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Development of Breed-Specific Markers using Single Nucleotide Polymorphisms in Feather Color of Korean Native Chicken, Korean Ogye, and White Leghorn

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ABSTRACT Korean Ogye (KO) is a valuable genetic resource in Korea for its complete black pigmentation (including feathers, muscles, and bones), which contributes to livestock biodiversity and scientific research on genes related to coloration. Although this unique physical characteristic of KO enables its distinction from other chicken breeds, markers that can distinguish KO from Korean Native Chicken (KNC) breeds of other colors at the molecular level provide versatility of identification across developmental stages and sample types. Therefore, this study aimed to identify single nucleotide polymorphisms (SNPs) in three genes associated with chicken feather coloration—melanocortin 1 receptor (*MC1R*), tyrosinase (*TYR*), and agouti-signaling protein (*ASIP*)—to develop breed-specific markers capable of differentiating White Leghorn (WL), KO, and a Korean Native Chicken Red Brown (KNC_Rb) line. While no SNPs in *TYR* and *ASIP* were found to be completely specific, those in the *MC1R* region showed complete breed specificity, making this locus a unique molecular marker for discriminating each breed through one-step PCR analysis. These breed-specific markers not only enable genetic authentication and traceability of Korean breeds (KNC_Rb, KO) relative to foreign breeds but also provide a crucial molecular framework for verifying donor-recipient relationships in primordial germ cell (PGC)-based germline chimera production.

(Key words: feather color, Korean native chicken, Korean ogye, PCR marker, SNP)

INTRODUCTION

Korean Ogye (KO) is a unique indigenous chicken breed characterized by its complete black pigmentation of the skin, feathers, muscles, and bones in Korea (Roh et al., 2018). In contrast, Korean Native Chicken (KNC) exhibits a broader range of feather colors—Gray-Brown (Gb), Black (B), Red-Brown (Rb), White (W), and Yellow-Brown (Yb)—as defined by the Domestic Animal Diversity Information System (DAD-IS) of the Food and Agriculture Organization (FAO). Both KO and KNC represent important components of Korea's agricultural heritage and serve as valuable resources for genetic research and sustainable breeding programs.

Breed identification is fundamental to the conservation and utilization of native livestock. However, physical assessment alone is limited by environmental influences and subjective interpretation, making molecular breed-specific markers indispensable for accurate differentiation in breeding programs (Beuzen et al., 2000). In scientific research, breed-specific markers enable efficient and non-invasive identification of donor-derived cells. This includes the transplantation of primordial germ cells (PGCs) into recipient embryos and the subsequent production of germline chimeras. Such markers allow verification of successful donor integration through simple polymerase chain reaction (PCR) analysis without histological staining or progeny testing (Kubo et al., 2009; Liu et al., 2012; Trefil et al., 2017).

Single nucleotide polymorphisms (SNPs), single-base variations between homologous DNA sequences, can be screened to identify potential breed-specific markers when phenotypic differences among chicken breeds are associated with specific genetic loci (Seo et al., 2021). Particularly, genes involved in

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melanogenesis— melanocortin 1 receptor (*MC1R*), tyrosinase (*TYR*), and agouti-signaling protein (*ASIP*)—have been extensively examined to explain the variation in chicken feather colors. *MC1R* encodes a seven-transmembrane domain G-protein-coupled receptor to which α-melanocyte-stimulating hormone (α-MSH) and ASIP competitively bind. While α-MSH activates MC1R to transduce signaling cascades that increase the concentration of tyrosinase, which promotes the production of the brown-to-black pigment eumelanin (Koga et al., 1995), ASIP leads to a higher concentration of cysteine, an inhibitor of tyrosinase activity, that promotes the production of the red-to-yellow pigment pheomelanin instead (Ozeki et al., 1997). Therefore, the *TYR*, *ASIP*, and *MC1R* were considered promising candidates for identifying breed-specific SNPs in chickens.

To date, numerous SNPs in the *TYR*, *ASIP,* and *MC1R* genes have been reported across multiple chicken breeds, including KO and KNC (Helsing et al., 2012; Hoque et al., 2012; Choi et al., 2014). However, no SNP among the three genes that can completely differentiate KNC and KO has been found, often sharing similar allele distributions at SNP sites. This indicates the need for more discriminative molecular markers that can distinguish them. The markers would enable breed discrimination even at a cellular or embryonic stage where physical examination of the animal is not available. Therefore, this study investigated SNPs in the *MC1R*, *TYR*, and *ASIP* genes among Red-brown KNC (KNC_Rb), KO, and WL samples to develop breed-specific markers that can completely distinguish them. These markers would allow cost- and time-efficient breed identification without Sanger sequencing or complex analyses that require advanced technical skills. Moreover, they hold broader potential for applications in both scientific research and the poultry industry.

