

## Discover of a Novel SNP Highly Associated with Chicken Blue Egg

<sup>1</sup>Z.P. Wang, <sup>2</sup>X.T. Wang, <sup>3</sup>R.F. Liu, <sup>4</sup>A.R. Wang, <sup>1</sup>J.Y. Li and <sup>1</sup>X.M. Deng

<sup>1</sup>College of Animal Science and Technology, China Agricultural University, 100193 Beijing, China

<sup>2</sup>Institute of Oceanology, Chinese Academy of Sciences, 266071 Qingdao, China

<sup>3</sup>Vocational and Technical College, Inner Mongolia Agricultural University, 014109 Baotou,

<sup>4</sup>DaBeiNong Group, 100080 Beijing, China

---

**Abstract:** Blue egg is caused by a dominant mutation, Oocyan (O) which is previous located on short arm of chicken chromosome 1 but precise genetic mechanism underlying blue egg is still unknown so far. In this study, we found a SNP (g.67419892-67419904del13) significantly associated with blue egg using 126 browned-shelled chickens and 202 blue-shelled chickens. The allelic and genotypic frequencies of g.67419892-67419904del13 are significantly higher in blue-shelled chickens (92.5 and 87.1%) compared with brown-shelled chickens (10.7 and 8.7%,  $p < 0.0001$ ). Moreover, the genetic distance between O and g.67419892-67419904del13 is estimated to be over 2 cM (LOD = 13.51) using 149 F<sub>2</sub> offspring from mating between blue-shelled males and brown-shelled females. Apparently, the marker is useful for the cloning of O and marker aid selection of blue-shelled chicken breed.

**Key words:** Chicken, blue egg, SNP, association, linkage, chromosome

---

### INTRODUCTION

In domestic fowl, blue egg is caused by an autosomal dominant mutation, Oocyan (O) and be haves as normal Mendelian inheritance (Punnett, 1933).

O is localized on the proximal end of the short (p) arm of chromosome 1 and shows close linkage with pea comb (P) (Bruckner and Hutt, 1939; Zartman, 1973; Bitgood *et al.*, 1980). A variant map distances between P and O has been reported by several groups. Bruckner and Hutt (1939) first reported a map distance of 6 cM after testing 35 birds. A refined linkage value was given to be 4.28±1.3 cM by Bitgood (Bitgood *et al.*, 1983).

Crawford (1986) calculated this distance to be 2.4 cM. Bartlett *et al.* (1996) reported 4.5±0.1 cM between them. In these linkage analysis, a range of markers including some phenotypic traits, chromosomal translocations and endogenous viral element 1 (ev1) were utilized (Bitgood *et al.*, 1980, 1983, 2000; Bitgood, 1985; Bartlett *et al.*, 1996).

As little is known about precise physical locations of these markers on chromosome 1, it is therefore very difficult to locate O with these markers. In the current study, we found a novel SNP (g.67419892-67419904del13) which is significantly associated with blue egg on chicken chromosome 1. Additional linkage analyses revealed a genetic distance of 2 cM between g.67419892-

67419904del13 and O. This study markedly narrows down candidate region harboring O which may be very helpful to carry out studies of candidate genes.

### MATERIALS AND METHODS

**Animals:** In this study all hens were from a local breed protection farm in DongXiang town, JiangXi province of China. It is noteworthy that not all females produce blue egg in this population where minor females laying brown-shelled eggs are found. Now, 202 DongXiang blue-shelled chickens and 126 DongXiang brown-shelled chickens from the same population were employed in the association analyses of g.67419892-67419904del13 with blue egg.

In order to calculate genetic distance between g.67419892-67419904del13 and O, a three generation pedigree was generated by crossing five blue-shelled males (O/O) with 20 brown-shelled females (O/O, O correspondingly indicates the recessive allele causing brown egg phenotype at O locus). Genotype of the five male founders at O locus has been conformed to be dominant homozygote (O/O) by test-crossing with the White Leghorn females. Ten F<sub>1</sub> males and 46 F<sub>1</sub> females were intercrossed. Finally, 149 F<sub>2</sub> females with shell color records were used for linkage analyses.

