Genome-wide DNA methylation differences between conservation and breeding populations of Shaoxing ducks

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HIGHLIGHTS

- The methylation level of a breeding population of Shaoxing ducks was higher than that of the two preserved populations.
- Hundreds of differentially methylated regions were compared among three Shaoxing duck populations.
- Different genes related to the breeding process of Shaoxing ducks were identified.
- Two differentially methylated genes related to egg quality were found.

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ABSTRACT

The genome-wide DNA methylation assay was used to analyze the difference in methylation between the breeding and conservation populations of Shaoxing ducks. The methylation level of the breeding population was higher than that of the two conservation populations, and the proportion of CG methylation sites was the largest in the three populations. Most of the methylation sites were located in the exon region. There were 1247 different methylation regions in the two populations (group A and B), and 927 different methylation regions in the two groups (group A and group C). The differential methylation regions of the three groups were evenly distributed in the gene and intergene regions. GO and KEGG enrichment analysis showed that the differentially expressed genes in the A and B groups were mainly involved in synaptic and cell connections, and the signaling pathways were significantly enriched in the cAMP and oxytocin signaling pathways. The results showed that the group C was significantly enriched in eight signaling pathways, including the cAMP signaling pathway and long-term enhancement. There were thirty-five differentially methylated genes, including CACNA1C, GRIA1, GRIA2, GABBR2, PDE10A, BRAF, GRM5, CPEB3, FMN2, GABRB2, PTK2, and CNTN1. These genes were involved in the development and ovulation of ovaries and follicles and were closely related to the excellent eggshell quality of the breeding population. In addition, ATP2B1, ATP2B2, and other genes related to eggshell quality were identified, which can be used as molecular markers to improve eggshell quality in the future.
1. Introduction

Epigenetic inheritance refers to stable genetic changes that do not change the DNA sequence but affect gene expression. Epigenetics has become the focus of studies on gene expression regulation. DNA methylation, as one of the most widely studied epigenetic modifications, is an important variation in DNA that affects gene expression, usually involving the addition of a methyl group to the fifth carbon of cytosine in a DNA sequence [1, 2, 3]. DNA methylation can regulate a variety of biological processes, including genetic imprinting, X-inactivation, cell differentiation, and embryonic development, which are manifested as differences in the growth and development of organisms [4, 5, 6, 7]. Studies in eukaryotes have shown that methylation mostly occurs in so-called CpG island, CHH, CHG, gene body, and transcription region, and that methylation levels are negatively correlated with transcription levels [8, 9, 10].

Various detection methods for methylation have been developed. Currently, there are four mainstream detection methods available, namely whole-genome bisulfite sequencing (WGBS) [11], reduced representation bisulfite sequencing (RRBS) [12], bisulfite amplicon sequencing (BSAS) [13], and the MassARRAY flight mass spectrometry technique [14]. The choice of method usually depends on the purpose of the study and usually involves the detection of the whole DNA methylation level or the site methylation level, and if there is no specific site or study region, the whole genome level should be tested [15]. WGBS is based on the analysis of genome methylation level as a whole. The principle of this method is that DNA sequences treated with disulfate are amplified by PCR, and methylated sites are identified by high-throughput sequencing technology. Bisulfite processing can convert unmethylated C bases in the genome into U and then turn into T after PCR amplification, which can be distinguished from originally methylated C bases. Combined with high-throughput sequencing technology, single-base resolution genome-wide DNA methylation maps can be drawn.

DNA methylation has been widely used to study mammalian growth and development, providing insights into epigenome evolution and complex traits [16]. For example, it has been used to investigate the breeding and genetic breeding of poultry [17]. DNA methylation technique was used to confirm the influence of the environment on poultry growth, and to reveal some candidate genes during development [18, 19, 20, 21]. However, there has been little research on laying performance and seed conservation, especially laying ducks, which need more attention.

The Shaoxing duck is one of the most widely cultivated laying duck breeds in China, and it has the characteristics of early sexual maturity, high egg production, low feed consumption, and strong disease resistance [22, 23]. The excellent production performance of Shaoxing ducks is of great significance to the development of the poultry industry, therefore efforts must be made to preserve the species and breed better populations.

