

Effects of *melanocortin 1 receptor (MC1R)* gene polymorphisms on plumage color in mule ducks

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ABSTRACT - The objective of the present study was to investigate the effect of single nucleotide polymorphism (SNP) of the melanocortin 1 receptor (*MC1R*) gene on plumage coloration in mule ducks. PCR-high-resolution melting analysis (PCR-HRM) and DNA sequencing were used to identify the SNP variability of the *MC1R* gene in white common ducks. Three non-synonymous SNP (*MC1R* gene exon 1, c.52G>A, c.376G>A, and c.409G>A) were identified in white Tsaiya ducks. Mating test (white Tsaiya ducks × white Muscovy drakes) in combination with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed to investigate the effect of non-synonymous SNP of different maternal lines on plumage coloration in mule ducks. Genotyping results from 58 white Tsaiya ducks revealed the significant associations between genetic variations (c.52G>A, c.376A>G, and c.409G>A) and plumage color in two maternal populations. After genotyping of 266 mule ducks, these three non-synonymous SNP identified in white Tsaiya ducks were significantly associated with plumage color of mule ducks. Therefore, the polymorphisms of *MC1R* gene at c.52G>A, c.376A>G, and c.409G>A in white Tsaiya duck could be used in marker-assisted selection to improve the plumage color of mule ducks.

Keywords: Muscovy drakes, polymerase chain reaction-restriction fragment length polymorphism, single nucleotide polymorphism, Tsaiya ducks

Introduction

Animal coat and plumage color are determined by the pigment distribution or proportion of eumelanin and pheomelanin (Prota, 1980). The content of eumelanin and pheomelanin is regulated by the melanocortin 1 receptor-mediated melanogenesis pathway (Schiöth et al., 2003). Melanocortin 1 receptor, encoded by the *MC1R* gene, is a G protein-coupled receptor that is mainly expressed in melanocytes (Ollmann et al., 1998). Melanocortin 1 receptor is activated by the binding of α -melanocyte stimulating hormone (α -MSH), which triggers eumelanin biosynthesis (Mundy, 2005). Constitutively active melanocortin 1 receptor caused by dominant gene mutation is highly correlated with black color, while recessive mutation of *MC1R* gene leads to a red or yellow phenotype in animals (Klungland et al., 1995; Marklund et al., 1996; Everts et al., 2000; Newton et al., 2000; Kerje et al., 2003).

Mule ducks are sterile intergeneric hybrids that are produced by crossing female common ducks (*Anas platyrhynchos*) with Muscovy drakes (*Cairina moschata*). The mule duck is a major meat-type duck in Taiwan, and white feather color has economic advantages over black feather color because of consumer

preference. Therefore, selection of mule ducks with white plumage by breeding is still an unmet need. Since mule ducks are an infertile hybrid, selection approaches have to be performed on parental lines.

Over the past decade, traditional selection of maternal lines of mule duck, including Tsaiya and Pekin ducks, have successfully improved mule ducks whose white plumage is generally accepted by consumers in Taiwan. Many studies have demonstrated the polymorphisms of genes involved in the melanogenesis pathway associated with plumage coloration between avian species (Hoque et al., 2013; Zhang et al., 2015; Ran et al., 2016; Sultana et al., 2017; Sultana et al., 2018). However, little is known about the relationship between gene polymorphism and plumage coloration in intergeneric hybrids.

It has been demonstrated that the several polymorphisms of genes involved in the melanocortin 1 receptor-mediated melanogenesis pathway, such as melanogenesis-associated transcription factor and dopachrome tautomerase (DCT), have an influence on plumage color in Asian duck breeds (Sultana et al., 2018). However, the single nucleotide polymorphism (SNP) variations of *TYP* gene (tyrosinase), a rate-limiting enzyme in the production of melanin pigment, are not associated with plumage color in Cherry Valley and Liancheng white hybrid ducks (Zhang et al., 2015).