MATERIALS AND METHODS

1. Experimental Animals and Animal Care

The management and experimental procedures involving chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-240105-1). All experimental animals (WL, KO, and the KNC_Rb line) were maintained under the standard management program at the University Animal Farm, Seoul National University. Animal care and feather follicle collection followed the established standard operating protocols of our laboratory (Lee et al., 2017).

2. gDNA Extraction, PCR, and PCR Purification

Genomic DNA (gDNA) was extracted from each feather follicle or cell line sample, and DNA concentration was measured using NanoDrop 2000 (Thermo Scientific, MA, USA). The final DNA concentration for every sample was diluted to 50 ng/μL. Primers targeting *MC1R* (each covering the first and second halves of the sequence), *TYR*, and *ASIP* were designed using Primer3 according to the reference chicken genome (Gallus gallus, ID: GCF_016700215.2) available on the National Center for Biotechnology Information (NCBI) and synthesized by Bionics (Seoul, South Korea) (Table 1).

PCR reactions were performed with the total PCR mixture volume of 20 μL containing 2 μL of 10X buffer, 0.4 μL of dNTPs (10 mM each), 0.1 μL of *Taq* polymerase (5 units/μL), 4 μL of 5X Band Helper (Biofact, Seoul, South Korea), 1 μL of each primer (10 μM each), 2 μL of extracted gDNA, and 9.5 μL of ultrapure water (UPW). PCR was conducted under the following thermocycling conditions: initial denaturation at 95℃ for 5 minutes; 35 cycles of denaturation at 95℃ for 30 seconds, annealing at the appropriate temperature (Table 1) for 30 seconds, and extension at 72℃ for 30 seconds; followed by a final extension at 72℃ for 10 minutes. After confirming successful amplification of the target sites on a 1.5% agarose gel via gel electrophoresis, each amplicon was purified using the Wizard™ SV Gel and PCR Clean-Up System (Promega, WI, USA).

3. SNP Identification

Purified PCR products underwent Sanger sequencing (Bionics, Seoul, South Korea). Sequence data were analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST, NCBI, National Library of Medicine, United States) based on the following reference sequences of *MC1R* (ID: NM_001031462.2), *TYR* (ID: NM_204160.2), and *ASIP* (ID: AB518066.1). Sequence differences from the reference sequences were manually annotated for all samples, and the

Table 1. List of primers and optimized annealing temperatures for PCR of targeted genes in chicken (*Gallus gallus*)

Name	Sequence (5'−3')	Use	Annealing temperature (℃)
TYR	F: ACT CTG AGC CTT CCA GTG TTA R: TCA CAT GGA TTG GGC TGT GG	Sequencing	60
ASIP	F: ATT CTG TCA CAT CCA GCA GTT R: GAA TTG TGG TTT GCC GCA TTG		58
MC1R_1st	F: GGC TGG ACA TCC CCA ATG AG R: TTA AGA CGG TGC TGG AGA CG		67
MC1R_2nd	F: CAC CTA CTA CCG CAA CAA CG R: CTG TCC ATC CAC CCA TCT GT		67
Control*	F: GAG TGT AGA CAG TAG TGT ATC R: CTC AGG GCA CCA TTT TCA CTG	PCR marker	60
WL-specific**	F: CTG CCT CAA CGT CTC GTT GGC R: AGC AGC GGC GAT GAG CGG TG		60
KO-associated**	F: CTG CCT CAA CGT CTC GTT GGC R: AGC AGC GGC GAT GAG CAG GA		60
KO-specific	F: CCA GCA TCG TCC GCC ACA TG R: CTG CGC ACG TGG TGG CAC		67
KNC_Rb-specific	F: GGC TGG ACA TCC CCA ATG AG R: CGC AGC GCA TAG AAG ATG GC		68

*Li et al. (2001).
**Choi et al. (2007).

distribution of nucleotides at each SNP site was compared among the three breeds.