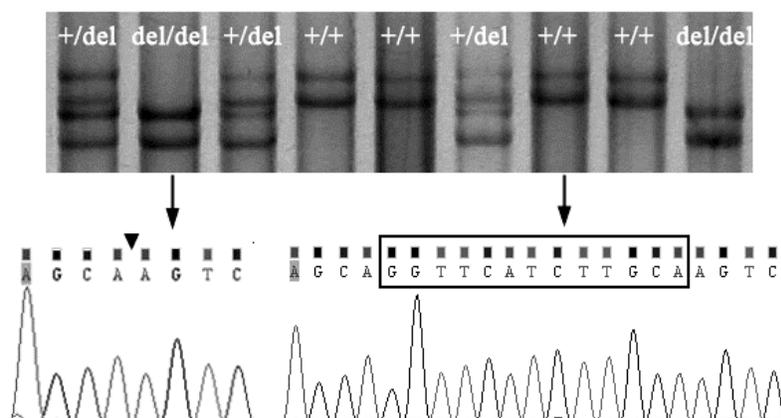


Fig. 1: PCR-SSCP assay for analyzing the g.67419892-67419904del13 locus polymorphism and sequencing results corresponding to +/+, +/del and del/del genotypes. ▼Cindicates site of deletion and open box indicates deleting sequence

**Genotyping method:** A 342 bp fragment containing g.67419892-67419904del13 was amplified by PCR from 50 ng of genomic DNA. The forward and reverse primers were 5'-ATCTATAAAGGAGCAAGG-3' and 5'-ATGAGGGTAAGAGGACAC-3'. PCR (25  $\mu$ L) was performed in 10 mM of Tris-HCl (pH 8.4), 50 mM of KCl, 3.5 mM of MgCl<sub>2</sub>, 2.5 mM of each dNTP, 20 pmol of each primer and 1 U Taq polymerase (Takara) using the following conditions: 94°C for 5 min followed by 36 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec followed by a final extension at 72°C for 7 min. PCR-based single-strand conformation polymorphism was used to test genotype of the SNP.

This method was carried out as follows. About 2  $\mu$ L of PCR product were mixed with 8  $\mu$ L of denaturing buffer consisting of 98% (v/v) formamide deionized (Amresco) and 2% (v/v) 0.5 M pH = 8 EDTA. Samples were heat denatured at 98°C for 10 min, snap-cooled in ice bath for 10 min. Finally, samples were resolved on 12% nondenaturing polyacrylamide gels at 150 V for 15 h and visualized after silver staining (Fig. 1). About 5% of results were further confirmed with sequencing at random (Fig. 1).

**Statistical analysis:** Allelic and genotypic association of g.67419892-67419904del13 with blue egg was tested using  $\chi^2$ -test.  $p < 0.05$  was considered statistic significance. The SAS v8.02 software was used for calculation. The TWO-POINT option in CRI-MAP v2.4 was used to calculate the recombination fractions between loci and corresponding LOD-scores (Green *et al.*, 1990). A LOD score value above 3 was set as a criterion for significant linkage.

## RESULTS AND DISCUSSION

Results of association analysis were shown in Table 1. We identified that + (+ is defined as normal sequence) allele was significantly associated with blue egg with 92.5% of frequency in blue-shelled chickens compared with brown-shelled chickens (10.7%,  $p < 0.0001$ ). Moreover, a genotypic association was found between +/+ genotype and blue egg (87.1 vs. 8.7% in brown-shelled chickens,  $p < 0.0001$ ). These data from association analyses implicated that there is close linkage relationship between g.67419892-67419904del13 with O locus.

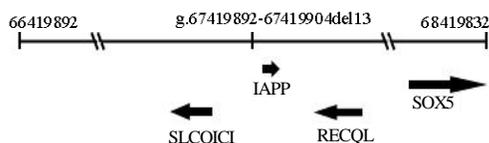
Therefore linkage analyses were further performed in a three-generation pedigree with the TWO-POINT option of CRI-MAP 2.4 software. Results showed that O was closely linked to g.67419892-67419904del13 with 0.02 of recombination fraction (LOD score = 13.51). Blue egg is colored by biliverdin which is deposited on the surface of eggshell by shell gland (Kennedy and Vevers, 1973). Physiologically, the eggshell pigment is derived from oxidative degradation of heme.

Therefore, Kennedy and Vevers (1973) believed that O is most likely to be a member of a specific enzyme system catalyzing oxidation degradation of heme which is present in bird laying blue egg but is absent from the rest. Recently, Heme Oxygenase (HO) is well established to be the rate-limiting enzyme in the catabolism of heme into biliverdin, free iron and carbon monoxide (Maines, 1997). In previous study, we detected that blue-shelled chickens indeed express more HO-1, one of HO isozymes, transcript and protein than brown-shelled chicken in shell gland.

