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Original Article

Detection of Snps in the Melanocortin 1-Receptor (MC1R) and Its Association with Shank Color Trait in Hs Chicken

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ABSTRACT

The melanocortin 1 receptor (*MC1R*) gene plays a key role in controlling the deposition of melanin. In mammals, the *MC1R*gene is regarded as a major candidate gene in the control of melanin formation. In domestic animals, the *MC1R* gene mainly controls the expression of coat, skin, and plumage color in mammals and birds. In order to breed chickens with dark-green shank faster, we screened the molecular markers for shank color in a HS chicken population by exploring the relationship between polymorphism of the *MC1R* gene and three different shank colors (light green, dark green and yellow). Two primer pairs for code region of the *MC1R* gene were designed in the basic of chicken genomic sequence. DNA sequencing was performed to detect the polymorphisms and PCR was used to amplify DNA fragment. Sequences analysis indicated that 7 SNPs were predominant the three HS chicken populations with different shank color, including g.18,287,945C>T, g.18,288,088T>C, g.18,288,150G>A, g.18,288,303A>G, g.18,288,512G>A, g.18,288,513T>C, and g.18,288,520A>C. Association analysis revealed that the dark-green shank population showed moderate polymorphism, whereas the light-green shank population showed low polymorphism among overall 7 SNPs and that SNP6 (g.18,288,513T>C) may be significantly associated with three different shank colors in HS chickens. The haplotype CTGGACA had the largest haplotype frequencies, accounting for 56.22%, and the haplotype combination H1H1 is mainly distributed in the dark-green shank population, and may be used as molecular maker for marker-assisted selection of shank color in HS chickens.

INTRODUCTION

Animals display a wide variety of coat or skin colors, which depend on the black-brown eumelanin to yellow-reddish phaeomelanin in the skin (Wang & Hebert 2006). More than 100 genes have been fully confirmed to regulate the pigmentation in mammals (Yang *et al.,* 2008). However, the melanocortin-1 receptor (*MC1R*) gene, with its ligands melanocortins and ACTH, is the main positive regulator (Slominski *et al.*, 2004). The cytogenetic location of the *MC1R* gene is the long (q) arm of chromosome 16 at position 24.3 and mainly controls which type of melanin is produced by melanocytes (García-Borrón *et al.*, 2005). When the *MC1R* gene is activated by external factors, a series of chemical reactions are triggered inside melanocytes, stimulating the production of eumelanin (Ha *et al.*, 2003). As early as 2001, it was found that *MC1R* gene variants predispose to cutaneous melanoma (Kennedy *et al.*, 2001). In recent years, most studies on the *MC1R*gene linked to cancer showed that *MC1R* germline mutations that determine light skin color and red hair phenotypes increase the risk of melanoma (Mundra *et al.,* 2017). The study of Tagliabue *et al.* (2018) determined

that the presence of any *MC1R* gene variant was connected with the melanoma risk independently of phenotypic characteristics, indicating that measuring the *MC1R* genotype may aid melanoma prediction.

The above studies demonstrated that the *MC1R* gene polymorphism is of great importance in human melanoma. Nevertheless, in standardized domestic breeds, coloration is one of the basic phenotypic characters under artificial selection used in morphological evaluation. In mammals, pigmentation, including coat and skin color, are closely related to the levels of melanin and carotenoids. Sequence analysis already revealed that *MC1R* alleles in seven porcine breeds were required for the expression of the wild-type coat color (Kijas *et al.*, 1998). A 2-bp insertion in *MC1R* gene leads to recessive white coat pigmentation in Bama miniature pigs (Jia *et al.*, 2017). The two independent and nonsynonymous Met73Lys and Asp121Asn mutations in the *MC1R* gene are associated with black or red coat colors in Saudi indigenous sheep populations (Mahmoud *et al.*, 2017). The single nucleotide polymorphism (901C/T) found in the coding region of *MC1R* was linked to the white coat color in in the Arabian camel (Almathen *et al.*, 2018). As for birds, *MC1R* gene was firstly cloned in chickens by Takeuchi *et al.* (1996) and the authors pointed out the accurate mechanism of the *MC1R* function, which is possibly shared both by mammals and birds. The abundant polymorphism of the *MC1R* gene determined in local Chinese Hebei chicken strain was associated with their rich plumage pigmentation diversity (Guo *et al.*, 2010). Zhang *et al.* (2017) found that both the C allele of c.212T>C and the A allele of c.644A>C differentiates 39 homozygous and heterozygous individuals for breeding of pure black plumage Chinese chicken.

