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Polymorphism of the SCNN1g Gene and its Association with Eggshell Quality

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Abstract

Eggshell quality is the main trait to assess egg quality. Marker assisted selection can be used to improve this trait. During eggshell formation, a mass of inorganic minerals is deposited. The Sodium Channel (SCNN1) gene family plays an essential role in cation transportation and SCNN1g is a member of this gene family. The objective of this study was to estimate the frequency of SCNN1g gene variants and to find its associations with eggshell quality in Hy-Line breed. 100 hens were randomly selected and their eggs and blood samples were collected. DNA was extracted and purified using the phenol-chloroform method and genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. GLM procedure of SAS software was used to evaluate the association of SCNN1g gene polymorphism with egg weight, specific gravity, eggshell strength, eggshell weight, and eggshell thickness. Based on the polymorphism of SCNN1g gene, three genotypes were observed including AA, AG, and GG with frequencies of 0.26, 0.57, and 0.17, respectively. Genotype only had a significant effect on eggshell strength ($P < 0.05$). Other traits were not significantly influenced by genotypes of this gene. Therefore, introducing this gene in marker-assisted selection programs may improve eggshell strength of Hy-Line breed.

Introduction

Chicken egg has high nutritive value as a human food source and provides inexpensive animal protein for consumers relative to other foodstuffs such as meat and milk (Nys *et al*., 2011). The eggshell is essential for propagation of all avian species with a sophisticated structure that functions to: (a) protect the contents of the egg from the microbial and physical environment; (b) control the exchange of water and gases through pores during the extra-uterine development of the chick embryo; and (c) to provide calcium for embryonic development once the yolk stores are depleted. In order to meet these requirements, the eggshell must be a porous ceramic material. The thickness, form, size, structural elements, and features of the porous of the eggshell system varies among different species. However, its general structure is similar across all birds (Romanoff and Romanoff, 1949; Tyler, 1964; Board, 1982; Mikhailov, 1997; Panhéleux *et al*, 1999). Vetter and O'Grady (2005) described that plasma levels of ionized Ca^{2+} did not significantly change in hens between 33 and 122 weeks of age. Thus, the decreased eggshell thickness in older hens may involve changes in

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shell gland function such as declining ability for epithelial transport of Ca^{2+} rather than availability of Ca^{2+} for secretion.

Ion transportation plays a very important role in the process of eggshell formation. The ion channel super family includes voltage-gate K^+ channels, voltage-gated Ca^{2+} channels, Na^+ channels, and non-voltage gated Na^+ channels, etc. Amiloride-sensitive Na^+ channels are a diverse group of ion channels essential for controlling the regulation of Na^+ transport into cells and across epithelia (Benos and Stanton, 1999). Na^+ is actively transported across the uterine epithelium into the plasma while net Ca^{2+} secretion progressively increases with increasing concentrations of Na^+ perfusions. These observations suggested a positive influence of Na^+ absorption on net Ca^{2+} secretion in the avian uterus (Eastin and Spaziani, 1978). Furthermore, new evidence suggests that the concentration and transfer of Na^+ can directly influence the transportation of calcium and bicarbonate ions in chicken uterus (Jonchère *et al*., 2012). The amiloride-sensitive Na^+ channels are made up of four subunits (α, β, γ, and δ) encoded by the SCNN1a, SCNN1b, SCNN1g and SCNN1d genes, respectively (Canessa *et al*., 1994). Three subunits (SCNN1a, 1b and 1g) of the Na^+ channel are overexpressed in the uterus compared to the duodenum and magnum, suggesting the involvement of these transporters in Na^+ absorption by the uterine glandular cells at the apical membrane. The γ subunit (SCNN1g) was overexpressed during shell calcification in contrast to α and β subunits (SCNN1a, 1b) suggesting its predominant involvement in the uterus. It has been shown that SCNN1g is expressed at a higher level in the presence of eggshell calcification than in its absence (Jonchère *et al*., 2012). SCNN1g is expressed highly in uterus of poultry compared to magnum, duodenum, liver, and kidney (Fan *et al*., 2013).Thus, the development and function of specific tissues contribute to the specific expression of its genes (Enuka, 2012).

Based on the evidence supporting a role for Na^+ in Ca^{2+} transport during eggshell formation, we hypothesized that the SCNN1g gene could affect eggshell quality. In the current study, the goal was to identify polymorphisms of this gene and study its association with chicken eggshell quality.

