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Exploring the distribution of polymorphism across diverse breeds Worldwide in the bovine *NR5A2* gene and its correlation with number of mature follicles and corpus albicans

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ABSTRACT

The *Nuclear receptor subfamily 5 group A member 2* (*NR5A2*) gene plays a pivotal role in ovarian development, ovulation, and reproductive traits. There is a lack of studies on its impact on ovarian traits and reproductive traits in cattle. This study aimed to explore *NR5A2* gene polymorphisms associations with reproductive traits and investigate the distribution of *NR5A2* gene polymorphisms across diverse bovine breeds worldwide. We identified a novel 17-bp deletion within the *NR5A2* gene specifically in Chinese Holstein cows (n = 1033) leading to the observation of two genotypes DD and ID. Subsequent association analysis revealed a significant correlation between the 'ID' genotype at this locus and a larger number of corpus albicans ($p = 0.042$) in diestrus, as well as a higher number of mature follicles ($p = 0.038$) in estrus. In addition, we also found that the distribution of this deletion exhibits strong regionality across different cattle breeds globally. These findings indicate that the 17-bp deletion mutation within the *NR5A2* gene is significantly associated with an increased corpus luteum diameter and a greater number of mature follicles, suggesting its potential utility as a valuable DNA marker for enhancing cow fertility.

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Cow; *NR5A2* gene; deletion; ovary; reproduction

1. Introduction

In recent decades, there has been a notable emphasis on milk production which has overshadowed the importance of fertility in dairy cow breeding, resulting in a gradual decline in reproductive efficiency.[1] Until the late twentieth century, dairy selection indices predominantly prioritized milk fat and protein production, often neglecting other critical traits.[2] However, low fertility significantly impacts dairy herd regeneration and cows' ability to conceive seasonally. Failure to maintain a one-year calving interval can result in substantial economic losses, often outweighing those incurred due to decreased milk production.[3] The fertility of dairy cows plays a pivotal role in determining the economic sustainability of dairy farming. As a result, modern selection indices globally no longer rely solely on milk fat and protein production; milk production traits now typically account for approximately 50% of the overall index.[2] Therefore, improving the reproductive performance of dairy cows through selective breeding is crucial for the dairy industry.

In the process of breed selection, molecular marker-assisted selection (MAS) has garnered widespread adoption due to its efficiency and precision.[4] The initial step in utilizing the MAS method involves the identification of candidate genes, particularly those with essential functions in the female ovary. In this context, *the nuclear receptor subfamily 5 group A member 2* (*NR5A2*) gene emerges as a significant candidate gene that has been extensively studied in various female organisms. *NR5A2*, also known as liver receptor homolog-1 (LRH-1), belongs to the nuclear

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receptor NR5A subfamily.[5,6] It encodes a protein that belongs to the subfamily of orphan nuclear receptors, possessing a DNA-binding zinc finger.[7,8] *NR5A2* is expressed in various endoderm-derived organs including the liver, pancreas, and intestine.[9] *NR5A2* is expressed in granulosa cells[10] where it modulates their proliferation. Research has shown that the depletion of *NR5A2* retards the proliferation of granulosa cells in the ovary, arresting them in the S phase, inducing anovulation in mice.[11–13] Moreover, the *NR5A2* gene plays crucial roles in sex hormone secretion. In the process of gonadotropin synthesis, *NR5A2* promotes the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior pituitary gland and gonadotropic cell lines by regulating the expression of gonadotropin-related genes in rats.[14,15] *NR5A2* knockout mice (*NR5A2*+/-) exhibit significantly lower fertility.[16] Collectively, these findings suggest that the *NR5A2* gene is instrumental in regulating reproductive traits in females. Therefore, exploring the indel mutations of *NR5A2* could be beneficial for breeding cows with superior reproductive performance.

The ovary, a key indicator of fertility, performs various functions encompassing ovulation, reproductive hormone secretion, and regulation of the female estrus cycle.[17,18] In dairy cattle breeding, ovarian functions directly influence reproduction traits with reproductive failure constituting a primary criterion for culling individuals.[19] Therefore, it's imperative to investigate the factors influencing ovarian function and traits. However, the ovaries exhibit distinct morphological characteristics during the various estrus stages (proestrus, estrus, postestrus, and diestrus): during proestrus, follicles initiate their development; during estrus, these follicles undergo rapid development; in the postestrus stage, follicles rupture to complete ovulation, marking the beginning of the formation of a new corpus luteum; and during diestrus, the corpus luteum enlarges, reaching its maximum size.[20] Therefore, in this study, we collected ovaries from cows at different estrus cycles for research (Figure 1).

