Polymorphism in *MC1R*, *TYR* and *ASIP* genes in different colored feather chickens

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Abstract

Coat color genetics successfully adapted and applied to different animal species, which provides a good demonstration of the concept of comparative genetics. In this study, we sequenced 945 bp fragments of *melanocortin 1 receptor* (*MC1R*) gene, 421 bp fragments of exon 1 of *tyrosinase* (*TYR*) gene and 266 bp fragments of exon 3 of *agouti signaling protein* (*ASIP*) gene for 250 individuals with five plumage color patterns. We detected a total of three SNPs (T398A, T637C, and G920C) in *MC1R* and built six haplotypes (H1–H6) based on the three SNPs. H5 and H6 haplotypes were mainly concentrated in white and grey chicken. And diplotypes H2H3 occurred in white feather and black-speckle feather with the same frequency. Moreover, a total of three SNPs (C47G, T120C, and T172C) in *TYR* were found and built six haplotypes (P1–P6) based on the three SNPs. Among them, haplotype P2, P3 and P6 were not occurred in black chicken, the diplotypes P1P6 and P4P6 were only distributed in white, gray and black-speckled feather. We only detected one SNP (T168C) in *ASIP* gene and found that genotype TT was advantage genotype in the different plumage color groups of chickens. Collectively, our study suggested an association between plumage color and genetic variation of *MC1R*, *TYR* and *ASIP* in chicken.

Keywords Chicken · *MC1R*, *TYR* and *ASIP* genes · SNP · Plumage Color

Introduction

With the development of society and the increase of population, the number of chickens is increasing and the distribution area is also growing. The chicken's plumage is gradually enriched by continuous natural selection and artificial selection. People have different preference for different color of chicken feathers. Thus, studying the relationship between feather phenotype and genotype paves the way for appropriate products that meet consumers' demands (Pang 2001).

The plumage color of birds is mainly related to the pigment distribution or proportion of eumelanin and pheomelanin (Giuseppe 1980). The relative ratio of eumelanin and pheomelanin is regulated by melanocortin receptor-1 (*MC1R*) and its antagonist agouti protein (Schiöth et al. 2003). *MC1R* encodes a seven-transmembrane domain G-protein-coupled receptor, which expressed primarily in melanocytes of developing feathers and hair (Mundy 2005). The process of eumelanin synthesis is triggered by the binding of α -melanocyte stimulating hormone (α -MSH) to *MC1R* (Scherer and Kumar 2010). Then, it will lead to an increase in intracellular cAMP which activates tyrosinase

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and catalyzes the first step of melanogenesis (Mountjoy et al. 1992).

Agouti, now known as Agouti signaling protein (ASIP), is a ligand to the MC1R, and plays an important role in coat colour (Schütz 2015). ASIP acts as an antagonist of MC1R by nullifying the action of α -melanocyte-stimulating hormone (α -MSH). Loss-of-function of MC1R will result in yellow pigment (pheomelanin), whereas gain-of-function of MC1R or loss-of-function of ASIP seems to result in the production of black pigment—eumelanin (Barsh 1996). In some animals, members of the defensins, a protein family previously implicated in immunity, have also been found to involved in melanocortin pathway by binding competitively to the MC1R (Candille et al. 2007; Zhang et al. 2019).

The tyrosinase, encoded by tyrosinase (*TYR*) gene, is known to be the rate-limiting enzyme affecting the production of melanin pigment. The functions includes oxidizing tyrosine to dihydroxyphenylalanine (DOPA) and determines which type of melanin (eumelanin or pheomelanin) could be synthesized (Ito 2000). Researcher found that the C locus is the structural site for tyrosinase in fowl, and *TYR* gene was cloned from chickens for the first time on this basis (Mochii et al. 1992).

In previous studies, it has been confirmed that mutations of the *MC1R*, *TYR* and *ASIP* genes were associated with melanin trait variation or skin cancer and other diseases in a number of mammalian species, such as human, mouse, cattle, horse, fox, pig, sheep, and dog (Oetting

2010; Robbins et al. 1993; Klungland et al. 1995; Schmutz et al. 2004; Yokoyama et al. 1990; Lu et al. 1994; Våge et al. 1999; Zdarsky et al. 1990). In birds, studies on molecular mechanism of melanin deposition are relatively rare at present. The aim of the current study is to detect SNPs in *MC1R*, *TYR* and *ASIP* genes and explore their phenotypes association with plumage color in chicken.