The HS chicken is a high-quality hybrid between the Tetra layer breed, with yellow shanks, and a local chicken breed of Sichuan, China, with dark-green shanks. This hybrid has been bred for two generation in Sichuan Agricultural University. However, shank color is still not stable, and birds have shown different shank colors, including dark green, light green, yellow and white.

Considering the preference of local consumers for chickens with dark-green shanks, it has become a top priority to quickly screen the dark-green shank population by molecular-assisted breeding. In the present study, we investigated the genetic polymorphisms of the *MC1R* gene in a HS chicken population with different shank colors. The association of the SNPs with shank color traits were then investigated to potentially provide a theoretical basis for molecular-aided breeding of HS chicken.

MATERIALS AND METHODS

Ethical Statement

This study was performed with permission of the Committee on Experimental Animal Management of Sichuan Agricultural University, permit number 2017-18, which was issued on the basis of the Regulation for the Administration Affairs Concerning Experimental Animals of the State Council of the People's Republic of China. All chicken involved in this study were sacrificed as painless as possible.

Chicken population and data collection

A total number of 180 HS chickens (male:female = 1:1), with 33.42±1.54 g initial body weight, reared in the experimental poultry breeding farm of Sichuan Agricultural University (Ya'an, China), was evaluated. All individuals were divided, according to shank color, into 60 yellow(Y), 60 light green(L), 60 dark green(D) (Figure 1). All chickens were housed on deep-litter bedding and moved to the growing house at 6 weeks of age. Birds had *ad libitum* access to feed (commercial corn-soybean diets meeting the NRC requirements) and water.

Figure 1 – The three shank colors in the HS chicken population.

DNA extraction

All of the 180 chickens were slaughtered at 90 days of age after 12-hour fasting. Blood samples were collected during bleeding. We used the method of standard phenol/chloroform to isolate the genomic DNA (Cao & Mo, 2009). The concentrations and purity of all DNA samples were assessed by a NanoVuePlus™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Based on the concentration of DNA samples determined by the machine, the appropriate amount of Tris-EDTA (TE) buffer was added to achieve a target concentration of 100 ng/μL and all DNA samples were stored at -20 °C until use (Cui *et al.*, 2017).

MC1R gene amplification and genotyping

The primers (Table 1) were designed to amplify the entire coding region based on the sequence of the *MC1R*gene (NM_001031462.1). The primers were synthesized by Shanghai Yingjun Biotechnology Co. Ltd. (Shanghai, China). Sequences were obtained from HS chicken DNA pool (10 random yellow-shank chickens, 10 random light-green shank chickens, 10 random dark-green chickens in a DNA pool).

Table 1 – *MC1R* primer sequence for SNP detection.

Primer name	Primer sequence (5′ → 3′)	Product length (bp)	Primer sites*
MC1R-1F	AAATCAGGACAGAGAAAGGG	861	130-150
MC1R-1R	TTAAGACGGTGCTGGAGA		972-990
MC1R-2F	CGCTACATCACCATCTTCTA	570	876-886
MC1R-2R	GTCCATCCATCCATCCATC		1426-1445

*Primer sites of the *MC1R* gene (D78272), 459-1403 is the coding sequence.

To amplify the DNA fragment of the *MC1R* gene, EasyCycler 96 PCR detection system was used for SNP genotyping of all individuals of the three shank colors (Analytik Jena, Germany). A PCR reaction was performed in 15 μL containing 1 μL of pooled DNA, 1.5 μL (10 pmol/μL) of each primer, 7.5 μL 2×Master mix (including Mg^{2+}, dNTPs, *Taq* DNA polymerase; Beijing TIAN WEI Biology Technique Corporation, Beijing, China). We adjusted the volume up to 15 μL by adding ultrapure water. A PCR protocol was used under the following conditions: initial denaturing at 94°C for 5 min, followed by 38 cycles of denaturing at 98°C for 40 s, annealing for 30s at 55°C, and extension at 71°C for 1min. The final extension was performed at 72°C for 5 min (Cui *et al.*, 2017). The PRC products were sequenced by Tsingke Biological Technology (Chengdu, Sichuan). Sequences were analyzed with the DNASTAR software and the CodonCode Aligner software (http: //www. codoncode.com/aligner).

