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SNPs in the 5'-regulatory region of the tyrosinase gene do not affect plumage color in ducks (*Anas platyrhynchos*)

N.N. Zhang, J.W. Hu, H.H. Liu, H.Y. Xu, H. He and L. Li

Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agricultural University, Ya'an, Sichuan, China

Corresponding author: L. Li
E-mail: liliang@sicau.edu.cn

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ABSTRACT. Tyrosinase, encoded by the *TYR* gene, is the rate-limiting enzyme in the production of melanin pigment. In this study, plumage color separation was observed in Cherry Valley duck line D and F1 and F2 hybrid generations of Liancheng white ducks. Gene sequencing and bioinformatic analysis were applied to the 5'-regulatory region of *TYR*, to explore the connection between *TYR* sequence variation and duck plumage color. Four SNPs were found in the 5'-regulatory region. The SNPs were in tight linkage and formed three haplotypes. However, the genotype distribution in groups with different plumage color was not significantly different, and there were no changes in the transcription factor binding sites between the different genotypes. In conclusion, these SNP variations may not cause the differences in feather color observed in this test group.

Key words: Duck; Tyrosinase gene; Promoter; SNP; Plumage color

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INTRODUCTION

Animal coat and plumage color is determined mainly by the content of eumelanin and pheomelanin in the skin. Eumelanin biosynthesis and distribution is regulated by a large number of genes. Mutations in these genes may affect color variation, but interactions between genes can also control plumage color. Tyrosinase, encoded by *TYR*, is the rate-limiting enzyme in the production of melanin pigment (Sanchez-Ferrer et al., 1995). It is a copper-containing enzyme that catalyzes the first two steps in the melanin biosynthesis pathway, converting tyrosine to L-dihydroxy-phenylalanine (DOPA), which is subsequently converted to DOPA quinone (Cooksey et al., 1997). The enzyme determines which type of melanin (eumelanin or pheomelanin) is synthesized (Kwon et al., 1987; Ito et al., 2000).

Close to 200 mutations have been found in *TYR* (Stenson et al., 2009). Non-synonymous SNPs occurring in coding regions result in single amino acid polymorphisms, which may affect protein function and lead to pathogenic phenotypes (Capriotti et al., 2011). Previous studies have shown that *TYR* mutations are associated with albinism in many vertebrates, including humans (Oetting, 2000), rats (Blaszczyk et al., 2005), cattle (Schmutz et al., 2004), cats (Imes et al., 2006), chickens (Liu et al., 2010), and geese (Wang et al., 2014). Peking ducks have a mutation in exon 1 of *TYR*, but whether this affects the color traits of duck feathers has not been determined. To investigate whether variations in the 5'-regulatory region of *TYR* are related to the plumage color of ducks, and to determine possible mechanisms of variations in this region, Cherry Valley line D (white) and Liancheng white (white) F1 and F2 hybrid generation ducks were studied.

MATERIAL AND METHODS

Animals

Cherry Valley line D and Liancheng white hybrid F1 and F2 generation ducks were obtained from the Sichuan Agricultural University Waterfowl Breeding Experimental Farm. Ducks were raised in a poultry house by specialized staff and the different lines were under the same nutrition and management conditions. The F1 generation animals all had gray plumage, while the plumage colors of the F2 generation were black, white, gray, and spotted (black and white). Venous blood samples were collected from 109 ducks displaying the four different plumage colors, including eight gray F1 and 16 black, 35 white, 24 gray, and 26 spotted F2 ducks. Genomic DNA was prepared from blood using a standard phenol/chloroform method (Wang et al., 2014) and stored at -20°C for sequencing of the *TYR* promoter.

Sequencing and genotyping of *TYR*

Primers were designed based on the genome sequence of *TYR* from Peking duck (NCBI Reference Sequence: NW_004678775.1) using the Primer 5.0 (PREMIER Biosoft International, Canada) and the Oligo 6.0 (Molecular Biology Insights Inc., Cascade, CO, USA) softwares. The primers were as follows: 5'-AACAATGACAGCCGAAG-3' (forward) and 5'-CTGCTACACTACACCCTACAAT-3' (reverse). Polymerase chain reactions (PCRs) were performed using 1X Taq Master Mix (Takara Biotech Co., Dalian, China); 50 ng genomic DNA; and 0.2 µM of each primer, in a volume of 25 µL. Thermal cycling parameters were as follows: 95°C for 5 min; 36 cycles of 95°C for 45 s, 53.4°C for 45 s, and 72°C for 1 min; and 72°C for 10 min. Completed reactions were stored at 4°C until directly sequencing using an ABI Prism 3700 DNA sequencer (Shanghai Biotech Co., Shanghai, China).