It has been reported that exon 1 of *MC1R* gene contributes to plumage coloration in Magelang ducks (Rahayu et al., 2015). Previous studies all focused on the effect of polymorphisms of gene involved in melanogenesis pathway on plumage color in different breeds of common ducks (*Anas platyrhynchos*) (Zhang et al., 2015; Rahayu et al., 2015; Sultana et al., 2018). However, the potential association between the *MC1R* gene and plumage color phenotypes in intergeneric hybrids, such as mule ducks, has not been validated. Therefore, the objective of the current study was to analyze the association between the polymorphisms of the *MC1R* gene and plumage color in mule ducks.

Material and Methods

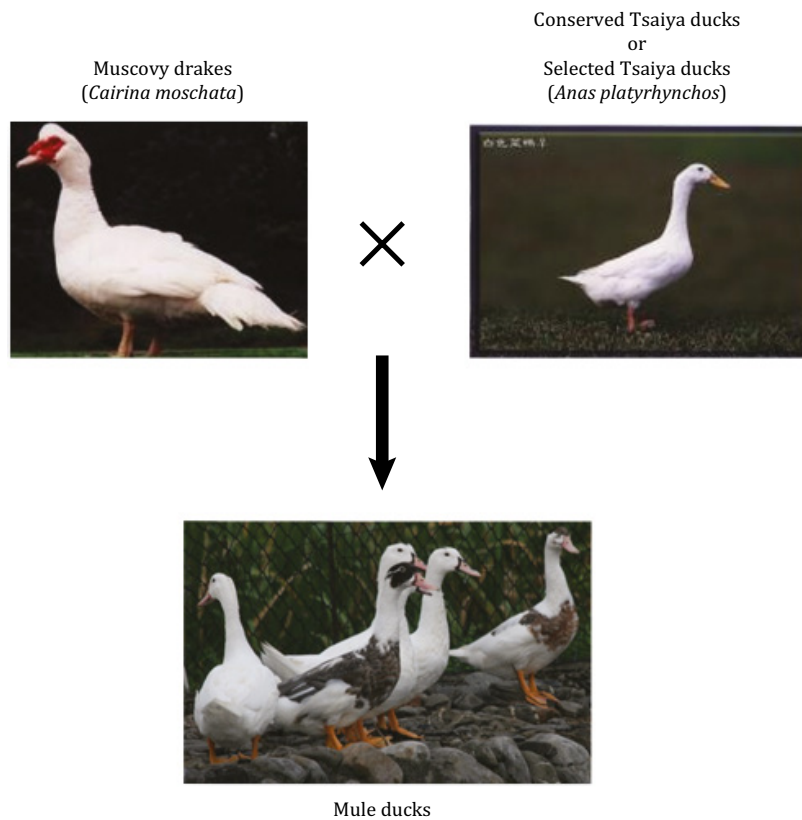
Research on animals was conducted according to the institutional committee on animal use (IACUC case No. LRIL IACUP 105002 and 106004). All experiments were conducted in Yilan, Taiwan (latitude 24°46'00" N, longitude 121°45'00" E, and 7.38 m altitude). The experimental period was carried out from September 1, 2015 to July 1, 2017.

Thirty-nine female white Tsaiya ducks (*Anas platyrhynchos*) from a conservation population (the 18th generation of a natural mating program without selection) and nineteen female white Tsaiya ducks from a selection population (30th generation of a plumage color selection program using traditional phenotype-based estimated breeding) were mated by artificial insemination with pooled semen from three white Muscovy drakes (*Cairina moschata*) (Figure 1).

The plumage color of intergeneric hybrid ducks, mule ducks, was graded based on the area of black spots on the head and back (Figure 2): 1 to 3 indicates ducks with black spot on head; 4 to 7 indicates ducks with a black head and a little spot on back; 8 to 10 indicates ducks with black head, black back, and black tail; and 11 to 15 indicates ducks with a mottled to pure black coat (Lee and Kang, 1997).