Materials and methods

Samples

Blood samples were collected from 250 chickens with five plumage color phenotypes, including 50 Jiuyuan chickens (pure black feather), 50 Guanyuan gray chickens (completely gray plumage), 50 Lohmann Laying Hens (pure white plumage), 50 barred Plymouth Rock chicken (horizontal spot feather), and 50 HS1 chickens (black-speckled feather) (Fig. 1). The chickens came from the poultry breeding farm of Sichuan Agricultural University. The protocol was approved by the Committee on the Care and Use of Laboratory Animals of the State-Level Animal Experimental Teaching Demonstration Center of Sichuan Agricultural University. All samples were refrigerated in drikold before stored at -20°C .

Fig. 1 Completely gray plumage (a), pure white plumage (b), pure black feather (c), horizontal spot feather (d) and black-speckled feather (e)

DNA isolation and PCR amplification

Total genomic DNA was extracted from chicken blood using the TIAN amp Genomic DNA Kit in accordance with the manufacturer's instructions. DNA was eluted in a final volume of 200 µl using AE buffer and then stored at -20°C . The primers were designed by Oligo 6.0 and Primer 5.0 software based on the conserved sequence of *MC1R* gene (Gene ID: 427562), exon 1 of *TYR* gene (Gene ID: 373971), and exon 3 of *ASIP* gene (Gene ID: 419147) in *Gallus gallus* (Table 1).

PCR (25 µl) consists of 12.5 µl $2\times$ Taq Master Mix, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl DNA and 8.5 µl ddH₂O. PCR cycles included 95°C for 5 min; 35 cycles of 95°C for 30 s, annealing temperature for 30 s, and 72°C for 90 s; and a final extension at 12°C for 10 min ending with incubation at 4°C . PCR products were checked on 1% agarose gel and sequenced by direct sequencing in TSINGKE Biological Technology Corporation (Beijing, China).

Sequence analysis

The general linear model (GLM) procedure of SAS 6.12 (Statistical Analysis Systems Institute Inc. Cary, NC, USA) was built to test associations between the genotype and feather color; significant associations were declared when $p < 0.05$. The mixed model is as follows:

$$Y = \mu + G + S + B + F + e_{ijkf}$$

where Y is the dependent variable, μ is the population mean, G is the genotype value, S is the fixed effects of sex, B is the fixed effects of breed, F is the family effect, and e_{ijkf} is the random error.

The identified SNPs in these genes were tested for Hardy–Weinberg equilibrium, when $p > 0.05$ indicated the genetic balance of population gene (Wigginton et al. 2005). The linkage disequilibria D' and r^2 value of the SNPs were estimated by Haploview (Barrett et al. 2005). Significance of the least squares means was tested with the Duncan's Multiple Range test. The polymorphism information content (PIC) was established (PIC > 0.5 is high polymorphism,

$0.25 < \text{PIC} < 0.5$ is the intermediate polymorphism, and $\text{PIC} < 0.25$ is low polymorphism) (Elston 2005).

Haplotypes were constructed based on each SNP of these genes in all experimental animals using the PHASE program v. 2.0. The function of this program is to reconstruct haplotypes from the population data. The genetic status of the subjects was expressed as the combination of two haplotypes. The SAS 6.12 (Statistical Analysis Systems Institute Inc., Cary, NC, USA) was used to analyze the associations between the haplotypes and feather color. Significant associations were declared when $p < 0.05$.

Results

Sequence polymorphism in the *MC1R*, *TYR* and *ASIP* genes

In this study, we obtained 945 bp fragments of *MC1R* gene, 421 bp fragments of exon 1 of *TYR* gene and 266 bp fragments of exon 3 of *ASIP* gene. Three SNPs (T398A, T637C, and G920C) were detected from *MC1R* gene; all of them were leading to amino acid substitution (Leu133Gln, Cys213Arg and Arg307Pro). Three SNPs (C47G, T120C, and T172C) were detected from *TYR* gene: two of them were leading to amino acid substitution (Pro16Arg and Phe58Leu), and only one synonymous SNP (T168C) was detected from *ASIP* gene.