Data analysis

Sequences were spliced and aligned using DNAMAN 6.0.3.99 (Lynnon Biosoft, USA). TYR haplotypes were deduced from the sequencing traces. Allele and genotype frequency determinations in the F2 generation, along with Chi-square tests for Hardy-Weinberg equilibrium, were performed using the PopGen32 software (Version 1.31) . Potential associations between TYR alleles and plumage color were evaluated with a Chi-square test for independence, using the TBM SPSS 19 software (International Business Machines Corp, USA). CpG islands (cytosine-guanine) were predicted using the CpGPlot software (http://www.bioon.com.cn/doc/showarticle.asp?newsid=1115). Promoter regions, TATA boxes, and transcription start sites were predicted by Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), and the Web Promoter Scan Service (http://www-bimas.cit.nih.gov/molbio/proscan/). The effects of TYR mutations on transcription factor binding sites were predicted using Signal Scan (http://www-bimas.cit.nih.gov/molbio/signal/).

RESULTS

We amplified 967 bp fragments (1094 to 128 bp) of the 5'-regulatory region of *TYR*. DNA sequencing detected four SNPs (570T>C, 495C>A, 366_365TG>CA, and 293A>C), all of which were found in F1 ducks, and in F2 ducks of each plumage color. There was evidence of tight linkage between the four mutations, with only two combinations of variations, defined as A (T-C-TG-A) and B (C-A-CA-C). Three haplotypes (AA, AB, and BB) were observed in the F1 and F2 generations (Figure 1).

The F2 generation group was in Hardy-Weinberg equilibrium for the variant sites (Chi-square analysis; P = 0.189). Allele and genotype frequency distribution in the F1 and F2 generations are shown in Table 1. Chi-square tests showed that the distribution of genotypes in F2 ducks with different plumage colors was not significantly different [$\chi^2(6) = 3.15$; P = 0.790].

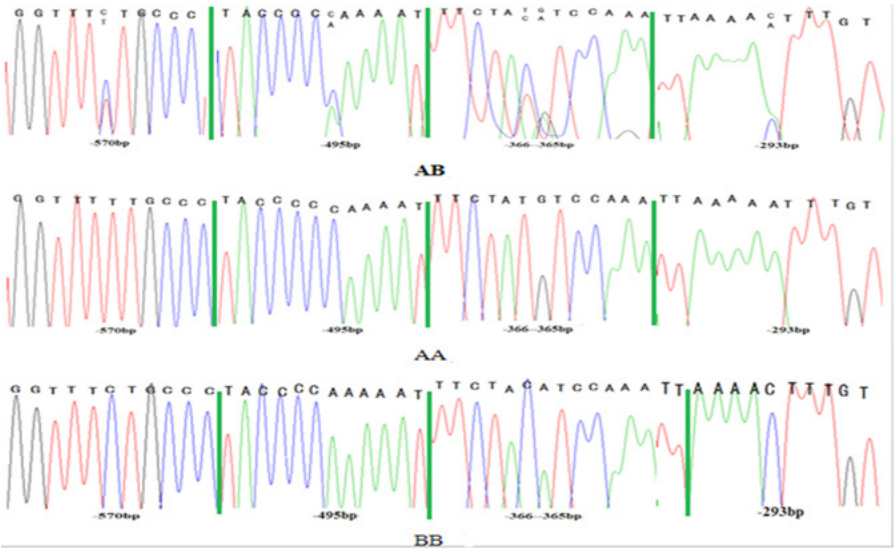

Figure 1. Sequencing results of four SNP loci from different genotypes. There were three haplotypes in the F1 and F2 generations. The four SNPs were in tight linkage, and with only two combinations of variations, defined as A (T-C-TG-A) and B (C-A-CA-C).

Table 1. Allele frequency and genotype frequency in ducks with different plumage colors.

Generation	Colour	Genotype frequency			Total	Allele frequency		Total
		AA	AB	BB		A	B	
F1	Gray	12.5% (1)	75.0% (6)	12.5% (1)	100% (8)	50.00%	50.00%	100%
F2	Black	31.3% (5)	50.0% (8)	18.8% (3)	100% (16)	53.33%	46.67%	100%
	White	14.3% (5)	48.6% (17)	37.1% (13)	100% (35)	36.76%	63.24%	100%
	Gray	20.8% (5)	45.8% (11)	33.3% (8)	100% (24)	41.30%	58.70%	100%
	Spotted	19.2% (5)	42.3% (11)	38.5% (10)	100% (26)	38.00%	62.00%	100%

*P > 0.05.

