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SNP screening of the *HSD3B1* gene and its association with laying performance in Taihang chickens

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ABSTRACT

The 3 beta- and steroid delta-isomerase 1 (*HSD3B1*) gene has a great influence on reproductive traits and therefore has been widely studied. However, little is known about the relationships between polymorphisms of the *HSD3B1* gene and laying traits in Taihang chickens. Therefore, the *HSD3B1* gene was selected for polymorphism detection and analysis of its relationship with laying performance traits. The mRNA expression levels of *HSD3B1* in 16 tissues of Taihang chickens were detected, and the *HSD3B1* gene was SNP screened and genotyped, and associated with laying traits. The results showed that the *HSD3B1* gene was widely expressed. The *HSD3B1* expression level was highest in F1 follicles, lowest in small white follicles, and differentially expressed in large follicles with small yellow follicles. The *C274T* allele was moderately polymorphic in Taihang chickens, and the age of opening day was significantly earlier for the CC genotype than for other genotypes. The present results demonstrate that the homozygous CC genotype can be considered a genotype for molecular marker-assisted selection (MAS) to improve the breeding efficiency of Taihang chickens.

Keywords: *HSD3B1*, Laying performance, SNP, Taihang chicken

Understanding the regulatory mechanisms of hormones is important for reproductive control in the animal industry. Steroid hormones are the most important gonadal hormones in animals (Groothuis and von Engelhardt 2005, Samardzija *et al.* 2018). The pathway of ovarian steroid production, which includes numerous genes involved in encoding synthesizing steroid enzymes in the ovary, is strongly linked to ovarian function and follicular development (Guo *et al.* 2018). Among them, *HSD3B1* is one of the key members of the steroid hormone family and contributes significantly to the transversion of steroid hormone synthesis (Simard *et al.* 2005). Along with in-depth studies, a few studies have found a relationship between *HSD3B1* and the reproductive performance of different animal species (Shimodaira *et al.* 2012). In pigs, it has been found that *AMH* inhibits LH-mediated expression of steroidogenic genes, including *HSD3B1*, and androstenedione production in theca cells (Li *et al.* 2020). OXA is one of the elements that can modify uterine steroidogenesis genes, including *HSD3B1*, by regulating the expression of significant steroidogenic enzyme genes and the secretion of steroid hormones in pigs (Rytelewska *et al.* 2020). In sheep, transcriptional analysis of follicular development after *FSH* superstimulation at the pre-recruitment, dominant, and mature stages confirmed that *HSD3B1* could be a key gene involved in follicular development (Yao *et al.* 2021). Polymorphisms in the sheep *HSD3B1* gene have little effect on its protein structure (Hu *et al.* 2021). In addition, it has been reported that the *HSD3B1* gene affects follicular development and ovulation in chickens by regulating ovarian secretion of steroid hormones in gallus, thus serving as a functional gene affecting reproductive performance in poultry (Sechman *et al.* 2020).

Taihang chickens are a domestic breed of poultry resources in China. This breed has advantages such as small body size, relatively low feed consumption and good egg quality. However, Taihang chickens still have low production performance overall, and it is urgent to improve the breeding of Taihang chickens. Only the *HSD3B1* gene of 3β-HSD family members was found in chickens, including Taihang chickens. *HSD3B1* plays an important function in follicular development in chickens (Zimmer *et al.* 2018, Huang *et al.* 2021).

Nevertheless, the SNPs of the *HSD3B1* gene in chickens have not been reported. In this study, the connection between *HSD3B1* polymorphism and laying traits was examined in domestic Taihang chickens. The study of chicken *HSD3B1* gene SNPs is helpful in terms of identifying meaningful molecular markers, laying a theoretical foundation for marker-assisted selection of chickens, and providing new approaches for improving chicken reproduction.

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MATERIALS AND METHODS

Sample collection: All experiments were approved by the animal welfare department of the Institute of Animal Science, Hebei University of Engineering. Taihang laying hens raised in single cages were provided by Hebei Tiankai Food Co., Ltd.

A total of 175 Taihang chicken were fed in the same layer and same feed. Five Taihang chickens were killed at the age of 43 weeks. Pterygoid veins were used to draw blood samples. The heart, liver, spleen, lung, kidney, ovary, large white follicle (LWF), small white follicle (SWY), large yellow follicle (LYF), small yellow follicle (SYF) and F6-F1 follicle samples were collected, wrapped in tin foil and stored in an -80℃ ultralow-temperature refrigerator for future use.

