

# A retroviral insertion in the tyrosinase (*TYR*) gene is associated with the recessive white plumage color in the Yeonsan Ogye chicken

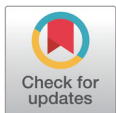
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Received: Mar 11, 2021

Revised: May 4, 2021

Accepted: May 9, 2021

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## Abstract

The recessive white (locus *c*) phenotype observed in chickens is associated with three alleles (recessive white *c*, albino *c<sup>a</sup>*, and red-eyed white *c<sup>re</sup>*) and causative mutations in the tyrosinase (*TYR*) gene. The recessive white mutation (*c*) inhibits the transcription of *TYR* exon 5 due to a retroviral sequence insertion in intron 4. In this study, we genotyped and sequenced the insertion in *TYR* intron 4 to identify the mutation causing the unusual white plumage of Yeonsan Ogye chickens, which normally have black plumage. The white chickens had a homozygous recessive white genotype that matched the sequence of the recessive white type, and the inserted sequence exhibited 98% identity with the avian leukosis virus *ev-1* sequence. In comparison, brindle and normal chickens had the homozygous color genotype, and their sequences were the same as the wild-type sequence, indicating that this phenotype is derived from other mutation(s). In conclusion, white chickens have a recessive white mutation allele. Since the size of the sample used in this study was limited, further research through securing additional samples to perform validation studies is necessary. Therefore, after validation studies, a selection system for conserving the phenotypic characteristics and genetic diversity of the population could be established if additional studies to elucidate specific phenotype-related genes in Yeonsan Ogye are performed.

**Keywords:** Genotyping, Plumage color, Recessive white, *TYR* gene, Yeonsan Ogye

## INTRODUCTION

Feather and skin color distinguish poultry breeds and are important morphological characteristics. The plumage color of breeds is strongly related to economic traits such as growth rate, egg production, and carcass traits [1,2]. For example, white plumage is preferred in broiler chickens because feathers from white chickens are easier to remove than those from colored chickens [3]. The Yeonsan Ogye is a Korean chicken that is known for its black feathers, skin, bones, and comb [4]. This is an uncommon

**Competing interests**

No potential conflict of interest relevant to this article was reported.

**Funding sources**

This work was supported by the research fund for Industry-University research collaboration, entitled "DNA marker analysis in Yeosan Ogye population for Pedigree design and inbreeding control" with Yeosan Ogye Foundation through Nonsan, Korea.

**Acknowledgements**

Not applicable.

**Availability of data and material**

Upon reasonable request, the datasets of this study can be available from the corresponding author.

**Authors' contributions**

Conceptualization: Kim M, Lee SS, Lee JH.  
 Data curation: Cho E, Kim M, Manjula P, Lee SS.  
 Formal analysis: Cho E, Kim M.  
 Methodology: Cho E, Kim M, Manjula P, Cho SH.  
 Software: Cho E, Kim M.  
 Validation: Manjula P, Cho SH, Seo D, Lee JH.  
 Investigation: Cho E, Kim M.  
 Writing - original draft: Cho E.  
 Writing - review & editing: Cho E, Kim M, Manjula P, Cho SH, Seo D, Lee SS, Lee JH.

**Ethics approval and consent to participate**

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) of Chungnam National University (202103A-CNU-061).

phenotype, and the Yeosan Ogye has been registered as a natural heritage of Korea (no. 265). Selection is used to maintain the standard phenotype of the Yeosan Ogye chicken, as individuals with various other plumage colors are occasionally observed [5]. Exceptional individuals are eliminated from the breeding pool to maintain the natural heritage phenotype.

Plumage color in birds is determined by pigments such as carotenoid and melanin [6,7]. Melanin is involved in the expression of black (eumelanin) and red (pheomelanin) and is synthesized by tyrosinase [8]. Two autosomal loci are associated with white plumage color in chickens [9]. The *PMEL17* gene gives rise to a dominant white (locus *I*) and has three alleles (dominant white *I*, smoky *I<sup>s</sup>*, and dun *I<sup>P</sup>*) [10]. The tyrosinase (*TYR*) gene is associated with a recessive white (locus *c*) and also has three alleles (recessive white *c*, albino *c<sup>l</sup>*, and red-eyed white *c<sup>r</sup>*) [11]. The recessive white mutation (*c*) is reported to involve insertion of the avian leukosis virus *ev-1* sequence at the end of intron 4 in the *TYR* gene [12], which inhibits the transcription of exon 5 (Fig. 1). Therefore, this study examined whether the white plumage phenotype in the black Yeosan Ogye population is due to this change in the *TYR* gene.