Marker-assisted selection (MAS), particularly through the utilization of insertion/deletion (indel) variations, has recently emerged as a widespread practice in livestock breeding, encompassing pigs, chickens, goats, and cattle.[21–26] MAS holds promise in enhancing ovarian functions in cows, thereby optimizing their reproductive potential.

The polymorphism of *NR5A2* has been applied to the research of various animal diseases and traits. In human, it has often been found that polymorphisms in the *NR5A2* gene are significantly associated with a range of conditions, including pancreatic cancer, bone density issues and spontaneous abortion.[27–29] In cattle, through genome-wide association analysis, it was found that *NR5A2* may be associated with bull reproductive traits.[30] In sheep, multiple single nucleotide polymorphisms in the *NR5A2* gene are significantly associated with litter size. One of these SNPs, T > G, can regulate upstream transcription factors to promote the expression of the *NR5A2* gene, thereby affecting litter size in sheep.[5,31] However, there remains a paucity of studies regarding their associations with ovary traits in cows. Therefore, this study was conducted to detect possible novel indels within the *NR5A2* gene and evaluate their effects on reproductive traits in dairy cows, to support some theoretical basis for the fertility breeding of cows.

2. Materials and methods

Experimental protocols were approved by the Faculty Animal Policy and Welfare Committee of Northwest A&F University under contract (protocol no. NWAFU-314020038).

2.1. Bioinformatic analysis

Nucleotide sequences from Bos taurus (NM_001206816.1), Ovis aries (NM_001246280.1), Capra hircus (XM_018060819.1), Sus scrofa (NM_001267893.1), Homo sapiens (NM_001276464.2) and Gallus gallus (NM_205078.1) were obtained from the NCBI database. The Blast database (blastn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect nucleotide sequence similarity, and the MEGA7 MUSCLE program was used to compare multiple nucleotide sequences and perform evolutionary analysis.

2.2. Samples and related data collection

In this study, ovary samples were obtained from 1033 Chinese Holstein cows in the same breeding farm. Based on the external behavior of the cows and the morphology of the corpus luteum in the ovary, we were able to determine the estrus period in which each cow was located, including interestrus, proestrus, estrus, and postestrus (Figure 1).[32,33] Subsequently, these samples were used to extract genomic DNA for further experiments.

In details, ovary samples were obtained from Chinese Holstein cows of the same age (5-6 years old). The ovary morphological features were measured by

