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The Identification of SNPs in BCDO2 Gene for Skin Color in Chinese Indigenous Chicken

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ABSTRACT

A complete linkage disequilibrium between the SNP (SNP B) in *BCDO2* gene and the *yellow skin* phenotype in European domestic chicken has been reported. Here, we genotyped the reported SNPs (SNP A, SNP B, and SNP C) of the *BCDO2* gene in 183 Chinese Indigenous chickens from 11 breeds/populations, including 57 yellow, 17 white, and 109 black skin chickens. The frequency of all three SNPs were significantly different between yellow and white skin chickens ($p<0.01$). In black skin chickens, a high frequency of the heterozygous genotype (AG) in SNP A (0.51) and SNP B (0.48) was observed. A total of three haplotypes (AAA, AGA, and GAA) from these three SNPs were obtained. Frequencies of the proposed yellow skin-associated haplotype AGA in yellow skin, white skin, and black skin chickens were 0.81, 0.35, and 0.56, respectively. The results showed that the yellow skin phenotype of the evaluated birds has not been under selection, and that the *BCDO2* gene in black skin chickens, evolutionally may undergo a transition phase from yellow to white skin chicken. We concluded that, the SNPs of *BCDO2* gene not only can be used to determine whether the chicken was subjected to selection, but may also be used as a marker when selecting for the preferred skin color in chicken breeding programs.

INTRODUCTION

The skin color of chicken is an important phenotype, and may influence consumers' decision. Chickens with the yellow skin phenotype are widely preferred in USA, Mexico, and China. It is one of the first traits in animals described to exhibit Mendelian inheritance, and it is caused by one or more cis-acting and tissue-specific regulatory mutations that inhibit expression of β-carotene dioxygenase 2 (*BCDO2*) in the skin (Eriksson *et al.,* 2008).

The *β-carotene dioxygenase 2* gene (*BCDO2*), located in chromosome 24, has been proposed as a candidate gene for the yellow skin phenotype in chickens (Schmid *et al.,* 2000), and was validated by Yang *et al.,* (2012). *BCDO2* gene encodes β-carotene dioxygenase 2, an enzyme that cleaves colorful β-carotenoids into colorless apocarotenoids by an asymmetric cleavage reaction, leading to white skin (Kiefer*et al.,* 2001). Based on the linkage mapping within families and identical-by-descent analysis across breeds with yellow skin phenotype in domestic chickens, Eriksson *et al.* (2008) reported an association of the three single nucleotide polymorphisms (SNPs: A, B, and C) around *BCDO2* gene with the yellow skin phenotype, and also that the *BCDO2* gene presented fairly strong expression in the liver and the skin. In addition to skin color in chickens, the *BCDO2* gene was also reported to play important roles in carotenoid metabolism in different farm animals, such as cattle and sheep. Berry *et al.* (2009) and Tian *et al.* (2010) showed

that the loss of function of the *BCDO2* gene increased the levels of β-carotene, resulting in the yellow color of milk and serum in cows. A nonsense mutation (c.196C>T) of the *BCDO2* gene was also found to be strongly associated with yellow fat in sheep (Våge DI & Boman IA, 2010).

In southwest China, the black skin chicken is one of the most important genetic lines. The black skin chicken (also known as the Silkie chicken) has been long used in natural medicine. Its genetic control and biology of dermal hyperpigmentation have been studied (Dorshorst *et al.,* 2010). Black skin was shown to be caused by a mutation of the *MC1R* gene (Kerje *et al.,* 2003). The *BCDO2* allele was thought to be acquired through hybridization with the Gray Jungle fowl of South Asia, which means that the domestic chicken has a hybrid origin. It was reported that the black skin trait was first found in India and then transferred to China and Japan (Roberts, 2009), which indicates that Chinese black skin Silkieis crossbred of many different genetic lines of chickens (Fu, 2002; Liu *et al.,* 2006; Zhou *et al.,* 2010). However, the SNPs of *BCDO2* gene in black skin chicken have not been identified yet. The aim of this study was to detect the three SNPs around the *BCDO2* gene in Chinese indigenous chickens, especially in the black skin chicken.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from 183 individuals, including 57 chickens with yellow skin from four breeds, 17 chickens with white skin from one breed, and 109 chickens with black skin from six breeds/ populations. All birds were older than 12 weeks of age at the time of sampling, and their skin color was optically recognized (Table 1). Blood samples were collected and stored at -80 °C freezer.