In the current study, the genome-wide DNA methylation detection technique was used to analyze the differential methylation in conservation population (A, B) and breeding population (C) of Shaoxing duck, and to screen out the differential candidate genes and related signal pathways related to production. Such data can help improve the breeding and production of this species.

2. Materials and methods

2.1. Animals and treatment

Nine 300-day-old Shaoxing ducks were randomly selected from three groups, the number of experimental samples referred to Zheng et al.'s epigenetic study [24]. Three ducks from a national conservation farm in Zhiyuan, Zhejiang, China, were included in group A (preservation period of 10 years), and three ducks from a national conservation farm in Shaoxing, Zhejiang, China, were included in group B (preservation period of 40 years). Group C was A breeding population based on group A (Shendan 2 was the terminal male parent). In this experiment, the Shaoxing ducks in the conservation group were all from the net feeding mode, and the breeding group was from the cage rearing mode, were free feeding. Blood samples were taken from subwing veins and stored at -20 °C.

2.2. DNA extraction

DNA extraction of blood samples was performed using the biomechanical DNA extraction kit (OMEGA, USA) according to the instructions of the kit. The concentration and purity of DNA were determined via UV spectrophotometry, and the integrity of DNA fragments was confirmed by agarose gel electrophoresis.

2.3. Methylation library preparation

The construction process of genome-wide DNA methylation detection library was as follows: (1) Genomic DNA of each sample was extracted and randomly interrupted to a fragment of 450bp; (2) Repair the end of DNA fragment and connect the sequencing connector; (3) EZ DNA Methylation-Gold™ kit (ZYM, USA) was used for bisulfite treatment; (4) After desalting, the glue was cut and recycled, and the library fragment size was selected and amplified by PCR. Qualified libraries were used for machine sequencing.

2.4. Quality control and comparison of sequencing data

Raw sequences need to be filtered and quality-controlled before they can be used for subsequent analysis. The main operations of quality control are as follows: (1) Adaptor sequences in reads were removed; (2) Reads with a proportion of N (N indicates that the base information could not be determined) greater than 10% were removed; (3) Low quality reads (reads with quality score Q < 10 accounting for more than 50% of the entire read length) were removed.

Trimmomatic software was used for quality control of sequencing data. Bsam software was used to compare the filtered data with the pre-selected reference genome, and the IGV browser was used to visually process Wig files.

2.5. Differentially methylated regions and gene identification and enrichment

DMRs were searched using MOABS (V1.3.7.7) with a 200-bp sliding-window and a 50-bp step-size [25]. DNA methylation levels were conducted using Fisher's exact test and the p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Windows with FDR \(< 0.05\) and \(\geq 1.5\)-fold change in the methylation level were retained for further analysis. Moreover, the p value of each cytosine in the selected regions was calculated by Fisher's exact test and it was considered as differentially methylated cytosine (DMC) if its p value was not more than 0.01 and FC \(\geq 2\) with an absolute methylation difference of 0.4, 0.2, and 0.1 for CG, CHG, and CHH, respectively. A region was retained only if it contained at least seven DMC. Finally, neighboring DMRs were combined if the gap was \(< 100\)bp. Gootools and KOBAS software were used for GO and KEGG enrichment analysis of differentially methylated genes, and Fisher's method was used to calculate the false positive rate. Four multiple tests (Bonferroni, Holm, Sidak, and false discovery rate) were used to correct the p values, with 0.05 set as the significance threshold.

3. Results

3.1. Sequencing data comparison

A total of 20,738.38 Mb of filtered data was obtained from the nine individual ducks studied, and the average rate of groups A, B, and C was 0.6903, 0.7605, and 0.6988, respectively. The average sequencing depth of groups A, B, and C was 19.31, 18.18, and 16.92, respectively. The maximum sequencing depth was 25.18 and the minimum was 14.48. The methylation conversion rates of groups A, B, and C were 95.6%, 99.5%, and 99.1%, respectively (Additional file 1: Table S1).
3.2. Statistics of different types of methylation sites

The average number of methylation sites was 43,749,575 in group A, 50,415,260 in group B, and 45,867,533 in group C. Group B had the highest number of methylated sites, while group A had the lowest number of methylated sites. There was a significantly higher ratio of CG type methylation than of CHG and CHH types among the methylation sites. Our results indicate that methylation sites mainly occur at CG sites (Additional file 1: Table S2 and Figure S1).