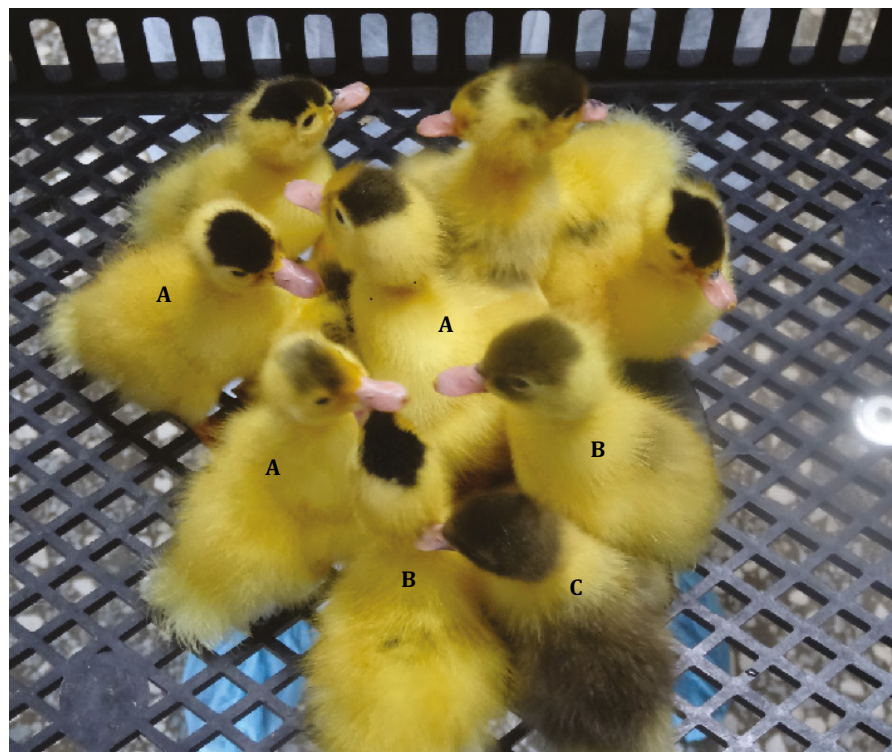
Venous blood samples were collected from the maternal line (58 white Tsaiya ducks) and intergeneric hybrid ducks (266 mule ducks). The genomic DNA was extracted from blood using a standard phenol/chloroform method, and the concentration of purified DNA was measured by a spectrophotometer (NanoVue Plus™, GE Healthcare, UK). Stock DNA samples were then stored at -20 °C for analysis of genetic polymorphisms of *MC1R* gene.

Blood samples from five white Tsaiya ducks of conservation population were subjected to genomic DNA extraction. For detection of potential SNP, three primer pairs targeting exon 1 of the *MC1R* gene were evaluated using the HRM (high-resolution melting) assay (Table 1). Primers were designed based on the genome sequence of exon 1 of *MC1R* from Pekin duck (NCBI Reference Sequence: NW_004676862) using the Vector NTI 9.1 (Thermo Fisher Scientific, Waltham, MA, USA) software. Polymerase chain reaction (PCR) was performed by G-Storm GS4 Thermal Cycler (GRI, Rayne, UK) and One Taq™ polymerase (New England BioLabs, Beverly, MA, USA). The PCR cycle was pre-denaturation at 95 °C



For examination of the association between the polymorphisms of the *MC1R* gene and plumage color in mule ducks, female white Tsaiya ducks (*Anas platyrhynchos*) from a conservation population or female white Tsaiya ducks from a selection population are mated by artificial insemination with pooled semen from white Muscovy drakes (*Cairina moschata*).

Figure 1 - Illustration of the mating test.



A indicates grade 1-3, B indicates grade 4-7, and C indicates grade 11-15.

Figure 2 - Plumage color grading in mule ducks.

Table 1 - Primer and restriction enzymes for detection of *MC1R* gene polymorphism by HRM, sequencing and PCR-RFLP

cDNA position ¹	Primer sequence (5'→3')	Annealing temperature	Product size	Polymorphism	Restriction enzyme	Allele size
52F	TGCTC CGTGA GCCCT GGAAC GCCAT C	55.0°C	250 bp	G→A	<i>Taq^α-I</i>	A: 168 bp, 82 bp
52R	TCCGC CAGGT TGCTG ACGCT					G: 168 bp, 57 bp, 25 bp
376F	ATCCA CGCCA GCATC ATC	56.4°C	106 bp	G→A	<i>Bcc-1</i>	G: 106 bp
376R	AGCGG TCCAC GGCGA TCA					A: 67 bp, 39 bp
409F	GACGC TCTTC ATGCT GCTGA TG	63.4°C	400 bp	G→A	<i>Bcl-1</i>	G: 400 bp
409R	GTGCT TCTGC TGGCT GGAGA TG					A: 271 bp, 129 bp

