shown a low genetic diversity in the population which might result from the cause of selection.

Association Analysis Between the SNP Genotypes in Chromosomes 3 and 13 With Fertility Rate in Chinese Jing Hong Hens Breed

Statistical analyses were performed to test the significance of the difference of genotype effect on fertility rate among the 20 (twenty) SNP in chromosomes 3 and 13 in Chinese Jing Hong layer chickens. The association analysis results were shown between the SNP genotypes in chromosomes 3 and 13 and fertility rate by 51 D of laying period in the Chinese Jing Hong layer chickens, and the least square means and standard error of means (LSM \pm SEM) of different genotypes for each SNP are listed in [Table 1](#). The results showed that the significant ($P < 0.0001$) association with 51 D fertility rate was found at the loci AX-75769978, AX-76582632, AX-75730546, AX-75730496, AX-75730588, AX-76530282, AX-76530329, AX-76529310, AX-75769906, AX-75755394,

Table 1. Association analysis between the genotypes of 20 genetic markers and fertility rate in the Chinese Jing Hong chicken population.

SNP	Location in chromosome	Population	N	Genotype frequency (LSM ± SEM)			F-value, <i>P</i> -value, and level of significance		
							F-value	<i>P</i> -value	Level of significance
SNP-01 (AX-75769978)	13			AA	AG	GG			
		P1	858	82.11 ± 0.65 ^a (250)	85.19 ± 0.41 ^b (608)	-	16.11	<0.0001	***
		P2	1,042	74.88 ± 0.70 ^a (312)	84.79 ± 0.46 ^b (730)	-	141.207	<0.0001	***
		P1+P2	1,900	78.10 ± 0.49 ^a (562)	84.97 ± 0.32 ^b (1338)	-	139.370	<0.0001	***
SNP-02 (AX-75769993)	13			AA	AG	GG			
		P1	858	86.82 ± 3.44 (9)	83.97 ± 0.61 (288)	84.41 ± 0.44 (561)	0.448	0.6390	NS
		P2	1,042	87.05 ± 7.59 (3)	81.91 ± 0.69 (365)	81.75 ± 0.51 (674)	0.256	0.7740	NS
		P1+P2	1,900	86.88 ± 3.46 (12)	82.82 ± 0.47 (653)	82.96 ± 0.34 (1235)	0.682	0.5060	NS
SNP-03 (AX-76582632)	3			CC	CT	TT			
		P1	858	84.87 ± 0.62 ^b (274)	85.76 ± 0.61 ^b (284)	82.37 ± 0.59 ^a (300)	8.667	<0.0001	***
		P2	1,042	83.07 ± 0.67 ^b (360)	85.22 ± 0.67 ^c (354)	76.79 ± 0.70 ^a (328)	40.409	<0.0001	***
		P1+P2	1,900	83.85 ± 0.47 ^b (634)	85.46 ± 0.47 ^c (638)	79.46 ± 0.47 ^a (628)	47.435	<0.0001	***
SNP-04 (AX-75730546)	13			CC	CT	TT			
		P1	858	-	86.14 ± 0.49 ^b (429)	82.44 ± 0.49 ^a (429)	28.427	<0.0001	***
		P2	1,042	-	85.84 ± 0.55 ^b (516)	77.88 ± 0.55 ^a (526)	105.513	<0.0001	***
