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Identification of Polymorphisms in Plumage Color Related Genes in Korean Native Ducks

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Identification of genetic polymorphisms in the genes related to plumage color and elucidation of their associations with plumage phenotypes are important for the selection of desirable plumage colors. The aim of this study is to identify candidate genetic polymorphisms conferring white plumage color to select ducks with desirable plumage colors. Single nucleotide polymorphism (SNPs) in 4 candidate genes, namely the Agouti signaling protein gene (ASIP), Dopachrome tautomerase gene (DCT), Melanocortin 1 receptor gene (MC1R), and Microphthalmia–associated transcription factor gene (MITF), were investigated in forty birds, of which 20 were colored Korean native ducks and 20 were white–colored commercial ducks. Twenty–one primer pairs were designed to amplify entire exons along with partial introns of the 4 genes, and the PCR products were sequenced. A total of 83 polymorphisms were identified in these genes. We identified 8 and 36 SNPs in the ASIP and MITF genes, respectively. Thirty polymorphisms, comprising of 4 non–synonymous SNPs, were identified in the DCT gene. Two non–synonymous SNPs were identified in the MC1R gene. Two genetic variations [(c.726C>T and g.10585–88–>AATC (4–bp indel)] in the DCT gene revealed significant associations with the 2 different colored breeds ($P<0.001$). The results of this study, after further verifications of the candidate SNPs, can be helpful for the selection of Korean native ducks with desirable plumage colors.

Key words: Korean native duck, plumage color, polymorphism

INTRODUCTION

Identification of genes related to plumage colors and their inheritance pattern are important in poultry breeding; this identification also forms an interesting topic for research from the genetic point of view. Plumage color variation is also important in geographical differentiation, sexual selection for bright–colored feather plumes, and the evaluation and maintenance of genetic variations in color (Roulin, 2004). The production of birds bearing uniform plumage color is a vital issue in the commercial poultry meat–production industry. Melanogenesis is one of the important phenotypic variations affecting plumage color, and it is controlled by complex regulatory processes involving multiple genes.

Genes involved in the melanogenesis of domestic species, including chicken, are often selected to identify genes involved in pigmentation in ducks. For example, the Agouti signaling protein (ASIP) responsible for yellow pigmentation, encodes a paracrine signaling molecule needed for synthesizing pheomelanin, instead of eumelanin. Besides, the ASIP gene also regulates melanin synthesis, the process of endogenous antagonism of the melanocortin system in the central nervous system, and has a possible involvement in the peripheral system (Nadeau *et al.*, 2008). The Melanocortin 1 receptor (MC1R) gene is well known for its effect on the pigmentation process in many vertebrate species (Hoekstra, 2006). The Microphthalmia–associated transcription factor (MITF) gene encodes a transcription factor of the Tyr family genes, which have important roles in pigmentation process (Tsujimura *et al.*, 1996). The MITF gene has a downregulating effect with a resulting loss of pigmentation and patterns on skin like white spots in both dogs and cattle (Karlsson *et al.*, 2007; Hayes *et al.*, 2010; Li *et al.*, 2011; Fontanesi *et al.*, 2012). The expression of this gene can be regulated by Scf–Kit signaling and can activate the transcription of the Tyr genes (Hou *et al.*, 2000). A 6–bp deletion that inactivated Tyrosinase (Tyr) in a line of albino chickens was reported (Tobita–Teramoto *et al.*, 2000). Chang *et al.* (2006) found that the causal mutation for the recessive white allele in chickens was a result of insertion of a complete avian retroviral sequence in intron 4 of the tyrosinase gene. The tyrosinase gene family encodes the tyrosinase, tyrosinase–related protein–1 (TRP–1) and DCT (also known as tyrosinase related protein–2 (TRP–2)). The enzymatic function of DCT is to convert DOPAchrome to 5,6,dihydroxyindole–2–carboxylic acid during eumelanin biosynthesis in melanocytes (Kobayashi *et al.*, 1994; Tsukamoto *et al.*, 1992). The melanocytes originating in the neural

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crest migrate from population specific regions of the developing mouse embryo, including the epidermis and hair follicles, to the inner ear and choroid of the eye (Jiao *et al*., 2006). Among them, 3 genes of the tyrosinase family, namely, Tyr, Tyrp1, and DCT were also identified by analysis of coat color mutants (Green, 1972).