Based on the sequence obtained from the DNA pool, polymorphisms were identified. Genotyping was performed using all DNA samples extracted from the blood of the 180 HS chickens. PCR was performed as described above for analyzing the mutations. Amplified products were electrophoresed and purified with a gel extraction kit (Takara, Dalian, China) according to the manufacturer's protocol, and sequenced by Shanghai Sangon Biology Technique Corporation.

Data analysis

We counted the genotypes and alleles in each SNP for genotypic and allelic frequencies. Hardy-Weinberg equilibrium was established with chi-square test at 5% significance level. The observed number (Ho) and expected allelic heterozygosity (He)were determined using POPGENE version1.31 (Pashaei *et al.*, 2009). The polymorphism information content (PIC) was established following a previously described method (Botstein *et al.*, 1980). PIC>0.5 indicates highly polymorphism, 25<PIC<0.5 indicates moderate polymorphism, and PIC<0.25 indicates low polymorphism. The PIC was calculated according to Bolstein *et al.* (1980) as:

$$PIC = 1 - \left(\sum_{i=1}^{n} P_i^2 \right) - \left(\sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2 \right)$$

Heritabilities of yellowness(Y), light greenness(L) and dark greenness(D) were estimated. The model is as follows:

$$Y_{ijk} = \mu + S_i + G_j + B_k + G_j \times S_i \times B_k + e_{ijk}$$

Where μ is the population mean, S_i is the fixed effect of sex, G_j is the fixed effect of *MC1R* polymorphism, B_k is the fixed effect of line, $G_j \times S_i \times B_k$ means the interaction among genotype, sex and line, and e is for random error. The values were presented as least square means±se. The PROC REG procedure of SAS (version 6.12, SAS Institute Inc.) was used to evaluate the statistical significance and P values were considered significant when lower than 0.05 (Lanjouw, 1992).

RESULTS

Identification of SNPs in the chicken MC1R gene

Nucleotide sequences were detected directly and we found 7 SNPs from all the individuals of the random population genotypes by utilizing Sanger-sequencing of the four amplicons, including a C/T mutation (g.18,287,945C>T), a T/C mutation (g.18,288,088T>C), a G/A mutation (g.18,288,150G>A), a A/G mutation (g.18,288,303A>G), a G/A mutation (g.18,288,512G>A), a T/C mutation (g.18,288,513T>C), a A/C mutation (g.18,288,520A>C). Table 2 shows the

Table 2 – Primer amplification polymorphism loci fragment screening.

	g.18,287,945 C>T	g.18,288,088 T>C	g.18,288,150 G>A	g.18,288,303 A>G	g.18,288,512 G>A	g.18,288,513 T>C	g.18,288,520 A>C
D*	+	+	+	+	+	+	+
Y*	+	+	+	+	+	+	+
L*	+	+	+	+	+	+	-

Note: + indicates a polymorphic locus, - indicates no polymorphic loci.

primer amplified polymorphic loci fragment screening of the *MC1R* gene among the HS chicken populations. There were sevenmutations in yellowshank individuals, sevenmutations in dark-green shank individuals, while light-green shank individuals had sixmutations.

Genetic diversity analysis of mutated sites

The genetic diversity analysis of the 7 SNPs of the *MC1R* gene in the HS chicken population was conducted and the results showed in Table 3. The data indicates that the genetic homogeneity (*Ho*) among the 7 SNPs was higher than genetic heterogeneity (*He*). The polymorphism information content (PIC) results revealed that the dark-green shank population and light green shank population showed moderate polymorphism (0.25<*PIC*<0.50) among all 7 SNPs, while the yellow shank population showed moderate polymorphism (0.25<*PIC*<0.50) ing.18,287,945C>T, g.18,288,150G>A, g.18,288,303A>G and g.18,288,520A>C, and low polymorphism (*PIC*<0.25) in g.18,288,088T>C, g.18,288,512G>A and g.18,288,513T>C.