		P1+P2	1,900	-	85.98 ± 0.38 ^b (945)	79.93 ± 0.38 ^a (955)	128.824	<0.0001	***
SNP-05 (AX-75730496)	13			AA	AG	GG			
		P1	858	-	87.09 ± 0.46 ^b (456)	81.11 ± 0.49 ^a (402)	78.404	<0.0001	***
		P2	1,042	-	85.68 ± 0.52 ^b (566)	77.24 ± 0.57 ^a (476)	119.06	<0.0001	***
		P1+P2	1,900	-	86.31 ± 0.36 ^b (1022)	79.01 ± 0.39 ^a (878)	192.351	<0.0001	***
SNP-06 (AX-75730588)	13			AA	AG	GG			
		P1	858	84.89 ± 0.64 ^b (257)	86.08 ± 0.61 ^b (278)	82.27 ± 0.57 ^a (323)	11.125	<0.0001	***
		P2	1,042	83.96 ± 0.71 ^b (317)	85.31 ± 0.66 ^b (358)	76.58 ± 0.66 ^a (367)	50.485	<0.0001	***
		P1+P2	1,900	84.38 ± 0.49 ^b (574)	85.65 ± 0.46 ^b (636)	79.24 ± 0.44 ^a (690)	56.193	<0.0001	***
SNP-07 (AX-76530282)	3			AA	AG	GG			
		P1	858	82.84 ± 0.52 ^a (75)	85.96 ± 0.51 ^b (401)	82.73 ± 1.18 ^a (382)	10.152	<0.0001	***
		P2	1,042	80.99 ± 1.18 ^b (114)	85.35 ± 0.55 ^c (526)	77.45 ± 0.63 ^a (402)	44.940	<0.0001	***
		P1+P2	1,900	81.68 ± 0.85 ^a (189)	85.61 ± 0.38 ^b (927)	80.08 ± 0.42 ^a (784)	48.714	<0.0001	***
SNP-08 (AX-76530329)	3			AA	AC	CC			
		P1	858	-	86.20 ± 0.50 ^b (416)	82.49 ± 0.48 ^a (442)	28.761	<0.0001	***
		P2	1,042	-	85.53 ± 0.54 ^b (540)	77.84 ± 0.56 ^a (502)	97.563	<0.0001	***
		P1+P2	1,900	-	85.82 ± 0.38 ^b (956)	80.02 ± 0.38 ^a (944)	118.166	<0.0001	***
SNP-09 (AX-76530331)	3			CC	CT	TT			
		P1	858	84.40 ± 0.61 (286)	84.24 ± 0.43 (572)	-	0.023	0.9780	NS
		P2	1,042	82.81 ± 0.70 (348)	81.38 ± 0.50 (694)	-	2.361	0.1250	NS
		P1+P2	1,900	83.47 ± 0.48 (634)	82.67 ± 0.34 (1266)	-	1.863	0.1720	NS
SNP-10 (AX-76530309)	3			AA	AG	GG			
		P1	858	84.40 ± 0.37 (768)	83.37 ± 1.08 (90)	-	0.799	0.3720	NS
		P2	1,042	81.90 ± 0.43 (938)	81.12 ± 1.29 (104)	-	0.328	0.5670	NS
		P1+P2	1,900	83.03 ± 0.29 (1706)	82.16 ± 1.16 (194)	-	0.892	0.3450	NS
SNP-11 (AX-76529310)	3			CC	CT	TT			
		P1	858	84.73 ± 0.66 ^b (240)	85.76 ± 0.59 ^b (302)	82.55 ± 0.58 ^a (316)	7.921	<0.0001	***
		P2	1,042	84.09 ± 0.72 ^b (302)	85.31 ± 0.66 ^b (360)	76.72 ± 0.64 ^a (380)	50.185	<0.0001	***
		P1+P2	1,900	84.37 ± 0.50 ^b (542)	85.52 ± 0.45 ^b (662)	79.37 ± 0.44 ^a (696)	52.653	<0.0001	***
SNP-12 (AX-75730343)	13			CC	CT	TT			
		P1	858	84.11 ± 0.38 (728)	85.30 ± 0.90 (130)	-	0.737	0.4790	NS
		P2	1,042	82.07 ± 0.44 (874)	80.55 ± 1.01 (168)	-	1.891	0.1690	NS
		P1+P2	1,900	83.00 ± 0.30 (1602)	82.62 ± 0.70 (298)	-	0.244	0.6210	NS