3.3. Distribution of methylation levels across different regions of the genome

The number and proportion of different types of methylation sites can reflect the relevant information and characteristics of species and individual methylation map to a certain extent. In this study, the distribution of methylation levels in different regions of the genome was statistically analyzed, and the results were shown in Table 1: The methylation levels in the gene region, the 2 K promoter region upstream of the gene, the downstream region of the gene and the exon region of all individuals were significantly higher in CG type than in CHG type and CHH type. For all methylation types, the levels were higher in exon region than in gene region, and the lowest in 2 K promoter regions upstream of genes and in gene regions downstream. The methylation level of group C was generally higher than that of groups A and B.

3.4. Linkage disequilibrium analysis

To evaluate the linkage disequilibrium (LD) of two conservation populations and breeding populations, the average R2 values of the two conservation populations and breeding populations of Shaoxing ducks were calculated. Groups C, B, and A were arranged from low to high. It can be seen from the results that the breeding population has a high degree of linkage, and the linkage imbalance between the two conservation populations decreases rapidly (Additional file 1: Figure S2).

3.5. Distribution of methylation level at 1 K upstream and downstream of the gene

The overall methylation level of group C at 1 K upstream and downstream of the gene was larger than that of the groups A and B, while the overall methylation level of A and B at 1 K downstream of the genome was similar. This may suggest that high-intensity breeding has resulted in elevated methylation levels. The methylation levels of CG, CHG, and CHH type in the breeding population were higher than those of the two preserved populations (see Figure 1).

3.6. The distribution of methylation levels on chromosomes

The methylation levels on chromosomes are shown in Figure 2. The methylation density of different sequences differed among the three groups, and the methylation level in the CG sequence environment was the highest, significantly higher than that of the CHG and CHH sequences.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Distribution of methylation levels 1 K upstream and downstream of the gene. The red denotes group A, blue is group B, and green is group C, the vertical axis represents the methylation level and the horizontal axis represents different gene regions.

| Sample | Region | C type | CG type | CHG type | CHH type |
|--------|--------|--------|---------|----------|----------|
| A      | Gene   | 0.0308 | 0.5776  | 0.0046   | 0.0046   |
| A      | Prom   | 0.0351 | 0.4463  | 0.0048   | 0.0047   |
| A      | Down   | 0.0353 | 0.5898  | 0.0047   | 0.0047   |
| A      | Exon   | 0.0545 | 0.6193  | 0.0049   | 0.0047   |
| B      | Gene   | 0.0314 | 0.5824  | 0.0047   | 0.0047   |
| B      | Prom   | 0.0354 | 0.4422  | 0.0048   | 0.0047   |
| B      | Down   | 0.0358 | 0.5915  | 0.0048   | 0.0048   |
| B      | Exon   | 0.0551 | 0.6201  | 0.0048   | 0.0046   |
| C      | Gene   | 0.0334 | 0.5894  | 0.0073   | 0.0073   |
| C      | Prom   | 0.0384 | 0.4835  | 0.0078   | 0.0077   |
| C      | Down   | 0.0378 | 0.6068  | 0.0075   | 0.0075   |
| C      | Exon   | 0.0568 | 0.6349  | 0.0080   | 0.0077   |

Table 1. Distribution of methylation levels in different regions of the genome.
3.7. Sequence characterization of 9 bp bases near methylated C in CHG and CHH