Analysis of the correlation betweenMC1R gene SNPs and shank color

The genotype distribution of the 7 *MC1R* gene SNPs in the three different shank color chicken populations was determined. The allele and genotype frequencies were compared by chi-square test. Table 4 shows that the CC genotype was dominant compared with the CT in SNP1 and SNP6, the CC genotype among yellow shank light green shank populations, and C was the advantageous allele. As for the dark-green shank population, T was the advantageous allele in SNP1 and SNP6. In SNP2, T was the advantageous allele and TT were predominant in the yellow and light-green shank populations, while CC genotype was dominant in the dark-green shank population. The GG genotype predominated in SNP3 and SNP4 relative to AA genotype in yellow and light-green shank populations. Dark-green shank populations were opposite to other two populations and the AA genotype predominated. In SNP5, AA genotype predominated in yellow and light green shank populations compared with the dark-green shank population. The CC genotype predominated in the dark-green population while A was the advantageous allele in the other two populations in SNP7.

Least-squares analysis was performed to analyze the correlation of shank color among the three chicken populations.

According with the results, the dark-green shank and yellow shank populations were significantly

Table 3 – Allele frequencies and average polymorphism information content (PIC) of each locus.

SNPs		Frequency		*Ho*	*He*	*Ne*	*PIC*
		A	B				
g.18,287,945(C>T)	D	0.2667	0.7333	0.6089	0.3911	1.6423	0.3146
	Y	0.8500	0.1500	0.7450	0.2550	1.3423	0.2225
	L	0.8833	0.1167	0.7939	0.2061	1.2596	0.1849
g.18,288,088 (T>C)	D	0.3167	0.6833	0.5672	0.4328	1.7630	0.3391
	Y	0.8000	0.2000	0.6800	0.3200	1.4706	0.2688
	L	0.9000	0.1000	0.8200	0.1800	1.2195	0.1638
g.18,288,150 (G>A)	D	0.3333	0.6667	0.5556	0.4444	1.8000	0.3457
	Y	0.8500	0.1500	0.7450	0.2550	1.3423	0.2225
	L	0.9500	0.0500	0.9050	0.0950	1.1050	0.0905
g.18,288,303 (A>G)	D	0.7167	0.2833	0.5939	0.4061	1.6838	0.3236
	Y	0.1667	0.8333	0.7222	0.2778	1.3846	0.2392
	L	0.1000	0.9000	0.8200	0.1800	1.2195	0.1638
g.18,288,512 (G>A)	D	0.7333	0.2667	0.6089	0.3911	1.6423	0.3146
	Y	0.2667	0.7333	0.6089	0.3911	1.6423	0.3146
	L	0.1167	0.8833	0.7939	0.2061	1.2596	0.1849
g.18,288,513 (T>C)	D	0.7000	0.3000	0.5800	0.4200	1.7241	0.3318
	Y	0.2000	0.8000	0.6800	0.3200	1.4706	0.2688
	L	0.0333	0.9667	0.9356	0.0644	1.0689	0.0623
g.18,288,520 (A>C)	D	0.3167	0.6833	0.5672	0.4328	1.7630	0.3391
	Y	0.9167	0.0833	0.8472	0.1528	1.1803	0.1411
	L	1.0000	0.0000	1.0000	0.0000	1.0000	0

Note: PIC>0.5 is considered as a high level of polymorphism, 0.25<PIC<0.5 is considered a medium level of polymorphism, while PIC<0.25 is considered as a low level of polymorphism.

Table 4 – The relations of*MC1R*genotype distributions with shank color among chickens with different shank color.