SNP-13 (AX-75769906)	13			CC	CT	TT			
		P1	858	-	86.45 ± 0.54 ^b (350)	82.80 ± 0.45 ^a (508)	26.769	<0.0001	***
		P2	1,042	-	85.92 ± 0.56 ^b (504)	77.97 ± 0.54 ^a (538)	104.426	<0.0001	***
		P1+P2	1,900	-	86.14 ± 0.40 ^b (854)	80.33 ± 0.36 ^a (1046)	117.081	<0.0001	***
SNP-14 (AX-75755394)	13			GG	GT	TT			
		P1	858	84.69 ± 0.67 ^b (234)	86.29 ± 0.64 ^b (255)	82.65 ± 0.53 ^a (369)	9.824	<0.0001	***
		P2	1,042	84.45 ± 0.71 ^b (302)	86.15 ± 0.67 ^b (338)	76.21 ± 0.62 ^a (402)	69.116	<0.0001	***
		P1+P2	1,900	84.55 ± 0.50 ^b (536)	86.21 ± 0.48 ^c (593)	79.30 ± 0.42 ^a (771)	66.803	<0.0001	***
SNP-15 (AX-75755413)	13			CC	CT	TT			
		P1	858	84.34 ± 0.38 (740)	83.96 ± 0.95 (118)	-	0.142	0.7070	NS
		P2	1,042	81.79 ± 0.44 (902)	82.03 ± 1.11 (140)	-	0.040	0.8420	NS
		P1+P2	1,900	82.94 ± 0.30 (1642)	82.91 ± 0.75 (258)	-	0.001	0.9710	NS
SNP-16 (AX-75755447)	13			AA	AG	GG			
		P1	858	84.41 ± 0.40 (677)	83.85 ± 0.77 (181)	-	0.415	0.5190	NS
		P2	1,042	81.96 ± 0.46 (815)	81.35 ± 0.87 (227)	-	0.385	0.5350	NS
		P1+P2	1,900	83.07 ± 0.31 (1492)	82.46 ± 0.59 (408)	-	0.832	0.3620	NS
SNP-17 (AX-75755457)	13			CC	CT	TT			
		P1	858	84.35 ± 0.38 (735)	83.91 ± 0.93 (123)	-	0.198	0.6570	NS
		P2	1,042	82.03 ± 0.44 (893)	80.63 ± 1.08 (149)	-	1.445	0.2300	NS
		P1+P2	1,900	83.08 ± 0.30 (1628)	82.11 ± 0.73 (272)	-	1.509	0.2200	NS
SNP-18 (AX-75730254)	13			AA	AG	GG			
		P1	858	84.27 ± 0.93 (122)	84.91 ± 1.00 (106)	84.19 ± 0.41 (630)	0.221	0.8010	NS
		P2	1,042	81.41 ± 1.05 (156)	82.80 ± 1.21 (117)	81.76 ± 0.47 (769)	0.409	0.6650	NS
		P1+P2	1,900	82.67 ± 0.72 (278)	83.80 ± 0.80 (223)	82.85 ± 0.32 (1399)	0.684	0.5050	NS
SNP-19 (AX-80813697)	3			AA	AT	TT			
		P1	858	83.84 ± 0.63 ^a (262)	86.30 ± 0.56 ^b (330)	82.25 ± 0.62 ^a (266)	12.055	<0.0001	***
		P2	1,042	84.00 ± 0.73 ^b (302)	85.08 ± 0.66 ^b (369)	76.81 ± 0.65 ^a (371)	46.279	<0.0001	***
		P1+P2	1,900	83.93 ± 0.49 ^b (564)	85.66 ± 0.44 ^c (699)	79.08 ± 0.46 ^a (637)	55.833	<0.0001	***
SNP-20 (AX-76582809)	3			AA	AG	GG			
		P1	858	84.08 ± 0.61 ^b (284)	85.98 ± 0.55 ^c (344)	82.03 ± 0.67 ^a (230)	10.428	<0.0001	***
		P2	1,042	83.62 ± 0.72 ^b (311)	84.73 ± 0.68 ^b (354)	77.62 ± 0.66 ^a (377)	32.795	<0.0001	***
		P1+P2	1,900	83.84 ± 0.48 ^b (595)	85.34 ± 0.44 ^c (698)	79.88 ± 0.48 ^a (607)	45.841	<0.0001	***

Abbreviations: P1, first population; P2, second population; P1+P2, both first and second population; N, number of individual; SNP, single nucleotide polymorphism; LSM, least squares of mean; SEM, standard error of mean; G = genotypes (i.e., AA, AG, GG).

The values in the parenthesis indicates the number of chicken of corresponding group, * = Significant at $P < 0.1$, ** = Significant at $P < 0.01$, *** = Significant at $P < 0.001$, ^{a, b, c} = LSM values bearing different letters in each column are significantly different at *, ** and ***, NS = Non-significant at $P \geq 0.5$.

AX-80813697, and AX-76582809 in all populations of Chinese Jing Hong layer chicken which are treated as SNP-01, SNP-03, SNP-04, SNP-05, SNP-06, SNP-07, SNP-08, SNP-11, SNP-13, SNP-14, SNP-19, and SNP-20, respectively. There was no significant association found for the SNP, that is AX-75769993, AX-76530331, AX-76530309, AX-75730343, AX-75755413, AX-75755447, AX-75755457, and AX-75730254, in fertility rate by 51 D of laying period in any group of population studied ($P \geq 0.05$).