The 9 bp bases near CHG and CHH sites in groups A, B, and C were counted and the sequence bias characteristics of CHG and CHH sites when methylation occurred were determined by comparing the composition of the bases with and without methylation in CHG and CHH. The following Figure 3 indicates the methylation sites that adjust the base height according to the sequence bias characteristics. In the sequence type of mCHG, the H tends to be represented as A base. In the sequence type of mCHH, the first H tends to be represented as A base, and the other H tends to be represented as T base.
3.8. Statistics of differentially methylated regions

DMRs play an important role in the regulation of gene expression. Comparing the groups A and B, we identified 1,247 DMRs, including 355,450 differentially methylated sites. Compared with group A, 633 were up-regulated methylation regions in group B, 315 were in gene regions, and 318 were in intergene regions; 614 were down-methylated regions, 301 were in gene regions, and 313 were in intergene regions.

927 DMRs were found in the groups A and C, including 262,500 differentially methylated sites. Compared with group A, 550 were up-regulated methylation regions in group C, 266 were in gene region, and 284 were in the intergene region; 377 were down-regulated methylation regions, 198 were in the gene region and 179 were in the intergene regions. There were fewer DMRs between groups C and A than between groups B and A, and the same trend was also observed for differentially methylated sites. This may be due to the fact that group C was bred from group A, and the difference in genetic diversity between the two populations was small (Table 2 & Table 3).

3.9. GO enrichment analysis of differentially methylated genes

In this study, we identified 389 differentially methylated genes between groups A and B, and GO enrichment analysis indicated a total of 131 GO entries. Compared with group A, 74 items were found to be involved in the synapse, neuronal development, neuronal differentiation, central nervous system development, cell junction, cell development, cytoskeleton organization, and other related biological activities.

GO enrichment analysis of differentially methylated genes between groups A and C identified 298 GO items, including 205, 55, and 28 items in biological process, cell component, and molecular function, respectively. The regulation of axon extension is involved in axon guidance and nervous system development, neurogenesis, generation of neurons, cell adhesion molecule binding, membrane potential regulation of membrane potential) and other related biological processes. According to the enrichment ratio of differential DMR genes, it can be concluded that high-intensity breeding may affect the related biological processes in ducks to a certain extent. There were more DMRs in the biological processes than cellular components and molecular functions. In addition, the number of GO enrichment items in group C compared to group A was significantly higher than that in group B compared with group A,
which again may have been caused by high-intensity selection (Table 4, Figures 4 and 5).

3.10. **KEGG enrichment analysis of differentially methylated genes**

In total 188 signaling pathways were detected in KEGG analyses of differentially methylated genes comparing groups A and B, significantly enriched in the cAMP signaling pathway, oxytocin signaling pathway, focal adhesion, insulin secretion, platelet activation, and cGMP-PKG signaling pathway ($P < 0.05$), a total of 26 genes were enriched.

KEGG enrichment results of differential methylation genes in groups A and C indicates that the differential genes were significantly enriched in the following categories: cAMP signaling pathway, long-term potentiation, dorso-ventral axis formation, cell adhesion molecules (CAMs), glutamatergic synapse, thiamine metabolism, retrograde endocannabinoid signaling, and focal adhesion ($P < 0.05$). Thirty-five genes including \textit{CACNA1C, ATP2B1, ATP2B2, GRIA1, GRIA2, GABBR2, PDE10A, BRAF, GRM5, CPEB3, FMN2, GABRB2, PTK2, and CNTN1}, were significantly enriched (Figures 6 and 7).

4. **Discussion**

In this study, genome-wide DNA methylation was detected in nine individuals Shaoxing ducks from two conserved populations and breeding population, and a total of 20 738.38 Mb of data were generated. The average sequencing depth was $18 \times$, the sequencing quality was good, and the genome coverage was comprehensive.

The number of methylation sites was 43,749,575 and 45,867,533 in groups A (conserved population) and C (breeding population), respectively. The number of methylation sites in group C was higher than that in group A, which might be related to high-intensity breeding. CG type

| Info       | GO enrich num | BP | CC | MF |
|------------|---------------|----|----|----|
| B vs A.CG  | 131           | 74 | 29 | 28 |
| C vs A.CG  | 298           | 205| 55 | 38 |

Table 4. GO enrichment analysis statistical table ($P < 0.05$).
methylation accounted for 84%, 84%, and 76% of all methylation in groups A, B, and C. We can find that the degree of methylation in the CG type is much higher than that in other types, indicating that CG methylation is the main methylation type in ducks, and this result has also been found in pigs and sheep, and other animals [26, 27].