SNPs	genotype	D	Y	L	P		
					D vs.Y	D vs.L	Y vs.L
g.18,287,945 (C>T) SNP1	CC	4 (0.1333)	22 (0.7333)	24 (0.8000)	0.0004	0.0002	0.7681
	CT	8 (0.2667)	7 (0.2333)	5 (0.1667)	0.7963	0.4054	0.5637
	TT	18 (0.6000)	1 (0.0333)	1 (0.0333)	9.617E-06	0.3173	1
	C	16 (0.2667)	51 (0.8500)	53 (0.8833)	9.476E-07	2.784E-07	0.8713
	T	44 (0.7333)	9 (0.1500)	7 (0.1167)			
g.18,288,088 (T>C) SNP2	TT	5 (0.1667)	19 (0.6333)	24 (0.8000)	0.0043	0.0004	0.4458
	CT	9 (0.3000)	10 (0.3333)	6 (0.2000)	0.8185	0.4386	0.3173
	CC	16 (0.5333)	1 (0.0333)	0	0.0003	6.334E-05	0.3173
	T	19 (0.3167)	48 (0.8000)	54 (0.9000)	2.194E-05	4.923E-07	0.2516
	C	41 (0.6833)	12 (0.2000)	6 (0.1000)			
g.18,288,150 (G>A) SNP3	GG	8 (0.2667)	22 (0.7333)	27 (0.9000)	0.0106	0.0013	0.4751
	AG	4 (0.1333)	7 (0.2333)	3 (0.1000)	0.3657	0.7055	0.2059
	AA	18 (0.6000)	1 (0.0333)	0	9.617E-06	2.209E-05	0.3173
	G	20 (0.3333)	51 (0.8500)	57 (0.9500)	1.261E-05	1.701E-08	0.1806
	A	40 (0.6667)	9 (0.1500)	3 (0.0500)			
g.18,288,303 (A>G) SNP4	AA	18 (0.6000)	1 (0.0333)	0	9.617E-05	2.209E-05	0.3173
	AG	7 (0.2333)	8 (0.2667)	6 (0.2000)	0.7963	0.7815	0.5930
	GG	5 (0.1667)	21 (0.7000)	24 (0.8000)	0.0017	0.0004	0.6547
	A	43 (0.7167)	10 (0.1667)	6 (0.1000)	3.504E-06	2.353E-07	0.5520
	G	17 (0.2833)	50 (0.8333)	54 (0.9000)			
g.18,288,512 (G>A) SNP5	GG	18 (0.6000)	1 (0.0333)	0	9.617-06	2.209E-05	0.3173
	AG	8 (0.2667)	14 (0.4667)	7 (0.2333)	0.2008	0.7963	0.1266
	AA	4 (0.1333)	15 (0.5000)	23 (0.7667)	0.0116	0.0003	0.1944
	A	16 (0.2667)	44 (0.7333)	53 (0.8833)	9.096E-06	1.491E07	0.0596
	G	44 (0.7333)	16 (0.2667)	7 (0.1167)			
g.18,288,513 (T>C) SNP6	TT	18 (0.6000)	1 (0.0333)	0	9.617E-07	2.209E-05	0.3173
	CT	6 (0.2000)	10 (0.3333)	2 (0.0667)	0.3173	0.1573	0.0209
	CC	6 (0.2000)	19 (0.6333)	28 (0.9333)	0.0093	0.0002	0.1893
	T	42 (0.7000)	12 (0.2000)	2 (0.0333)	1.028E-05	7.6E-10	0.0103
	C	18 (0.3000)	48 (0.8000)	58 (0.9667)			
g.18,288,520 (A>C) SNP7	AA	7 (0.2333)	26 (0.8667)	30 (1)	0.0009	0.0002	0.5930
	AC	5 (0.1667)	3 (0.1000)	0	0.4795	0.0254	0.0833
	CC	18 (0.6000)	1 (0.0333)	0	9.617E-06	2.209E-05	0.3173
	A	19 (0.3167)	55 (0.9167)	60 (1)	1.853E-07	1.741E-10	0.1124
	C	41 (0.6833)	5 (0.0833)	0			

Note:D indicates dark-green shank chicken populations, Y indicates yellow shank chicken populations, and L indicates light-green shank chicken populations.

different (p<0.01) in all SNPs. Moreover, the differences between dark-green shank and light-green shank populations were also significantly different (p<0.01) in all SNPs. NoSNP differences were detected between the yellow shank and the light-green shank populations (p>0.05), except for SNP6, which was significantly different (p<0.05) between the yellow shank and the light-green shank populations. This result indicates that SNP6 may be significantly associated with the different shank colors in HS chickens.

The Hardy-Weinberg Equilibrium

Using the Phase 2.0 software package, haplotype typing was performed on seven SNPs of all individuals. In total, 35 haplotypes were obtained, defined as H1-H35 (Table 5). The haplotypes H1 and H35 had the largest frequencies, accounting for 21.10% and 56.22%, respectively, butthe other haplotypes corresponded to less than 5%.