An association analysis between the haplotypes (TTAA, TTGG, TTAG, CTAA, CTGG, and CTAG) inferred based on 2 significant loci AX-75730546 and AX-76530282 and fertility rate by 51 D in P1, P2, and P1+P2 population were shown in Table 2. In the case of P1, among the haplotypes, haplotype CTAG was found to be correlated with the significantly highest ($P < 0.0001$) fertility rate (86.42 ± 0.59) % by 51 D of laying period, followed by haplotype CTGG (85.55 ± 1.04), CTAA (85.28 ± 1.74), TTAG (84.61 ± 1.01), and TTGG (81.94 ± 0.60) and with the lowest levels associated with haplotype TTAA (80.61 ± 1.58). In the P2, among the 6 haplotypes, conversely, haplotype CTAG was shown to be markedly associated with the significantly highest ($P < 0.0001$) fertility rate (85.98 ± 0.59) by 51 D of laying period, followed by haplotype CTAA (85.08 ± 1.82), CTGG (84.75 ± 2.55), TTAG (81.84 ± 1.32), and TTAA (78.11 ± 1.51) and with the lowest levels associated with haplotype TTGG (76.99 ± 0.64). Finally, in case of P1+P2 population, among the 6 haplotypes, haplotype CTAG was shown to be markedly associated with the significantly highest ($P < 0.0001$) fertility rate (86.16 ± 0.42) by 51 D of laying period, followed by haplotype CTGG (85.39 ± 1.06), CTAA (85.16 ± 1.29), TTAG (83.38 ± 0.86), and TTGG (79.13 ± 0.49) and with the lowest levels associated with haplotype TTAA (79.06 ± 1.11).

LD Analysis of SNP in Chromosomes 3 and 13 in Chinese Jing Hong Chicken Population

Haplotype block and LD structures were generated from the 10 SNP genotyped in chromosomes 3 and 13 from chicken populations which are represented in Figures 3A–3C. Pairwise coefficients of LD (D') values are shown between polymorphisms, which were calculated from the genotypic data of P1 = 858, P2 = 1,042, and P1+P2 = 1,900 chickens. The haplotypes block was defined by using the default setting of the Haploview software (Broad Institute, Cambridge, MA). In the P1 population, 2 variants, that is rs317994379 A/G and rs15696982C/T, showed significant LD with each other with high D' ($D' = 82$) with spanning 17 kb in block 1, and 2 variants, that is rs313789133 A/T and rs313740316 C/T, showed significant LD with each other with D' ($D' = 58$) with spanning 2 kb in block 3. For the P2 population, 2

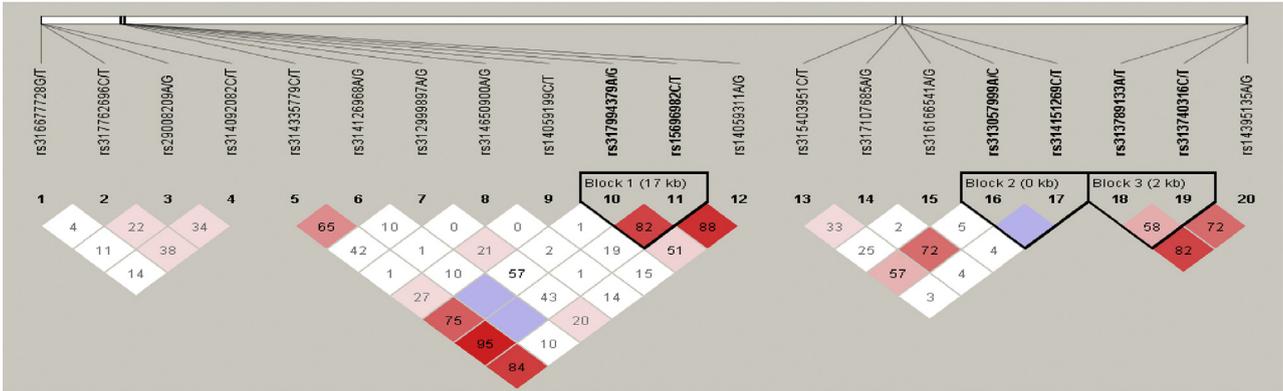
Table 2. Association analysis between the haplotype inferred through 2 SNP in chromosome 13 at AX-75730546 (C10203243 T) and 3 at AX-76530282 (A66244496 G) and fertility rate of Chinese Jing Hong chicken population.

