Expression analysis and single-nucleotide polymorphisms of *MLPH* and *PMEL17* genes

associated with melanin deposition in Xuefeng black-bone chickens

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Abstract: Melanin deposition related genes such as *MLPH* and *PMEL17* play an important role in black-bone chicken. This study was aimed to identify and associate SNPs in the *MLPH* and *PMEL17* genes with melanin content of pectoral muscle (MCPM) in Xuefeng black-bone chicken. A total of 120 Xuefeng black-bone chickens at 120-day-old were randomly selected to measure blackness of pectoral muscle (BPM), according to the degree of BPM selected 22 high blackness (HB) and 22 low blackness (LB) chickens to determine the MCPM, and extract DNA and mRNA. The results indicated that the MCPM in the HB group was higher than in the LB group ($P < 0.01$), and the L value in the HB group was lower than in the LB group ($P < 0.01$). And we measured the mRNA expression levels of *MLPH* and *PMEL17* genes in pectoral muscle by quantitative real-time PCR. The results revealed that the mRNA expression levels of *MLPH* gene ($P < 0.05$) and *PMEL17* gene ($P < 0.01$) in the HB group was higher than in the LB group, and the mRNA relative expression level of *MLPH* and *PMEL17* genes with MCPM was positive correlation ($P <$

0.01). And the sequencing results found that a total of 17 SNPs were found in *MLPH* gene, the C-1411T was associated with MCPM ($P < 0.05$), there was no difference in MCPM among other locus ($P > 0.05$). And there were 10 SNPs in *PMEL17* gene, the G-1843C, C-2812T, and G-2794A were associated with MCPM ($P < 0.05$), there was no difference in the MCPM among other locus ($P > 0.05$). These SNPs could be molecular markers for breeding selection of blackness traits.

Keywords; SNPs, *MLPH*, *PMEL17*, melanin deposition, Xuefeng black-bone chicken

Blackness is an important economic trait of black-bone chicken. Consumers generally judge the quality of black-bone chicken based on blackness. The blackness darker, the chicken better. Therefore, in order to satisfy requirement of consumers, blackness trait has always been an important selection index in the breeding of black-bone chicken. However, the blackness depends on the melanin which is synthesized by melanocytes and deposited in nearby tissues. It has been revealed that melanin could scavenge free radicals (Bustamante *et al.* 1993), improve antioxidant capacity (Blarzino *et al.* 1999), provide photoprotection (Premi *et al.* 2015), and antibacterial (Nosanchuk & Casadevall 2003).

Melanin deposition is a complex process which is regulated by multiple genes, and the formation and transportation of melanosomes could affect melanin deposition, since melanosomes are the places where melanin is synthesized and stored (Lv *et al.* 2020). Melanophilin (*MLPH*) is a Rab27a and myosin Va binding protein that regulates melanosomes transport in melanocytes, which could accumulate melanosomes in melanocyte dendritic endings to affect melanosomes transfer from melanocytes to adjacent epidermal keratinocytes (Hume *et al.* 2007; Uyen *et al.*

2008). At present, the lightening of coat color caused by *MLPH* gene mutations has been discovered in animals, like rabbits (Lehner *et al.* 2013), quails (Bed'hom *et al.* 2012), and chickens (Vaez *et al.* 2008). It has also been reported that *PMEL17* gene is involved in melanosomes formation and transport in humans (Singh *et al.* 2008). Melanosomes development involves four steps. *PMEL17*, serves as the structural basis of fibrils in stage II, is mainly through proteolytic cleavage and forms amyloid fiber structure to maintain homeostasis within melanocytes, thereby in order to facilitate the synthesis and deposition of melanin in stage III and IV (Raposo *et al.* 2001; Slominski *et al.* 2004; Hellström *et al.* 2011). There have been a number of studies observed that *PMEL17* mutations could cause the change of coat or skin color in animals, such as mouse (Kwon *et al.* 1995), horse (Brunberg *et al.* 2006), cattle (Mészáros *et al.* 2015), and chicken (Kerje *et al.* 2004).