Table 6 shows the haplotype groups for each individual obtained by Phase 2.0, with 24 haplotype combinations: H1H1, H1H20, H2H20, H3H20, H4H4, H5H20, H6H7, H6H20, H7H7, H7H12, H7H15, H7H20, H8H8, H9H20, H10H20, H11H20, H12H20, H13H20, H14H20, H16H17, 17H17, H18H20, H19H20, H20H20. Among these combinations, H20H20 had the largest proportion in the population and was mainly found in the yellow and the light green shank populations, followed by H1H1, mainly distributed in dark-green shank population. Nevertheless, only H5H20 was present in all three different shank colors.

Table 5 – The statistics analysis of haplotype in chickens.

Haplotype	Sequence	Frequency	Haplotype	Sequence	Frequency
H1	TCAAGTC	0.210997	H19	CCGAGTA	0.005055
H2	TCAAGTA	0.010149	H20	CCGAGCA	0.004794
H3	TCAAGCA	0.022054	H21	CCGAACA	0.000111
H4	TCAAACA	0.000351	H22	CCGGGTA	0.006822
H5	TCAGGTC	0.022278	H23	CCGGGCA	0.000166
H6	TCGAGTA	0.014782	H24	CCGGACA	0.009333
H7	TCGAGCA	0.010495	H25	CTAGGTC	0.005554
H8	TCGAACA	0.000222	H26	CTAGACA	0.000113
H9	TCGGGTA	0.002499	H27	CTGAGTA	0.003730
H10	TCGGGCA	0.000332	H28	CTGAGCC	0.003966
H11	TCGGACA	0.001057	H29	CTGAGCA	0.002414
H12	TTGAGTA	0.002172	H30	CTGAACC	0.001590
H13	TTGAACA	0.000389	H31	CTGAACA	0.017114
H14	TTGGGTA	0.000483	H32	CTGGGTA	0.009195
H15	TTGGACA	0.035072	H33	CTGGGCA	0.017001
H16	CCAAGTC	0.011168	H34	CTGGATA	0.000112
H17	CCAAGTA	0.006112	H35	CTGGACA	0.562203
H18	CCAAACA	0.000110			

Table 6 – The disposition grouping among haplotype combinations in chickens with different shank skin color.

Block	Shank color			Block	Shank color		
	D	Y	L		D	Y	L
H1H1	15	1	0	H8H8	1	0	0
H1H20	4	2	0	H9H20	0	1	0
H2H20	0	1	1	H10H20	0	1	0
H3H20	0	2	2	H11H20	0	0	1
H4H4	2	0	0	H12H20	0	1	0
H5H20	1	1	1	H13H20	1	0	0
H6H7	0	0	1	H14H20	0	1	0
H6H20	1	0	0	H16H17	1	0	0
H7H7	1	0	0	H17H17	1	0	0
H7H12	0	1	0	H18H20	0	1	0
H7H15	1	0	0	H19H20	0	2	1
H7H20	1	0	1	H20H20	0	15	22

DISCUSSION

Shank color is the very important phenotypic quantitative trait in Chinese indigenous chicken breeding, as it directly determines the competitiveness of new chicken species in the consumer market. However, shank color may be regulated by a major gene and several minor genes, and it is difficult to be rapidly selected using traditional phenotypic value selection (Yin *et al.,* 2011). Nowadays, with the fast development of molecular breeding technology, the candidate gene approach is a powerful and cost-effective method to find the quantitative trait loci (QTL) for accelerating the selection process (Yin *et al.,* 2012).

The difference in skin pigmentation, such as shank color, is due to the different types and the levels of melanin and carotenoids (Smyth, 1990). The *MC1R* gene has been extensively studied in human melanoma. Research show that the risk ofmelanoma which associated with *MC1R* gene is ascribe to the increased risk of developing melanomas with BRAF mutations (Landi *et al.,* 2006). However, it has been repeatedly shown that the *MC1R* gene regulates skin color in various mammals, as well as plumage and skin color in birds (Kerje, *et al.*, 2003; Klungland & Våge 2003; Lin & Fisher 2007). Therefore, we investigated the genetic association of the *MC1R* gene polymorphism with the skin pigmentation, and in particular, with shank color, to determine if it may be used as a selection tool in HS chickens.

