Study on the Crystal Structure and Genetic Structure of Eggshell

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Abstract. Eggshell provides a relatively independent and stable internal environment for embryo growth and development, and is also a source of calcium ions required for embryo development. Eggshells of good quality can reduce the risk of food safety to a certain extent, and can also reduce the economic losses caused by broken eggshells and improve economic efficiency. At the same time, the process of eggshell formation is also a classic biominerlization model. In this study, the 600K high-density SNP chip of chicken was used to perform SNP typing on several groups obtained by the reciprocal cross between Bailaihang and Dongxiang green-shell hens. Scanning electron microscopy and X-ray crystal diffraction were used to determine the ultrastructure and eggshell ultrastructure. Crystal structure, explain its genetic basis, and explore the relationship between eggshell ultrastructure and crystal structure. This study believes that the DERA gene plays an important role in regulating the growth of eggshell crystals. There are three possible ways of action: one is to provide energy for the synthesis of uterine tissue cell matrix protein and its transport with ions; the second is to reduce the stress of high Ca²⁺ concentration stress, maintain cell activity, and for the growth of eggshell crystals Stably provide the required Ca²⁺; third, by regulating the concentration of ATP in the uterine fluid, the effect on the growth of eggshell crystals.

Keywords: Egg shell, crystal structure, genetic inheritance, X-ray crystal diffraction.

1. Introduction
Eggs are one of the most important sources of protein for humans. Eggs, like the eggs of other birds, contain all the nutrients needed for embryonic growth and development. Biologically speaking, on the one hand, the eggshell provides calcium ions for embryo growth and development, on the other hand, it has a certain fixed shape and pressure resistance, provides a relatively independent and stable internal environment, and isolates some from the outside. Environmental violations. From an economic point of view, on the one hand, a good-quality egg shell can significantly reduce the economic loss caused by the broken egg shell during the production, transportation, and processing of
eggs, on the other hand, it can increase the hatching rate of egg breeding and improve economic efficiency. In terms of food safety, the eggshell is a barrier that separates the inside and outside of the egg. A good-quality eggshell can effectively prevent external microorganisms from contaminating the egg content to a certain extent and reduce the risk of food safety. In addition, the process of eggshell formation is also a classic biomineralization model.

In order to explore the relationship between the eggshell ultrastructure and crystal structure, and to explain the genetic basis of the eggshell ultrastructure and crystal structure, this study formed several groups obtained from the reciprocal cross between Bai Laihang and Dongxiang Green Shell Laying Hens. The chicken's 600K high-density SNP chip was used to perform SNP typing. Scanning electron microscopy and X-ray crystal diffraction were used to determine the ultrastructure and crystal structure of the eggshell. A genome-wide association analysis was performed based on the mixed linear model. The phenotypic distribution of eggshell ultrastructure and crystal structure in large groups revealed by this study, the relationship between different structures and their genetic mechanism help us to analyse eggshell and other biomineralized materials from different angles Deeper understanding of the structure and biomineralization process [1].

2. Materials and methods

2.1. Materials

With 240-day-old green-shell eggs, Taihe eggs, Langshan eggs and Hailan W-36 parent eggs (stored at 24°C for 11 weeks), 240-day-old green-shell eggs ♂×green-shell eggs ♀ (Group I), Green-shell layer hen ♂×Taihe chicken ♀ (II), Green-shell layer hen ♂×Wolf Mountain chicken ♀ (Group III), Green-shell layer hen ♂×Hailan W-36 parent chicken ♀ (Group IV) Eggs produced by F1 generation (stored at 24°C for 1 week) are used as materials, and 4 randomly selected samples are sent to the test centre for observation and analysis [2].

2.2. Test method

Each chicken collects one normal egg, and after the measurement following the routine egg quality measurement procedure, retain the eggshell and let it dry for 1 day at room temperature. The eggshell of each chicken is individually sealed in a Ziplock bag and stored at room temperature for Follow-up test.