LD decay velocity (A > B > C) was an indicator of LD degree (A < B < C) and genetic diversity (A > B > C). There were a lot of linkage sites in the genome of group C possibly due to the high-intensity breeding. The degree of LD decay velocity in group B was lower than that of group A because the group B had been preserved for 40 years, and the near intersection resulted in the decrease of genetic diversity and the decline of production performance.

The GO and KEGG enrichment analysis revealed that the differentially expressed genes in groups A and B were mainly involved in synapse and cell junction processes, and the signaling pathways were significantly enriched in the cAMP signaling pathway and the oxytocin signaling pathway. These results also confirmed that the extension of inbreeding years will lead to an increase in epigenetic differences between the two populations, and ultimately affect the growth and production performance of ducks.

GO analyses of differentially methylated genes between groups A and C showed that the differential genes were more involved in nervous system development, neuronal development, nerve cell differentiation, reproductive neurons, and other neurological activities. It is also enriched in synapses, membrane potential regulation, cell differentiation, and tissue development. KEGG showed that differentially methylated genes were mostly involved in eight signaling pathways including cAMP signaling pathway, long-term potentiation, dorsoventral axis formation, cell adhesion molecules, glutamate synapse, thiamine metabolism, retrograde endocannabinoid signaling, and focal adhesion. The results of enrichment convey that the genes of the nervous system, signal transduction, metabolism, and signal molecule interaction were mainly affected by the breeding process. There were thirty-five differentially methylated genes including CACNA1C, ATP2B1, ATP2B2, GRIA1, GRIA2, GABBR2, PDE10A, BRAF, GRM5, CPEB3, FNX2, GABRB2, PTK2, and CNTN1.

The cAMP signaling pathway is one of the most studied pathways, and as a second messenger, it plays a role in information transmission in cells. When cells are stimulated by external signals, those signals go to G-protein-coupled receptors (GPCRs) on the corresponding membrane to
activate adenylate cyclase (AC) and promote the conversion of ATP into cAMP to the corresponding biological functions [28]. At the end of this biological process, phosphodiesterase (PDE) converts cAMP back into ATP. The content of cAMP is regulated by both AC and PDE. As a second messenger, iT activates protein kinase A (PKA), phosphorylates downstream target genes, and ultimately achieves intracellular and intracellular signal transmission [29]. It has been confirmed that the cAMP signaling pathway is widely involved in biological processes such as nerve regeneration, memory repair, emotional awareness, and plasticity regulation of the synaptic system [30]. It plays a role in ovarian development, follicular maturation, ovulation, and other processes.

Compared with the group A, 35 differentially methylated genes were significantly enriched in group C. Most of these genes were involved in the reproductive regulation pathway of the body and played an important role in ovarian and follicular development, ovulation, gametogenesis, embryo development, and eggshell formation.

*CACNA1C* was identified as a key gene in the process of breeding in the analysis of genomic selection signals. It was involved in the serial regulation process of reproduction and had a positive effect on the egg production performance of the breeding population. The analysis results of Su et al. [31] confirmed that the methylation level of the gene would affect its expression level.

*GABBR2* is a member of the G-protein coupled receptor family, which is widely involved in immune regulation, behavior, and other signaling pathways after binding with corresponding ligands. In this study, *GABBR2* was identified as a differential methylation gene in the two populations before and after breeding, suggesting that the *GABBR2* gene may have a certain effect on the egg production performance of the breeding population.

*PDE* is the key to regulating the level of cAMP and plays an important role in the cAMP signaling pathway. Thus, the regulation of *PDE*-related genes can indirectly promote ovarian development and ovulation, and other reproductive activities. In this study, *PDE10A* was the differentially methylated gene between the two populations before and after breeding, and it was speculated that *PDE10A* might promote ovulation and ovarian development to improve egg production of the breeding population.