Materials and Methods

Animal sampling and data collection

100 hens were randomly selected from a population of Hy-Line breed and their eggs (one from each hen) and blood samples were collected at 18 weeks of age. An electronic scale with an accuracy of 0.01 g was used to weigh the eggs (egg weight, EW). The eggs were broken using an Egg Shell Strength Tester to measure egg shell strength (ESS). Egg shell weight (ESW) was measured after 72 hrs of exposure to dry air. Egg shell thickness (EST) was measured with a Shell Thickness Meter (calibrated in mm) at the pointed end, equator, and blunt end of shells and average values were used. Specific gravity (SG) was calculated using the following formula:

$$SG = [EW/ (EW - EW_1)]$$ (Hempe *et al*., 1988)

where EW_1 is the egg weight in water)

Blood samples were kept in 3 mL tubes containing EDTA as coagulant agent and stored in -20^{0}C. Genomic DNA was isolated from 20 µL blood samples using a phenol-chloroform kit (Fermentas, #k0512).

PCR-RFLP Analysis

The National Center for Biotechnology Information (NCBI) SNP bank (www.ncbi.com) was used to search for potential SNPs in the SCNN1g gene DNA sequences. The SCNN1g gene (GenBank accession No. BC059391) is located on chromosome 14. The SNP rs15009191 (SNP location (Chr: bp) is 14:7018954) was used in current study. This SNP is a silent mutation located in exon 10 of SCNN1g and corresponds to the substitution of adenine/guanine (A/G) according to Fan et al. (2013).

Primers (Table 1) were designed using CLC Main Workbench 5 and Primer Premier 6.1 software. The PCR was performed in 25 µL mixture containing 100 ng genomic DNA, 10X PCR buffer, 0.5 µL of each primer (5 pmol) and 12.5 µL of Farazist Avaran Sorengostar master mix and deionized water. The PCR conditions were conducted in a thermocycler as follows: an initial denaturation step at 94^{0}C for 10 min followed by 35 cycles of denaturation at 94^{0}C for 30s, annealing at 55^{0}C for 30s, extension at 72^{0}C for 45s, and a final extension at 72^{0}C for 10 min.

An amplified fragment was subsequently digested with *Ssi*I (*Aci*I) enzyme (Thermo Scientific). The restriction enzyme digestions were performed using 3 µL of PCR product mixed with 2 U of the appropriate restriction

enzyme, followed by incubation at 37^{0}C for 16 hrs. Gel monitoring and spectrophotometry were used to visualize the genotypes.

Statistical analyses

The frequency of alleles and genotypes were calculated using POPGENE software (Yeh *et al*., 1997). A chi-square (χ^2) test was performed to test the goodness of fit to Hardy–Weinberg equilibrium expectations for the distribution of genotypes. In order to test the association of SCNN1g genotypes with egg quality traits, statistical analysis was performed using GLM procedure of the SAS program and least squares means of the genotypes were compared by the Tukey–Kramer test (SAS, 2001). The following model was used:

$$y_{ijk} = \mu + G_i + H_j + e_{ijk}$$

Where y_{ijk} is egg quality traits for each hen, μ is mean of the population, G_i is the fixed effect of genotype (I = 1, 2, 3), H_j is the fixed effect of hatch (j = 1, 2) and e_{ijk} is the random residual error.

Results

The results of SCNN1g gene digestion are shown in Figure 1. Allelic and genotype frequencies of this gene are shown in Table 2. Based on present results, frequencies of A and G alleles were 0.545 and 0.455, respectively. Frequencies of AA, AG and GG genotypes were 0.26, 0.57 and 0.17, respectively. The χ^2 was not statistically significant ($P > 0.05$), suggesting that the population was in Hardy-Weinberg equilibrium (Table 3). The observed heterozygosity in the studied population was 57% which was higher than the expected heterozygosity for this gene. The higher frequency of heterozygotes in this strain suggests an adaptive advantage. Comparison of least squares means of the different genotypes of SCNN1g gene for eggshell quality traits are shown in Table 4. SCNN1g gene polymorphism showed a significant association ($P < 0.05$) with eggshell strength where heterozygous birds had greater average eggshell strength compared to homozygous birds, indicating that A and G alleles cooperated to increase the strength of the shell. No significant associations were found between genotypes and other egg quality traits.