Trait	Population (N)	Haplotype frequency (LSM \pm SEM)						F-value, P-value, and level of significance		
		H1 (TTAA)	H2 (TTGG)	H3 (TTAG)	H4 (CTAA)	H5 (CTGG)	H6 (CTAG)	F-value	P-value	Level of significance
Fertility rate	P1 (858)	80.61 \pm 1.58 ^a (41)	81.94 \pm 0.60 ^{ab} (287)	84.61 \pm 1.01 ^{b,c} (101)	85.28 \pm 1.74 ^{b,c} (34)	85.55 \pm 1.04 ^c (95)	86.42 \pm 0.59 ^c (300)	7.203	<0.0001	***
	P2 (1042)	78.11 \pm 1.52 ^{a,b} (67)	76.99 \pm 0.64 ^a (378)	81.84 \pm 1.39 ^{b,c} (81)	85.08 \pm 1.82 ^c (47)	84.75 \pm 2.55 ^c (24)	85.98 \pm 0.59 ^c (445)	23.338	<0.0001	***
	P1+P2 (1900)	79.06 \pm 1.11 ^a (108)	79.13 \pm 0.49 ^a (665)	83.38 \pm 0.86 ^b (182)	85.16 \pm 1.29 ^b (81)	85.39 \pm 1.06 ^b (119)	86.16 \pm 0.42 ^b (745)	30.170	<0.0001	***

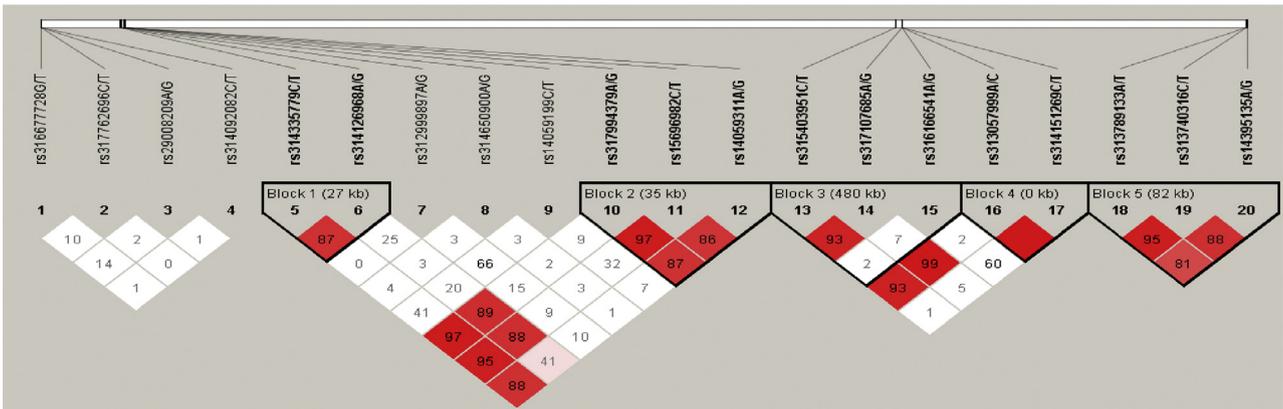
Abbreviations: P1, first population; P2, second population; P1+P2, Total (Both first & second) population; N, number of individual; SNP, single nucleotide polymorphism, LSM, least squares of mean; SEM, standard error of mean; H, haplotypes (i.e., TTAA, TTGG, TTAG, CTAA, CTGG, and CTAG).

The values in the parenthesis indicates the number of chicken of corresponding group, * = Significant at $P < 0.01$, ** = Significant at $P < 0.001$, *** = Significant at $P < 0.0001$, ^{a, b, c} = LSM values within a row for each haplotype lacking a common superscript differ significantly $P < 0.05$.