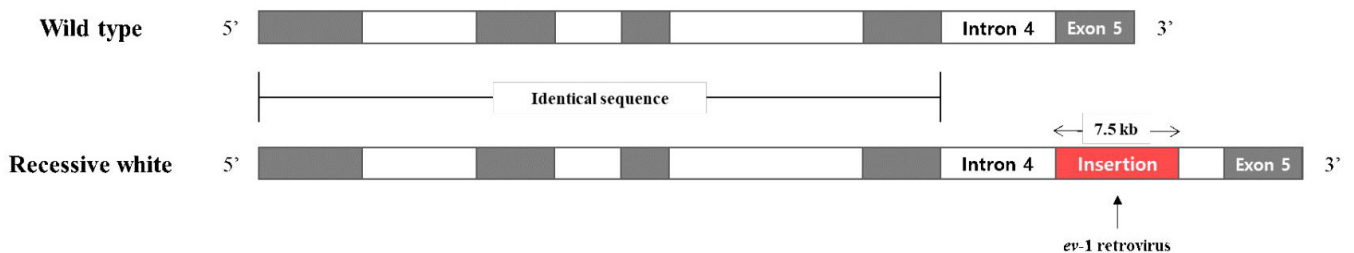
**MATERIALS AND METHODS**

**Animals**

The study examined 19 of 625 Yeosan Ogye chickens from the same generation: seven white, four brindle, and eight normal black chickens (Fig. 2). Genomic DNA (gDNA) was extracted from blood collected from the brachial vein. After mixing 20 µL of blood with 180 µL of phosphate-buffered saline, gDNA was extracted using the PrimePrep Genomic DNA Extraction Kit from Blood (GeNetBio, Daejeon, Korea), following the manufacturer's instructions. The purity and concentration of the extracted DNA were confirmed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The final gDNA was diluted to a concentration of 25 ng/µL and stored at -20°C before use.

**Genotyping**

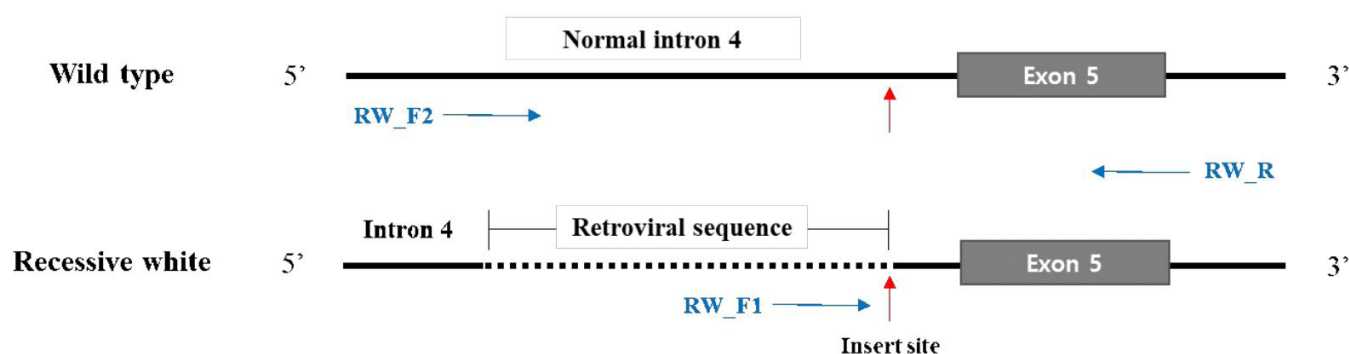
A primer set consisting of two forward primers and one reverse primer was prepared to identify the recessive white genotype based on published information (Fig. 3 and Table 1) [12]. Polymerase chain reaction (PCR) was performed in a total volume of 20 µL, which consisted of 2 µL of gDNA (25 ng/µL), 10 µL of HS Prime Taq Premix (2×) (GeNetBio), 0.5 µL each of the three primers (20 pmol/µL), and distilled water to make up the remaining volume. PCR consisted of an initial 3 min at 95°C, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and a final 5 min extension at 72°C. The product was examined via electrophoresis at 130V for 40 min using 2% agarose gels.



**Fig. 1. Structural differences in the tyrosinase (*TYR*) gene between the wild-type and recessive white phenotypes.** The recessive white allele has a 7.5-kb *ev-1* retroviral insertion in intron 4.