Primer design and synthesis: Using Primer 5.0 software, primers were designed according to the sequences of the chicken *HSD3B1* gene (GenBank accession no. NM_205118.1). P-*HSD3B1* was used for PCR, and *HSD3B1* was used for qPCR to amplify the *HSD3B1* gene. The primers were synthesized by Suzhou GENEWIZ Biotechnology Co., Ltd. (GENEWIZ, Suzhou, China) and given in Table 1.

Total RNA extraction and cDNA synthesis: Different tissue samples and follicles of Taihang chickens were ground in liquid nitrogen. As stated in the instructions, total RNA was extracted using an EasySPIN Plus Tissue Cell RNA Rapid Extraction Kit (Aidlab, Beijing, China). The integrity, concentration and purity of the total RNA were determined by 1% agarose gel electrophoresis and stored at -80°C. cDNA was synthesized by HiFiScript gDNA Removal cDNA Synthesis Kit (Kangwei, Beijing, China).

DNA extraction and detection: DNA was extracted by a blood genomic DNA extraction kit (Tiangen, Beijing, China). Agarose gel electrophoresis was used to verify the DNA's purity and concentration.

qPCR and PCR amplification: Using cDNA of each tissue and follicle as template and *GAPDH* as an internal reference gene, the *HSD3B1* gene was detected by qPCR to analyze its expression in different tissues. qPCR was carried out using SYBR® qPCR Master mix (Vazyme, Nanjing, China).

The genomic DNA of randomly selected Taihang chickens with known laying numbers was used as the template. Primers P-*HSD3B1* were used for PCR amplification, as given in Table 1. The PCR was carried out with 2×TaqMaster Mix (Vazyme, Nanjing, China) and the amplification were sent to Sangon Bioengineering Co., Ltd. (Shanghai, China) for custom sequencing. DNASTAR software (http://www.biologysoft.com/) was used for sequence alignment and SNP selection.

Enzyme identification: PCR products of primers P-*HSD3B1* were digested by the restriction endonuclease *MvaI* (TaKaRa, Beijing, China) at 37°C for 4 h. The digestion system was as follows: total volume 10 μL, including 0.5 μL of *MvaI* restriction enzyme (10 U/μL), 3 μL of PCR product, 1 μL of 10× Buffer and 5.5 μL of ddH_2O. The enzyme products were tested by using 1% agarose gel electrophoresis.

Statistical analysis: The *HSD3B1* gene's relative mRNA levels were calculated as $2^{-\triangle\triangle CT}$ using *GAPDH* for normalization. Calculations were made for the genotype and allele frequency, polymorphism information content (PIC), heterozygosity (He) and effective number of alleles (Ne). The Hardy-Weinberg law was used to test whether the distribution of SNP genotypes in the studies population deviated from Hardy-Weinberg equilibrium. SPSS 20.0 software was used for correlation analysis of different genotypes with traits (age of opening day (AFE), egg production at 300 days (E300), and egg production at 500 days (E500)).

RESULTS AND DISCUSSION

Expression of HSD3B1 in different tissues and follicles: The relative expression levels of the *HSD3B1* gene were analyzed by qPCR in sixteen tissues of Taihang chickens. The experimental results indicated that the gene was widely expressed in all tissues, with tissue expression specificity. Daily selection of one SYF to LYF from the SYF pool is called follicle selection, which is an important process and is directly associated with laying performance in hens (Johnson 2015). We found that the expression level of *HSD3B1* in LYF was significantly higher than that in the SYF ($P<0.05$), *HSD3B1* expression in the LYF was approximately 2.2 times that in the SYF, and the expression level increased significantly with increasing follicle diameter ($P < 0.05$), indicating that the *HSD3B1* gene could be involved in the chicken follicle selection process. The result is consistent with previous research (Kozubek *et al.* 2020, Huang *et al.* 2021).