A



B



C

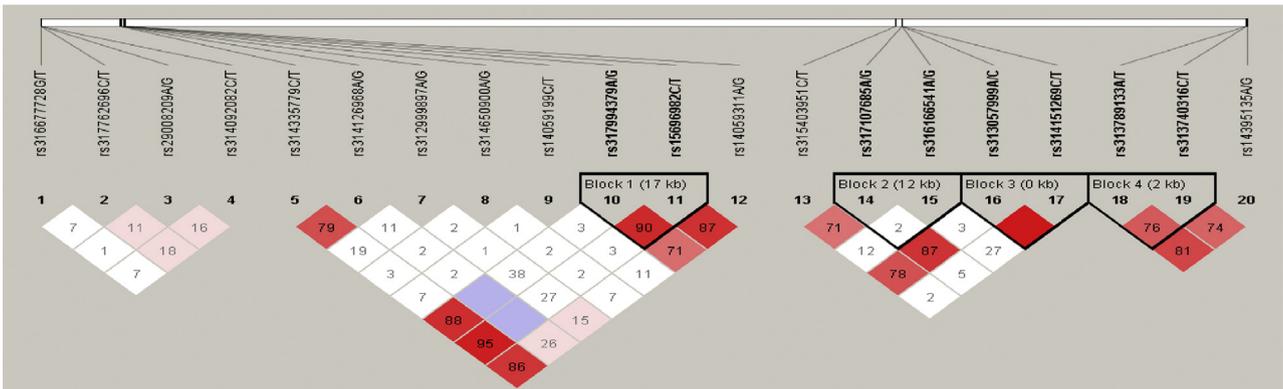


Figure 3. (A–C). Linkage disequilibrium (LD) of single nucleotide polymorphisms (SNP) in chromosomes 3 and 13 in Chinese Jing Hong Chicken. Pairwise correlation (D') values are shown between polymorphisms, which were calculated from the genotypic data of 858, 1042 and 1900 chicken for P1, P2, and P1+P2 respectively. **Figures 3A–3C** indicate P1, P2, and P1+P2 respectively. The color of the block indicates the LD status of SNP; deep red means high linkages between 2 SNP. The haplotypes block was defined by using the default setting of the Haploview software.

variants, that is rs314335779 C/T and rs314126968 A/G, showed significant LD with each other with high D' ($D' = 87$) with spanning 27 kb in block 1; 3 variants, that is rs317994379 A/G and rs15696982 C/T and rs14059311 A/G, showed significant LD with each other

with high D' ($D' = 97$ and 86 , respectively) with spanning 35 kb in block 2; 2 variants, that is rs315403951 C/T and rs317107685 A/G, showed significant LD with each other with high D' ($D' = 93$) with spanning 480 kb in block 3; 2 variants, that is

rs313057999 A/C and rs314151269 C/T, showed significant LD with high D' ($D' = 100$) with spanning 82 kb in block 4; and 3 variants, that is rs313789133 A/T and rs313740316 C/T and rs14395135 A/G, showed significant LD with each other with high D' ($D' = 95$ and 88 , respectively) with spanning 82 kb in block 5. Finally, in the P1+P2 population, 2 variants, that is rs317994379 A/G and rs15696982 C/T, showed significant LD with each other with high D' ($D' = 90$) with spanning 17 kb in block 1, 2 variants, that is rs313057999 A/C and rs314151269 C/T, showed significant LD with each other with high D' ($D' = 100$) with spanning 0 kb in block 3, and 2 variants, that is rs313789133 A/T and rs313740316 C/T, showed significant LD with each other with high D' ($D' = 76$) with spanning 2 kb in block 4.

DISCUSSION

The importance of fertility in hens cannot be overemphasized, and spermatozoa are known to be stored within the female genital tract after AI or natural mating in various species to optimize the timing of reproductive events such as copulation, fertilization, and ovulation. The mechanism supporting high and low sperm storage in hens is still unclear. In this study, the fertility rate of Chinese Jing Hong layer chicken, as well as molecular markers, were explored. We chose AI as a reproductive method. Because females were the focus of interest, we used pooled ejaculates from several males and decided to use a higher than recommended in practice number of spermatozoa per insemination dose (Beaumont et al., 1992) such that a high number of spermatozoa would fill SST completely. The assessment of qualitative and quantitative parameters of ejaculates obtained from previously selected top males showed that they are within the normal range reported for breeder roosters (Soller, et al., 1965; Kirby, et al., 1998), and also hens achieved good productive results. The results of fertility presented here are similar to those studies performed on other egg type lines, in which the fertility rate is medium repetition rate trait and shows high individual variability among hens (Kosba, et al., 1983; Liu, et al., 2008). Therefore, relative genetic variability was expected to exist. Similarly, several studies have reported that the biological basis of fertility rate is related to sperm storage, and sperm storage is dependent on sperm immune privilege. This mechanism may be realized suppressing local immune function by upregulating *TGF β s* expression in the UVJ (Das, et al., 2010). To sum up, the fertility rate was a complex and systematic process as follows: surviving sperms from the UVJ environment and immunological response, retaining sperms in the SST, activating and taxiing sperm toward oocytes, and removing nonfertilizing sperm (Stephen, et al., 2007; Das et al., 2010). Fertility and hatchability are major parameters of reproductive performance which are most sensitive to environmental and genetic influences (Stromberg and Stromberg, 1975). Fertility in poultry is traditionally