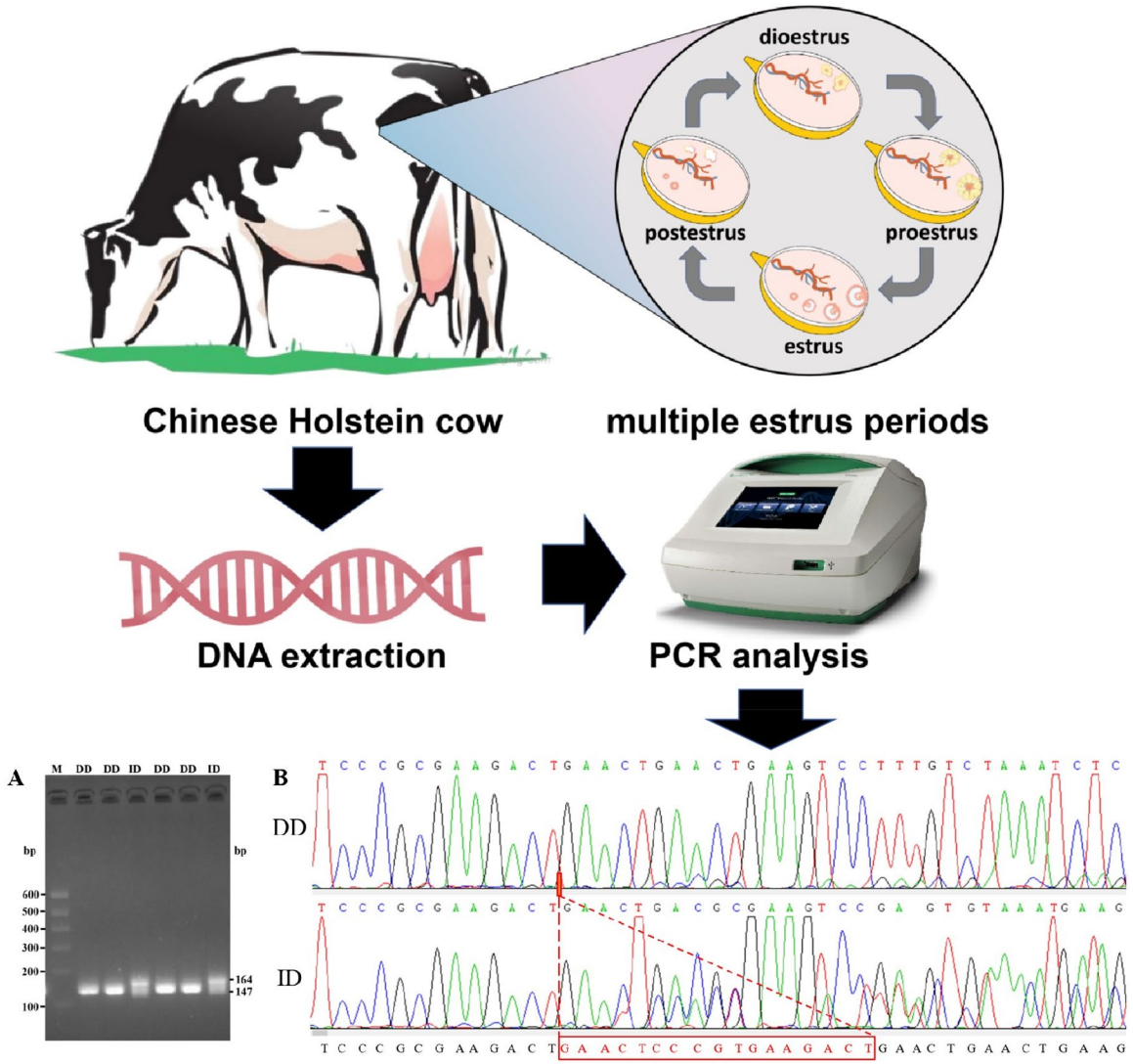

Figure 1. Agarose gel electrophoresis of Chinese Holstein cow *NR5a2* gene loci P7. (A) Agarose gel electrophoresis of bovine *NR5a2* gene P8 locus. (B) DNA sequencing diagram of the 17-bp deletion of *NR5a2* gene (ID and DD genotypes).

the same standard, including the length: the length of the long axis of the ovary, width: the length of the short axis of the ovary, height: the thickness of the ovary, weight of ovary; the number of corpus albicans, corpus luteum, mature follicles; the diameter of corpus luteum, corpus albicans, and mature follicles. In this process, sterile electronic scales and a vernier caliper were used to measure weight, length, width, and height of the ovaries (Figure 2).[32,33]

2.3. Genomic DNA isolation and DNA pool construction

According to Aljanabi's method, whole genome DNA was extracted from blood tissues.[34,35] Next, DNA was quantified by Nanodrop 1000 and diluted to 50 ng/μL. In addition, 50 DNA samples were selected randomly to construct a genomic DNA pool to explore genetic variation in the candidate gene.[32,36] The purpose of the DNA mixing pool was to detect whether these mutations were polymorphic in Chinese Holstein cows.[37]

2.4. Primer design and PCR amplification

A total of eight indels of the *NR5A2* gene were derived from the NCBI (https://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) databases. The specific forward and reverse primers were designed using the Primer Premier 5.0 software (Premier, Canada). Primers for each mutation site were designed to maximize the amplification of the target fragment to make sure that each pair of primers was the most appropriate for each