2.3. Measurement indicators and methods

2.3.1. Detection of physical properties of eggshells. On the 16th weekend of the test, egg quality indicators were tested. Weigh the egg weight and eggshell weight, and examine the egg shape index (egg shape index = long diameter/short diameter), eggshell thickness, and eggshell strength. Place the pigeon eggs upright on the eggshell strength measuring instrument, and the instrument will automatically read the eggshell strength after pressing the pigeon eggs. The pigeon eggs are knocked to take the eggshells, washed and dried to remove the shell membrane, and the thickness of the top, middle and bottom of the pigeon eggs is measured using a spiral micrometre, and the average value is the thickness of the eggshell.

2.3.2. Detection method of eggshell ultrastructure. After the egg quality was determined, 6 eggs were randomly selected, and the eggshell (shell removal membrane) was taken, washed and dried repeatedly. Take a large piece of eggshell for each sample, with the outer surface facing upwards, and gently press the thumb to break it into small pieces. Select small pieces with fresh sections, with the fresh sections facing upwards, and stick them vertically on the sample table. After gold spraying, the ultrastructure of each layer of eggshell cross section was observed using XL30 SEM environmental scanning electron microscope. The thickness of each part of the ultrastructure can be calculated according to the distance and scale on the photo.
2.3.3. Blood sample collection and DNA extraction. The test chickens were all collected by subbing
vein blood collection. The blood volume was about 1.0 mL and placed in a centrifuge tube containing
0.5 mL of anticoagulant (ACD). After mixing, it was stored in a -20 °C refrigerator for subsequent
extraction of genomic DNA. Thaw frozen blood samples at room temperature and take about 30 ACD
anticoagulated chicken blood samples into a prepared 1.5mL centrifuge tube, then add 600μL of cell
lysat (STE with 0.5% SDS), then add 20% 20 μL of proteinase K, shaking for 20 minutes, and
shaking overnight in a water bath at 550C and 220 rpm. Add 600 horses of Tris-saturated phenol
(pH=8.0) to the digested blood sample, invert the centrifuge tube slowly for 10 minutes, and then
centrifuge at 1200rpm for 10 minutes. Finally, carefully aspirate the supernatant with a sheared
pipette tip to another a clean 1.5mL centrifuge tube. Add 600 μL of phenolform to a new centrifuge
tube, invert the centrifuge tube slowly for 10 minutes, and then centrifuge at 1200 rpm for 10 minutes.
Finally, carefully aspirate the supernatant into another clean 1.5 mL centrifuge tube with a sheared tip.
Add 500 μL of chloroform to the new centrifuge tube, invert the centrifuge tube slowly for 10 minutes,
then centrifuge at 12000r for 10 minutes, and then carefully aspirate the supernatant into another clean
1.5 mL centrifuge tube with a sheared tip. Add 1.0 mL of absolute ethanol to the new centrifuge tube,
gently shake the centrifuge tube until a white flocculent precipitate appears in the tube, centrifuge at
12000r for 10 minutes to precipitate DNA, and discard the waste solution. Then add 300 μL of 75%
ethanol to the centrifuge tube and centrifuge at 12000r for 3 minutes to precipitate DNA. Carefully
aspirate the ethanol, leave the centrifuge tube at room temperature and wait for the ethanol to
evaporate, then add an appropriate amount of ddH2O or TE buffer to dissolve the DNA. After
dissolution, use Nano Drop nucleic acid analyser to detect its concentration. The DNA band integrity
was tested under 1.5% agarose gel electrophoresis. Store the dissolved DNA in the refrigerator at
-20°C.

2.4. Whole genome association analysis
This test uses a linear mixed model to test the degree of association between each SNP and traits. The
model is as follows:

\[ y = Wa + Xp + \mu + \epsilon \] (1)

Where Y is an n×1 vector of n individual phenotypic values; W is an n×c covariance matrix,
consisting of fixed effects and column vectors 1; a is a corresponding coefficient including intercept
x× 1 vector; X is the n×1 vector composed of the genotypes of the SNP to be tested; p is the effect
value of the corresponding SNP; u is the random multigene effect n×1 vector, and its covariance
structure is \( u \sim N(0, K V_g) \), K represents nxn inheritance Correlation matrix, Vg is the additive
variance of multiple genes; \( \epsilon \) is the random residual n×1 vector, subject to \( \epsilon \sim N(0, I V_e) \) distribution,
I is the nxn identity matrix, and Ve is the residual variance.