Selection and foreign blood were imported in the HS chicken, accounting for the rate of recombination and we sequenced the different variant PCR products

in this study. Six SNPs were the main mutations in the *MC1R* gene and present in the overall evaluated HS chickenpopulation, and the light-green shank population lacked the loci g.18,288,520A>C. We then analyzed genetic diversity. The index to estimate the level of gene mutation, PIC, is ideal for detecting allele polymorphism, and can be classified as highly informative (PIC>0.5), medium polymorphic (0.5>PIC>0.25), and slight variation (PIC<0.25) (Yang *et al.*, 2015). In the present study, the PIC of overall SNPs was considered medium polymorphic in the dark-green shank and light-green shank populations, whereasa low level of polymorphism of SNP5 and SNP6 was detected in the yellow shank population. Higher PIC indicates higher heterozygosis within animal populations and results in more genetic variation, may benefit the improvement of relevant traits. A previous report indicated the site T69C of the *MC1R* gene showed a high-level polymorphism and was significantly associated with plumage color in Chinese domestic chickens (Yang *et al.,* 2008). However, none of the SNPs detected in the present study showed high polymorphism level in the HS chicken population, which is consistent with Xi *et al.* (2012), who did not find high polymorphism level in any SNP in apopulation of Chinese yakow (Bos grunnien × Bos taurus) of 84 individuals. Therefore, we speculate that the reason for this result is that the evaluated population was too small.

Many research findings demonstrated that the SNPs of the *MC1R* gene are mainly involved in plumage color variations in birds (Baião *et al.*, 2007; Nenzhu*et al.*, 2009; Hoque *et al.*, 2013). It was also shown that the *MC1R* gene is highly correlated with shank color of chickens, such as the studythat reported that two investigated SNPs of the *MC1R* gene were significantly associated with the yellow color of the shanks of Korean native chickens (Jin *et al.*, 2014). In our study, highly significant differences among all SNPs in shank color were detected between dark-green and yellow shank populations, as well as between dark-green and light-green shank populations (p<0.01). Although no differences were detected between the yellow and the light-green shank populations (p>0.05), interestingly, a higher number of individuals in the dark-green shank populations presented the mutant genotype in SNP1, SNP2, SNP3, SNP7, but the reverse was found in other two populations. Therefore, we speculate that SNP1, SNP2, SNP3, and SNP7 are closely associated with the dark-green shank trait. As for SNP6, the difference in shank color between any two chicken populations was significant, which indicates that SNP6 is closely linked with the three shank colors. These results show that SNPs detected in *MC1R* are relevant for shank color inHS chickens, although their specific regulation still needs to be determined.

In general, phenotypic traits are controlled by the interaction of multiple loci, especially in a haplotype block, which result in the interaction among a set of mutations in thespecific chromosome regions, therefore, the correlation of the multiple loci in linkage disequilibrium (LD) and phenotypic traits is more effective than a single locus analysis (Liu *et al.,* 2015). Previous research carried outonhaplotype analysis of the *MC1R* gene in *Canidae* found the highest numbers of missense polymorphisms in the dog and red fox (Nowacka *et al.*, 2013). In addition, *MC1R* gene haplotypes of plumage color in Nageswari ducks were reconstructed and the haplotype AAGC showed the highest frequency (Sultana *et al.,* 2017). The 35 haplotypes of *MC1R* gene found in the three HS chicken populations in the present study in agreement with those studies. The frequency of the CTGGACA genotype was the highest, accounting for half of all haplotype frequencies found, indicating it plays a major role in HS chicken shank color.

Linkage disequilibrium in a population is affected by many factors, including mutation and recombination rates, population stratification, artificial selection, genetic drift, etc., and in addition, some amorphs can also be combined with QTL, causing linkage disequilibrium and leading to false positive results (Yin *et al*, 2012). No linkage between loci were detected in the present study, and the results showed that 2^7 haplotypes should be generated from 7 SNPs, whereas only 35 haplotypes were detected in 180 samples, indicating that three loci were in tight linkage disequilibrium. Then the 24 groups among haplotypes were obtained. The combinations of H12H20, H13H20, H14H20, H16H17, 17H17, and H18H20 were not distributed in light green shank population and we inferred that it may be caused by the small numbers of sample selected or that these combinations do not exist at all, which indicates the need to increase sample size in order to study and discuss further these results.

In conclusion, the present study showed that the haplotype combination H1H1 may serve as molecular maker for the selection of shank color in HS chickens. Furthermore, the molecular maker should be validated in a larger population before the *MC1R* gene could be used for commercial selection.

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