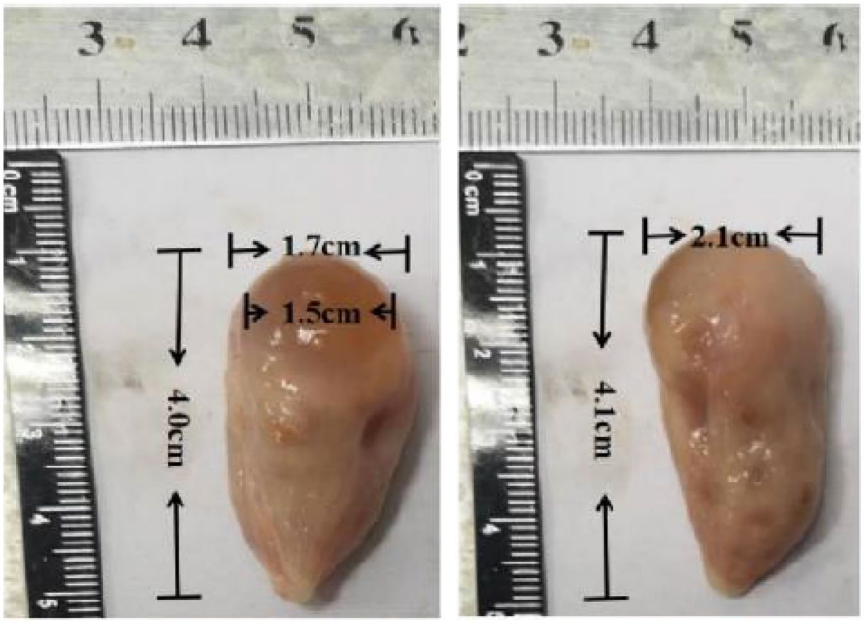

Figure 2. Diagram of measured ovaries of Chinese Holstein cow.

Table 1. Primers used for detecting *NR5A2* indel variants.

Loci	Primer sequences (5′-3′)	Product Size (bp)	Region	Variant ID
P1	F: CTTCTGATGGGCTTTTCGAGT R: GTGCTTTGGAGAAACCGGAA	205/191	Intron 1	rs433404285
P2	F: AACGCCATGTTTCCGACCT R: TGGGGCGCTCTTTTAGTCTT	167/157	Intron 4	rs721454859
P3	F: AAGCTCTTATTTGCCTGAGTGTC R: CGGAAGTAAGGGTGTGGTTCT	288/271	Intron 4	rs482206194
P4	F: GCCGTGTAGAGGGGAATCAG R: CGGACTAATCGGGTCTCTGC	295/280	Intron 5	rs450027422
P5	F: TAGGGTACGTGGAGAGCTGTT R: AAGAGCTGCCACGATGGATT	260/248	Intron 6	rs717775415
P6	F: CGTTGTCCTCTTGCCCGTT R: CAAAAGACTGAACTCACCGTCC	237/230	Intron 6	rs718234858
P7	F: GCAAGTTGTACAGGCTCATTTCTA R: CCACTTCGCATCCGGAGAA	297/280	Intron 6	rs470625485
P8	F: TTTTACATGCAGTTCCCGCCAAA R: TCCCGCGAAGACTGAACTG	164/147	Intron 6	rs135422978

variation (Table 1). All primer pairs were synthesized by Tsingke Biotech (Xian, Shaanxi, China). In this study, a touch-down polymerase chain reaction (TD-PCR) program was performed. The reaction mixture contained genomic DNA, primers, Master Mix (Vazyme, Shaanxi, China). Eventually, fragments of PCR products were identified by electrophoresis; then homozygous and heterozygous variants were sequenced directly by Tsingke Biotech (Xian, Shaanxi, China).[38,39] TD-PCR was performed in 12.5 μL reaction volume, containing 1.5 μL (10 ng/μL) of genomic DNA, 0.5 μL of each primer, 6.0 μL 2× Taq Master Mix, 4.0 μL ddH_2O. The thermal cycling program was 5 min at 95 °C for pre-denaturation, then 18 cycles at 94 °C for 30 s, then annealing for 30 s at 68 °C (with a decrease of 1 °C per cycle), 15 s at 72 °C, and a final extension at 72 °C for 10 min. Finally, 4.0 μL PCR products were directly assayed by electrophoresis on 3.0% agarose gels stained with nucleic acid dye.

2.5. Potential transcription factor binding site prediction

We used JASPAR (http://jaspar.genereg.net/collection/core/) and Alibaba2 (gene-regulation.com/pub/-programs/alibaba2/index.html) online databases to predict the transcription factors (TFs) that bind to mutations.

2.6. Worldwide bovine breeds' sample information

Our data were retrieved from the BGVD database (accessed on July 3rd, 2022, via http://animal.omics.pro/code/index.php/BosVar), encompassing 54 bovine breeds and 432 samples. The comprehensive sample set comprised individuals from diverse geographical backgrounds: 108 from West Europe, 28 from Northeast Asia, 9 from the Middle East, 9 from Tibet, 47 from North-Central China, 21 from Northwest China, 33 from South China, 24 from Indo-Pakistan, 83 from Central-South Europe, and 70 from Africa.