Xuefeng black-bone chicken, a valuable race native breed in the Hunan Province of China, is a dual-purpose chicken bred for its meat and eggs, which is loved by people due to its high nutritional value. At present, the blackness of Xuefeng black-bone chicken varies greatly among individuals. The conventional breeding methods are slow and cost, genetic marker could effectively speed up the breeding selection process of blackness traits. Therefore, the objective of this study was to screen SNP (single nucleotide polymorphism) related to melanin deposition and provide a theoretical basis for the breeding of black-bone chickens. We selected *MLPH* and *PMEL17* genes as candidate genes for this experiment, analyzed its mRNA expression level in different blackness chickens, and evaluated the relationship between the SNP in candidate gene and melanin deposition.

1. Materials and Methods

All the birds and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Hunan Agricultural University, Hunan, China.

1.1 Chickens and sample collection

In this study, the Xuefeng black-bone chicken was obtained from Hunan Yunfeifeng Agricultural Commercial Company (Hunan, China). A total of 120 Xuefeng black-bone chickens at 120-day-old were randomly selected to measure blackness of pectoral muscle (BPM). According to the degree of BPM selected 22 high blackness (HB) and 22 low blackness (LB) chickens, and slaughtered (half male and female) them to get the pectoralis major tissue on the same side, meanwhile, put samples quickly in cryogenic vials with RNase-free equipment and then stored in liquid nitrogen. Within 24 h, the samples were transferred to -80°C storage for subsequent determination.

1.2 Determination of blackness and melanin content in pectoral muscle

The BPM of Xuefeng black-bone chicken (measured twice for each chicken) was measured with colorimeter (Hunter Associates Laboratory, Inc. Virginia, USA), which performs white board calibrated before the measurement. The measurement scale is set to L*a*b mode, and the L value represents the brightness of the sample, which range is 0 to 100. Determination of melanin content reference to Ozeki (Ozeki *et al.* 1995).

1.3 DNA extraction, RNA isolation and cDNA preparation

Genomic DNA was extracted by the Phenol/chloroform method (Sambrook et al. 1989), and total RNA was isolated from pectoral muscles sample using Trizol reagent (Invitrogen Life Technologies, Carlsbad, USA). The cDNA samples were prepared from 1 µL total RNA per reaction using a reverse transcription kit (Takara Biotechnology, Tokyo, Japan) and stored at —

20°C for qRT-PCR assays. The concentration and purity of genomic DNA and total RNA were determined by NanoDrop ND-2000 UV spectrophotometer (Thermo Fisher Scientific, Inc. Massachusetts, USA).

1.4 Polymerase chain reaction (PCR) primers and amplification

The primer pairs for the amplification of *MLPH*, *PEML17* genes, and *GAPDH* gene (served as an endogenous reference gene) were used as listed in Table 1, Full-length sequences of *MLPH* and *PEML17* genes, 1000 bp upstream sequences and 1000 bp downstream sequences were obtained from GenBank. PCR primers were designed by Primer Premier 5.0 software, the primers were synthesized by Tsingke Biotechnology Co., Ltd (Beijing, China). The detailed information for PCR of *MLPH* and *PEML17* genes has been presented in Table 2, respectively.

Using the SYBR Premix Ex Taq™ II kit (Takara Biotechnology, Tokyo, Japan) to perform the quantitative real-time PCR assay with CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). The RT-PCR amplification was carried out in 25 µL reaction volume, containing 2.0 µL cDNA, 1 µL of each primer, 12.5 µL of SYBR Premix Ex Taq™ (2×) and 8.5 µL double-distilled H₂O. And the PCR amplification of *MLPH* and *PEML17* genes was carried out in 20 µL reaction volume, containing 1.0 µL DNA, 0.8 µL of each primer, 12 µL of 2×Master mix and 5.4 µL double-distilled H₂O.