Allele and genotype frequency of the mutated loci

The results of the allele and genotype frequency of the 7 SNPs in population are shown in Tables 2, 3 and 4. The Chi-squared test was used to compare the allele frequencies in the *MC1R*, *TYR* and *ASIP* genes between the different plumage color groups. The genotype distribution of all mutations in five chicken populations of different plumage color reached significant level ($p < 0.05$). In the different plumage color groups of chickens, SNP1 (T398A) and SNP2 (T637C) showed a homozygous genotype TT for advantage genotype, while SNP3 (G920C) showed GG genotype for advantage. We analyzed the relationship between different

Table 1 Primers of PCR in chicken *MC1R*, *TYR* and *ASIP* genes

Gene	Sequence (5'–3')	Annealing temperature ($^{\circ}\text{C}$)	Fragment (bp)
<i>MC1R</i>	F:GTAGGTGCTGCAGTTGTGCT	59.4	1187
	R:CTTTATTTGGGAGCGCGAGG	59.4	
<i>TYR</i>	F:TCACATGGATTGGGCTGTGG	57.5	667
	R:ACTCTGAGCCTTCCAGTGTTA	57.5	
<i>ASIP</i>	F:GAATTGTGGTTTGCCGCATTG	59	451
	R:AACTGCTGGATGTGACAGAAT	59	

Table 2 The genotype distribution and allele frequencies of *MC1R* gene SNPs locus

SNPs	Genotype	Phenotype class					Total	Frequency/%	χ^2 value
		Black	White	Gray	Black-speckle	Horizontal spot			
SNP1 (T398A)	TT	50 (0.2)	15 (0.06)	47 (0.188)	50 (0.2)	24 (0.096)	186	74.4	$\chi^2 = 6.56$ $p < 0.05$
	TA	0 (0)	26 (0.104)	3 (0.012)	0 (0)	19 (0.076)	48	19.2	
	AA	0 (0)	9 (0.036)	0 (0.00)	0 (0)	7 (0.028)	16	6.4	
	Total	50	50	50	50	50	250	100	
	T	100	56	97	100	67	420	84.0	
	A	0	44	3	0	33	80	16.0	
SNP2 (T637C)	TT	46 (0.184)	8 (0.032)	32 (0.128)	43 (0.172)	22 (0.088)	151	60.4	$\chi^2 = 7.25$ $p < 0.05$
	TC	4 (0.016)	29 (0.116)	14 (0.056)	6 (0.024)	18 (0.072)	71	28.4	
	CC	0 (0.00)	13 (0.052)	4 (0.016)	1 (0.004)	10 (0.04)	28	11.2	
	Total	50	50	50	50	50	250	100	
	T	96	45	78	92	62	373	74.6	
	C	4	55	22	8	38	127	25.4	
SNP3 (G920C)	GG	50 (0.2)	31 (0.124)	39 (0.156)	41 (0.164)	41 (0.164)	202	80.8	$\chi^2 = 6.03$ $p < 0.05$
	GC	0 (0)	12 (0.048)	8 (0.032)	9 (0.036)	9 (0.036)	38	15.2	
	CC	0 (0)	7 (0.028)	3 (0.012)	0 (0)	0 (0.08)	10	4	
	Total	50	50	50	50	50	250	100	
	G	100	74	86	91	91	442	88.4	
	C	0	26	14	9	9	58	11.6	

Table 3 The genotype distribution and allele frequencies of *TYR* gene SNPs locus

SNPs	Genotype	Phenotype class					Total	Frequency/%	χ^2 value
		Black	White	Gray	Black-speckle	Horizontal spot			
SNP4 (C47G)	CC	44 (0.176)	45 (0.18)	41 (0.164)	39 (0.156)	50 (0.2)	210	84.0	$\chi^2 = 4.15$ $p < 0.05$
	CG	5 (0.02)	5 (0.02)	6 (0.024)	9 (0.036)	0 (0)	25	10.0	
	GG	1 (0.004)	9 (0.036)	3 (0.012)	2 (0.008)	0 (0)	15	6.00	
	Total	50	50	50	50	50	250	100	
	C	93	77	88	87	100	445	89.0	
	G	7	23	12	13	0	55	11.0	
SNP5 (T120C)	TT	50 (0.2)	45 (0.18)	45 (0.18)	34 (0.136)	48 (0.192)	222	88.8	$\chi^2 = 6.88$ $p < 0.05$
	TC	0 (0)	5 (0.02)	5 (0.02)	11 (0.044)	2 (0.008)	23	9.2	
	CC	0 (0)	0 (0)	0 (0)	5 (0.02)	0 (0)	5	2.0	
	Total	50	50	50	50	50	250	100	
	T	100	95	95	79	98	467	93.4	
	C	0	5	5	21	2	33	6.6	
SNP6 (T172C)	TT	35 (0.14)	45 (0.18)	35 (0.14)	30 (0.12)	0 (0)	145	58.0	$\chi^2 = 6.71$ $p < 0.05$
	TC	11 (0.044)	5 (0.02)	8 (0.032)	15 (0.06)	27 (0)	66	26.4	
	CC	4 (0.016)	0 (0)	7 (0.028)	5 (0.02)	23 (0)	39	15.6	
	Total	50	50	50	50	50	250	100	
	T	81	95	78	75	27	356	71.2	
	C	19	5	22	25	73	144	28.8	