The aim of this study was to identify polymorphisms in 4 well–known melanogenesis–related genes (ASIP, DCT, MITF, and MC1R) and investigate their possible relationships with plumage color in Korean native ducks.

MATERIALS AND METHODS

Animals and DNA sample preparation

Forty experimental animals consisted of two duck breeds (20 Korean native ducks and 20 commercial ducks) and six samples (three samples in each breed) were used for verified polymorphisms. Blood samples for all individuals were collected from National Institute of Animal Science (NIAS) in Korea. Genomic DNAs were extracted using PrimePrep™ Genomic DNA Isolation Kit (GeNetBio, Korea) in according to the manufacturer's instructions.

PCR amplification and sequencing

Primers were designed from National Center for Biotechnology Information (NCBI) reference data using Primer3 program (v. 0.4.0) (Table 1). The PCR reactions included approximately 100 ng of genomic DNA, 2.5 ul of 10X buffer [Tris–HCl (pH 9.0), PCR enhancers, $(NH4)_2SO_4$, 20 mM $MgCl_2$], 2.0 ul of 10 mM dNTPs mixture (2.5 mM each of dATP, dCTP, dGTP and dTTP), 1 ul of 10 pmol of each primer and 1 U HS Prime *Taq* (GeNet Bio, Korea) in a 25 ul reaction volume. PCR was performed in a My–Genie 96 Thermal Block (Bioneer) with an initial denaturation step at 95°C for 10 min fol-

Table 1. Primer information for plumage color genes in Korean native duck

Gene name	Primer sequence (5' to 3')	Primer size (bp)	Exon (Size)
ASIP	P1_F: GCCTCTGCCTCTGTTCGCTATC	519	5' UTR (176 bp)
	P1_R: GCCATTCTTGGGTACTGCGC		Exon 1 (175 bp)
	P2 _F: CTGTTTTACATGAGCAACAGCGAC	645	Exon 2 (62 bp)
	P2_R: CATGGCAAGACTGGGAATGGTAT		
	P3_F: GCTACCTAACAGAGGTCCCA	439	Exon 3 (177 bp)
	P3_R: CCAACCTACCAGCTTCAAAGC		3' UTR (115 bp)
MC1R	P1_F: CCATGTCCCCTTGACCTCG	512	5' UTR (52 bp)
	P1_R: CTGCAGATGAGCATGTCGATG		Exon 1 (945 bp)
	P2_F: GCTCTTCATGCTGCTGATGG	515	Exon 1 (945 bp)
	P2_R: GGCAGGTGACGATGAGGATG		
	P3_F: TGCTGGTGCTCTACATCCAC	479	3' UTR (232 bp)
	P3_R: CCACTGCAAAGAGCCTTTATTCG		
MITF	P1_F: GCATTTGGAAACATTCGGTCCC	580	5' UTR (175 bp)
	P1_R: GCAAATCAAGCCACAGTGCAGC		Exon 1 (198 bp)
	P2 _F: GCCACTGAACGTGAATTAGTGAAGC	553	Exon 2 (228 bp)
	P2_R: GGCAGGCAAAGAAGCATTAATTGC		
	P3_F: GCCTCAAGCTTTGTTGTGGTC	424	Exon 3 (96 bp)
	P3_R: GTGCGCTAACTGCTTAACTGC		
	P4_F: GGTTTATTCCTCATGCCCATGC	493	Exon 4 (118 bp)
	P4_R: CCTTAATTTGTCACAGTGAATGGACGC		
	P5 _F: CGTTCTCATCACAGAAGTCAGC	554	Exon 5 (57 bp)
	P5_R: CTTGGAATTAGACCACCCAGACC		
	P6_F: GATCTTGCCACGTCAACAGTCC	500	Exon 6 (76 bp)
	P6_R: CCTACCAAGATGCTGAGCTGC		
	P7_F: GGAAGGAATGTTGTATTAGCTGGC	407	Exon 7 (148 bp)
	P7_R: CTGCAGATATCTCAGTACAGGTCAC		
	P8_F: CAGCGTAGAAGTGTACAGGGTGC	832	Exon 8 (402 bp)
	P8_R: GGCGAATCCAGTTCACAGATACTG		3' UTR (124 bp)
DCT	P1_F: GGCTTTTGAACCACATTCAAGGC	562	5' UTR (118 bp)
	P1_R: GCCTTATGCCATCTGGGATG		Exon 1 (301bp)
	P2_F: CTCAGCTGCTGTCCCTTCC	473	Exon 2 (300 bp)
	P2_R: CCTCTCCTTTACCTGCAAAGC		
	P3_F: CTTGTCCCCTGTAAGTGTTGC	614	Exon 3 (101 bp)
	P3_R: GCCTTCCTGCTTGCTGTCTG		
	P4_F: GCAGAATACAGGATGCCCAGC	414	Exon 4 (166 bp)
	P4_R: GCTATAGTTTCCATAGTCCCAAAGC		
	P5_F: CACCACCTTTCCCTAACTTGTGC	500	Exon 5 (180 bp)
	P5_R: TTGAAGCCACAGCCACTGAC		Exon 6 (136 bp)
	P6_F: CTCTCAGGTCATGAGTGTCTCTGC	449	Exon 7 (202 bp)
	P6_R: CAGACCCATGCTTTTGTGAGCAG		
	P7_F: CTGTGGGATATTTGCTTTGTTCCAAG	787	Exon 8 (182 bp)
	P7_R: GAGCAAACTTTAACCATGGTGTTCC		3' UTR (446 bp)