3. Results

3.1. Eggshell ultrastructure and eggshell quality
The eggshell is a highly precise and ordered mineralized structure, which is mainly formed by the
deposition of calcite crystals (CaCO₃) on the eggshell membrane. An environmental scanning electron
microscope was used to observe the eggshell. The cut planes were shell membrane, mastoid layer,
barrier layer, crystal layer and epidermal layer from inside to outside (see Figure 1).
Although the outer surface of the parent eggshell of each hybrid combination of green-shell laying hens has turtle-shaped cracks, and the pores and particles of different sizes are distributed on it, the turtle-shaped cracks of green-shell eggs are thin, many, and the lines are connected; green shell eggs The outer surface structure of egg shells of F1 generation chickens is basically similar among the four groups, only the turtle-shaped cracks in group IV are less, and the outer surface of egg shells in the four groups are rarely covered with particles. Eggs are not fresh and the outer membrane of the shell is damaged. As shown in picture 2.

**Figure 1.** Schematic diagram of eggshell ultrastructure

**Figure 2.** Eggshell structure observed under scanning electron microscope
The inner surface of the eggshell observed under the scanning electron microscope is the part where the outer layer of the inner eggshell membrane closely adhered to the inner wall of the eggshell is closely connected with the nipple layer of the eggshell. Therefore, the inner surface structure of the eggshell is similar to the outer structure of the inner eggshell membrane. The fibres seen are keratin fibres, and their network structure is formed by these fibres being slightly infiltrated by lime salt. Rock-like structures can be seen in the deep layer of the fibre web, which may be the upper part of the eggshell papillary layer or the lower part of the sponge layer. According to the size of the particle body, it can be considered that many fine particles attached to the fibre are calcite-type CaCO₃ crystals; while the slightly larger particles attached to the fibres are calcite crystal particles composed of many CaCO₃ crystals. All chicken breeds are interwoven with fibres on the inner surface structure of the eggshell. Green-shell eggs: relatively regular stacking of dendritic; Taihe eggs: fusion of lactometer and fibres of varying sizes; Wolf Mountain eggs: random branching Stacked; Hyacinth Eggs: Randomly arranged and piled up in branches. In the internal surface structure of the F1 hybrid eggshell, group I and II are similar, and group III and IV are similar. Whether the difference in ultrastructure between the eggshells of the above-mentioned varieties leads to green shell eggs, Hailan eggs, eggshell strength and other qualities that are better than Taihe eggs and Wolf Mountain eggs remains to be discussed [3].

3.2. Descriptive statistical analysis of phenotypes

Table 1 lists some basic statistics of eggshell ultrastructure 4 traits. The coefficient of variation of the total thickness of eggshell EST is the smallest, ET is second, and the coefficient of variation of MT is larger. This may be due to the short duration of the papillary formation period corresponding to MT, but as the initial stage of eggshell mineralization, the factors involved in regulating mineralization are more complicated, and then enter the rapid mineralization period, which lasts for a long time, but the participation of regulation Various factors tend to stabilize, making the variation of ET relatively small. Figure 3 shows the distribution of four traits and their phenotypic correlations. The bar graph shows that the four traits are close to the normal distribution, which is used for subsequent correlation analysis after normal transformation. The heritability of EST and ET estimated based on genome-wide SNP is at a medium level (0.36-0.39). The heritability of MT and MD is relatively low (0.17-0.19) (see Table 2). Since ET and MT are part of EST, they have a high genetic correlation. The phenotypic and genetic correlations between the remaining traits are low. This shows that different stages of eggshell mineralization may have different regulatory factors involved.

| Character | Mean | SD  | CV (%) | Min  | Max  |
|-----------|------|-----|--------|------|------|
| EST       | 318.33 | 38.73 | 12.17  | 186.32 | 433.81 |
| ET        | 244.65 | 36.28 | 14.83  | 128.69 | 361.98 |
| MT        | 73.5  | 15.02 | 20.44  | 30.64  | 135.98 |
| MD        | 284.76 | 67.87 | 23.83  | 98.63  | 581.05 |

| Character | EST     | ET       | MT       | MD       |
|-----------|---------|----------|----------|----------|
| EST       | 0.39(0.06) | 0.96(0.02) | 0.60(0.15) | -0.29(0.16) |
| ET        | 0.36(0.06) | 0.35(0.20) | -0.26(0.15) |
| MT        | 0.17(0.05) | -0.26(0.21) |
| MD        | 0.19(0.05) |
3.3. Screening for key genes that affect the ultrastructure of eggshells