**Fig. 2. Comparison of plumage color in Yeonsan Ogye chickens.** (A) A normal black Yeonsan Ogye chicken (wild type). (B) A chicken with black plumage and white patches with a red comb (brindle). (C) A chicken with white plumage with a red comb (recessive white).



**Fig. 3. Primer locations for genotyping wild-type and recessive white alleles.** Two forward primers were used: RW\_F2 for the wild-type allele (C) and RW\_F1 for the recessive white allele (c). One reverse primer, RW\_R, was used.

**Table 1. Primers used for genotyping alleles and sequencing wild-type and recessive white individuals**

| Primer | Direction | Sequence (5' to 3')         | Product size (bp) |
|--------|-----------|-----------------------------|-------------------|
| RW_F1  | Forward   | CCTCTGGCTCTATTTGACTACACAGT  | 345               |
| RW_F2  | Forward   | CAAAACCATAAATAGCACTGGAAATAG | 481               |
| RW_R   | Reverse   | TTGAGATACTGGAGGTCTTTAGAAATG | -                 |
| Seq_F1 | Forward   | TACCCAGGTGCACACCAATG        | 865               |
| Seq_R1 | Reverse   | CATGGATTGGGCTGTGGGATT       |                   |
| Seq_F2 | Forward   | ATGGGTACCCCATCCCTGAA        | 860               |
| Seq_R2 | Reverse   | TCCTTGGGTGGCATGGTAAC        |                   |

The same reverse primer was used for genotyping.

### Sequencing

The PCR products were sequenced and compared with sequences of the *TYR* gene (NCBI accession: NC\_006088.5) and intron 4 of the *TYR* gene with the *ev-1* insertion (GenBank: DQ118701.1) published in the NCBI database (<https://www.ncbi.nlm.nih.gov>). Another primer set with two forward and two reverse primers was produced from these sequences using Primer-BLAST on the NCBI website (Table 1). PCR amplification was performed at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, and a final 5 min extension at 72 °C. The amplified DNA fragments were purified with a PrimePrep PCR Purification Kit (GeNetBio) and sequenced using an automatic genetic analysis machine (ABI3730XL, Applied Biosystems, Foster

City, CA, USA).

## RESULTS AND DISCUSSION

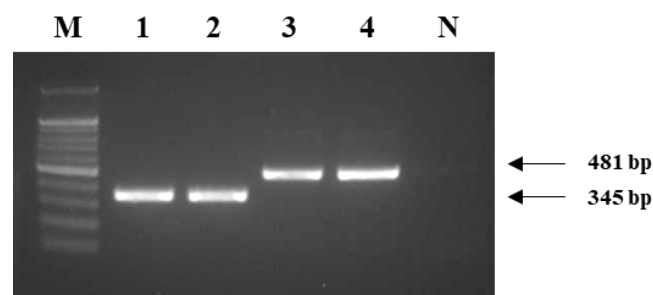
Only a few chicken breeds are entirely black like the Yeonsan Ogye. These include Ayam Cemani from Indonesia, H'Mong from Vietnam, Black Silkie from China, and Svarthona from Sweden [13,14]. In Asia, the nutritional value of black-bone chickens has long been known. Many studies have reported on the antioxidant and anti-inflammatory effects of immuno-active substances in black-bone chickens, such as carnosine and cytokine [15–19]. The Yeonsan Ogye is functional medicinal food. In the classical medical books “Dongeuibogam” by Heo and “Bonchogangmok” by Lee, the Yeonsan Ogye was considered effective for treating stroke, arteriosclerosis, and heart disease [20,21].

To maintain the Yeonsan Ogye population, the Yeonsan Ogye Foundation is cooperating with various livestock research institutes and universities [5]. However, there is no established system for selecting which males and females to breed other than selecting completely black individuals. Consequently, individuals with exceptional phenotypes continue to appear in the population.