1.4 Screening of SNPs in *MLPH* and *PEML17* genes

The PCR products included all exon and some nearby intron sequences in *MLPH* and *PEML17* genes, and sent it to Tsingke Biotechnology Co., Ltd (Beijing, China) for sequencing. The sequencing results were analyzed by Chromas and DNASTar software to screen out each SNPs site, and then sequenced a single sample and typed.

2. Statistical Analysis

The relative quantitative results were calculated using the $2^{-\Delta\Delta C_t}$ method after normalization against the reference gene *GAPDH*. The correlation analysis between the melanin content and the expression level of mRNA was performed by pearson correlation and significant differences among the melanin content of different genotype were analyzed using one-way ANOVA with duncans multiple comparison test, above all using SAS 9.2 version 21.0 statistical software (version 9.2, SAS Institute Inc., Cary, USA). All data are presented as mean $\pm$ standard error, considered statistically significant at *P*-Value less than 0.05 ($P < 0.05$).

Genotype frequencies and allelic frequencies were determined by direct counting, genotype frequency of SNPs was measured through chi-square test by Excel 2016, and checked it if consistent with the Hardy-Weinberg equilibrium. The least squares analysis was performed on the MCPM and carried out Tukey's test, and significant level was set to be 0.05, the model used in the study was: $Y = \mu + G + e$

Where: Y was the phenotype value of melanin, μ is the overall mean, G was the effect of genotype, and e was the effect of random residual errors.

3. Result

3.1 Correlation analysis of BPM and MCPM.

Fig 1 showed a good linear relationship which is a melanin standard curve drawn by the absorbance of different concentrations of melanin standard solutions at 500 nm. According to Fig 2, the MCPM in HB group was higher than in LB group ($P < 0.01$), and the L value in HB group was lower than in LB group ($P < 0.01$).

3.2 Correlation analysis of the mRNA expression level of *MLPH*, *PMEL17* gene and MCPM.

As showed in Fig 3-5, the mRNA relative expression level of *MLPH* ($P < 0.05$) and *PMEL17* ($P < 0.01$) in the HB group was higher than in the LB group. Through the correlation analysis found that the mRNA relative expression level of *MLPH* and *PMEL17* with MCPM was a positive correlation ($P < 0.01$). The results indicated that *MLPH* and *PMEL17* genes were closely related to MCPM of Xuefeng black-bone chicken.

3.3 Association of genetic variants of *MLPH*, *PMEL17* gene with MCPM.

As presented in Fig 6-7, a total of 17 SNPs were found in the *MLPH* gene, 3 of which are in exon, and there were 10 SNPs in the *PMEL17* gene, 8 of which were in exon. All the SNPs in exon and their effects were summarized in Table 3. Allelic and genotypic distribution of the *MLPH* and *PMEL17* genes and MCPM in different genotypic were listed in Table 4-5, respectively. In the *MLPH* gene, the G-1414A were not in Hardy-Weinberg equilibrium ($P < 0.05$), and others were in Hardy-Weinberg equilibrium ($P > 0.05$). Meanwhile, the analysis revealed that the C-1411T was associated with MCPM ($P < 0.05$), and MCPM with genotype CC was higher than CT ($P < 0.05$). There was no difference in MCPM among other locus ($P > 0.05$). In the *PMEL17* gene, the G-2794A and G-3478A locus were not in Hardy-Weinberg equilibrium ($P < 0.05$), and others were in Hardy-Weinberg equilibrium ($P > 0.05$). And the analysis revealed that the G-1843C, C-2812T, and G-2794A locus were associated with MCPM ($P < 0.05$), there was no difference in MCPM among other locus ($P > 0.05$). In the G-1843C, MCPM of genotype GG was higher than GC and CC ($P < 0.01$), MCPM with genotype CC was higher than CT ($P < 0.05$) in the C-2812T, and MCPM with genotype AA was higher than GA and GG in the G-2794A ($P < 0.05$).