lowed by 35 cycles of 30 s at 95˚C, 30 s at 63˚C, 30 s at 72˚C and a final extension step at 72˚C for 10 min. The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide and DNA fragments were visualized under ultraviolet light. Purification of PCR products was performed using an AccuPrep® PCR purification kit (Bioneer) according to the manufacturer's instructions. Purified PCR products were also confirmed by using agarose gels for sequencing. Initially, 3 samples were selected from each duck breeds for DNA fragment amplification and sequencing. All the purified DNA fragments were sequenced by Cosmogenetech Company (www.cosmogenetech.co.kr). The duck genes nucleotide sequences were aligned using the ClustalW program (Thompson *et al*., 1994) and saved as bioedit format. Nucleotide replacement export data from gene were carried out polymorphisms (Kumar *et al*., 2008).

Genotyping

PCR–restriction fragment length polymorphism (RFLP) was used for genotyping of three genes (Table 2). Also, identified polymorphisms and PCR–RFLP genotyping were confirmed in Figure 1. The PCR product was digested with two units of each restriction enzyme in a 20 μL reaction volumes with approximately 15 μL of PCR product based on the protocol recommended by company (Biolabs® Inc., USA). Finally, RFLP products were electrophoresed on 3% agarose gels stained with ethidium bromide, and DNA fragments were visualized under ultraviolet light.

Statistical Analysis

Seven SNPs were genotyped and Fisher's exact test ($P<0.05$) was used for comparisons of genotype frequencies between two breeds.

RESULTS

Twenty–one primer sets were used to identify 83 polymorphisms in 4 genes associated with plumage color in Korean native and commercial duck breeds. The 6,290–bp long ASIP gene (NCBI Gene ID: 101795392) was sequenced using 3 primer sets, encompassing all 3 exons (414–bp), partial introns, and flanking regions (5' and 3'). Based on the resulting sequences, 8 polymorphisms (6 intronic and 2 in 3' –UTR) were identified. The 1,229–bp long MC1R gene (NCBI Gene ID: 101796989) was sequenced using 3 primer sets, encompassing 1 exon (945–bp) and flanking regions (5' and 3'). Based on the resulting sequences, 9 polymorphisms, 2 in the exon and 7 in the 3' –untranslated region, were detected. The 17,316–bp long DCT gene (NCBI Gene ID: 101792699) was sequenced using 7 primer sets, which encompassed 8 exons (1568–bp), partial introns, and flanking regions (5' and 3'). Based on the resulting sequences, 30 polymorphisms, of which 2, 11, 13, 1, and 3 were found in the 5' –untranslated region, exons, partial introns, insertion–deletion (indel), and 3' –untranslated region, respectively. Lastly, the 45,505–bp long MITF gene (NCBI Gene ID: 101795047) was sequenced using 8 sets of primer, encompassing 8 exons (1323–bp), partial introns and flanking regions (5' and 3'). Based on the resulting sequences, 8, 5, 22, and 1 polymorphisms were identified in 5' –untranslated region, exons, partial introns, and indels, respectively.