Detection of HSD3B1 gene polymorphisms: Amplification length to detect *HSD3B1* gene polymorphisms was 586 bp (Marker DL 2000) (Fig. 1). The target fragment obtained by PCR amplification presents a single, bright band consistent with the predicted fragment

Table 1. *HSD3B1* gene primers

Primer	Sequence (5′→3′)	Annealing temp (°C)	Product size (bp)
P-*HSD3B1*	F: GCCCAAGGTGTCAATGAT R: AAGACCCTCTGTGCCATT	63	586
HSD3B1	F: GCAAGAGGCTGGCAGAGGAATG R: TGGATCTCAGGGCACAGGTCAC	60	90
GAPDH	F: GCCATCACAGCCACACAGA R: TTTCCCCACAGCCTTAGCA	60	120

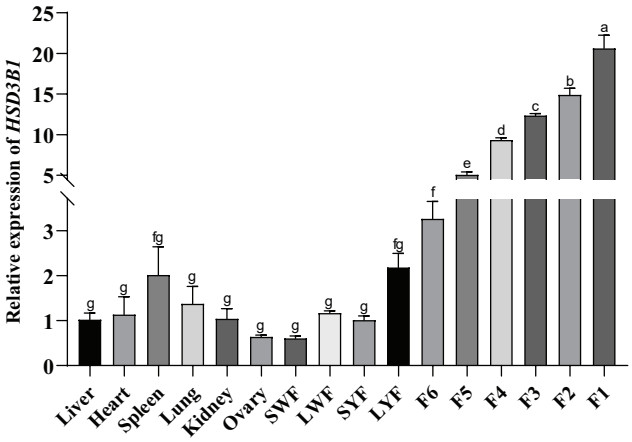

Fig. 1. Expression of *HSD3B1* in different chicken tissues and follicles (mean±SEM). Different letters indicate significant differences (P<0.05).

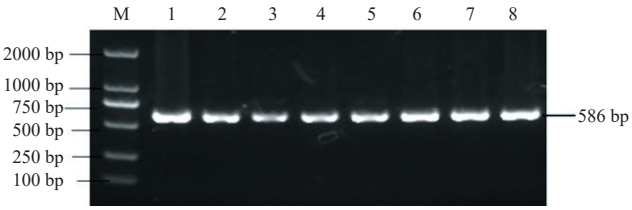

Fig. 2. PCR amplification of *HSD3B1* gene.

size; therefore, this product was used for further study (Fig. 2).

SeqMan software was used to analyze the sequencing results, and SNPs were found in the *C274T* site (Exon 3) of the *HSD3B1* gene amplified from Taihang chicken population, producing the CC, CT and TT genotypes, as shown in Fig. 3.

The target PCR fragment was digested by the endonuclease *MvaI*, resulting in 586 bp, 493 bp and 93 bp fragments. The three genotypes were detected according to different band patterns, as follows: CC (493 bp, 93 bp), CT (586 bp, 493 bp and 93 bp) and TT (586 bp). Genotyping results are shown in Fig. 4.

Analysis of HSD3B1 gene polymorphism and laying performance in chickens: The results showed that the CT genotype frequency (0.47) was higher than the CC genotype (0.24) and TT genotype (0.29) frequencies, and the T allele frequency (0.52) was higher than the C allele frequency (0.48). The *C274T* mutation in Taihang chickens was moderately polymorphic (0.25<PIC<0.5). SHEsis online software was used for linkage imbalance analysis. The results indicated that the *C274T* site was in a linkage equilibrium state, which could be because the domestic Taihang chickens were not under continuous selection, as shown in Table 2.