The GC content of the amplified *TYR* regulatory region was 35%, and no CpG island was predicted by CpGPlot. Using the Neural Network system, promoters were predicted at 791 to 741 and 200 to 150 bp on the forward strand, and at 411 to 472, 467 to 517, and 635 to 685 bp on the reverse strand (Table 2). The Web Promoter Scan Service predicted a promoter region on the reverse strand at 207 to 557, a TATA box at 432, and a transcription start site at 464 bp. Transcription factor binding sites predicted by Signal Scan did not differ for any of the described SNPs.

Table 2. Neural network promoter prediction.

Promoter predictions for sequence			Promoter sequence
Start	End	Score	
-791 bp	-741 bp	0.68	AAATTGCCTGTATTCTCCAAAAAACTGCAGAGATTCGGGTGCAGATTTGA
-200 bp	-150 bp	0.72	GTGTATTCTGTGTGTTAATGTGCCCAGGGTAGGAATGGGGAAGGACATTG
Promoter predictions for the reverse strand of sequence			
-411 bp	-472 bp	0.98	AATGATTGTTTAAAAAAACTCAGAAGAATCTGACCAATTAAATAAGCACA
-467 bp	-517 bp	0.67	GCACAGTGAATATAAACATGAGAAAAATATTTTTGGGGTAGACTACATTT
-635 bp	-685 bp	0.69	AGGCTAGCATATAACAAAGTGTCTTCACAAGAGAGCTGACAAACTTTTCA

DISCUSSION

TYR is an important candidate for the regulation of melanin biosynthesis. *TYR* mutations have been confirmed in mammals including humans, rats, mice, rabbits, and cattle, and are associated with albinism (Yokoyama et al., 1990, Aigner et al., 2000, Schmutz et al., 2004, Blaszczyk et al., 2005, Imes et al., 2006). Balu and Rituraj (2013) proposed that the structural and functional behavior of tyrosinase, upon mutation, could lead to skin albinism. Using an albino hybrid strain, researchers produced transgenic rabbits expressing the murine tyrosinase gene, and tyrosinase-transgenic rabbits showed a greater variety in hue, intensity and extent of coat pigmentation, which is caused by the diversity in the loci affecting the melanization (Aigner et al., 1996). The expression level of *TYR* in dark-iris rabbits was significantly higher than that in non-pigmented rabbits, and the study concluded that the 1118C>A substitution affected tyrosinase function, and that the level of *TYR* expression is related to iris color in rabbits. Studies in humans have found that tyrosinase activity in the skin is higher for black people, compared to white people (Pomerantz and Ances, 1975, Iwata et al., 1990). A study in Suffolk sheep demonstrated that albinism displays autosomal recessive inheritance. Histochemical tests of sheep revealed defective melanin synthesis at the tyrosine to DOPA conversion step, but not the subsequent reactions that lead to melanin (Rowett et al.,1993).

Mochii et al. (1992) cloned and analyzed chicken TYR, and observed a significant difference in the expression level of TYR mRNA in feather pulp between black and grayish-white Bian chickens. Real-time quantitative PCR experiments showed that *TYR* expression levels influence feather color of both half- and full-Muscovy ducks (Zheng et al., 2013). Wang et al. (2014) detected three SNPs in a cloned 588 bp fragment of the *TYR* exon 1, and suggested an association between *TYR* polymorphisms and plumage color in domestic geese.

In mammals, TYR structure is regulated by the 5'-regulatory region, and shows different pigment deposition phenotypes (Methot et al., 1995). Single-strand conformational polymorphism analysis was applied to the upstream regulatory region of chicken *TYR*, from 641 to 2125 bp. Three SNPs were found in this region and results indicated that these mutations were significantly associated with shank and body skin color in chickens (Chen et al., 2005). Correlations between SNPs and pigment traits in parental and F2 generations of China Agricultural University chicken populations were analyzed by Chi-square test; these results also indicated that the variants were significantly associated with shank and skin color. In our study, four SNPs that were in tight linkage were found in the 5'-regulatory region of *TYR* from 1094 to 128 bp. These SNPs formed three haplotypes. However, there was no significant difference in genotype distribution between F2 ducks with different plumage colors. Bioinformatic analysis of these sequences showed that, although promoter regions and a TATA box may exist in this segment, transcription factor binding sites did not differ between the genotypes. We speculate that variations in this region do not cause changes in expression of *TYR*, and are thus not the cause of the differences in feather color seen in this study group. Melanin synthesis is an extremely complicated process, and the molecular mechanisms of plumage color determinants in Cherry Valley and Liancheng white hybrid ducks remain to be determined.

Conflicts of interest

The authors declare no conflict of interest.

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