Sixty–five non–coding polymorphisms from the 5' –untranslated region, partial introns, and indel are listed in Table 3. Two indels, AATC in intron 4 of the DCT gene and GCTGCAAACAGATG in intron 7 of the MITF gene, for the discrimination between Korean native duck and commercial duck, were found. For the coding polymorphisms, 18 SNPs were detected from MC1R, DCT,

Table 2. The information for PCR–RFLP of the SNPs in DCT, MITF and MC1R genes in Korean native duck

Gene name	Primer sequence (5' to 3')	Amplicon Size in bp (amplified region)	Annealing temp(˚C)	SNP position	Restriction enzyme
DCT	P2_F:CTCAGCTGCTGTCCCTTCC P2_R:CCTCTCCTTTACCTGCAAAGC	473 (exon–2)	63˚C	c.468A>G	*Nci*I
	P4_F:GCAGAATACAGGATGCCCAGC P4_R:GCTATAGTTTCCATAGTCCCAAAGC	414 (exon–4)	65˚C	c.726C>T	*HpyAV*
	P4_F:GCAGAATACAGGATGCCCAGC P4_R:GCTATAGTTTCCATAGTCCCAAAGC	414 (exon–4)	65˚C	c.762C>T	*Hpy*CH4IV
	P4_F: GCAGAATACAGGATGCCCAGC P4_R:GCTATAGTTTCCATAGTCCCAAAGC	414 (intron–4)	65˚C	g.10585–88–> AATC	*Xmn*I
	P5_F:CACCACCTTTCCCTAACTTGTGC P5_R: TTGAAGCCACAGCCACTGAC	500 (exon–5)	63˚C	c.905C>T	*Bsr*DI
MITF	P1_F: GCATTTGGAAACATTCGGTCCC P1_R:GCAAATCAAGCCACAGTGCAGC	580 (exon–1)	63˚C	c.147C>T	*Tsp*RI
MC1R	P2_F: GCTCTTCATGCTGCTGATGG P2_R: GGCAGGTGACGATGAGGATG	515 (exon–1)	60˚C	c.409A>G	*Bcl*I