plumage color pattern and the genotypes of SNP1 and SNP2; the results showed that TT genotype was the advantageous genotype in black, gray, black-speckle and horizontal spot chicken in both sites, while TA and TC genotypes was predominated in white feather chicken in SNP1 and SNP2,

respectively. For SNP3 loci, GG genotype was the advantage genotype in all groups.

In addition, SNP4 (C47G) showed a homozygous genotype CC for advantage genotype in all groups, while SNP5 (T120C) and SNP6 (T172C) showed TT genotype

for advantage. However, for SNP6 loci, there was no TT genotype in horizontal spot chicken, and TC genotype was predominated. Homozygous genotype TT was the advantage genotype of SNP7 (T168C) in all groups, while there was only advantage genotype in white chicken.

Haplotype analysis

Six haplotypes (H1–H6) were obtained based on the three SNPs (T398A, T637C, and G920C) of the *MC1R* gene (Table 5); six haplotypes (P1–P6) of the *TYR* gene are shown in Table 6 and the corresponding diplotypes are displayed in Tables 7 and 8, separately. The results showed that

haplotypes H1 and H3 were found in all kinds of feather colors with a high frequency, and speculated haplotype H1 was the advantage haplotype. The H5 and H6 haplotypes were mainly concentrated in white and gray chicken, but not found in black chicken. We guessed that two haplotypes may partially inhibit the production of melanin. Haplotype P1 was found in all kinds of feather colors with a high frequency, and speculated that it was the advantage haplotype. Haplotypes P2, P3 and P6 were not occurred in black chicken.

Furthermore, the H1H1 combination was distributed in all groups with the largest frequency. Black chicken only had a haplotype combination of H1H1. Particularly, diplotypes

Table 4 The genotype distribution and allele frequencies of *ASIP* gene SNP locus

SNPs	Genotype	Phenotype class					Total	Frequency/%	χ^2 value
		Black	White	Gray	Black-speckle	Horizontal spot			
SNP7 (T168C)	TT	44 (0.176)	50 (0.2)	43 (0.172)	44 (0.176)	46 (0.184)	227	90.8	$\chi^2 = 5.96$ $p < 0.05$
	TC	6 (0.024)	0 (0)	7 (0.028)	6 (0.024)	4 (0.016)	23	9.2	
	CC	0 (0.00)	0 (0)	0 (0.00)	0 (0.00)	0 (0.00)	0	0.00	
	Total	50	50	50	50	50	250	100	
	T	94	100	93	94	98	477	95.4	
	C	6	0	7	6	4	23	4.6	

Table 5 Distribution of the *MC1R* gene haplotypes

Haplotype	Number	Frequency	Phenotype class				
			Black	White	Gray	Black-speckle	Horizontal spot
H1 (TTG)	156	0.624	26	32	28	31	39
H2 (TCC)	21	0.084	0	6	5	3	7
H3 (TCG)	35	0.140	6	10	9	5	5
H4 (ATG)	17	0.068	0	7	3	2	5
H5 (ATC)	12	0.048	0	6	2	0	4
H6 (ACC)	9	0.036	0	5	2	0	2
Total	250	1.0000	32	66	49	41	62

Table 6 Distribution of the *TYR* gene haplotypes

Haplotype	Number	Frequency	Phenotype class				
			Black	White	Gray	Black-speckle	Horizontal spot
P1 (CTT)	127	0.508	26	28	16	23	34
P2 (CCT)	34	0.136	0	6	9	8	11
P3 (CCC)	26	0.104	0	4	8	6	8
P4 (GTT)	30	0.120	7	9	8	6	0
P5 (GTC)	21	0.084	6	8	1	6	0
P6 (GCC)	12	0.048	0	3	4	5	0
Total	250	1.0000	39	58	46	54	53