*CPEB3* is an important member of the cytoplasmic polyadenylate element-binding protein (*CPEB*) family, which currently includes *CPEB1, CPEB2, CPEB3*, and *CPEB4*. Some studies have exhibited that this gene is involved in the regulation of reproductive mechanisms [32, 33, 34], and
this gene was identified as a differential methylation gene before and after breeding, suggesting that it may improve egg production performance through the regulation of related reproductive processes.

GRIA has also been identified as a differentially methylated gene, which promotes ovarian development and plays an important role in reproductive regulation [35, 36].

A large number of studies have shown that thyroid-stimulating hormone plays an important role in the seasonal reproduction of animals [37, 38]. In this study, the BRAF gene was identified as a differentially methylated gene, which may participate in reproductive regulation and improve the performance of laying ducks by regulating thyroid signal transduction.

The Formin gene family is considered to be an oogenesis-related gene and has been shown to play an important role in the meiosis of oocytes [39]. In this study, FMN2 was identified as a differentially methylated gene, which may result in a change in gene expression and thus affect its egg production performance.

PTK2, a protein tyrosine kinase, plays an important role in the phosphorylation of oocytes. Activated PTK2 in oocytes regulated spindle assembly during meiosis and impeded oocyte maturation [40, 41]. In addition, differentially methylated genes such as CNTN1, GABRB2, IGSF11, and GRM5 were also found in this study, and all of these are involved in reproductive processes such as ovarian development and ovulation.

We also found differentially methylated genes related to egg quality were also identified. Studies have shown that the calcium ion transport ATPase gene family (ATP2) has a major influence on eggshell strength [42]. The differentially methylated genes ATP2B1 and ATP2B2 found in this study have certain effects on the eggshell quality of the breeding population, which may be used to develop molecular markers to improve eggshell strength and quality.

Differences in DNA methylation between breeding population and conserved populations were identified in this study, and some differentially methylated genes related to reproduction and egg quality were screened. However, the expression levels of these differential genes were not measured in this study, and their mechanisms of action are unclear. Further studies on the mechanisms of related genes involved in reproductive regulation can be conducted to improve the production performance of laying ducks.
5. Conclusion

In conclusion, we found that the methylation levels were higher in a breeding population of Shaoxing ducks than in two conserved populations, and the proportion of CG methylation sites in the three populations was the largest, and most of the methylation sites were located in exon regions. There were 1247 DMRs between the two populations (groups A and B). There were 927 DMRs between the two groups (groups A and group C) before and after breeding. The DMRs were evenly distributed in the gene and intergene region. GO and KEGG analysis results showed that compared with the conserved population, the breeding population was significantly enriched in eight signal pathways, such as cAMP signal pathway and long-term potentiation. There were 35 differentially methylated genes, including CACNA1C, GRIA1, GRIA2, GABBR2, PDE10A, B Raf, GRM5, CPEB3, FMN2, GABBR2, PTK2, and CNTN1, which are involved in reproductive processes such as ovarian and follicular development and ovulation. In addition, ATP2B1, ATP2B2, and other genes related to eggshell quality were identified, and may be possible to use as molecular markers to improve eggshell quality in the future.

Declarations

Author contribution statement

Ligen Xu and Zhenzhen Shi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Haiying Li, Jun He, Bindan Chen, Zeng Tao, Yong Tian, Li Chen, Guoqin Li, Zhengrong Tao and Tianiant Gu: Performed the experiments; Wenwu Xu and Lizhi Lu: Conceived and designed the experiments.

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Declaration of competing interest

We declare that we have no financial or personal relationships with other people or organizations that could have inappropriately influenced our work; there is also no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Institutional review board statement

The animal care and use protocol was approved by the Institutional Animal Care and Use Committee of the Zhejiang Academy of Agricultural Sciences (Protocol number: 2021ZAASLA15), in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China).

Data availability statement

Data associated with this study has been deposited at Sequence data accession number CRA006223.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

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