4. Breed-Specific Marker Development

Using the SNPs identified in the BLAST results for *MC1R*, a reverse primer targeting the KO-specific nucleotide region (nucleotide positions 937 and 938) was designed and named KO-specific_R. For the forward primer, common nucleotides of *MC1R* among the three breeds were targeted. Control was the Chicken-specific Primer (CSP) set (Li et al., 2001) that served as the positive control for each sample. For the KNC_Rb-specific PCR, a reverse primer targeting the KNC_Rb-specific SNP region (nucleotide position 728) in *MC1R* was designed and termed KNC_Rb-specific_R. For the forward primer, the MC1R_1st primer from the earlier sequencing analysis of *MC1R* was used.

RESULTS

1. SNPs in *TYR* and *ASIP* were not Breed-Specific

We initially evaluated whether the existing method for distinguishing KO from other breeds is also effective at separating KO from KNC breeds. In a previous study (Choi et al., 2007), a 9-bp deletion in the premelanosome protein 17 (*PMEL17*) gene of KO was used to differentiate KO from WL. Although the WL-specific marker developed from this study was specific to WL, without showing the band for KNC_Rb and KO, the KO-Associated marker could not successfully distinguish KO from KNC_Rb (Fig. 1). Therefore, genotype analysis of KNC_Rb, KO, and WL was performed to identify SNPs within the three target genes that provide complete discriminatory power among the breeds.

TYR encodes tyrosinase, a key rate-limiting enzyme in melanin biosynthesis, and therefore was selected as the first gene of interest (Fig. 2A). Analysis of *TYR* identified a single SNP at nucleotide position 1837 (Chromosome 1, Exon 5). KNC_Rb and KO displayed different genotype distributions at this site−KNC_Rb predominantly homozygous T (93%), and KO either homozygous C (42%) or heterozygous C/T (48%) −and the variant was synonymous (both translated to Glu) (Fig. 2B and Table 2). Although a Chi-square test of independence (df = 2) revealed a statistically significant difference

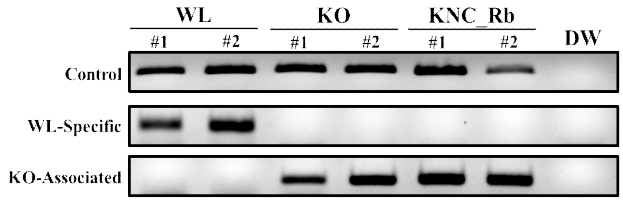

Fig. 1. Differentiation PCR results for WL, KO, and KNC_Rb using the 9-bp deletion in the *PMEL17* gene of KO (Choi et al., 2007). Control confirmed the quality of PCR. WL-specific marker resulted in bands specific to WL, while KO-associated marker resulted in bands for both KO and KNC_Rb.

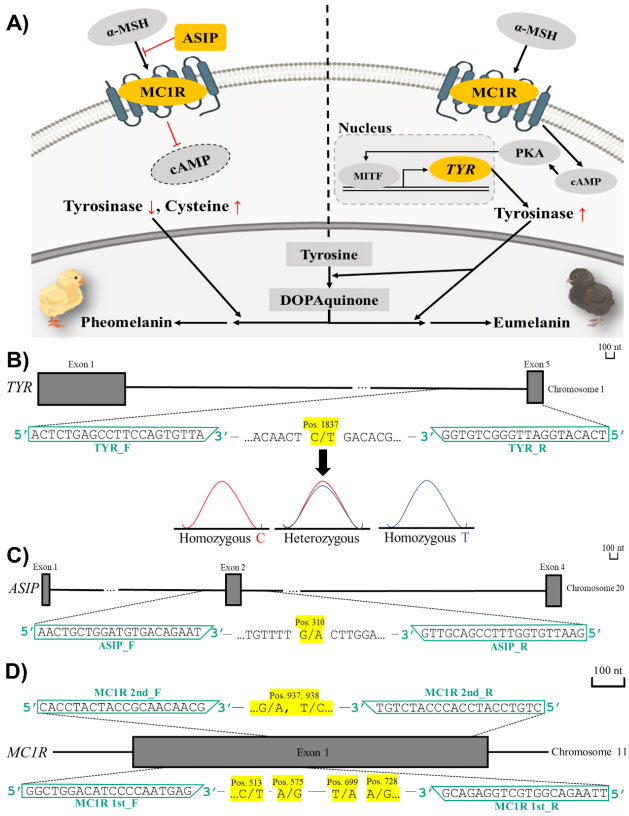

Fig. 2. SNPs found in *TYR*, *ASIP*, and *MC1R* genes (A) α-MSH and *ASIP* competitively bind the receptor *MC1R*, wherein *ASIP* inhibits the synthesis of cAMP. cAMP activates PKA, activating the transcription factor MITF, which then induces the transcription of the *TYR* gene. (B) A mutation occurred at nucleotide position 1837 for *TYR* with different patterns of homo- and heterozygosity for KNC_Rb, KO, and WL. (C) A mutation occurred at nucleotide position 310 for ASIP. (D) A total of six synonymous and nonsynonymous mutations occurred at positions 513, 575, 699, 728 (detected by the *MC1R*_1st primer) and consecutively at positions 937 and 938 (detected by the *MC1R*_2nd primer) for *MC1R*.