¹F = forward primer; R = reverse primer.

for 5 min, denaturation at 95 °C for 30 s, annealing at 55-63.4 °C for 30 s, elongation at 72 °C for 30 s, and post-elongation at 72 °C for 7 min and then a 35-cycle replication. The amplified cDNA was melted in a high-resolution melting device (HR-1 instrument, Idaho Technology, Salt Lake City, UT, USA) using LCGreen Plus + Melting Dye (Idaho Technology, Salt Lake City, UT, USA). High resolution melting curve acquisition was performed from 40 to 95 °C in 0.2 °C increments for 1 s and then normalized by the LightScanner Software with CALL-IT 2.0 (Idaho Technology, Salt Lake City, UT, USA).

Potential variations in the *MC1R* gene determined by HRM analysis were further sequenced. We performed the PCR using a SelectCycler II Thermal Cycler (Select BioProducts, Edison, NJ, USA) and One TaqTM polymerase (New England BioLabs, Beverly, MA, USA). The PCR cycle was pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 55-63.4 °C for 30 s, elongation at 72 °C for 35 s, and post-elongation at 72 °C for 7 min and then a 35-cycle replication. The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and sequenced with ABI Prism 3700 DNA sequencer (Applied Biosystems). Sequences were analyzed using the AlignX software (Thermo Fisher Scientific, Waltham, MA, USA) to identify potential polymorphisms. The PCR-RFLP analysis was used for genotyping each SNP of the *MC1R* gene coding regions in white Tsaiya ducks and mule ducks (Table 1). The enzymatic reactions were performed in 10 µL reaction mixtures containing 8 µL of PCR products, two units of each restriction enzyme and reaction buffer. The digested products were separated by 3% agarose gel electrophoresis and then visualized with ethidium bromide.

Each duck formed an experimental unit. Chi-square test (χ^2) was used to determine a significant difference in potential associations between *MC1R* SNP and plumage color using SAS software (Statistical Analysis System, version 9.2).

The statistical model applied for the genotypic and SNP analysis of *MC1R* was:

$$\chi^2 = \sum \frac{(o - e)^2}{e} \text{ with degrees of freedom } (r - 1) \times (c - 1),$$

in which o = observed frequency of genotype and SNP, e = expected frequency of genotype and SNP, r = number of rows of the contingency table, and c = columns of the contingency table. The degrees of freedom are the number of values in the final calculation of a statistical model that are free to vary.

Chi-square test of independence was performed following null and alternative hypotheses, H_0 (independent, no association) and H_1 (not independent, association), respectively. The decision is to reject the H_0 (and infer the alternative hypothesis) if the calculated value is greater than the critical value. P-values of less than 0.05 were considered statistically significant.

Haploview software (Barrett et al., 2005) was used to calculate haplotype numbers and their frequencies among three SNP from the five duck populations.

Results

Three synonymous and three non-synonymous SNP (c.52G>A, p.Glu18Lys; c.376G>A, p.Val126Ile; c.409A>G, p.Thr137Ala) were detected in exon 1 of the *MC1R* gene in conserved female white Tsaiya ducks by HRM and sequencing (Table 2). Next, we examined the genotype and allele frequency of the three non-synonymous SNP in white Tsaiya ducks from a conservation population and selection population. As expected, a normal genotype distribution and allele frequency of three SNP (c.52G>A, c.376G>A, and c.409A>G) were observed in the conservation population (Tables 3 and 4). In contrast, the selected white Tsaiya ducks were fixed for one allele at each of the SNP ($P < 0.05$) (Tables 3 and 4). Two SNP (c.52G>A and c.376G>A) showed a sole homozygous genotype AA and allele frequency in white Tsaiya ducks from the selection population compared with conservation population (Table 3), whereas c.409A>G SNP showed a sole homozygous genotype GG and allele frequency in selected white Tsaiya ducks (Table 4). Taken together, AA genotype of two non-synonymous SNP loci (c.52G>A and c.376G>A) and GG genotype of c.409A>G SNP in female white Tsaiya ducks were the advantage genotype in white plumage coloration of mule ducks.