Table 1. Primer sequences used in-PCR-RFLP for SCNN1g gene

Primer name	Sequence (5′→3′)	Tm(°C)	Product size (bp)	Position
SCNN1g-F	GCGGGATATGCCATTCATTACTGC	61	589	EXON 10, 2607
SCNN1g-R	GCTCCGTGTCGGGATAGAAG			2019

Table 2. Allelic and genotypic frequencies of SCNN1g gene in the studied population

Frequency	Allele		Genotype		
	A	G	AA	AG	GG
	0.545	0.455	0.26	0.57	0.17

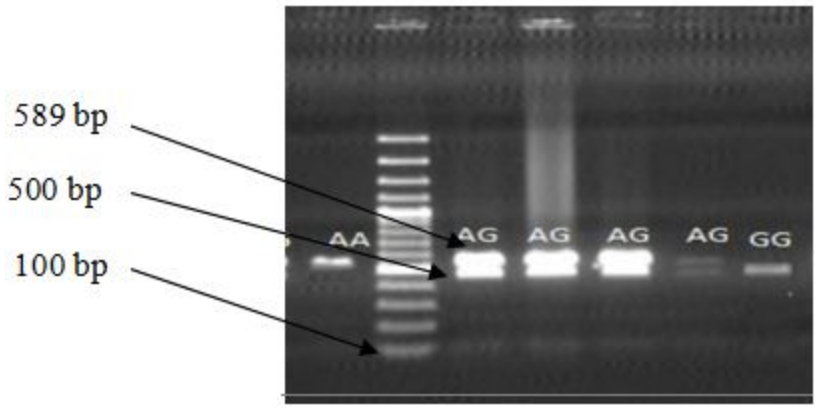

Figure 1. Results of a 589 bp fragment of SCNN1g gene digestion with *Ssi*I (*Aci*I) enzyme on agarose gel

Table 3. Chi-Square test for Hardy-Weinberg equilibrium in SCNN1g genotypes

Genotypes	Observed (O)	Expected (E)	$(O-E)^2/E$	$\chi 2$
AA	26	29.32	0.37	1.82^{ns}
AG	57	49.34	0.89	
GG	17	20.32	0.54	

Table 4. Least squares means comparison of different genotypes of SCNN1g gene for egg quality traits

Trait	AA	AG	GG	*P*-value
EW (g)	55.01 ± 0.66	55.28 ± 0.44	55.73 ± 0.8	0.79
SG (g/cm^3)	1.079 ± 0.001	1.079 ± 0.007	1.079 ± 0.001	0.38
ESS (kg/cm^2)	3.63 ± 0.13[b]	4 ± 0.09[a]	3.51 ± 0.16[c]	0.01
SW (g)	5.3 ± 0.14	5.53 ± 0.09	5.33 ± 0.17	0.32
ST (mm)	0.42 ± 0.007	0.43 ± 0.004	0.42 ± 0.008	0.37

EW = egg weight, SG = specific gravity, ESS = eggshell strength, SW = eggshell weight, ST = eggshell thickness. Least square means with different letters in each row have significant difference ($P < 0.05$).

Discussion

Polymorphism of the eggshell organic matrix genes was considered to be related to eggshell breaking strength, eggshell thickness, and dynamic stiffness (Dunn *et al*., 2009). These types of investigations are helpful to identify loci which are potentially useful for breeding layers with higher egg quality. Previous studies showed that SCNN1a, SCNN1b and SCNN1g are highly expressed in the uterus during the eggshell formation while SCNN1g expression increases quickly in the stage of eggshell formation (Jonchère *et al*., 2012, 2010). In this study, we found that SCNN1g gene had a significant effect on eggshell strength. It seems that birds with the heterozygous genotype have more active sodium-calcium channels. Therefore, higher sodium absorption and calcium secretion in the uterus of these birds lead to increased eggshell strength. This advantage in eggshell quality may be associated with the higher frequency of the heterozygous genotype in this population relative to the homozygous genotypes.

Fan *et al* (2013) reported that sodium channels can affect eggshell quality, especially eggshell strength and eggshell thickness. The SCNN1g gene is located on chromosome 14 and comprises of seven SNPs. One SNP (rs15009191) of this gene was shown to be associated with eggshell percentage and eggshell thickness (Fan *et al*., 2013). Also, they three genotypes (i.e. CC, CT and TT) were observed for this gene with frequencies of 0.69, 0.27 and 0.04, respectively. Birds with the CT genotype had the highest eggshell percentage. Birds with CC genotype had the highest shell thickness while CT and TT genotypes had similar values for this trait. Duan *et al* (2015) found that rs15009190 (SCNN1g) had a significant effect on eggshell weight. The identification of ion transporters related to eggshell mineralization could improve our understanding of the mechanisms and regulation for ionic precursors of calcium carbonate (CaCO3), and enable us to find new potential genes effectively.

In the present study, the SNP of the SCNN1g gene was not associated with eggshell thickness but instead was associated with eggshell strength. Therefore, introducing this gene as a marker in marker-assisted selection program can improve eggshell strength of Hy-Line breed and build understanding of the process of ion transport during eggshell formation. These potential markers may genetically improve eggshell quality.

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