Genotyping analysis

Genomic DNA was extracted by using standard phenol/chloroform method. Three primer pairs were designed to independently amplify three fragments encompassing the three proposed SNPs (Table 2), including SNP A (g6,264,085G→A), SNP B (g6,273,428A→G), and SNP C (g6,287,900G→A) in the *BCDO2* gene (GenBank no. NC_006111.3). The primer pairs were all synthesized by Shanghai Invitrogen Biology Technique Corporation.

PCR amplification was performed in a 50 μL reaction mixture containing 100ng genomic DNA, 25μL 2×MasterMix (Beijing Tianwei Biology Technique Corporation, Beijing, China), and 2μL of each primer (10μM). PCR conditions included an initial denaturation cycle at 95°Cfor 5 min, followed by 35 cycles of 95°C for 1 min, appropriate annealing temperature (51.4°C for primer pair A, 53.3°C for primer pair B and 54.7°C for primer pair C) for 1 min, and extension at 72°C for 2 min. PCR products were detected in 1% agarose gel and were visualized on Gel TMEQ170-8060 and then photographed. PCR products were purified and sequenced by Shanghai Invitrogen Biology Technique Corporation (Shanghai, China).

Statistical analysis

Sequences were aligned by using DNASTAR package (DNASTAR, Madison, WI) with manual inspection. After the genotype and allele frequencies were directly counted, Hardy-Weinberg equilibrium (HWE) was

Table 1 – Skin color and samples evaluated in this study and allele frequencies at SNPs around the BCDO2gene with yellow skin haplotype.

Skin color	N	Breeds	Sample IDs	Location	SNP A (A)	SNP B (G)	SNP C (A)
Yellow skin	12	Fengkaixinghua	FX	Guangdong, China	1	0.72	1
	10	Huxu[1]	HX	Guangdong, China	0.94	1	1
	20	Qinyuan[1,2]	QY	Guangdong, China	1	1	1
	15	Xiayan[1]	XY	Jiangxi, China	0.89	0.73	1
Black skin	20	Caoke[1,2]	CK	Sichuan, China	0.43	0.5	0.75
	14	Junlian Black-boned	JL	Sichuan, China	0.14	0.11	1
	20	Jiuyuan Black-boned[2]	JY	Sichuan, China	0.43	0.42	1
	22	Shandi Black-boned	SD	Sichuan, China	0.36	0.39	0.98
	22	Taihe Silky[1,2]	SK	Jiangxi, China	0.30	0.29	1.00
	11	Tibetan[1]	TB	Sichuan, China	0.41	0.45	0.95
White skin	17	Chahua[1]	CH	Yunnan, China	0.77	0.35	1.00
Total	183	—	—	—			

[1] These samples had been analyzed in our former study of the phylogeny and genetic diversity of domestic chicken (Liu *et al*., 2006).

[2] These breeds were collected from the local conservation farms.

Table 2 – Primers pairs of PCR amplification for direct sequencing

Primer pairs	Sequences (5'-3')	Annealing temperature (°C)	Fragment length (bp)
SNP A (g6,264,085G→A)	GAGAAGATGCCCTGGATTA TTGAGTGGTGGTGGTGATA	51	802
SNP B (g6,273,428A→G)	TTGGGCTAAGAAGTGGGAAG CATGAGCTCGTCATGGTCAA	53	983
SNP C (g6,287,900G→A)	CTGCTCGTCATCGTTACCT TGCGGACATCCATTTACAC	55	742

analyzed by the $\chi 2$ test. Fisher's exact test (two-tailed) was performed to quantify the statistical difference. The *p* value with Bonferroni correction of < 0.05 was regarded as statistically significant. Haplotypes were constructed by using Phase 2.0.