4. Discussion

The methods of determining melanin content include ultraviolet spectrophotometry and liquid chromatography, in this experiment, we chose the former. The result showed that the MCPM in the HB group was extremely significant higher than in the LB group and the L value in the HB group was extremely significantly lower than in the LB group. Hu et al and Du et al detected the melanin content at absorbance of A500, (Hu *et al.* 2019) reported that the melanin content of black Rex rabbit in skin was significantly higher than beaver, protein cyan, protein yellow, and white Rex rabbits. And (Du *et al.* 2017) found that the wild-type raccoon dog's melanin content of guard hair and fluff was significantly higher than white-type. (Muroya *et al.* 2000) also demonstrated that melanin deposition was different in silky fowl different tissue, and melanin content of pectoralis in silky fowl was higher than white leghorn chicken. It reveals that melanin content could affect the visible blackness, which was similar to our results.

Melanin deposition is regulated by multiple genes which related more than 125 genes have been identified to date (Bennett & Lamoreux 2003). In this study, we chose two genes that affect the maturation and transport of melanosomes were selected as target genes for research. As a key structural protein of melanosome, *PMEL17* gene is mainly involved in the formation and transport of melanosomes (Singh *et al.* 2008; Sitaram & Marks 2012), while *MLPH* gene affects the transport of melanosomes to adjacent cells (Hume *et al.* 2007). The results indicated that the mRNA expression level of *MLPH* and *PMEL17* genes in the HB group was significantly higher than in the LB group. We speculate that *MLPH* and *PMEL17* genes may correlation with MCPM. Therefore, we have done further correlation analysis. The results indicated that the mRNA expression level of *MLPH* and *PMEL17* genes with MCPM was a significant positive correlation. There are a few reports on the mRNA expression of these two genes and their correlation with

MCPM in black-bone chickens at present. and so as other animals. However, similar studies have shown that melanin-related genes are highly expressed in black phenotype, such as MITF (Lin *et al.* 2019), TYR (Anello *et al.* 2019), and MC1R (Li *et al.* 2019) genes. This suggested that *MLPH* and *PMEL17* genes are related to melanin deposition. However, the specific mechanism needs to be further studied.

SNPs, as molecular markers, have unique advantages in animals genetic breeding, and a single SNP could be directly used to research the association with related traits (Vandeputte *et al.* 2007; Ren *et al.* 2020). Missense mutation in exon could lead to amino acid sequences changes, which affect the structure and function of proteins, thereby causing phenotype differences between individuals (Cai *et al.* 2019; Bai *et al.* 2021). Meanwhile, the intron is involved in the splicing process of genes, which changes the coding of the amino acid through influencing the splicing efficiency or accuracy of gene translation, thereby altering the phenotype of individuals (Mayo 2008; Guo *et al.* 2016). This indicated that phenotype may be affected by multiple mutation locus at the same time. Research on Tianfu black rabbit and Sichuan white rabbit revealed that polymorphism distribution of three SNPs (c.693C < G, c.851A < G, c.911G < A) in the *MLPH* gene were significantly different (Jia *et al.* 2021). and Xu *et al.* (Xu *et al.* 2016) tested the relationship between polymorphism of *MLPH* gene and the dilution phenotype to demonstrate that a mutation (C.1909A > G) in the *MLPH* gene causing the development of five-gray phenotype in the anyi tile-like gray chicken. There also have the same reported in dogs (Welle *et al.* 2009; Santos *et al.* 2017; Dudášová *et al.* 2021). In this paper, the results revealed that a total of 17 SNPs were found in *MLPH* gene, and 3 of which are in exon, and the C-1411T in intron had significant association with MCPM, which MCPM in genotype CC was significantly higher than CT.