All sNPs that are significantly related to EST, ET, MT, MD are annotated by Ensemble database. We found 4 SNPs associated with EST and ET: rs316793137, rs312347405, rs314218674, rs312660069} are missense mutations. This study evaluated the effect of these missense mutant SNPs on the function of the corresponding protein using the predictive function of SIFT with VEP = in the Ensemble database. The SIFT score can evaluate the degree of effect of amino acid substitutions on protein function. This score is calculated by the SIFT algorithm. A score of less than 0.05 is considered to have an effect on protein function. The SIFT scores of the above four missense mutant SNPs are between 0.09 and 0.61, which means that these mutations are tolerable and have little effect on protein function [4].

After analysing the annotation information of all significant SNPs, we found that they correspond to 64, 73, 1 and 2 genes, respectively. GO annotation of these genes was adopted by Ensemble BioMart. Considering that the eggshell is mainly composed of minerals, combined with the annotation functions of these genes, we screened the four genes most likely to affect EST and ET, and their functions are related to ion transport. These genes are KCNJS, WNK1, ABCC9 and ITPR2. Only one TTM2C gene related to MT was located through significant SNP. The gene associated with MD is KNDC. Since there are many genes related to EST and ET, we explored whether there is some close relationship between these genes. However, we used the Functional Annotation and Gene Functional Classification tools of DAVID v6.7 software, and did not find that these genes were significantly enriched in any entry, signalling pathway or functional group. As shown in Figure 4.

Figure 3. Correlation matrix diagram of various traits of eggshell ultrastructure
3.4. Contribution of key SNPs to phenotypic variation

We selected the six genes most likely to have a greater impact on eggshell ultrastructural traits: KCNJ1, WNK1, ABCC9, ITPR2, ITM2C and KNDC 1. Next, we selected the SNPs with the smallest P values on these genes for further analysis. These SNPs are: rs15301807, rs313822026, rs314985144, rs316011750, rs314546938 and rs313308902 in this order. The first 4 SNPs explain the 3.2%-6.3% phenotypic variation between TEST and ET. rs314546938 explained the 3.34% phenotypic variation of MT, and rs313308902 contributed 3.91% of the phenotypic variation of MD. rs314985144 showed the greatest effect on EST and ET. One copy of EA replacement can produce changes of 13.98 μm and 13.28 μm. For rs313308902, a copy of the EA replacement can result in a 10% change in MD. The influence of these SNP genotypes on the four traits is shown in Figure 5. A common feature is that the phenotypic value corresponding to heterozygotes is in the middle, the smallest allele homozygote corresponds to the smallest phenotypic value, and the largest allele homozygote corresponds to the largest phenotypic value [5].

Figure 4. Conditional correlation analysis and linkage disequilibrium analysis of EST and ET
4. Conclusion

The papillae start to grow from the nucleation site on the eggshell membrane, so the density of the nucleation site determines the density (MD) of the papillae. Although the main process of mastoid formation is completed in the uterus, the start of this process is in the red tube of the fallopian tube. This part forms the eggshell membrane and the fibrous nucleus on the membrane responsible for initiating the mineralization of the eggshell. At present, there is still a lack of research on eggshell membrane fibre cores. This study found that KNDC1 gene is significantly related to MD. KNDC1 interacts with high molecular weight microtubule fibrin 2, leading to negative regulation of neuronal dendritic growth. 6i, 6aoGO annotations show that KNDC1 is involved in GTPase-mediated signal transduction and protein phosphorylation. Based on the above findings, we believe that KNDC1 may be involved in the modification of eggshell membrane fibrin and determine whether the modification site can become a nucleation site that can initiate eggshell mineralization. The exact regulation of KNDC1 on MD remains to be clarified. We propose two possible ways for KNDC1 to function: one is a direct way, KNDC1 interacts directly with fibro nuclear protein; the other is an indirect way, KNDC1 participates in signal transduction and regulates other modified proteins to modify fibrin clear protein.

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