This database provided signatures of selection specific to eight distinct groups, six of which are designated as the 'core' cattle groups and acknowledged as ancestral lineages. These included Indian indicine (IN), Chinese

Table 2. Genetic parameters of the 17-bp deletion within the *NR5A2* gene in Chinese Holstein cow.

	Genotypes frequencies			Alleles frequencies			Population indexes			
Sample size	DD	ID	II	D	I	*p* value (HWE)	Ho	He	Ne	PIC
1033	0.587 (n = 606)	0.413 (n = 427)	0 (n = 0)	0.793	0.207	5.6×10^{-17}	0.672	0.328	1.488	0.274

Note: HWE: Hardy-Weinberg equilibrium; Ho: observed homozygosity; He: heterozygosity; Ne: effective allele numbers; PIC: polymorphism information content.

indicine (CN), East Asian taurine (EA), Eurasian taurine (EUA), European taurine (EUR), and African taurine (AFR). Furthermore, this database boasts the identification of approximately 60.4 million autosomal SNPs and around 6.8 million autosomal INDELs. It offered a comprehensive display of the minor allele frequencies (MAF) of these genetic variations across the entire cattle population, along with allele frequencies specific to individual breeds and the designated 'core' cattle groups.

2.7. Statistical analyses

The genotypic and allelic frequencies, the population genetic parameters (HWE, Hardy–Weinberg equilibrium; He, heterozygosity; Ho, homozygosity; Ne, effective allele number, polymorphism information content, PIC, the polymorphism information content) of the mutation site was calculated according to[39] The independent sample *t*-test used in the SPSS software was to analyze the correlation between genotypes and traits (version 25.0, IBM, USA).

3. Results

3.1. Identification of a novel 17-bp deletion within the bovine NR5A2 gene

DNA genotyping revealed a novel 17-bp deletion (5′- GAACTACCGCGAAGACT −3′) in the intron 7 of the bovine *NR5A2* gene, located on chromosome 16 at position 78947846-78947862 (Figure 1B). Using agarose gel electrophoresis, we distinguished two genotypes: DD and ID. The DD genotype exhibited an amplified fragment size of 147 bp, while the ID genotype displayed a dual fragment size of 164 bp and 147 bp (Figure 1A). Notably, the deletion sequence significantly differed from that reported in the Ensembl database (GAAGACGCTGGTTTGTC; rs470625485), indicating a potential novel variant within the bovine *NR5A2* gene.

3.2. Genetic parameters of the 17-bp deletion within the NR5A2 gene

The detailed genotype and allele frequency distribution, including He, Ho, Ne, PIC, and other relevant parameters for this mutation, were presented in Table 2. In the Chinese Holstein cow population, only two genotypes, 'DD' and 'ID', were observed. The frequency of the 'DD' genotype (0.587) was higher than that of the 'ID' genotype (0.413). The frequency of the 'D' allele (0.793) was also higher than that of the 'I' allele (0.207). Since the *p*-value was 5.6×10^{-17} ($p < 0.05$), this deletion variation deviates significantly from HWE. The PIC value indicates that this variant exhibited medium genetic diversity in the population of interest.

Table 3. Association of the 17-bp deletion with ovarian morphological traits in multiple estrus.

Estrus periods	Traits	Genotypes (Mean ± SE) ID	Genotypes (Mean ± SE) DD	*p*-values
Diestrus	Number of Corpus albicans	1.68 ± 0.19 (n = 31)	1.24 ± 0.09 (n = 33)	0.042
	Corpus albicans diameter (mm)	4.35 ± 0.48 (n = 31)	4.64 ± 0.72 (n = 33)	0.749
Estrus	Number of mature follicles	1.44 ± 0.15 (n = 36)	1.10 ± 0.05 (n = 39)	0.038
	Mature follicle diameter (mm)	14.17 ± 0.61 (n = 36)	14.03 ± 0.77 (n = 39)	0.888

3.4. Association analysis between the 17-bp deletion and ovarian morphological characteristics

To explore the influence of the 17-bp deletion within the *NR5A2* gene on fertility, an independent-sample t-test analysis was used to detect associations between the deletion and ovarian morphological characteristics during various stages, including the diestrus, proestrus, estrus, and postestrus (Table 3; Tables S1–S4). We found that the 17-bp deletion was significantly associated with the number of Corpus albicans in diestrus ($p = 0.042$) and the number of mature follicles in estrus ($p = 0.038$) (Table 3). In addition, individuals carrying the 'DD' genotype exhibited superior traits compared to those with the 'ID' genotype. Although we tested the association between the 17-bp deletion and other ovarian morphological characteristics, such as ovarian length, ovarian width, and ovarian height, no significant correlation was observed ($p > 0.05$) (Tables S1–S4.).