Genotype TT was not detected, maybe it was eliminated in the artificial selection process and the C allele was more conducive to the melanin phenotype. And there were 10 SNPs in *PMEL17* gene, 8 of which were in exon. And the analysis revealed that the G-1843C, C-2812T, and G-2794A had significant association with MCPM. Previous studies have demonstrated that three different types of *PMEL17* gene mutations associated with plumage color in chickens (Kerje *et al.* 2004; Alhateemi *et al.* 2018). And it has also been reported in other animals that *PMEL17* gene mutation was related to phenotypic color changes (Brunberg *et al.* 2006; Hellström *et al.* 2011; Mészáros *et al.* 2015). In this study, one intron mutation (G-1843C) and two sense mutations (C-2812T and G-2794A) were found in *PMEL17* gene, and nonsense mutation in this gene can also cause phenotypic changes (Ishishita *et al.* 2018). This indicated that any mutation may change the phenotype. Meanwhile, three SNPs were in the Hardy-Weinberg equilibrium in the population, which indicated this SNPs have become stable in the long-term evolution and selection, and those SNPs could be molecular markers for the breeding of Xuefeng black-bone chicken.

5. Conclusion

In conclusion, the results indicate that *MLPH* and *PMEL17* genes are associated with melanin deposition in the pectoral muscle of Xuefeng black-boned chicken. And one SNP in *MLPH* gene and three SNPs in *PMEL17* gene were discovered, which could be as molecular markers for the melanin deposition in pectoral muscle. This study provides a theoretical basis for the breeding of Xuefeng black-boned chicken, and has enriched the information on breed protection and selection for local varieties.

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Table 1 RT-PCR¹ primer sequences

Gene ²	Primer Sequence (5'→3')	Fragment Size (bp)	Tm (°C)
<i>MLPH</i>	F:GAATGCCTCTGGTTGTCACCTAG	185	60
	R:TCTTGCTGCTTTCCTGGTCT		
<i>PEML17</i>	F:GGGGGGACTGGGATATGGGA	177	60
	R:GCAGCAAACGCAGGGTAGCA		
<i>GAPDH</i>	F:AGAAGGCTGGGGCTCATCT	195	60
	R:CAATGCCAAAGTTGTCATG		

¹RT-PCR: real time polymerase chain reaction

²F: forward primer, R: reverse primer, bp: base pairs.

Table 2 PCR¹ primer sequences of *MLPH* and *PMEL17* genes

Gene ²	Primer Sets	Primer Sequence(5'→3')	Fragment Size(bp)	Tm(°C)
<i>MLPH</i>	1	F:TCAATGCTTCCTTTACCCAG	598	57.6
		R:ATCCCTTCCAACCTAAGACATCC		
	2	F:GCAGTTTTACCCCGTGATAGG	1274	57.3
		R:TGTGTGTGCCAGACAGAGACG		
	3	F:ACAGAACCTGCCGTAGCCAT	1158	56.5
		R:GCATGCTGCACTGTACCTCCTA		
	4	F:AACTGCTTTGCCCCAACTG	1213	56.4
		R:CTCCCTTCACATCCTCATCG		
	5	F:CTGGTTGCTGCCTGTCTT	1166	55.7
		R:TTTCCTCTGTGGCTGTGC		
	6	F:AGGAAGTGAGGGGATTGC	1867	55.5
		R:GGTTCTGGACCCGATGTT		
<i>PMEL17</i>	1	F: TGTGAGCGGGGAGCAGTGGC	580	63.2
		R: GATCCCTCCTTGACTGCGGACTC		
	2	F: ATCGCATCCCCGTCCCTTATC	1718	62.6
		R: GGACCCAACCATCCCCTTGC		
	3	F: GGATGGTTGGGTCCCTCCGTA	616	61.4
		R: CCCTCGCATGTCACCGTCAG		
	4	F: GCTGGAGCCGGGCAGGAACT	647	63.2

		R: GCAGCAAACGCAGGGTAGCA		
	5	F: GGGGAGACACGGGGAGAACA	673	62.1
		R: AACCAGGCACCCATGCTGATG		

¹PCR: polymerase chain reaction

²F: forward primer, R: reverse primer, bp: base pairs.