between the two breeds (P = 4.38 × 10^{-10}), complete breed separation could not be achieved due to the overlapping patterns: a small subset of KO also exhibited homozygous T (10%), the major genotype of KNC_Rb, while some KNC_Rb were heterozygous C/T as well (7%). Analysis of WL at the same site showed a similar pattern to KO [mainly either homozygous C (40%) or heterozygous C/T (33%)].

ASIP encodes agouti-signaling protein, a competitor against α-MSH that regulates the receptor's activity (Furumura et al., 1996) (Fig. 2A). At this second gene of interest, an SNP at nucleotide position 310 (Chromosome 20, Exon 2) from G to A resulted in a nonsynonymous substitution from Val to Ile (Fig. 2C and Table 2). Both KNC_Rb and KO were dominantly homozygous A (84% and 72%, respectively) rather than homozygous G (16%, and 28%, respectively), whereas WL was completely homozygous A (100%) (Table 2). Through the Chi-square test of independence (df = 2), the difference was proven to be statistically significant (P = 0.0071). However, because the A allele predominated across all three breeds, this SNP also lacked breed-specific discriminatory value. Therefore, results from *TYR* and *ASIP* analyses demonstrated that neither gene contains SNPs capable of fully distinguishing KNC_Rb, KO, and WL from one another.

2. SNPs in MC1R Showed Complete Breed-Specificity among KNC_Rb, KO, and WL

MC1R encodes the receptor to which ASIP and α-MSH competitively bind to either inhibit or activate the *TYR* gene and therefore was selected as the third gene of interest (Furumura et al., 1996) (Fig. 2A). In contrast to *TYR* and *ASIP*, analysis of *MC1R* revealed several SNPs that completely differentiated KNC_Rb and KO. Complete variants were found at the following nucleotide positions (Chromosome 11, Exon 5): 513 (KNC_Rb: T; KO: C), 575 (KNC_Rb: G; KO: A), 728 (KNC_Rb: G, KO: A), 937 (KNC_Rb: A; KO: G), 938 (KNC_Rb: C; KO: T) (Fig. 2D and Table 2). These polymorphisms were either nonsynonymous (513, C to T: Thr to Met; 575, A to G: Lys to Glu; 728, A to G: Ile to Ala; 938, T to C: Cys to Arg) or synonymous (937, G to A: Ala to Ala).

WL displayed mixed genotypic patterns relative to the other two breeds, sharing some alleles with KNC_Rb and others with KO, and frequently showing heterozygosity where

Table 2. *TYR*, *ASIP*, and *MC1R* Genotype Analysis for KNC_Rb line, KO, and WL

Gene	Nucleotide position	Amino acid change	Genotype	Genotype frequency		
				KNC_Rb	KO	WL
TYR	1837 C>T	Glu>Glu	T/T	0.93 (28/30)	0.10 (3/31)	0.26 (8/30)
			C/C	0.00 (0/30)	0.42 (13/31)	0.40 (12/30)
			T/C	0.07 (2/30)	0.48 (15/31)	0.33 (10/30)
ASIP	310 G>A	Val>Ile	G/G	0.16 (5/31)	0.28 (9/32)	0.00 (0/31)
			A/A	0.84 (26/31)	0.72 (23/32)	1.00 (31/31)
MC1R	513 C>T	Thr>Met	C/C	0.00 (0/30)	1.00 (30/30)	0.00 (0/30)
			T/T	1.00 (30/30)	0.00 (0/30)	1.00 (30/30)
	575 A>G	Lys>Glu	A/A	0.00 (0/30)	1.00 (30/30)	0.55 (16/30)
			G/G	1.00 (30/30)	0.00 (0/30)	0.00 (0/30)
			A/G	0.00 (0/30)	0.00 (0/30)	0.45 (14/30)
	699 T>A	Leu>Glu	T/T	1.00 (30/30)	1.00 (0/30)	0.37 (11/30)
			A/A	0.00 (0/30)	0.00 (0/30)	0.00 (0/30)
			T/A	0.00 (0/30)	0.00 (0/30)	0.63 (19/30)
	728 A>G	Ile>Ala	A/A	0.00 (0/30)	1.00 (30/30)	1.00 (30/30)
			G/G	1.00 (30/30)	0.00 (0/30)	0.00 (0/30)
	937 G>A	Ala>Ala	G/G	0.00 (0/30)	1.00 (30/30)	0.00 (0/30)
			A/A	1.00 (30/30)	0.00 (0/30)	1.00 (30/30)
	938 T>C	Cys>Arg	T/T	0.00 (0/30)	1.00 (30/30)	0.00 (0/30)
			C/C	1.00 (30/30)	0.00 (0/30)	1.00 (30/30)