RESULTS AND DISCUSSION

The *BCDO2* gene is an excellent candidate for explaining the variation in carotenoid-based pigmentation observed in many other vertebrate species. In this study, we identified the distribution of three SNPs (A, B, C) of the *BCDO2* gene in Chinese indigenous chickens presenting three skin colors (shown in Table 3) based on the study of Eriksson *et al*. (2008). All three SNPs were substitutions of A/G. The frequency distribution of three SNPs significantly differed among the three skin colors. The allele frequencies of the three proposed yellow skin-associated nucleotides A (SNP A), G (SNP B), and A (SNP C) were 0.96, 0.88, and 1.00 in yellow skin chickens; 0.77, 0.35, and 1.00 in white skin chickens; and 0.35, 0.37, and 0.95 in black skin chickens. For SNP A, the AA genotype (0.91) was predominant in yellow skin chickens, and was less abundant in white skin chickens (0.70) and black skin chickens (0.09). A similar genetic pattern was observed for SNP B, with the highest and lowest frequencies of the GG genotype in yellow skin chickens (0.81) and black skin chickens (0.12), respectively. For SNP C, the AA genotype presented complete penetrance in both yellow skin and white skin chickens, but in black skin chickens, it accounted for the highest frequencies

Table 3 – Genotype and allele frequencies of three SNPs of the *BCDO2* gene in Chinese indigenous chickens.

Genotypes / alleles	Yellow skin(Y, n=57)	White skin(W; n=17)	Black skin (B, n=109)	Pairwise comparison		
				Y vs. W	Y vs. B	W vs. B
SNP A						
GG	0 (0.0)	3 (0.18)	43 (0.40)	<0.01	<0.01	0.08
AG	5 (0.09)	2 (0.12)	56 (0.51)	0.71	<0.01	<0.01
AA	52 (0.91)	12 (0.70)	10 (0.09)	0.03	<0.01	<0.01
A	109 (0.96)	26 (0.77)	76 (0.35)	<0.01	<0.01	<0.01
G	5 (0.04)	8 (0.23)	142 (0.65)			
HWE[a]	0.73	0.01	0.17	—	—	—
SNP B						
AA	3 (0.05)	11 (0.65)	43 (0.40)	<0.01	<0.01	0.05
AG	8 (0.14)	0 (0.0)	52 (0.48)	0.10	<0.01	<0.01
GG	46 (0.81)	6 (0.35)	14 (0.12)	<0.01	<0.01	0.02
A	14 (0.12)	22 (0.65)	138 (0.63)	<0.01	<0.01	0.87
G	100 (0.88)	12 (0.35)	80 (0.37)			
HWE	0.07	<0.01	0.78	—	—	—
SNP C						
GG	0 (0.0)	0 (0.0)	0 (0.0)	—	—	—
AG	0 (0.0)	0 (0.0)	12 (0.11)	—	0.01	0.15
AA	57 (1.00)	17 (1.00)	97 (0.89)	—	0.01	0.15
A	57 (1.00)	34 (1.00)	206 (0.95)	—	0.07	0.16
G	0 (0.0)	0 (0.0)	12 (0.05)			
HWE	—	—	0.54	—	—	—
Haplotypes (SNP A- SNP B- SNP C)						
AAA	9 (0.19)	9 (0.53)	7 (0.06)	1.00	0.02	<0.01
AGA	38 (0.81)	6 (0.35)	60 (0.56)	<0.01	<0.01	0.12
GAA	0 (0.0)	2 (0.12)	41 (0.38)	0.02	<0.01	0.02

[a] Chi-square test values<0.05 are considered as deviated from HWE.