Table 7 Distribution of the *MC1R* gene diplotypes

Diplotype	Number	Frequency	Phenotype class				
			Black	White	Gray	Black-speckle	Horizontal spot
H1H1	148	0.592	29	31	22	30	36
H1H2	31	0.124	0	7	12	9	3
H1H5	26	0.104	0	10	8	4	4
H2H3	19	0.076	0	5	7	5	2
H3H4	16	0.064	0	4	3	2	7
H3H6	10	0.040	0	4	3	0	3
Total	250	1.0000	29	61	55	50	55

Table 8 Distribution of the *TYR* gene diplotypes

Diplotype	Number	Frequency	Phenotype class				
			Black	White	Gray	Black-speckle	Horizontal spot
P1P1	121	0.484	27	21	26	31	16
P1P2	41	0.164	0	5	14	7	15
P1P6	16	0.064	0	7	3	6	0
P2P3	29	0.116	0	8	11	4	6
P4P5	26	0.104	10	8	3	5	0
P4P6	17	0.068	0	6	8	3	0
Total	250	1.0000	37	55	65	56	37

H2H3 was occurred in white feather and black-speckled feather with the same frequency. Diplotype P1P1 was distributed in all groups with the largest proportion, and diplotype P1P6 with the smallest proportion. The diplotypes P1P6 and P4P6 were only distributed in white, gray and black-speckled feather.

Discussion

The plumage color of poultry has been widely used as a morphological marker for genetic selection and also considered as an important economic trait that caters to consumer preference. A functional melanocortin-1-receptor is fundamental for eumelanin expression. Gain-of-function mutations *MC1R* or loss-of-function mutations in the *MC1R* antagonist *ASIP* are believed to enhance this process, resulting in black coat color phenotypes. In our previous study, we found that there was significant association between the *MC1R* genetic variation with plumage colors of geese and pigeon (Huang et al. 2014; Ran et al. 2016).

In the past few years, lots of *MC1R*, *TYR* and *ASIP* genes mutation and the feather color difference relations research could form the perspective of the molecular to expound melanin synthesis regulative process, and for birds

and mammals the molecular mechanism on the formation of melanin provides a solid theoretical basis. In this study, we identified five missense and two synonymous mutations in 250 samples of three genes. The SNP1, SNP2, SNP3, SNP4 and SNP6 lead to a change in amino acids. In genetics, a missense mutation, a type of nonsynonymous substitution, could result in the changes of protein. When the amino acid loci 156 of *MC1R* protein mutated into proline, chicken plumage color occurs as albino (grey, white, black-speckle and horizontal spot), which is similar to Guernsey's research (Guernsey et al. 2013). In addition, although the two SNPs did not cause amino acid change, association analysis showed that they were significantly associated with chicken plumage colors ($p < 0.05$). Previous studies have shown that different synonymous degenerate codon on these loci would affect protein translation efficiency and structural conformation, which lead to phenotypic changes finally (Shastry 2009; Kurland 1991; Kimchi-Sarfaty et al. 2007). Anthony's study hypothesized that when frequent codons are changed to rare codons in a cluster of infrequently used codons, the timing of cotranslational folding is affected and may result in altered function (Anthony and Skach 2002). For the sample size which is relatively limited, further more studies should be carried forward on the relationship between loci and plumage color traits in chicken.

For the purpose of investigating the possible function of the mutations, we analyzed the association between *MC1R* and *TYR* genotypes with plumage color trait. Haplotype analysis provided a practical solution to resolve these problems. Haplotypes were constructed with the three SNPs and were used to analyze the association of haplotypes with plumage color traits. We found that haplotype H1 was distributed in all kinds of feather colors with a high frequency but the H5 and H6 haplotypes were mainly concentrated in white and gray chicken. We also found that haplotype P2, P3 and P6 were not occurred in black chicken. And we could obtain that haplotypes and diplotypes were significantly associated with plumage colors. These results demonstrated that haplotypes and diplotypes of the *MC1R* and *TYR* genes bear the characteristic of regional distribution and were associated with plumage color in chicken, although the plumage color control is a very complex trait.

Conclusion

On the basis of our results in this study, we speculated that there were significant associations between plumage colors and genetic variants of the *MC1R*, *TYR* and *ASIP* genes in chicken. However, as a complex trait, plumage color is determined by a complex pathway system and multiple interactive patterns; further studies would be helpful to confirm this conclusion.

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Compliance with ethical standards

Conflict of interest All authors have no declared conflict of interest.

Ethical approval All procedures carried out in this experiment were reviewed and approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University, China.

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