3.5. Prediction of potential transcription binding factors

To explore whether this deletion affects the binding of transcription factors to the *NR5A2* gene, the JASPAR online database was used. Specifically, the database predicted that a subsequence within the 17-bp deletion, namely ACTACCGCGAA, might bind to the E2F transcription factor 1 (E2F1) [MA0024.2] (Figure 3).

3.6. Analysis of NR5A2 gene distribution in 54 bovine breeds and 6 core bovine breeds Worldwide

To investigate the global distribution of the 17-bp deletion, we utilized the BGVD database (accessible at http://animal.omics.pro/code/index.php/BosVar) to analyze 54 bovine breeds, as depicted in Figure 4. Notably, the deletion's distribution appeared to be influenced by geographical location, with distinct patterns emerging between southern and northern regions (divided with a dashed line). Specifically, the 'I' allele was relatively rare in Indian indicine (0.174), Chinese indicine (0.132), and Africa taurine (0.100) breeds. Conversely, it was more prevalent in East Asian taurine (0.608), European taurine (0.803), and Eurasian taurine (0.737) breeds (Figure 5).

4. Discussion

In recent decades, the emphasis on milk production in dairy cow breeding has led to a decline in fertility rates.[1] Low fertility often indicated by failure to conceive can collapse milk productivity for dairy cows and result in significant economic losses for the dairy industry. Therefore, it is crucial to improve the

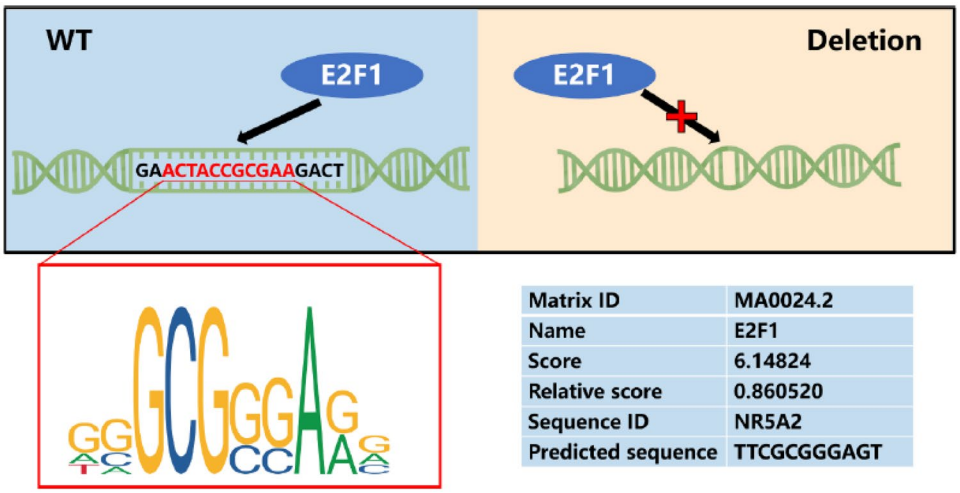

Figure 3. Predicted combination of transcription factors (TFs) and sequence of the *NR5A2* gene.

