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Interactions of allele *E* of the *MC1R* gene with *FM* and mutations in the *MLPH* gene cause the five-gray phenotype in the Anyi tile-like gray chicken

J.G. Xu[1,2,3]*, M.G. Xie[4]*, S.Y. Zou[1], X.F. Liu[1], X.H. Li[1], J.F. Xie[4] and X.Q. Zhang[1,2]

[1]College of Animal Science, South China Agricultural University, Guangzhou, Guangdong, China
[2]Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding, and Key Lab of Chicken Genetics, Breeding and Reproduction, Ministry of Agriculture, South China Agricultural University, Guangzhou, Guangdong, China
[3]Guangzhou Quancheng Bio-Technology Co., Ltd., Guangzhou, Guangdong, China
[4]Institute of Animal Husbandry&Veterinary, Jiangxi Academy of Agricultural Sciences, Nanchang, China

*These authors contributed equally to this study.
Corresponding author: X.Q. Zhang
E-mail: xqzhang@scau.edu.cn

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ABSTRACT. The Anyi tile-like gray chicken is a Chinese indigenous breed with a gray dilution phenotype, having gray feathers, comb, skin, shanks, and beak, which is valuable for genetic research on pigmentation. However, the genetic basis of the gray dilution phenotype remains unknown. The objective of this study was to investigate

the genetic basis of the gray dilution phenotype in the Anyi tile-like gray chicken. We found that all Anyi tile-like gray chickens tested in this study carried at least one *E* allele, which is responsible for the appearance of black feathers, and some of them carried the *FM* allele, which is responsible for the black skin phenotype. A single nucleotide polymorphism (C.1909A>G) was identified within the melanophilin (*MLPH*) gene and was significantly associated with the gray dilution phenotype. Our findings suggest that the *E* and *FM* alleles act together to cause the development of the "five-black" phenotype (black feather, comb, skin, shank, and beak), whereas the *MLPH* mutation results in defective melanosome transport, leading to the development of the "five-gray" phenotype.

Key words: Gray dilution phenotype; *MC1R*; *EDN3*; *MLPH*; *RAB27A*; *MYO5A*

INTRODUCTION

Pigmentation phenotypes in animals result from the interaction of a number of genes, and the diversity of such phenotypes associated with specific mutations or combinations of mutations can provide insight into the underlying actions of genes (Cieslak et al., 2011). Currently, 378 loci (including 171 cloned and 207 uncloned genes) have been associated with pigmentation phenotypes in mice (http://www.espcr.org/micemut/), but little is known about the genes and mutations that cause the highly variable pigmentation phenotypes observed in birds (Cieslak et al., 2011). The Anyi tile-like gray chicken, which has a pigment-dilution phenotype, is a rare indigenous breed in China and is characterized by gray feathers, shank, skin, beak, and comb. In addition, the muscle and periosteum of Anyi tile-like gray chickens are also gray (Xie et al., 2013). The formation of coloration phenotypes in animals involves melanocyte development, pigment production, and pigment distribution, and a defect in any of these processes can change the pigmentation phenotype of the animal (Cieslak et al., 2011). Studies in chicken and quail showed that the lavender plumage color dilution phenotype was caused by a mutation in the *MLPH* gene (Vaez et al., 2008; Bed'hom et al., 2012), and that the protein complexes encoded by *MLPH*, *RAB27A*, and *MYO5A* were required for melanosome transport. In fact, mutations in *MLPH*, *RAB27A,* and *MYO5A* can all lead to the dilution phenotype in animals (Matesic et al., 2001; Ménasché et al., 2003; Philipp et al., 2005; Ishida et al., 2006; Bed'hom et al., 2012; Cirera et al., 2013; Fontanesi et al., 2014). Presumably, the "gray" phenotype is derived from the black phenotype (i.e., black feathers, shanks, skin, beak, and comb). The black feather and lavender plumage colors are controlled by the *E* and *LAV* alleles, respectively, and the *FM* and *ID* genes are both involved in the development of black skin, comb, and shanks (Kerje et al., 2003; Ling et al., 2003; Dorshorst et al., 2010, 2011). Collectively, chickens with the gray dilution phenotype appear to carry the *E* and the *FM* alleles.

In this study, we tested whether this hypothesis is correct and investigated the relationship between polymorphisms of candidate genes (*MLPH*, *RAB27A*, and *MYO5A*) and the dilution phenotype.

MATERIAL AND METHODS

Animals

Thirty Anyi tile-like gray chickens and 30 Ba Wang chickens, which are characterized by partridge plumage color, black shanks, white skin, black beak, and red comb, were selected as the case and control group, respectively.