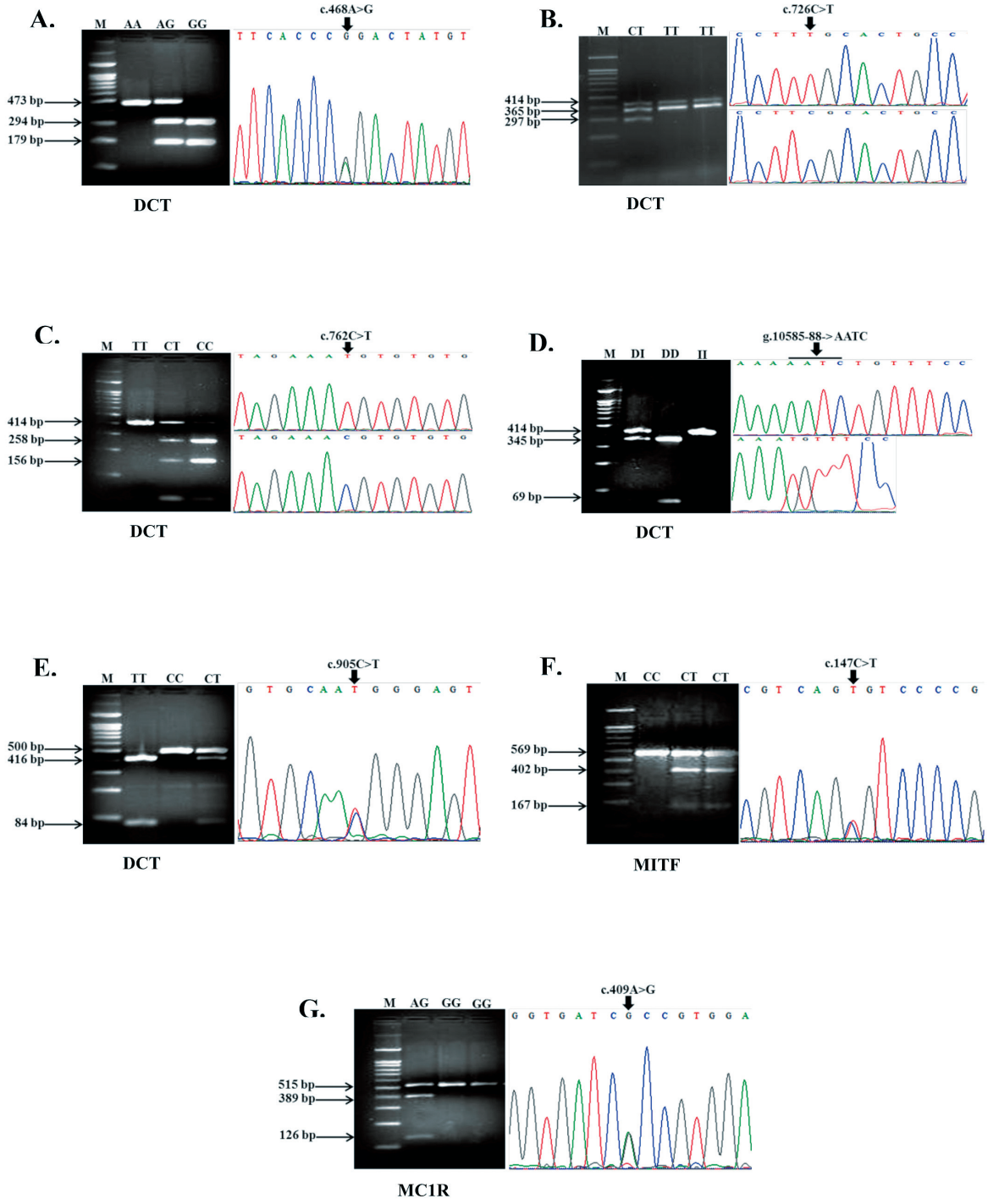

Fig. 1. PCR–RFLP genotyping results of six SNPs and one indel in three plumage color genes.

and MITF gene (Table 4). Two non–synonymous substitutions were identified in the MC1R gene. Thus, the DCT gene was verified to contain 11 exonic polymorphisms containing 7 synonymous and 4 non–synonymous substitutions. We observed 5 synonymous substitutions in the MITF gene. Among the coding polymorphisms, 6 SNPs and 1 indel was confirmed by PCR–RFLP in DCT, MITF, and MC1R (Table 5). Genotyping experiments of the DCT gene revealed significant associations between the 2 genetic variations of the DCT gene [(c.726C>T and g.10585–88–>AATC (4–bp indel)] in the 2 different breeds in this study ($P<0.001$). While c.468A>G, c.762C>T and c.905C>T SNPs in the DCT gene were not significantly associated between duck breeds. In addition, c.147C>T in MITF and c.409A>G in MC1R SNPs were also not associated with duck breeds.