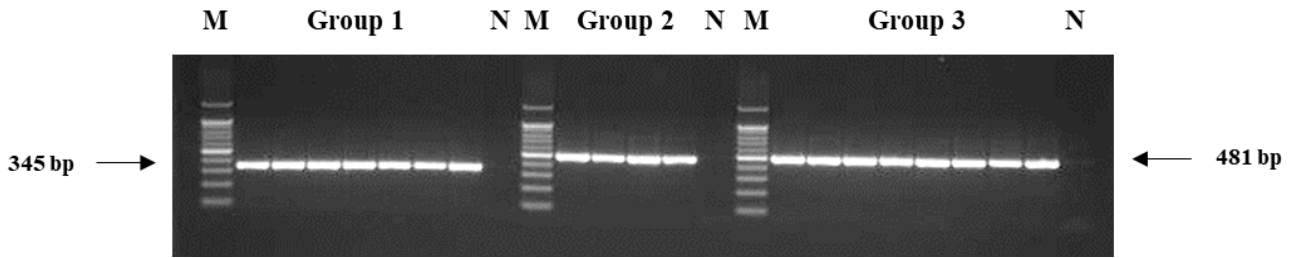
This study genotyped individuals with plumage that was not completely black, but either completely or partially white. PCR amplification products were confirmed using gel electrophoresis. Homozygous colored and recessive white individuals would produce a single 481 bp band on amplification with the primers RW\_F2 and RW\_R or a 345 bp band with the primers RW\_F1 and RW\_R, whereas heterozygous individuals would produce both bands (Fig. 4). The seven white chickens tested all produced one 345 bp band and were homozygous recessive white individuals. The four brindle and eight normal black chickens all produced one 481 bp band and were homozygous colored individuals. None of the individuals exhibited heterozygous patterns (Fig. 5).

The genotypes were confirmed by sequencing. On aligning the sequences with reference sequences (NCBI accession: NC\_006088.5 and GenBank: DQ118701.1), the white chickens had the same sequence as the recessive white type (GenBank: DQ118701.1). Similarly, the sequences of the normal and brindle chickens matched that of the wild type (Fig. 6).

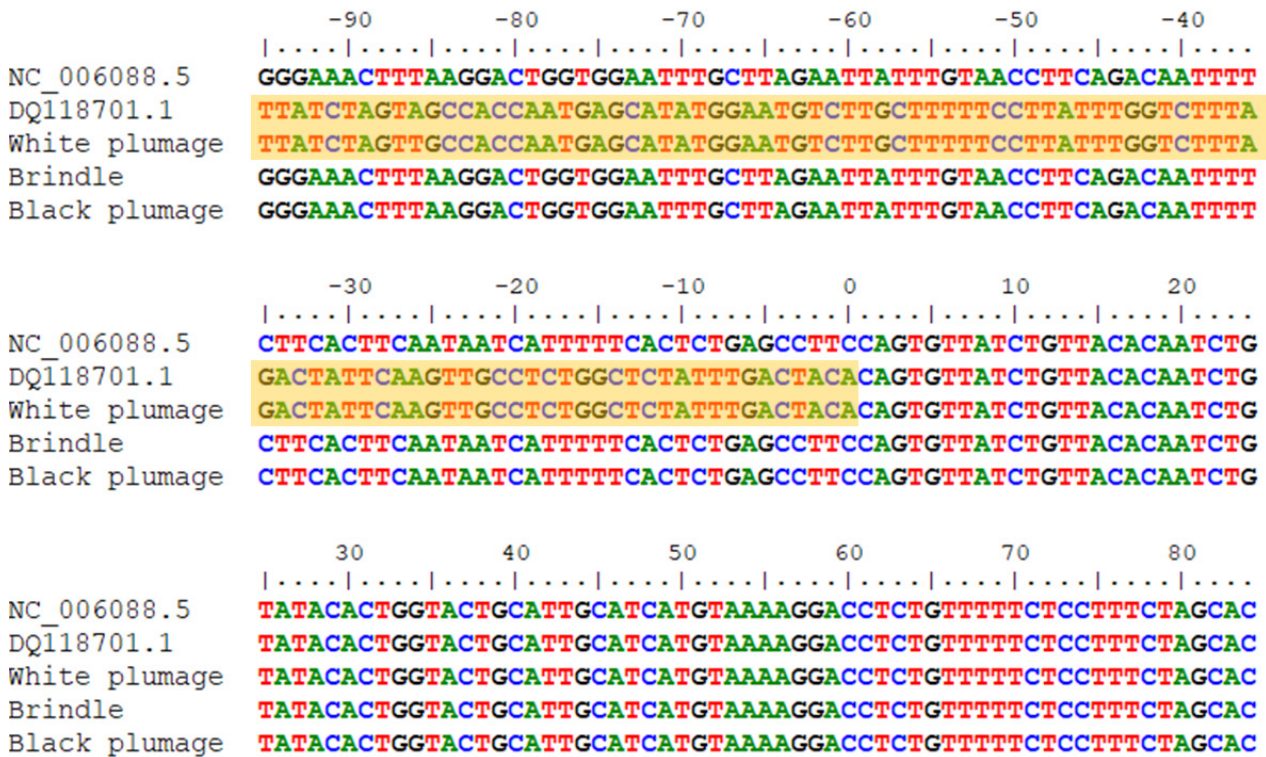
Comparing the white chicken sequences against the NCBI database using Nucleotide BLAST (BLASTN), the inserted retrovirus sequence exhibited 99% (278/279) identity (GenBank: DQ118701.1), as reported previously, and 98% (194/198) identity with the avian leukosis endogenous virus *ev-1* sequence (GenBank: AY013303). The other sequences were the same as the *TYR* mRNA reference sequence (NCBI accession: NM\_204160). Therefore, part of the retroviral