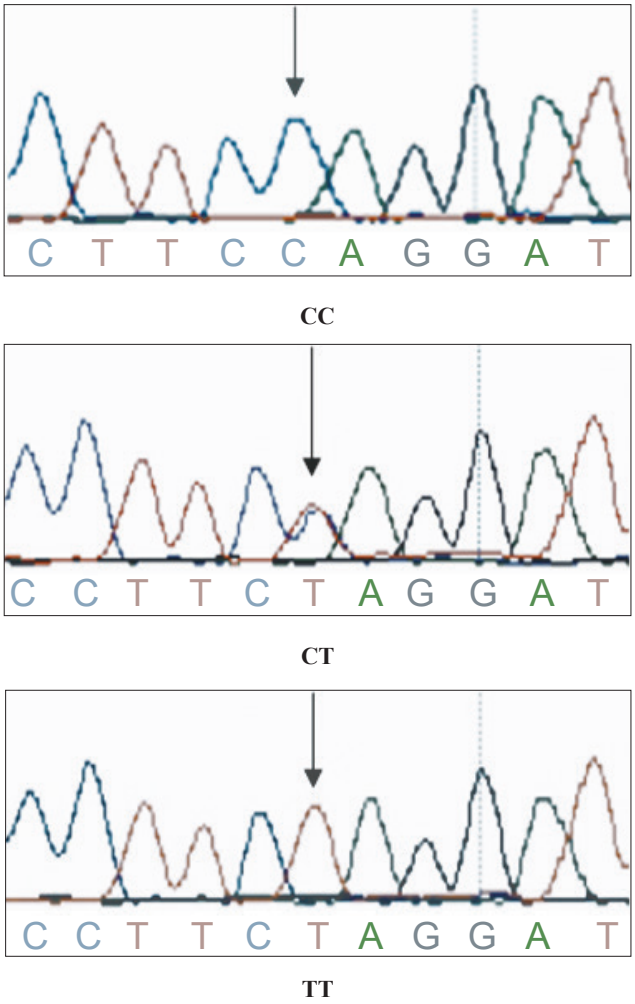

Fig. 3. Sequencing of *HSD3B1* gene mutation sites.

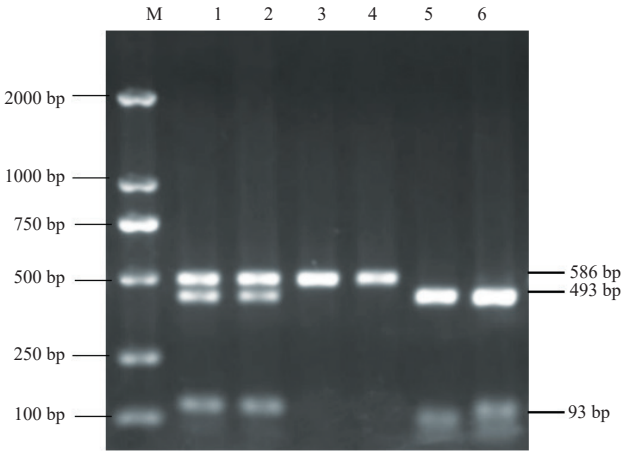

Fig. 4. Electrophoresis results of *MvaI* digestion of the *HSD3B1* gene (Line M: DL 2000 marker; Line 1 and 2: CT (586 bp, 493 bp and 93 bp); Line 3 and 4: TT (586 bp); Line 5 and 6: CC (493 bp, 93 bp)).

Table 2. Genotype and allele frequency distribution of SNPs in the exon 3 region of the chicken *HSD3B1* gene

SNP	Breed	Genotype frequency			Allele frequency		PIC	He	Ne	χ_2
C274T	Taihang chicken (175)	CC	CT	TT	C	T	0.37	0.5	2.0	0.64
		0.24	0.47	0.29	0.48	0.52				

Here, an association study between the *HSD3B1* gene and laying traits was also conducted. The three main laying performance traits, AFE, E300 and E500, of Taihang chickens with the three genotypes at the *C274T* locus of the *HSD3B1* gene were statistically analyzed, and the association of polymorphic exon 3 *C274T* with laying performance was analyzed. The results are shown in Table 3.

Table 3. Association analysis of polymorphisms at the *C274T* locus (exon 3) of the chicken *HSD3B1* gene and laying performance

SNPs	Trait	Genotype		
		CC (42)	CT (82)	TT (51)
C274T	AFE	169.57±2.11[a]	176.21±1.71[b]	174.6±2.49[ab]
	E300	96.31±2.29	90.66±1.70	91.90±2.01
	E500	182.29±6.72	175.29±4.10	179.31±5.72

Note: AFE refers to the age of opening day, E300 refers to egg production in 300 days, and E500 refers to egg production in 500 days.

There was no significant difference in E300 production or E500 among the 3 genotypes at this locus. However, the CC type showed earlier AFE than the heterozygous CT type and homozygous TT type ($P<0.05$). Therefore CC genotype had better AFE compared to the other genotype in Taihang chicken.

In conclusion, the *HSD3B1* gene was differentially expressed in sixteen chicken tissues and could be an important gene in follicle selection. Exon 3 of the *HSD3B1* gene is polymorphic, and the *C274T* allele showed a significant effect on AFE. The present results lay a foundation for the MAS breeding process and could provide a new way to improve the breeding efficiency of Taihang chickens. Further study will be necessary to confirm the SNP function in a large laying group before informing in the breeding procedure.

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