(0.89). The frequencies of SNP A, B and C detected in white skin chickens were not consistent with a previous study (Eriksson *et al*., 2008), but significant SNP A and SNP B differences between yellow skin and white skin chickens were detected (p<0.01). In addition, SNP A and SNP B also presented deviation from the HWE in white skin chickens (Table 3). Possible explanations for this inconsistency may be the involvement of additional genes in the biogenesis of the yellow skin in domestic chickens, or this skin color in chickens may be the result of artificial selection. However, because only one white skin breed with 17 samples was analyzed in the present study, no definitive conclusions can be made. Nevertheless, Yang *et al*. (2012) observed all 30 Chahua chickens from China presented homozygous AA in the SNP B locus, which was consistent with previous results (Eriksson *et al*., 2008), providing further support to our results.

A total of three haplotypes (AAA, AGA, and GAA, shown in Table 3 and Figure 1) was detected in all chickens. Only two haplotypes (AAA and AGA) were observed in yellow skin chickens, while all three haplotypes were detected in white and black skin chickens. The frequencies of the proposed yellow skin-associated haplotype (AGA) accounted for 0.81, 0.35 and 0.56 in yellow skin, white skin, and black skin individuals, respectively. In addition, the frequency of the haplotype AGA of the yellow skin chicken was significantly different compared with white and black skin chickens, but no statistical difference was detected between white skin and black skin chickens.

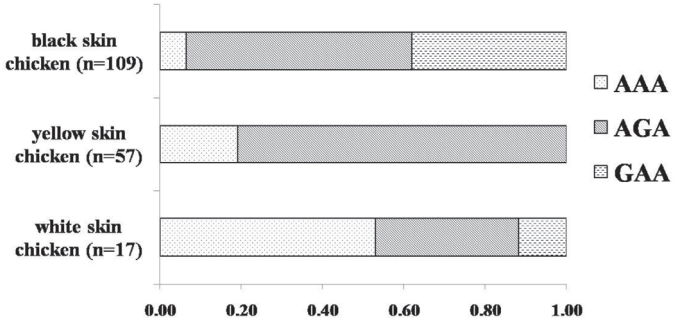

Figure 1 – Schematic illustration of the haplotype composition of the different skin color types. The axis represents the frequency of each haplotype.

Based on the sequence similarity, Eriksson *et al.* (2008) proposed the hybrid origin of the modern domestic chickens based on the fact that the yellow skin-associated haplotype was originated from the grey jungle fowl (*Gallus sonneratii*) rather than from the red jungle fowl (*Gallus gallus*), because the grey jungle fowl (*Gallus sonneratii*) has yellow skin, while the red jungle fowl has white skin (which is called Chahua chicken in China). Chicken skin color can be stably recognized until 12-14weeks of age under the same feeding conditions (Crawford, 1990). In this study, we found high frequencies of the heterozygous genotype (AG) in SNP A (0.51) and SNP B (0.48), but not in SNP C (0.11). Therefore, we hypothesize that the black skin chicken would be an intermediate type, that is, that the black skin is a transition between white and yellow, or may be a hybridization of yellow and white skin chickens. This would provide useful clues to investigate the biogenesis of black skin in these breeds/populations.

In summary, skin color, as a classic phenotypic trait, plays an important role in breed recognition and breeding selection in farm animals. We reconfirmed that the *BCDO2* gene is present in Chinese indigenous yellow skin chickens, and plays an important role in their skin color. However, its incomplete link with yellow skin also indicates a more complex role of the *BCDO2* gene underlying the biogenesis of skin color in domestic chickens.

CONCLUSION

Based on the results of this study, we speculate that there are significant associations between skin colors and genetic variants of the *BCDO2* gene in Chinese domestic chickens, and that the *BCDO2* gene may be used as a marker gene to identify skin color for chicken breeding. However, as it is a complex trait, skin color is determined by a complex pathway system and multiple interactive patterns, and therefore, further studies are warranted to confirm this conclusion.

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