**Fig. 4. Results of genotyping using primers to distinguish between wild-type and recessive white alleles.** The lanes included the negative control lane are labeled as follows. A 345 bp band was produced by white Yeonsan Ogye chickens, whereas a 481 bp band was produced by brindle and normal chickens. M, 100 bp ladder marker; 1 and 2, white Yeonsan Ogye chickens; 3, brindle chicken; 4, normal black Yeonsan Ogye; N, negative control.



**Fig. 5. Genotyping all of the chicken samples.** The seven white chickens in Group 1 produced only one 345 bp band. The brindle (Group 2) and normal (Group 3) chickens produced only the 481 bp band. M, 100 bp ladder marker, N, negative control.



**Fig. 6. Alignment of the sequences of the three differently colored chickens with reference sequences.** All sequences included the end of intron 4. The white chicken sequence matched the reference recessive white chicken sequence (DQ118701.1). From base 1, the white chicken sequence is the same as the wild-type reference sequence (NC\_006088.5) and the sequences of the brindle and normal chickens.

sequence was inserted in the normal *TYR* intron 4. Retroviral insertion causes phenotypic changes by affecting the host genetic mechanism [22,23]. Specifically, this can be involved in pigmentation and control the expression of coat color [24,25]. The *KIT* gene is a proto-oncogene that causes cancer, but when a mutation occurs, it induces partial piebaldism [26,27]. The retroviral insertion identified here is located in *TYR* intron 4 and affects the expression of exon 5, a membrane-spanning domain that plays an important role in determining the location of melanosome enzymes [28]. Retroviral insertion interfered with the transcription of exon 5, resulting in impeded melanogenesis.

The exceptional phenotypes observed in the Yeonsan Ogye population include the recessive white mutation and a few individuals with a mixed plumage phenotype including yellow and gold coloration, similar to a brindle chicken. According to Yu et al. [29], four homeobox genes (*HOXB9*,

*HOXC8*, *HOXA9*, and *HOXC9*), two glutathione metabolism genes (*CHAC1* and *GPX3*), and the transforming growth factor beta signaling pathway are associated with plumage melanogenesis in black-bone chickens. Campo and Alvarez [30] found that the birchen allele ( $E^R$ ) at the *E* locus is affected by the melanotic gene (*Ml*) in the development of black plumage. Kerje et al. [31] reported that the extended black (*E*) locus controls plumage color in the same way as *MC1R*. Based on these results, an investigation of plumage color in Yeonsan Ogye can help with establishing a selection system to maintain its unique phenotype. It may also elucidate the reasons for the medicinal use of black Yeonsan Ogye.

As explained above, the Yeonsan Ogye has value as a genetic resource. But because only black individuals are selected to breed and there is no systematic breeding method, pedigree management has been poor. Additionally, because the individuals are selected phenotypically, it is impossible to confirm the presence or absence of mutant alleles. Therefore, to preserve the unique characteristics of the Yeonsan Ogye, genetic studies of specific phenotypes are required, and a new selection system for maintaining genetic diversity must be established.

## CONCLUSION

This study genotyped Yeonsan Ogye chickens and confirmed that white chickens had the recessive white (*c*) *TYR* allele. Sequencing showed that an ev-1 retroviral sequence was inserted at the end of *TYR* intron 4 in this allele. Since the size of the sample used in this study was limited, further research through securing additional samples to perform validation studies is necessary. Therefore, after validation studies, a selection system for conserving the phenotypic characteristics and genetic diversity of the population could be established if additional studies to elucidate specific phenotype-related genes in Yeonsan Ogye are performed.

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