To further characterize the effect of non-synonymous SNP loci on plumage color in mule ducks, female white Tsaiya ducks from conservation or selection population were mated by artificial insemination with pooled semen from three white Muscovy drakes. Compared with mule ducks produced by crossing conserved female white Tsaiya ducks with Muscovy drakes, two SNP (c.52G>A and c.376G>A) in mule ducks produced by crossing selected female white Tsaiya ducks with Muscovy drakes showed

Table 2 - Identified exonic polymorphisms in *MC1R* gene of white Tsaiya ducks

SNP position	Amino acid change	Functional class
c.52G>A	p.Glu18Lys	Non-synonymous
c.189A>G	p.Lys36Lys	Synonymous
c.376G>A	p.Val126Ile	Non-synonymous
c.409A>G	p.Thr137Ala	Non-synonymous
c.420T>C	p.Arg140Arg	Synonymous
c.429G>C	p.Thr143Thr	Synonymous

Table 3 - Genotype and allele frequency of c.52G>A and c.376G>A in *MC1R* gene of white Tsaiya ducks

Maternal line	Number	c.52G>A and c.376G>A					Chi-square
		Genotype frequency			Allele frequency		
		GG	GA	AA	G	A	
Conservation	39	0.18 ₍₇₎ ^a	0.38 ₍₁₅₎	0.44 ₍₁₇₎	0.37	0.63	
Selection	19	0 ₍₀₎	0 ₍₀₎	1 ₍₁₉₎	0	1	13.65 ^b

^a Number of ducks.

^b Significance of Chi-square test comparing genotypic frequencies of the conservation and selection population. Chi-square in $df = 2$ and Probability < 0.01 , which means it must be bigger than 9.21.

Table 4 - Genotype and allele frequency of c.409G>A in *MC1R* gene of white Tsaiya ducks

Maternal line	Number	c.409G>A					Chi-square
		Genotype frequency			Allele frequency		
		GG	GA	AA	G	A	
Conservation	39	0.44 ₍₁₇₎ ^a	0.38 ₍₁₅₎	0.18 ₍₇₎	0.63	0.37	
Selection	19	1 ₍₁₉₎	0 ₍₀₎	0 ₍₀₎	1	0	13.65 ^b

^a Number of ducks.

^b Significance of Chi-square test comparing genotypic frequencies of the conservation and selection population. Chi-square in $df = 2$ and Probability < 0.01 , which means it must be bigger than 9.21.

a genotype GA for advantage of white plumage color (grade 1-3, $P < 0.01$) (Table 5). These two SNP (c.52G>A and c.376G>A) in male Muscovy drakes were all genotype GG (data not shown), indicating that only the G type of allele was produced in semen. Therefore, the A allele in the two SNP (c.52G>A and c.376G>A) from female white Tsaiya ducks contributed to the determination of the white plumage color in mule ducks.

The c.409A>G SNP in mule ducks produced by crossing selected female white Tsaiya ducks with Muscovy drakes showed genotype GG for the advantage of white plumage color ($P < 0.01$) (Table 6). The c.409A>G SNP in male Muscovy drakes was genotype GG (data not shown), implying that A allele from conserved female white Tsaiya ducks was unfavorable to white plumage color in mule ducks. Furthermore, haplotypes results showed that haplotype AAG had the highest frequency (100%) in selected female white Tsaiya ducks compared with conserved female white Tsaiya ducks (63.3%) and Muscovy drakes (0%) (Table 7). However, the haplotypes GGG were restricted to the Muscovy drakes (100%) (Table 7).