DISCUSSION

According to the melanogenesis pathway reported in the Kyoto Encyclopedia of Genes and Genomes (KEGG), ASIP, MC1R, DCT, and MITF have pleiotropic effects on

Table 3. The identified non–coding polymorphisms in ASIP, DCT, MITF and MC1R genes in Korean native duck

Gene name	Location (Gene ID)	SNP number	SNP position
ASIP	NW_004676760 (101795392)	Intron–1–01	g.246A>G
		Intron–1–02	g.3766A>G
		Intron–2–03	g.4071T>G
		Intron–2–04	g.4105A>G
		Intron–2–05	g.6018A>G
		Intron–2–06	g.6064A>G
		3' UTR–07	g. 6337A>G
		3' UTR–08	g.6358A>G
DCT	NW_004676654 (101792699)	5' UTR–01	g.16C>T
		5' UTR–02	g.94C>T
		Intron–2–07	g.9138A>T
		Intron–3–08	g.9300C>T
		Intron–3–09	g.9315C>G
		Intron–3–10	g.9325A>G
		Intron–3–11	g.9353A>T
		Intron–3–12	g.9376G>C
		Intron–3–13	g.9418G>C
		Intron–3–14	g.10314T>C
		Intron–4–19	g.10531G>A
		Intron–4–20	g.10579G>A
		Intron–4–21	g.10585–88–> AATC
		Intron–4–22	g.12832C>T
		Intron–5–26	g.13109A>G
		Intron–6–27	g.14972A>T
		3' UTR–28	g.16880A>G
		3' UTR–29	g.17078A>T
		3' UTR–30	g.17167A>C
MITF	NW_004676442 (101795047)	5' UTR–01	g.–149A>G
		5' UTR–02	g.–130A>G
		5' UTR–03	g.–113C>T
		5' UTR–04	g.–90C>T
		5' UTR–05	g.–86T>G
		5' UTR–06	g.–76A>G
		5' UTR–07	g.–57T>G
		5' UTR–08	g.–56C>T
		Intron–1–12	g.282T>C
		Intron–1–13	g.293C>T
		Intron–1–14	g.299T>C
		Intron–1–15	g.29408A>G
		Intron–2–16	g.29411T>C
		Intron–1–17	g.29482T>C
		Intron–2–18	g.29813T>G
		Intron–2–19	g.29855A>G
		Intron–2–20	g.32220C>T
		Intron–2–21	g.32295C>T
		Intron–3–24	g.36435G>T
		Intron–3–25	g.36438T>C
		Intron–3–26	g.36442T>C
		Intron–3–27	g.36452G>C
		Intron–3–28	g.36471G>C
		Intron–3–29	g.36504C>T
		Intron–4–30	g.36748C>T
		Intron–4–31	g.39118C>T
		Intron–5–32	g.39480T>G
		Intron–6–33	g.40535A>G
		Intron–6–34	g.42119G>A
		Intron–7–35	g.42294A>G
		Intron–7–36	g.42318–44–>GCTGCAAACAGATG
MC1R	HQ190952	3' UTR–03	g.1019C>T
		3' UTR–04	g.1020A>G
		3' UTR–05	g.1023C>T
		3' UTR–06	g.1024A>G
		3' UTR–07	g.1028A>G
		3' UTR–08	g.1033A>G
		3' UTR–09	g.1038A>G

Table 4. The identified exonic polymorphisms in DCT, MITF and MC1R genes in Korean native duck

Gene name	Exon number	Nucleotide position	Amino acid position	Substitution	Effect on protein due to amino acid change
MC1R	Exon–1	c.409A>G	Thr137Ala	Non–synonymous	Polar to nonpolar
	Exon–1	c.649C>T	Arg217Cys	Non–synonymous	Basic to polar
MITF	Exon–1	c.114T>G	Val38Val	Synonymous	
	Exon–1	c.117C>T	Pro39Pro	Synonymous	
	Exon–1	c.147C>T	Ser49Ser	Synonymous	
	Exon–3	c.457C>T	Leu153Leu	Synonymous	
	Exon–3	c.501A>G	Pro167Pro	Synonymous	
DCT	Exon–1	c.62A>G	His21Arg	Non–synonymous	Basic to basic
	Exon–1	c.198A>G	Val66Val	Synonymous	
	Exon–2	c.348A>G	Thr116Thr	Synonymous	
	Exon–2	c.468A>G	Pro156Pro	Synonymous	
	Exon–4	c.726C>T	Phe242Phe	Synonymous	
	Exon–4	c.735C>T	Pro245Pro	Synonymous	
	Exon–4	c.753A>G	Thr251Thr	Synonymous	
	Exon–4	c.762C>T	Asn254Asn	Synonymous	
	Exon–5	c.905C>T	Thr302Met	Non–synonymous	Polar to nonpolar
	Exon–5	c.935A>G	Asp312Gly	Non–synonymous	Acidic to polar
	Exon–5	c.938A>G	His313Arg	Non–synonymous	Basic to basic