regarded as an independent trait either of the male or the female, but genetic and nongenetic factors originating from both the male and female affect egg fertilization and embryo development (Brillard, 2003). Both the male and the female contribute to variation in fertility (Wolc, et al., 2009). The depression in vivo sperm-egg penetration and fertility in heat stressed roosters may be because of a decrease in a number of spermatozoa stored in the sperm nest gland in the hen's reproductive tract (Bakst et al., 1994; Bakst, 1998; Brillard, 2003). In the previous study, it is reported that the female sperm allergy is an important cause leading to the problem of sperm survival, and this allergy was present in the hens with short duration of fertility (Das et al., 2005; Jin-Chun, et al., 2008).

Poultry possesses a unique ability to store sperm for a prolonged period in the oviduct (Bakst et al., 1994; Bakst and Vinyard, 2002; Bakst, 2011). The SST are located in the UVJ and infundibulum of the hen oviduct, where sperm can be stored and survived for few days to few weeks after single AI or natural mating (Fujii, 1963). It is accepted that SST in the UVJ is the primary residence of spermatozoa, whereas SST in the infundibulum is generally considered as a secondary sperm storage site. Nowadays, the advantage of prolonged sperm storage and survival in the oviduct of laying hen is fully exploited and utilized in practical poultry production systems. This unique feature of prolonged sperm storage in the SST enables laying hens to produce a series of fertile eggs following a single copulation event or AI. Sperm storage function(s) of the SST is therefore directly correlated with the fertility of laying hens (D Tingari and E Lake, 1973). The SST, which are lined by a single layer of nonciliated cells, are formed by the investigation and differentiation of mucosal surface epithelium (Bakst, 2011). In contrast, in the low-fertility birds, the SST showed a swollen appearance, lymphocytes were invading the SST, and no sperm was observed in the SST (Das et al., 2005). Then, later studies suggested that other factors such as carbonic anhydrase (Holm and Ridderstråle, 1998), avidin (Long, et al., 2003), aquaporin's (Zaniboni and Bakst, 2004), and alkaline phosphatase (Bakst and Akuffo, 2007) might also facilitate the sperm storage in the SST. Spermatozoa and seminal proteins are antigenic to the female immune system and should, therefore, be promptly rejected (Das, et al., 2009). Moreover, immune-competent cells for acquired immunity, namely macrophages, antigen-presenting cells expressing MHC class II, CD⁴⁺ and CD⁸⁺ T cells, and premature B and plasma cells have been localized to the mucosal tissue of all avian oviductal segments (Das et al., 2010). Das et al., 2005 (Das et al., 2005) reported changes in the localization of antigen presenting cells and T cells in the UVJ after repeated AI in laying hens, and a significant increase was observed in the population of lymphocytes (CD4 + CD8 T cells) and antigen-presenting cells expressing MHC class ii in the stroma of UVJ in low fertility birds. The mRNA expression of estrogen receptors- α (Era) in the UVJ has been reported to

significantly decreased in low-sperm storage birds compared with high-sperm storage birds (Das, et al., 2006).

In addition, chi-square test results demonstrated that allelic and genotypic frequencies for the SNP in chromosomes 3 and 13 were not in HWE. These results suggest that the allelic and genotypic frequencies of the 20 polymorphic sites in chromosomes 3 and 13 of the Chinese Jing Hong layer chicken population do not remain constant from generation to generation because of the influence of selection, mate choice, migration, and mutation. Single nucleotide polymorphisms were tested and demonstrated remarkably genetic disequilibrium between alleles which might change the population structure and genetic drift in the studied populations. The Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant unless specific disturbing influences are introduced. Those disturbing influences contain nonrandom mating (including inbreeding, assortative mating, small population size), mutation, selection, and so on. The results revealed a significant deviation from HWE at the SNP in Hyline hens. The hens used in that study were from a commercial line (Tang, et al., 2012). In general, commercial lines are systematically selected as closed populations, and high selection intensity, small population size, and nonrandom mating could be the reason for the disequilibrium (Li, et al., 2006).