The highest frequency of haplotype GGG (50%) was identified in mule ducks produced by crossing conserved female white Tsaiya ducks with Muscovy drakes, followed by frequencies of AAG (28.9%) and GGA (21.1%) (Table 7). The equal frequency of haplotype AAG and GGA was found in mule ducks produced by crossing selected female white Tsaiya ducks with Muscovy drakes (Table 7). Collectively, these results demonstrate that the maternal genotype of these three *MC1R* SNP identified in white Tsaiya ducks has a significant impact on the determination of black or white coat color in mule ducks.

Table 5 - Association between plumage grading and *MC1R* genotypes (c.52G>A and c.376G>A) in mule ducks

Phenotype (Plumage grading)	Conservation ^a			Chi-square	Selection		
	Number	Genotype			Number	Genotype	
		GG	GA			GG	GA
1-3 (Black spot on head)	19	0	19		115	0	115
4-7 (Black head and a little spot on back)	25	3	22		5	0	5
8-10 (Black head, black back, and black tail)	46	30	16		1	0	1
11-14 (Mottled coat to pure black)	55	31	24		0	0	0
Total	145	64	81	32.79 ^b	121	0	121

^a Maternal line.

^b Significance of Chi-square test comparing plumage color grading and genotype. Chi-square in $df = 3$ and Probability < 0.01 , which means it must be bigger than 11.34.

Table 6 - Association between plumage grading and *MC1R* genotype (c.409G>A) in mule ducks

Phenotype (Plumage grading)	Conservation ^a			Chi-square	Selection		
	Number	Genotype			Number	Genotype	
		GG	GA			GG	GA
1-3 (Black spot on head)	19	19	0		115	115	0
4-7 (Black head and a little spot on back)	25	22	3		5	5	0
8-10 (Black head, black back, and black tail)	46	16	30		1	1	0
11-14 (Mottled coat to pure black)	55	24	31		0	0	0
Total	145	81	64	32.79 ^b	121	121	0

^a Maternal line.

^b Significance of Chi-square test comparing plumage color grading and genotype. Chi-square in $df = 3$ and Probability < 0.01 , which means it must be bigger than 11.34.

Table 7 - Reconstructed haplotypes and their frequencies for the *MC1R* gene in mule ducks

Haplotype	Haplotype frequency (%)				
	CT	ST	MD	CM	SM
GGG	-	-	100.0	50.0	50.0
GAG	-	-	-	-	-
GAA	-	-	-	-	-
GGA	36.7	-	-	21.1	-
AAG	63.3	100.0	-	28.9	50.0
AGG	-	-	-	-	-
AGA	-	-	-	-	-
AAA	-	-	-	-	-

CT - conserved female white Tsaiya ducks; ST - selected female white Tsaiya ducks; MD - Muscovy drakes; CM - mule ducks produced by crossing conserved female white Tsaiya ducks with Muscovy drakes; SM - mule ducks produced by crossing selected female white Tsaiya ducks with Muscovy drakes.

Discussion

The specific association of SNP involved in the melanogenesis pathway and plumage color in different breeds of common ducks has been reported (Rahayu et al., 2015; Sultana et al., 2018). However, to the best of our knowledge, the present study is the first to demonstrate that the intergeneric maternal SNP of *MC1R* gene are associated with the plumage color of their offspring. We demonstrated that three non-synonymous SNP (c.52G>A, c.376G>A, and c.409G>A) identified in white Tsaiya ducks were significantly associated with the plumage color of mule ducks.

It has been reported that the white plumage color in Magelang ducks displays a predominant GG genotype of c.376G>A SNP (Rahayu et al., 2015). However, the AA genotype of c.376G>A SNP in white Kaiya and white Liancheng ducks tend to the white plumage color (Yu et al., 2013).