plumage pigmentation. Among the diverse color patterns present in the plumage of Korean native ducks, the duck breeding industry prefers birds possessing white feather colors. Previous studies have reported concerning the role of the ASIP gene in the synthesis of pheomelanin (yellow pigment) instead of eumelanin (black/brown pigment), especially in the Japanese quail (Takahiro, 2007). Additionally, the ASIP gene preliminarily controls the function of the MC1R gene for further melanogenesis (Nadeau *et al*., 2008). The MC1R gene is well known for coat color pigment related melanogenesis polymorphisms in vertebrate and avian species (Roulin, 2013; Hoekstra, 2006). Roulin *et al*. (2013) reported 2 non–synonymous mutations (Glu18Lys and Val126Ile) for plumage color variations in domestic ducks. We also observed 2 non–synonymous substitutions (Thr137Ala and Arg217Cys) in the MC1R gene in Korean native ducks. Nine substitutions (3 synonymous and 6 non–synonymous) were reported to result in genetic variation in Korean native chickens (Hoque *et al*., 2013). Two known non– synonymous SNPs in the duck MC1R gene (c.409G>A and c.649C>T) were also identified from our study (Wenhua *et al*., 2012). Besides this, 2 non–synonymous substitutions (Thr137Ala and Arg217Cys) were found in Korean native ducks. Several reports concerning the association of the MITF gene with color in avian and mammalian species have been reported (Fontanesi *et al*., 2012; Li *et al*., 2011; Hayes *et al*., 2010; Karlsson *et al*., 2007). A causal mutation of the MITF gene has been shown to have a substantial effect on the "silver" plumage color in Japanese quail (Minvielle *et al*., 2010). Additionally, it is reported that 2 isoforms of MITF, M and B, exist. The B isoform was shown to be expressed in both black and white feather bulbs, while the M isoform was expressed only in black feather bulbs, regardless of being collected from ducks with pure black or black–white plumage (Li *et al*., 2012). Furthermore, our gene sequencing analysis identified 11 exonic mutations between white meat ducks and Korean native ducks. Also, 1 exonic SNP (c.726C>T) and 4–bp indel (g.10585–88–>AATC) were significantly associated with the difference in breed i.e. commercial or native Korean ducks. Three mutations and 1 targeted deletion at the DCT gene locus have been described to result in a dark grey coat color on a non–agouti black background (Guyonneau *et al*., 2004; Mouse Genome Database (MGD), 2005).

In conclusion, our study selected melanogenesis related genes to identify polymorphisms and genetic variations in the Korean native ducks. Variations in the plumage color genes were investigated for possible use in breed identification of different colored Korean native ducks.

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Table 5. Genotype distribution of the SNPs in DCT, MITF and MC1R genes and the test of association between two different colored Korean native ducks

Phenotype	Genotype (Number of animal)			P–value
	DCT c.468A>G			
	AA	AG	Arg217Cys	
Native duck	0	5	15	N.A.
White duck	3	7	10	
	DCT c.726C>T			
	CC	CT	TT	
Native duck	0	19	1	<0.001
White duck	0	3	17	
	DCT c.762C>T			
	CC	CT	TT	
Native duck	0	9	11	N.A.
White duck	1	18	1	
	DCT g.10585–88–> AATC (4 bp Indel)			
	DD	DI	II	
Native duck	11	8	1	<0.001
White duck	1	9	10	
	DCT c.905C>T			
	CC	CT	TT	
Native duck	6	7	7	N.A.
White duck	3	10	7	
	MITF c.147C>T			
	CC	CT	TT	
Native duck	14	6	0	0.20
White duck	10	10	0	
	MC1R c.409A>G			
	GG	GA	AA	
Native duck	18	2	0	N.A.
White duck	19	1	0	

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