KNC_Rb and KO were fixed for different alleles. Specifically, WL matched to KNC_Rb in the complete homozygosity for T, A, and C at nucleotide positions 513, 937, and 938, while they followed KO in the complete homozygosity for A at nucleotide position 728. On the other hand, at nucleotide position 575, WL was either homozygous A (55%) or heterozygous A/G (45%). Lastly, at nucleotide position 699, WL was either homozygous T (37%) or heterozygous T/A (63%) in contrast to KNC_Rb and KO which both showed complete dominance for T (100%) (Table 2).

3. Breed-Specific Markers are Developed and Validated among KNC_Rb, KO, and WL

The reverse complement sequence of the KO-specific marker (KO-specific_R) was designed to target the consecutive two-nucleotide difference in the *MC1R* nucleotide positions 937 and 938 of KO that distinguishes the breed from KNC_Rb and WL (KO: 937th G, 938th T; WL & KNC_Rb: 937th A, 938th C) (Fig. 3). Control bands were observed for all chicken breeds, and KO-specific bands were detected only in KO samples, whereas no amplification was observed in KNC_Rb or WL (Fig. 4). This confirmed that the KO-specific_R primer sequence is specific for KO and does not align with KNC_Rb or WL.

To develop a breed-specific marker that can differentiate KNC_Rb from WL and KO, the SNP specific to KNC_Rb at *MC1R* nucleotide position 728 (KNC_Rb: G; WL & KO: A) was used to design the reverse complement sequence of the KNC_Rb-specific marker (KNC_Rb-Specific_R) (Fig. 3). Bands were observed only in the KNC_Rb samples (Fig. 4), confirming that this marker can specifically distinguish the KNC_Rb line from both WL and KO.

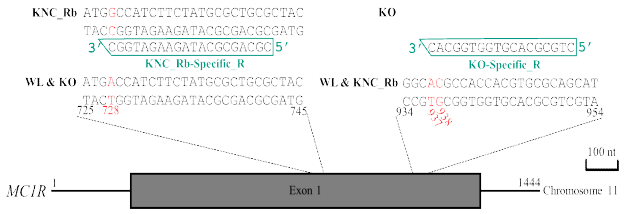

Fig. 3. Primer designs for KO-specific_R and KNC_Rb-specific_R. The two-nucleotide difference of KO from KNC_Rb and WL for MC1R at nucleotide positions 937 and 938 (highlighted in red, above) was targeted for the design of KO-specific R. The SNP found only among KNC_Rb for MC1R nucleotide position 728 (highlighted in red, above) was targeted for the design of KNC_Rb-specific_R.

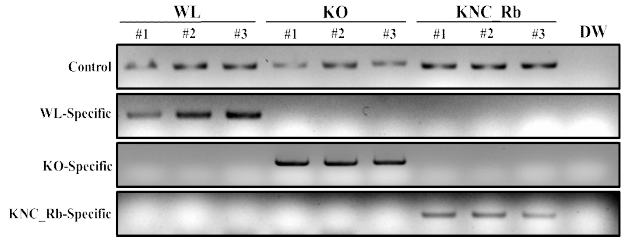

Fig. 4. Validation of *MC1R* SNP-based breed-specific markers Control confirmed the quality of PCR. WL-Specific marker (Choi et al., 2007) was specific to the WL samples (#1-#3 derived from feather follicle), while KO-specific marker showed bands only for KO samples (#1 and #2 gDNA derived from feather follicle; #3 gDNA derived from PGC). KNC_Rb-specific marker showed bands only for KNC samples (#1 and #2 gDNA derived from feather follicle; #3 gDNA derived from PGC), confirming their specificity to their targeting breeds.