DNA extraction and genomic analyses

High molecular weight DNA was obtained from blood samples according to the manufacturer protocol (OMEGA, Guangzhou, Guangdong, China). Primers for the *MC1R* gene were designed using Gene Tool and the genomic sequence of *MC1R* was used as a template for PCR (NC_018927.2) (Table 1). Primers used to detect the *FM* allele were as described by Dorshorst et al. (2011) (Table 1). *LAV* was genotyped using the Multiplex SNaPshot System with an ABI 3730XL genetic analyzer (Shanghai Generay Biotech Co., Ltd., China).

Table 1. Primer sequences used for genomic DNA PCR assays.

Name	Primer sequence	Length (bp)	TM (°C)
*MC1R*AF	5'-atcccaaggtacacagtgac-3'	1538	58
*MC1R*AR	5'-gggcacccaggggacacc-3'		
FMF1(232)	5'-agaaacaagggtcaaggtgagc-3'	379	58
FMR(234)	5'-tggatcattggaggaagtgttg-3'		
FMF2(200)	5'-gggatggctctcacataaaagg-3'	280	58
FMR(234)	5'-tggatcattggaggaagtgttg-3'		

Single nucleotide polymorphism (SNP) screening, selection, and genotyping

DNA sequences of Anyi chickens and breeds with a non-gray feather color were used as templates to amplify the coding regions and the 5'-flank regions of the candidate genes (i.e., *MLPH*, *RAB27A*, and *MYO5A*). All PCR products were sequenced using the same primers. Amplified sequences were assembled and analyzed for polymorphisms using the SeqMan program of DNAS-TAR. Finally, 10 identified SNPs were selected for genotyping using the SNaPshot method (Table 2). Of the 10 identified SNPs, SNP1-5 were missense mutations of *MLPH*, SNP6 and SNP7 were located within the 5'-flank region of *RAB27A*, and SNP8-10 were missense mutations of *MYO5A*.

Table 2. Single nucleotide polymorphisms used for genotyping.

ID	Gene	Chromosome	Position	Variation
SNP1 (*LAV*)	*MLPH*	*7*	103	c/t
SNP2	*(Chr7:4722132-4739842)*		447	g/t
SNP3			922	t/c
SNP4			1290	g/t
SNP5			1909	a/g
SNP6	*RAB27A*	*10*	-128	a/g
SNP7	*(Chr10:7484196-7512450)*		-70	a/g
SNP8	*MYO5A*	*10*	3517	c/t
SNP9	*(Chr10:8240552-8332115)*		3518	c/t
SNP10			3740	a/g

The first nucleotide of the translation start codon is designated +1, with the next upstream nucleotide designated -1.

Marker-trait association analyses

Haploview 4.2 was utilized to analyze the relationship between polymorphisms in candidate genes and the gray dilution traits. Moreover, SNPs that deviated from Hardy-Weinberg quilibrium, and whose allele frequencies were less than 1%, were removed.

RESULTS

Variations of *MC1R*

Seven SNPs were identified in the *MC1R* gene following the sequencing 27 Anyi tile-like gray chickens. Of these SNPs, one was located in the non-coding region, and the rest were located within the coding region. Two SNPs in the coding region were synonymous mutations, while the other four were missense mutations (Table 3).

Table 3. Variations in the *MC1R*sequence identified in Anyi tile-like gray chickens.

No.	SNP	Amino acid variation	No.	SNP	Amino acid variation
1	C-37T		5	A636G	Ala/Ala
2	C69T	Asn/Asn	6	C637T	Arg/Cys
3	C212T	Thr/Met	7	A644C	His/Pro
4	A274G	Lys/Glu			

The first nucleotide of the translation start codon was designated +1, with the next upstream nucleotide designated -1.

Haplotype analysis revealed four haplotypes for the birds used in this study (Table 4). Of these, Hap2 was the *E* allele, and the birds carrying the *E* allele had black feathers. In addition, except for animal No. 15, all the birds in this study were found to carry at least one copy of the *E* allele (Table 5).

Table 4. Distribution of haplotypes in Anyi tile-like gray chickens.

Name	-37	69	212	274	636	637	644	No.
Hap1	T	T	C	A	A	C	A	7
Hap2	T	T	C	A	G	T	A	34
Hap3	C	C	T	G	G	T	A	4
Hap4	T	T	C	A	G	T	C	5

Table 5. Haplotype status of every bird.