Consistently, we also found that white plumage color in Tsaiya ducks from a selection population displayed predominant AA genotype of c.376G>A SNP. A similar association between white plumage color and AA genotype of c.52G>A SNP was also found in white Kaiya and white Liancheng ducks (Yu et al., 2013). Our result is consistent with the previous finding that AA genotype of c.52G>A SNP in white Tsaiya ducks from a selection population associates with white plumage color. Further, we demonstrated that white plumage color in mule ducks displayed a predominant GA genotype of c.52G>A and c.376G>A SNP. These results imply that maternal A allele from Tsaiya ducks has a strong influence on white plumage coloration in their offspring. Additionally, the GG genotype of c.409G>A SNP is associated with white plumage color in white Kaiya and white Liancheng ducks (Yu et al., 2013). In the present study, white Tsaiya ducks from a selection population and mule ducks with white plumage color also displayed predominant GG genotype of c.409G>A SNP. Currently, white Tsaiya ducks from selection populations show a low plumage color grading result (grade 1-3, data not shown) after traditional breeding. Further, their offspring produced by crossing selected female white Tsaiya ducks with Muscovy drakes also display an identical plumage color grading result (grade 1-3), while high plumage color grading was observed in mule ducks produced by crossing conserved female white Tsaiya ducks with Muscovy drakes. These findings demonstrate that maternal effect on determination of plumage color in mule ducks is greater than paternal effect.

It has been demonstrated that several plumage color mutants of ducks are produced by the combined effect of controlled breeding and selection pressure from domestication (Gong et al., 2010). Here, we also observed that other SNP associated with the plumage color of mule ducks in selected white Tsaiya ducks may be simultaneously selected by traditional breeding. For example, mule ducks with GA genotype of c.52G>A and c.376G>A produced by crossing conserved female white Tsaiya ducks and white Muscovy drakes displayed a dispersed plumage color grading result (grade 1-14). However, mule ducks with GA genotype of c.52G>A and c.376G>A produced by crossing selected female Tsaiya ducks and Muscovy drakes displayed an extremely low plumage color grading result (grade 1-3).

In the present study, we found that these base changes of SNP in the *MC1R* gene sequence led to changes in the amino acid (Sultana et al., 2017). It has been reported that no significant difference is found in *MC1R* mRNA expression between white and black feather bulb of ducks (Li et al., 2012), implying that these amino acid substitutions caused by non-synonymous SNP mutations might lead to structural conformational change of MC1R protein and have an effect on enhancing MC1R activity in plumage coloration. The precise mechanisms governing conformational change of MC1R protein or alteration of α -melanocyte stimulating hormone binding activity in plumage coloration of the mule duck need to be further studied.

Mule ducks are the major meat duck breed in Taiwan. The selection of white plumage in mule ducks through breeding is in continuous demand in Taiwan due to the added economic value of such ducks. Selection of maternal lines for bleaching of mule ducks is a current breeding strategy as the mule duck is an infertile intergeneric hybrid. Over the past decade, traditional selection of maternal lines of mule duck, including Tsaiya and Pekin ducks, has successfully improved mule ducks whose white plumage is generally accepted by consumers.

In the present study, we further demonstrated that the maternal *MC1R* SNP genotypes in the selection population have a significant influence on white plumage coloration in mule ducks, indicating that our traditional breeding strategy for improving the plumage color of mule ducks is reliable.

Conclusions

The c.52G>A, c.376G>A, and c.409G>A amino acid substitutions in the *MC1R* gene of mule ducks have a significant association with white plumage color. Our findings provide a novel insight into the relationship between maternal *MC1R* gene polymorphisms and plumage color in the intergeneric hybrid ducks.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Y.-H. Yu and M.-C. Chen. Data curation: Y.-C. Tu, Y.-H. Yu and M.-C. Chen. Formal analysis: Y.-C. Tu, Y.-H. Yu and M.-C. Chen. Funding acquisition: Y.-H. Yu and M.-C. Chen. Investigation: Y.-C. Tu, H.-H. Lee, Y.-H. Yu and M.-C. Chen. Methodology: Y.-C. Tu, Y.-H. Yu and M.-C. Chen. Project administration: Y.-H. Yu and M.-C. Chen. Resources: L.-Y. Wei, Y.-Y. Chang, H.-C. Liu, Y.-H. Yu and M.-C. Chen. Supervision: H.-C. Liu, H.-H. Lee, Y.-H. Yu and M.-C. Chen. Validation: H.-C. Liu, H.-H. Lee, Y.-H. Yu and M.-C. Chen. Writing-original draft: Y.-H. Yu and M.-C. Chen. Writing-review & editing: Y.-H. Yu and M.-C. Chen.

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