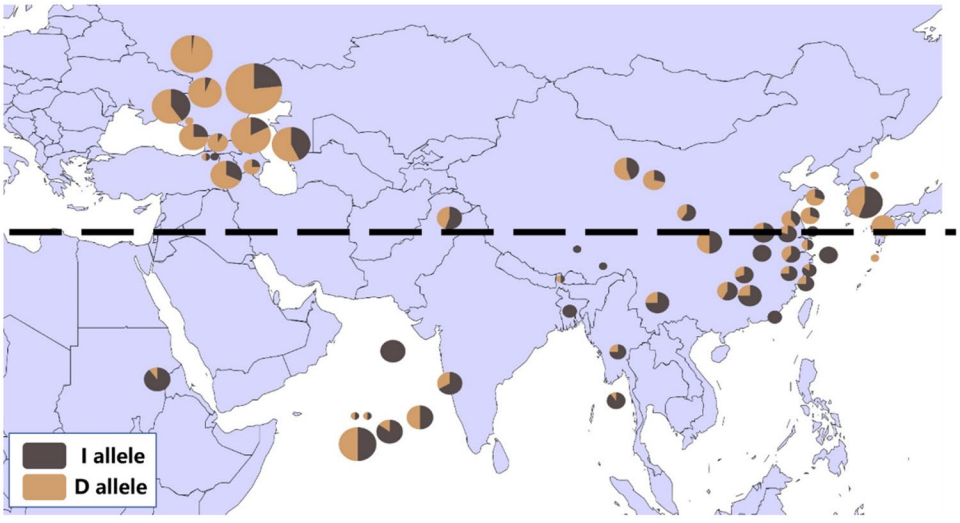

Figure 4. Allele frequency distribution of P7-$_{17bp}$ of *NR5A2* in world-wide 54 cattle breeds.

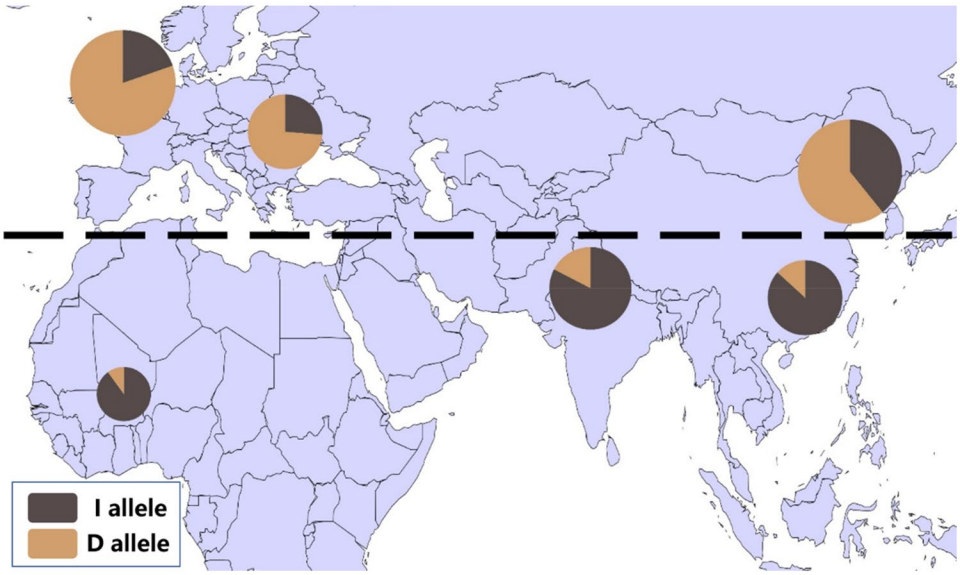

Figure 5. Allele frequency distribution of P7-$_{17bp}$ of NR5A2 in worldwide 6 core cattle breeds.

fertility of dairy cows. To achieve this, various reproductive traits, such as the estrous cycle,[40] litter size,[24,41] and ovarian status,[32,42,43] can be leveraged in breeding processes. Exploring polymorphisms within candidate genes associated with ovarian development and reproduction is imperative, and genetic selection based on these polymorphisms can yield significant benefits. Marker-assisted selection (MAS) method is a feasible approach to explore mutations in reproductive-related candidate genes, including the *NR5A2* gene (reference for MAS method).

This study explored a novel deletion mutation within the bovine *NR5A2* gene in a large population, including 1,033 Chinese Holstein cows. In the Chinese Holstein cow population, the 17-bp deletion showed moderate polymorphism. In addition, the population deviated from Hardy-Weinberg equilibrium, suggesting it was not at dynamic equilibrium probably due to artificial selection, migration, and genetic drift.[44] The 'D' allele corresponded to high frequencies in the Chinese Holstein cow population (0.793), which could be identified as a proto-gene. In addition, no individual with the genotype 'II' was found in this population. Furthermore, based on the distribution of this polymorphism across some global cattle breeds, the genotype frequency of II is generally low, and some breeds completely lack individuals with this genotype. This suggests that this breed may have undergone genetic drift, founder effects, and selective pressure leading to the absence of the II genotype.[45,46] Ovarian morphology characteristics serve as a reproductive indicator to select the best individuals. We conducted independent-sample t-test analyses to examine the association between the deletion and ovarian morphological traits. Our results indicated that the 17-bp deletion significantly affected the number of mature follicles in estrus and the corpus albicans in diestrus.