Table 3 Summary of SNPs identified in exon of *MLPH* and *PMEL17* genes

Genes	SNPs	Type of variant	Change in amino acid
<i>MLPH</i>	G-1351A	synonymous variant	Glutamine
	C-5745T	Missense variant	Arginine to Cystine
	T-15357C		Leucine to Serine
<i>PMEL17</i>	T-3184C	synonymous variant	Proline
	G-1987A		
	G-2794A	Missense variant	Serine to Leucine
	C-2812T		Arginine to Histidine
	G-3241A		Proline to Leucine
	C-1950T		Alanine to Valine
	T-3519C		Leucine to Serine
	C-3585T		Threonine to Methionine

Table 4 Allelic and genotypic distribution of the *MLPH* gene and MCPM in different genotypic¹

SNP	Genotype	Genotype frequency	Least-aquares	Allele	Allele frequency	Hardy-Weinberg equilibrium	
			MCPM			χ^2 value	<i>P</i> value
G-1351A	GG	34 (0.773)	0.312±0.027	G	0.886	0.72	0.395
	GA	10 (0.227)	0.229±0.028	A	0.114		
	AA	0 (0.000)					
C-1411T	CC	36 (0.818)	0.314±0.026 ^a	C	0.909	0.44	0.507
	CT	8 (0.182)	0.196±0.029 ^b	T	0.091		
	TT	0 (0.000)					
G-1414A	GG	31 (0.705)	0.281±0.029	G	0.807	5.20	0.023
	GA	9 (0.205)	0.358±0.042	A	0.193		
	AA	4 (0.091)	0.238±0.055				
G-1527A	GG	7 (0.159)	0.308±0.064	G	0.409	0.05	0.821
	GA	22 (0.500)	0.300±0.032	A	0.591		
	AA	15 (0.341)	0.275±0.041				
C-1554G	CC	22 (0.500)	0.305±0.036	C	0.716	0.17	0.683
	CG	19 (0.432)	0.284±0.033	G	0.284		
	GG	3 (0.068)	0.258±0.072				
T-1558G	TT	22 (0.500)	0.305±0.036	T	0.716	0.47	0.495
	TG	19 (0.432)	0.284±0.033	G	0.284		

	GG	3 (0.068)	0.258±0.072				
T-1560C	TT	30 (0.682)	0.274±0.025	T	0.845	1.37	0.241
	TC	14 (0.318)	0.334±0.047	C	0.155		
	CC	0 (0.000)					
T-1575C	TT	29 (0.659)	0.275±0.029	G	0.807	0.12	0.729
	TC	13 (0.295)	0.327±0.043	A	0.193		
	CC	2 (0.045)	0.328±0.043				
C-5021G	CC	10 (0.227)	0.244±0.039	C	0.489	0.09	0.760
	CG	23 (0.523)	0.333±0.035	G	0.511		
	GG	11 (0.250)	0.254±0.038				
C-5044T	CC	4 (0.091)	0.392±0.036	C	0.261	0.60	0.437
	CT	15 (0.341)	0.345±0.047	T	0.739		
	TT	25 (0.568)	0.246±0.025				
C-5952A	CC	5 (0.114)	0.353±0.061	C	0.364	0.28	0.594
	CA	22 (0.500)	0.313±0.033	A	0.636		
	AA	17 (0.386)	0.249±0.036				
A-5937G	AA	6 (0.114)	0.353±0.061	A	0.364	0.28	0.594
	AG	21 (0.500)	0.313±0.033	G	0.636		
	GG	17 (0.386)	0.249±0.036				
T-5931C	TT	6 (0.114)	0.353±0.061	T	0.364	0.28	0.594
	TC	21 (0.500)	0.313±0.033	C	0.636		
	CC	17 (0.386)	0.249±0.036				