Name	IDs
Hap1(e^{bc})	2, 8, 17, 20, 21, 24, 28
Hap2(*E*)	2-14, 16-22, 24, 25, 27-30
Hap3(e^{+T})	5, 15, 22, 25
Hap4	9, 13, 15, 29, 30

FM and *LAV* allele distribution in Anyi tile-like gray chickens

The *FM* allele was detected in all birds using the method reported by Dorshorst et al. (2011) and the *LAV* allele was genotyped by SNaPshot. The results of *FM* genotyping showed that all the birds with the "five-gray" phenotype carried the *FM* allele, whereas not all the birds

with the "three-gray" phenotype did (Table 6 and Figure 1). Furthermore, the *LAV* allele was not detected in all birds (Table 7).

Table 6. Genotypes of Anyi tile-like gray chickens with different phenotypes.

ID	Phenotype	Genotype	ID	Phenotype	Genotype
1	Five gray	*FM/_*	37	Three gray	*fm/fm*
2	Five gray	*FM/_*	38	Three gray	*fm/fm*
3	Five gray	*FM/_*	39	Three gray	*fm/fm*
4	Five gray	*FM/_*	40	Three gray	*fm/fm*
5	Five gray	*FM/_*	41	Three gray	*fm/fm*
6	Five gray	*FM/_*	42	Three gray	*fm/fm*
7	Five gray	*FM/_*	43	Three gray	*fm/fm*
8	Five gray	*FM/_*	44	Three gray	*fm/fm*
9	Five gray	*FM/_*	45	Three gray	*fm/fm*
10	Five gray	*FM/_*	46	Three gray	*fm/fm*
11	Five gray	*FM/_*	47	Three gray	*fm/fm*
12	Five gray	*FM/_*	48	Three gray	*fm/fm*
13	Five gray	*FM/_*	49	Three gray	*fm/fm*
14	Five gray	*FM/_*	50	Three gray	*fm/fm*
15	Five gray	*FM/_*	51	Three gray	*fm/fm*
16	Five gray	*FM/_*	52	Three gray	*fm/fm*
17	Five gray	*FM/_*	53	Three gray	*fm/fm*
18	Five gray	*FM/_*	54	Three gray	*fm/fm*
19	Five gray	*FM/_*	55	Three gray	*fm/fm*
20	Five gray	*FM/_*	56	Three gray	*fm/fm*
21	Five gray	*FM/_*	57	Three gray	*fm/fm*
22	Five gray	*FM/_*	58	Three gray	*fm/fm*
23	Five gray	*FM/_*	59	Three gray	*fm/fm*
24	Five gray	*FM/_*	60	Three gray	*fm/fm*
25	Five gray	*FM/_*	61	Three gray	*fm/fm*
26	Five gray	*FM/_*	62	Three gray	*fm/fm*
27	Five gray	*FM/_*	63	Three gray	*fm/fm*
28	Five gray	*FM/_*	64	Three gray	*fm/fm*
29	Five gray	*FM/_*	65	Three gray	*fm/fm*
30	Five gray	*FM/_*	66	Three gray	*fm/fm*
31	Five gray	*FM/_*	67	Three gray	*fm/fm*
32	Five gray	*FM/_*	68	Three gray	*fm/fm*
33	Five gray	*FM/_*	69	Three gray	*fm/fm*
34	Five gray	*FM/_*	70	Three gray	*fm/fm*
35	Five gray	*FM/_*	71	Three gray	*fm/fm*
36	Five gray	*FM/_*	72	Three gray	fm/fm

"_" represents "*FM*" or "*fm*".

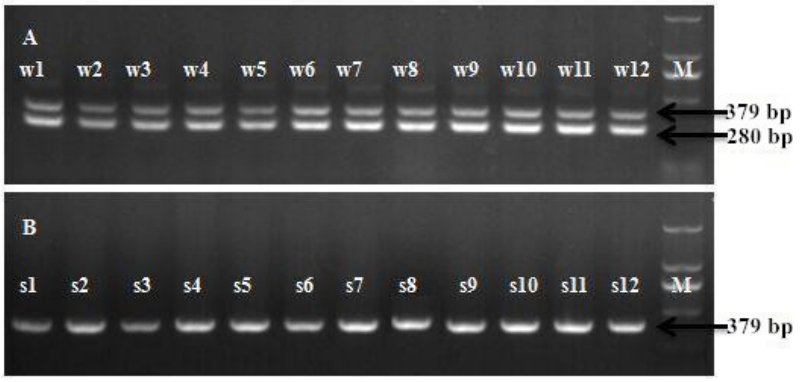

Figure 1. Agarose gel image of *FM* PCR diagnostic test products. W1-W8 are the birds with "five-gray" phenotype and s1-s8 are the birds with three-gray phenotype. We can draw the conclusion by the picture that all birds with five-gray phenotype carry the *FM* allele and all birds with three-gray phenotype do not carry the *FM* allele.