During the estrus cycle, follicles rapidly grow, develop, mature, and lead to ovulation during estrus, followed by the formation of the corpus luteum. The corpus luteum then gradually shrinks after ovulation and transforms into a white connective tissue scar known as the corpus albicans. During this process, individuals with ID genotype exhibit an increase in the number of mature follicles during estrus, indirectly leading to a tendency of increased corpus luteum in later stages, which in turn diestrus and increases the number of corpus albicans. This suggests that individuals with ID genotype have more active ovulation activities compared to those with DD genotype. This suggests that the *NR5A2* gene is involved in ovarian development given the significant relationship between its 17-bp deletion polymorphism and ovarian morphological traits.

In this study, we observed that ovarian morphological traits of cows were affected by the 17-bp deletion mutation, which might regulate the *NR5A2* gene function. *NR5A2* gene has been reported to affect fertility in several species such as mice,[47] rats,[48] goats,[24,49] and sheep.[5,31] Numerous studies have indicated that the *NR5A2* gene plays a pivotal role in follicular development and hormone secretion. In humans, large-scale meta-analyses have highlighted associations between the *NR5A2* gene and menarche timing.[50] In geese, RNA-seq and ATAC-seq analyses revealed that the *NR5A2* gene influences early goose

ovarian development.[51] *NR5A2* was highly expressed in mouse ovarian tissue particularly in ovarian granulosa cells.[52] During follicular formation, the *NR5A2* gene is expressed in the progranulosa cells of the primary follicle, granulosa cells of the primary follicle, and in the anaphase of follicle formation. *In vitro* studies have demonstrated its role in the differentiation of pluripotent and mesenchymal stem cells into steroid cells.[13,53] Various investigations have highlighted the functions of *NR5A2* in the ovary of laboratory animal models. For instance, the *NR5A2* depletion in granulosa cells resulted in ovulation failure[11] and decreased granulosa cell proliferation in mice.[9] Additionally, granulosa-specific *NR5A2* knockout females exhibited impaired cumulus expansion and ovulation.[11,54] Transgenic mouse models with depletion of *NR5A2* in granulosa cells of antral follicles generate similar outcomes.[16] Notably, specialized NR5A2 knockout mouse models capable of reproduction, ovulation, and fertilization were found to be sterile. In these models, the corpus luteum exhibited reduced size and impaired function. The expression of ovarian steroid factors, such as STAR and CYP11A1, was significantly reduced, underscoring the necessity of the *NR5A2* gene for corpus luteum function.[55]

Furthermore, JASPAR prediction suggests that the 17-bp deletion may disrupt the binding of the gene transcription factor E2F1. E2F1 is significant in the reproductive physiology of female animals, particularly in ovarian function. In bovine ovarian granulosa cells, E2F1 has been found to enhance cell proliferation and suppress estradiol production by inhibiting FGF9.[56] In human ovaries, LARS2/E2F1/Mfn-2 exhibits regulatory functions in mitochondrial dysfunction and endoplasmic reticulum stress within ovarian granulosa cells.[57] In porcine ovarian granulosa cells, miR-17-5p regulates cell proliferation and estradiol synthesis by targeting the E2F1 gene.[58,59] Collectively, these studies suggest that this deletion might influence bovine ovarian traits by altering the interaction between E2F1 and *NR5A2*.

Although our study elucidated the influence of the 17-bp deletion mutation on ovarian morphological and reproductive traits in dairy cows, the specific mechanism of the 17-bp deletion mutation affecting ovarian morphological and reproductive traits of dairy cows remains to be further explored.

5. Conclusion

In conclusion, the novel 17-bp deletion mutation within the bovine *NR5A2* gene was significantly associated with increased corpus luteum diameter, number of mature follicles and decreased age at first calving. All these outcomes suggest that it can be used as a candidate molecular marker for breeding high-fertility dairy cows.

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Authors' contributions

Enhui Jiang: Formal analysis, Software, Investigation, Writing - original draft. Xuanbo Chen: Investigation, Writing-review & editing; Project administration; Taotao Yan: Investigation, Writing-review & editing, Project administration, Yi Bi:Writing-review & editing. Juanshan Zheng: Formal analysis, Investigation. Haiyu Zhao: Software, Investigation. Yongsheng Wang: Writing-review & editing. Xiangchen Li: Conceptualization, Project administration. Xianyong Lan: Conceptualization, Validation, Funding acquisition, Supervision.

Disclosure statement

The authors declare no conflict of interest.

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Data availability statement

Data supporting the results of this study can be obtained from corresponding authors upon reasonable request.

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