**Table 1: Allelic and genotypic frequencies of g.67419892-67419904del13<sup>1</sup> in blue-shelled chickens and brown-shelled chickens**

Strain	Sample size	Allelic test (frequency, %)			Genotypic test (frequency, %)			
		+ <sup>2</sup> (%)	del <sup>3</sup> (%)	p-value	+/+ (%)	+/del (%)	del/del (%)	p-value
Blue-shelled chickens	202	374 (92.5)	30 (7.5)	<10 <sup>-5</sup>	176 (87.1)	22 (10.9)	4 (2.0)	<10 <sup>-5</sup>
Brown-shelled chicken	126	27 (10.7)	225 (89.3)	-	11 (8.7)	5 (4.0)	110 (87.3)	-

<sup>1</sup>The g.67419892-67419904del13 is available in dbSNP database under accession numbers ss244244378 (<http://www.ncbi.nlm.nih.gov/snp/>), 2+ and del, respectively represent normal sequence and 13 bp deletion



**Fig. 2: Genes in 2 Mb of region around g.67419892-67419904del13.** Four genes are depicted by horizontal block arrows. Gene names are given below the arrows. Arrows indicate direction of gene transcript. Gene positions are obtained from UCSC database v2.1 draft assembly (<http://genome.ucsc.edu/>). SLCO1C1, solute carrier organic anion transporter family, member 1C1; IAPP, islet amyloid polypeptide; RECQL, RecQ protein-like (DNA helicase Q1-like); SOX5, SRY (sex determining region Y)-box 5

It therefore, of great interest for us to clarify whether heme oxygenase (decycling) 1 (HMOX1, the gene encoding HO-1 protein) is the O. Here, the data from association and linkage analysis clear determined that O is in proximate to g.67419892-67419904del13. However, HMOX1 which is located on chr1:54136215-54141675 in chicken is far away g.67419892-67419904del13. Taken together we, therefore, propose the following model for explaining blue egg formation. HMOX1 do plays an important role in blue egg formation but it is not the O. It is more likely to act as a downstream gene regulated by O involving in formation of blue egg.

Based on the above knowledge, genes near g.67419892-67419904del13 should be particularly emphasized. Jacobsson *et al.* (2004) indicated that average recombination on chicken chromosome 1 is approximate 340 kb cM<sup>-1</sup> therefore, O is most like to be situated in about 640 kb region around g.67419892-67419904del13 in view of 0.02 of recombination rate between them. Here, we searched 2 Mb region of from 66419892-68419892 on chromosome 1 for potential candidate genes using UCSC database (<http://genome.ucsc.edu/>) and found that four genes are located in the region (Fig. 2).

As we know, SLCO1C1 encodes a member of the organic anion transporter family. It as a transmembrane receptor plays an important role in uptake of thyroid

hormones (Visser *et al.*, 2008). The protein encoded by RECQL is a member of the RecQ DNA helicase family.

DNA helicases are needed in maintaining genome stability and various processes of DNA metabolism including DNA replication, mismatch repair, recombination and transcription (Zhang and Xi, 2002; Wu and Brosh, 2010). SOX5 encodes a member of the SOX (SRY-related HMG-box) family of transcription factors (Lefebvre, 2010).

Its main function is involved in chondrogenesis (Smits *et al.*, 2001). Given current known functions of these genes, the precise biological relationship between them and HMOX1 is not well obvious. In contrast, Islet Amyloid Polypeptide (IAPP) also known as amylin is a 37-amino acid peptide of the calcitonin gene family (Macfarlane *et al.*, 2000). Huang *et al.* (2007) suggested that toxic oligomers of IAPP induce Endoplasmic Reticulum (ER) stress which plays a role in development of type 2 diabetes (Huang *et al.*, 2007). In another study, ER stress was found to stimulate HMOX1 gene expression (Liu *et al.*, 2005).

## CONCLUSION

Therefore, it is very necessary further to analyze association of IAPP gene polymorphisms and expression traits with blue egg. Although, shell color is not an indication of internal quality of eggs, blue eggs is preferable to brown egg and white egg in the Chinese egg market. Therefore, it is very meaningful to establish a blue-shelled population with marker aid selection. Recent research showed that blue-shelled individuals accounted for 94.1% of the 187 individuals with ++ genotype (Table 1). It is relatively reliable to identify blue-shelled chickens as soon as the birds are hatched according to ++ genotype.