T-5901C	TT	6 (0.114)	0.353±0.061	T	0.364	0.28	0.594
	TC	21 (0.500)	0.313±0.033	C	0.636		
	CC	17 (0.386)	0.249±0.036				
G-5745A	GG	7 (0.159)	0.337±0.061	G	0.455	1.62	0.204
	GA	26 (0.591)	0.295±0.031	A	0.545		
	AA	7 (0.250)	0.259±0.042				
T-15357C	TT	15 (0.341)	0.255±0.022	T	0.625	1.98	0.159
	TC	25 (0.568)	0.303±0.035	C	0.375		
	CC	4 (0.091)	0.370±0.103				
A-15464G	AA	32 (0.727)	0.308±0.029	A	0.830	3.37	0.066
	AG	9 (0.205)	0.289±0.036	G	0.170		
	GG	3 (0.068)	0.136±0.043				

¹ MCPM: melanin content of pectoral muscle, Means with different superscript differs

significantly

Table 5 Allelic and genotypic distribution of the *PMEL17* gene and MCPM in different genotypic¹

SNP	Genotype	Genotype frequency	Least-aquares	Allele	Allele frequency	Hardy-Weinberg equilibrium	
			MCPM			χ^2 value	<i>P</i> value
G-1843C	GG	19 (0.432)	0.376±0.036 ^A	G	0.614	2.39	0.122
	GC	16 (0.364)	0.239±0.034 ^B	C	0.386		
	CC	9 (0.205)	0.211±0.028 ^B				
C-1950T	CC	29 (0.659)	0.266±0.025	C	0.807	0.12	0.729
	CT	13 (0.295)	0.367±0.048	T	0.193		
	TT	2 (0.045)	0.192±0.066				
G-1987A	GG	21 (0.477)	0.343±0.033	G	0.693	0.01	0.920
	GA	19 (0.432)	0.247±0.035	A	0.307		
	AA	4 (0.091)	0.251±0.043				
A-2037G	AA	5 (0.114)	0.425±0.061	A	0.250	3.27	0.070
	AG	12 (0.273)	0.285±0.037	G	0.750		
	GG	27 (0.614)	0.272±0.030				
G-3241A	GG	14 (13.642)	0.301±0.048	G	0.557	0.05	0.827
	GA	21 (21.716)	0.322±0.034	A	0.443		
	AA	9 (8.642)	0.212±0.016				
T-3184C	TT	23 (0.523)	0.276±0.029	T	0.716	0.11	0.739
	TC	17 (0.386)	0.311±0.041	C	0.284		

	CC	4 (0.091)	0.315±0.090				
C-2812T	CC	38 (0.864)	0.315±0.025 ^a	C	0.932	0.24	0.627
	CT	6 (0.136)	0.152±0.011 ^b	T	0.068		
	TT	0 (0.000)					
G-2794A	GG	17 (0.386)	0.216±0.023 ^b	G	0.523	9.05	0.030
	GA	12 (0.273)	0.346±0.048 ^a	A	0.477		
	AA	15 (0.341)	0.338±0.043 ^a				
T-3519C	TT	13 (0.295)	0.264±0.041	T	0.511	0.81	0.367
	TC	19 (0.432)	0.340±0.035	C	0.489		
	CC	12 (0.273)	0.249±0.041				
C-3585T	CC	21 (0.477)	0.282±0.033	C	0.648	2.82	0.093
	CT	15 (0.341)	0.300±0.037	T	0.352		
	TT	8 (0.182)	0.309±0.067				

¹ MCPM: melanin content of pectoral muscle, Means with different superscript differs

significantly

Fig 1: The standard curve of melanin

Fig 2 Melanin content, L value, W₁ value, and W₂ value in pectoral muscle¹

¹HB: high blackness group; LB: low blackness group

Fig 3 The mRNA relative expression of MLPH and PMEL 17 gene¹

¹HB: high blackness group; LB: low blackness group

Fig 4 The linear relationship of relative expression of *MLPH* gene and MCPM¹

¹ MCPM: melanin content of pectoral muscle

Fig 5 The linear relationship of the mRNA relative expression of *PMEL 17* gene and MCPM¹

¹ MCPM: melanin content of pectoral muscle

Fig 6 Chromatogram showing SNPs in *MLPH* gene

Fig 7 Chromatogram showing SNPs in *PMEL 17* gene