Table 7. Distribution of *LAV* alleles.

LAV		
*LAV*N/LAV*N*	*LAV*L/LAV*N*	*LAV*L/LAV*L*
100% (30)	0	0

Number in parentheses represents the number of tested individuals.

Association of the SNP with Anyi tile-like gray phenotypes

Five of the SNPs identified met the statistical requirements. The results of an association analysis showed that only SNP4 located within *MLPH* was significantly associated with pigmentation phenotypes ($P = 0.0025 \leq 0.05$; Table 8).

Table 8. Results of the association analysis.

ID	Gene	Chromosome	Position	SNP	P value
SNP3	*MLPH*	*7*	*4731966*	C.922G>T	0.192
SNP4	***MLPH***		***4727070***	**C.1909A>G**	**0.0025**
SNP6	*RAB27A*	*10*	*7492725*	C.-128A>G	0.2958
SNP7	*RAB28A*		*7492783*	C.-70A>G	0.711
SNP8	*MYO5A*	*10*	8311840	C.3517C>T	0.455

Data in bold mean significant results.

DISCUSSION

The product of the *MC1R* gene affects the relative distribution of black (eumelanin) and red (phaeomelanin) pigments in animals. There are eight alleles of *MC1R*, namely, in decreasing order of dominance, *E*(Extended), E^R(Birchen), e^{wh}(Wheaten), e^+(Wild-type), e^b(Brown), e^s(Speckled), e^{bc}(Buttercup), and e^y(Recessive Wheaten). Importantly, birds that carry the *E* allele are all black in both sexes, and most of them have black melanin in the epidermis of their shanks (Ling et al., 2003). In this study, all chickens were found to carry at least one copy of the *E* allele, which implies that the gray plume is derived from the black plume.

In chicken, the *FM* allele is responsible for the dermal hyperpigmentation phenotype (fibromelanosis) and is dominant to the *fm* allele (Bateson and Punnett, 1911). The FM phenotype is characterized by hyperpigmentation of the dermal layer across the entire body; therefore, the clear epidermis of FM birds is dark blue (Dorshorst et al., 2011). Early studies showed that the *FM* phenotype was also controlled by the sex-linked inhibitor of dermal melanin (*ID*) locus as well as *FM* (Bateson and Punnett, 1911; Dunn and Jull, 1927). Thus, the genotype of *FM* birds can be *FM/FM*, *id/id*, or *FM/FM*, id/W. In this study, we detected the *FM* allele in the birds of different phenotypes. The results showed that all birds with the "five-gray" phenotype carried the *FM* allele, while the birds with the "three-gray" phenotype did not. These findings suggest that the gray skin and gray comb phenotype is controlled by the *FM* mutation in Anyi tile-like gray chickens, and that gray skin in Anyi tile-like gray chicken is derived from the black-skin (*FM*) phenotype.

Vaez et al. (2008) used a candidate-gene approach to demonstrate that a mutation (c.103c>t) in the *MLPH* gene leads to the development of the lavender plumage color phenotype in chicken (Vaez et al., 2008). Despite the phenotypic similarity between the lavender plumage

color and the gray feather color, we did not detect the *LAV* allele in any of the animals sampled in this study.

Because the *LAV* allele is not the causative allele of the gray feather color, we examined the associations between polymorphisms of other candidate genes (i.e., *MLPH*, *RAB27A*, and *MYO5A*) and the dilution phenotype. Studies have shown that the protein complexes encoded by *MLPH*, *RAB27A*, and *MYO5A* are required for melanosome transport (Ménasché et al., 2003). In several species, such as humans, cats, dogs, horse, mink, rabbit, and quail, defects in MYO5A, RAB27A, and MLPH were found to cause a phenotypically identical pigmentary dilution (Wilson et al., 2000; Matesic et al., 2001; Ménasché et al., 2000, 2003; Philipp et al., 2005; Ishida et al., 2006; Bed'hom et al., 2012; Cirera et al., 2013; Fontanesi et al., 2014). Results of the association analysis showed that only one SNP in *MLPH* was significantly associated with the gray plumage color phenotype, suggesting that the *MLPH* gene might be closely associated with the gray plumage color phenotype.

In conclusion, these results indicate that the "five-gray" phenotype is derived from the "five-black" phenotype. The results of the association analysis suggest that the *MLPH* gene is the best candidate gene for the gray dilute phenotype.

Conflicts of interest

The authors declare no conflict of interest

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