DISCUSSION

A previous study exploited a 9-bp difference in the *PMEL17* sequences of KO and WL to develop WL-specific and KO-associated markers for differentiating the two breeds (Choi et al., 2007; Jung et al., 2024). Although this marker successfully distinguished KO from WL, our results demonstrated that it could not discriminate KO from KNC_Rb. Therefore, the need for a truly breed-specific marker capable of reliably distinguishing not only foreign breeds from Korean breeds, but also one Korean breed from another, remains to be addressed.

Mutations in *TYR* and *ASIP* have been associated with albinism or diluted colors in several animal species, such as mice (Beermann et al., 2004), rabbits (Fontanesi et al., 2010; Jia et al., 2021), and pigs (Wu et al., 2016). Therefore, their potential as molecular markers for color-based differentiation has also been explored in avian species. In previous studies of KNC and KO, polymorphisms in these genes showed statistically significant differences among different feather color groups. However, these differences reflected only shifts in allele frequencies and did not include any SNPs that were completely breed-specific to a single group (Choi et al., 2014; Nam et al., 2021). Our results also exhibited similar patterns for *TYR* and *ASIP* SNPs that could not completely distinguish each breed. This incomplete differentiation likely reflects the fact that feather color in chickens is a polygenic trait influenced by the combined effects of multiple genes beyond *TYR* and *ASIP*, including genes such as *GNAS, EDN3,* and *MTAP* (Cha et al., 2023). Moreover, *TYR* and *ASIP* variations may contribute to expression-level differences rather than consistent coding-sequence polymorphisms, as their elevated expressions have been observed in darker-skinned chickens without having fixed SNPs within each group (Zhang et al., 2015; Yu et al., 2019).

MC1R triggers eumelanin biosynthesis (responsible for black or dark brown feather color) by activating the intracellular cAMP signaling pathway in melanocytes. Out of the six mutations found in this study, the four nonsynonymous mutations (Nucleotide positions 513, 575, 728, and 938) can be predicted to influence the amount of eumelanin deposited, resulting in darker coloration for KO. Since the heterozygosity of T/A was only observed for WL at position 699, this may also be related to the melanin synthesis pathway. Similar associations between *MC1R* and black pigmentation have been reported in other species, including sheep and pigs (Fontanesi et al., 2011; Zheng et al., 2023), where fixed polymorphisms in this gene are characteristic of specific black-coated breeds and have been proposed for breed authentication of products such as milk. In chickens, extended black plumage at the E (extension) locus in MC1R has likewise been linked to fixed variants in black Spanish breeds, underscoring its critical role in enhancing eumelanin deposition (Davila et al., 2014). This suggests that the strong discriminatory power of MC1R SNPs likely reflects both the central role of MC1R in melanogenesis and the selective breeding of KO for uniform hyperpigmentation traits, including fibromelanosis (Roh et al., 2018).

There have been numerous attempts to differentiate multiple KNC lines and KO by studying their genetic diversity using microsatellite (MS) markers, but they usually require at least 10 MS markers to be able to establish a concrete phylogenetic relationship (Kong et al., 2006; Seo et al., 2017). Although other molecular markers aside from microsatellites, such as restriction fragment length polymorphism (RFLP), are also available, SNPs in PCR analysis are preferred not only because they require less labor and cost but also because they are less influenced by environmental factors (Gaerke et al., 2012). Additionally, SNPs offer higher precision in estimating population diversity, stronger power in differentiating certain groups of different genotypes, and a better ability to relate certain mutations to adaptation (Zimmerman et al., 2020). Despite the advantages of using SNPs for breed identification, previous KO-associated markers failed to distinguish KO from KNC_Rb (Choi et al., 2007). In this study, breed-specific markers based on *MC1R* SNPs were newly established to reliably discriminate KO from KNC_Rb, enabling simple and cost-efficient differentiation between these two Korean breeds.

A limitation of this study that should be addressed in the future is that the detailed mechanisms by which the SNPs in *MC1R* lead to the phenotypic differences between KNC_Rb and KO still need to be further investigated. However, this study has significance in investigating the genetic relationship between the two different native chicken breeds through the fixed polymorphisms in the *MC1R* gene. It is especially important in that the application of KNC_Rb in research has been limited primarily by the lack of comprehensive knowledge on its genetic background, and the SNPs and their mechanism underlying feather color variation in KNC breeds have not been established. In addition, the ability to determine chicken breed at the molecular level creates new opportunities for research and industry in Korea by enabling verification of genetic consistency in genome-edited or selectively-bred lines and by providing an effective authentication system to support the commercialization of native chickens.

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