Furthermore, we demonstrated a low genetic diversity in the population which might result mainly because of the cause of selection, genetic drift, and founder effects. Moreover, we demonstrated that the allele frequency and the genotype frequency for the SNP, that is AX-75769978, AX-76582632, AX-75730546, AX-75730496, AX-75730588, AX-76530282, AX-76530329, AX-76529310, AX-75769906, AX-75755394, AX-80813697, and AX-76582809, in the Jing Hong layer chicken of all studied population but after all the population was found to exhibit significant genetic disequilibrium between the 2 alleles in chromosomes 3 and 13 ($P < 0.05$). This has shown a low genetic diversity in the population which result may mainly because of the cause of selection. Moreover, the gene *Ho* was higher than gene *He* for the SNP, that is AX-75769978, AX-76582632, AX-75730546, AX-75730496, AX-75730588, AX-76530282, AX-76530329, AX-76529310, AX-75769906, AX-75755394, AX-80813697, and AX-76582809, that were found also to be under genetic disequilibrium. The reason why this phenomenon occurred may be explained mainly by the following 2 aspects: (i) the mutation of alleles in these chromosomes 3 and 13 fragment were initially present in the original chicken population at a lower frequency and (ii) this substitution has occurred recently. Additionally, it cannot be ignored that the number of birds examined in each population was not enough to demonstrate the true event, and an extreme allele frequency was estimated as a result. There was no significant

association found for the SNP, that is AX-75769993, AX-76530331, AX-76530309, AX-75730343, AX-75755413, AX-75755447, AX-75755457, and AX-75730254, in fertility rate and duration of fertility traits (DN and FN) by 51 d of laying period in any group of the population studied ($P \geq 0.05$).

The results of the association study and the LD analysis revealed that the 12 significantly associated SNP were more or less closely linked together in this region. Linkage disequilibrium plays a vital role in mapping genes that affect complex diseases and identifying association among genetic markers and functional genes (Hazelett, et al., 2016). Understanding LD among SNP also avoids redundant inferences involving nonindependent genetic markers. Result of this study indicated that 12 significantly associated variants of chromosomes 3 and 13 were in more or less significant LD with each other, which implies that these polymorphisms are associated with our studies on the fertility rate trait. Therefore, haplotype formation from these mutations belonging to the LD blocks was consistent. Haplotype analysis is effective in LD studies to resolve unsatisfying and noisy effects than analysis of single marker which is caused by diverse marker history and statistical methods and results in the monotonic and step-like breakdown of LD by recombination (Daly, et al., 2001).

Collectively, the results of the present study strongly suggested the conclusion that the 12 novel SNP are associated with the fertility rate and thus are potential molecular markers for fertile egg productivity in Chinese Jing Hong chicken breeding. To the best of our knowledge, the first evidence of these 12 genetic markers and its association with the fertility rate trait may help deepen our understanding of the function of these SNP as the markers for genetically improving fertility in hens.

In conclusion, 20 SNP that are associated with fertility rate were selected by GWAS in parent population. Out of them, 12 SNP were confirmed in 2 commercial populations with PCR-RFLP genotyping data and fertility rate. Based on the results of association analysis, it has been shown that the A allele at SNP AX-75769978, C allele at SNP AX-76582632, C allele at SNP AX-75730546, A allele at SNP AX-75730496, A allele at SNP AX-75730588, A allele at SNP AX-76530282, A allele at SNP AX-76530329, C allele at SNP AX-76529310, C allele at SNP AX-75769906, G allele at SNP AX-75755394, A allele at SNP AX-80813697, and A allele at SNP AX-76582809 in chromosomes 3 and 13 are the most potential candidate molecular genetic markers that can be used to improve the fertility rate in marker-assisted selection programs. Our present study therefore put a new insight on SNP in chromosomes 3 and 13 function in Chinese Jing Hong chickens. This study not only provides the candidate genetic markers for marker-assisted selection of Chinese Jing Hong hens but also provides a basic knowledge for further studies on SNP detection on chromosomes 3 and 13 in other chicken breeds or any other animal species.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2019.12.068>.

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