## ACKNOWLEDGEMENTS

The researchers thank Xu JianSheng, Chen YunLong for providing samples. This research was fund by the National Nature Science Foundation of China (No. 31072024), National Science and Technology Pillar Program (2008BADB2B01) and the Major State Basic ResearchDevelopment Program of China (2006CB102100).

**REFERENCES**

- Bartlett, J.R., C.P. Jones and E.J. Smith 1996. Linkage analysis of endogenous viral element1, blue eggshell and pea comb loci in chickens. *J. Hered.*, 87: 67-70.
- Bitgood, J.J., 1985. Locating pea comb and blue egg in relation to the centromere of chromosome 1 in the chicken. *Poult. Sci.*, 64: 1411-1414.
- Bitgood, J.J., J.S. Otis and R.N. Shoffner, 1983. Refined linkage value for comb and blue egg: Lack of effect of pea comb, blue egg and naked on age at first egg in the domestic fowl. *Poult. Sci.*, 62: 235-238.
- Bitgood, J.J., R.N. Shoffner, J.S. Otis and W.E. Briles, 1980. Mapping of the genes for pea comb, blue egg, barring, silver and blood groups A, E, H and P in the domestic fowl. *Poult. Sci.*, 59: 1686-1693.
- Bitgood, J.J., R.W. Briles and W.E. Briles, 2000. Further tests for genetic linkages of three morphological traits, three blood groups and break points of two chromosome translocations on chromosome one in the chicken. *Poult. Sci.*, 79: 293-295.
- Bruckner, J.H. and F.B. Hutt, 1939. Linkage of pea comb and blue egg in the fowl. *Science*, 90: 88-88.
- Crawford, R.J., 1986. Linkage between pea comb and melanotic plumage loci in chickens. *Poult. Sci.*, 65: 1859-1862.
- Green, P., K. Falls and S. Crook 1990. Documentation for CRIMAP, Ver. 2.4. University School of Medicine, St Louise.
- Huang, C.J., C.Y. Lin, L. Haataja, T. Gurlo, A.E. Butler, R.A. Rizza and P.C. Butler, 2007. High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes*, 56: 2016-2027.
- Jacobsson, L., H.B. Park, P. Wahlberg, S. Jiang, P.B. Siegel and L. Andersson, 2004. Assignment of fourteen microsatellite markers to the chicken linkage map. *Poult. Sci.*, 83: 1825-1831.
- Kennedy, G.Y. and H.G. Vevers, 1973. Eggshell pigments of the Araucano fowl. *Comp. Biochem. Physiol. B*, 44: 11-25.
- Lefebvre, V., 2010. The SoxD transcription factors-Sox5, Sox6 and Sox13-are key cell fate modulators. *Int. J. Biochem. Cell Biol.*, 42: 429-432.
- Liu, X.M., K.J. Peyton, D. Ensenat, H. Wang, A.I. Schafer, J. Alam and W. Durante, 2005. Endoplasmic reticulum stress stimulates heme oxygenase-1 gene expression in vascular smooth muscle. *J. Biol. Chem.*, 280: 872-877.
- Macfarlane, W.M., S.C. Campbell, L.J. Elrick, V. Oates and G. Bermano *et al.*, 2000. Glucose regulates islet amyloid polypeptide gene transcription in a PDX1- and calcium-dependent manner. *J. Biol. Chem.*, 275: 15330-15335.
- Maines, M., 1997. The heme oxygenase system: A regulator of second messenger gases. *Ann. Rev. Pharmacol. Toxicol.*, 37: 517-554.
- Punnett, R.C., 1933. Genetic study in poultry: The blue egg. *J. Genet.*, 27: 465-470.
- Smits, P., P. Li, J. Mandel, Z. Zhang and J.M. Deng *et al.*, 2001. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev. Cell*, 1: 277-290.
- Visser, W.E., E.C. Friesema, J. Jansen and T.J. Visser, 2008. Thyroid hormone transport in and out of cells. *Trends Endocrinol. Metab.*, 19: 50-56.
- Wu, Y. and R.M. Jr. Brosh, 2010. Distinct roles of RECQ1 in the maintenance of genomic stability. *DNA Repair*, 9: 315-324.
- Zartman, D.L., 1973. Location of the pea comb gene. *Poult. Sci.*, 52: 1455-1462.
- Zhang, A.H. and X. Xi, 2002. Molecular cloning of a splicing variant of human RECQL helicase. *Biochem. Biophys. Res. Commun.*, 298: 789-792.