

A genome-wide association study identifies novel single nucleotide polymorphisms associated with dermal shank pigmentation in chickens

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ABSTRACT Shank color of domestic chickens varies from black to blue, green, yellow, or white, which is controlled by the combination of melanin and xanthophylls in dermis and epidermis. Dermal shank pigmentation of chickens is determined by sex-linked inhibitor of dermal melanin (*Id*), which is located on the distal end of the long arm of Z chromosome, through controlling dermal melanin pigmentation. Although previous studies have focused on the identification of *Id* and the linear relationship with barring and recessive white skin, no causal mutations have yet been identified in relation to the mutant dermal pigment inhibiting allele at the *Id* locus. In this study, we first used the 600K Affymetrix Axiom HD genotyping array, which includes ~580,961 SNP of which 26,642 SNP were on the Z chromosome to perform a genome-wide association study on pure lines of 19 Tibetan hens with dermal pigmentation shank and 21 Tibetan hens with yellow shank to refine the *Id* location. Association analysis was conducted by

the PLINK software using the standard chi-squared test, and then Bonferroni correction was used to adjust multiple testing. The genome-wide study revealed that 3 SNP located at 78.5 to 79.2 Mb on the Z chromosome in the current assembly of chicken genome (galGal4) were significantly associated with dermal shank pigmentation of chickens, but none of them were located in known genes. The interval we refined was partly converged with previous results, suggesting that the *Id* gene is in or near our refined genome region. However, the genomic context of this region was complex. There were only 15 SNP markers developed by the genotyping array within the interval region, in which only 1 SNP marker passed quality control. Additionally, there were about 5.8-Mb gaps on both sides of the refined interval. The follow-up replication studies may be needed to further confirm the functional significance for these newly identified SNP.

Key words: chicken, dermal pigmentation, shank

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INTRODUCTION

Shank color of domestic chickens varies from black to blue, green, yellow or white, which is determined by loci inhibitor of dermal melanin (*Id*), extended black (*E*), yellow legs (*W*), and other regulatory genes including sex-linked barring (*B*), dominant white (*I*), and the recessive allele at the sex-linked yellow skin locus (*y*; Knox, 1935) through affecting the presence or absence of melanin and xanthophylls in the dermis and epidermis. The loci *Id*, *B*, and *y* were found to be linked and the linear order was *B-Id-y*, with *B* on the long arm

of the Z chromosome distal from the centromere and *Id* was 13.7 cM from *B* (Bitgood, 1988). Therefore, the *Id* locus is located on the distal end of the long arm of Z chromosome (Dorshorst et al., 2010) controlling dermal melanin pigmentation. The *Id* gene expressed progressively in the recessive homozygote (Wang et al., 2006) and the shank color changed gradually from light to dark with increased age up to 8 wk (Kang et al., 2002). The *E* locus affects the relative distribution of melanin pigmentation in epidermis, making chicken shank gray to black. Yellow pigmentation in epidermis is determined by the *W* locus, at which the *W* allele inhibits epidermal xanthophyll pigmentation and is completely dominant to *w* (Knox, 1935). Alleles such as *B* have an inhibiting effect on shank melanin pigmentation (Dorshorst and Ashwell, 2009). Dominant white and the recessive allele at the sex-linked *y* will remove epider-

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mal shank pigment and dilute dermal pigment (Knox, 1935). Multiple loci affect shank pigmentation making shank color a complex genetic trait. Previous studies of genome-wide SNP-trait association analysis in Silkie chickens demonstrated that 2 SNP located at 68.6 and 78.8 Mb on chromosome Z in the chicken genome (galGal4) were significantly associated with dermal pigmentation, suggesting that the *Id* locus was located at 78.8 Mb on chromosome Z (Dorshorst et al., 2010). However, no causal sequence variations have yet been identified in relation to the mutant dermal pigment inhibiting allele at the *Id* locus.

Tibetan chickens, a unique breed native to high altitude, have dermal pigmentation shank and yellow shank. Tibetan chickens have higher gene diversity and higher numbers of alleles because little selection has been performed on this breed (Chen et al., 2008). The 600K Affymetrix Axiom HD genotyping chicken array is a more powerful platform for polymorphism detection in the whole genome of the chicken (Kranis et al., 2013) to identify sequence variations influencing dermal shank pigmentation of chickens. In this study, we first used the 600K chicken SNP array to perform genome-wide association studies on pure lines of Tibetan hens with dermal pigmentation shank and yellow shank to refine genomic regions responsible for chicken dermal shank pigmentation.

MATERIALS AND METHODS

Birds and Sample Collection

Tibetan pure-line hens were raised in individual cages. Forty hens without extended black, sex-linked barring, and white feather phenotypes were selected in which 19 hens exhibited dermal pigmentation shank and 21 hens exhibited yellow shank. The blood samples were collected from brachial veins of chickens by standard venipuncture along with the regular quarantine inspection of the experimental station of China Agricultural University. Genomic DNA was isolated from blood samples using standard phenol-chloroform extraction. The whole procedure was carried out in strict accordance with the protocol approved by the Animal Welfare Committee of China Agricultural University (Permit Number: XK622).

Genotyping and Quality Control

The 600K Affymetrix Axiom HD genotyping array, which includes ~580,961 SNP of which 26,642 SNP were on the Z chromosome, was used to genotype samples. The genotyping work was carried out by Bioassay Laboratory of CapitalBio Corporation (Beijing, China). Samples were removed due to low (<85%) call rate. The SNP were excluded if SNP call rates were less than 90% or Z chromosome minor allele frequencies were <0.5% or *P*-values for Hardy-Weinberg equilib-

Table 1. Single nucleotide polymorphisms (SNP) showing significant association with dermal shank pigmentation of chickens

SNP	Position (bp)	<i>P</i> -value	Bonferroni correction
rs315631308	78,518,678	6.14E-09	0.0027
rs317250881	79,161,331	3.78E-08	0.0166
rs315850000	79,164,223	5.04E-08	0.0221

rium tests were <1.00E-13. After quality control, all samples passed the filter, and a final set of 439,000 SNP was selected for the final association analysis of which 24,932 SNP were on Z chromosomes.

Statistical Analysis

Association analysis was conducted by the PLINK software (Purcell et al., 2007) using the standard chi-squared test, and then Bonferroni correction (Benjamini and Hochberg, 1995) was used to adjust for multiple testing.

RESULTS AND DISCUSSION

The genome-wide study revealed that 3 SNP (Table 1) located at 78.5 to 79.2 Mb on the Z chromosome in the current assembly of the chicken genome (galGal4) were significantly associated with dermal shank pigmentation of chickens. However, there were about 5.8-Mb gaps in SNP coverage flanking the refined interval (Figure 1), and only 15 SNP markers developed within this interval on the array of which only one SNP marker passed quality control. The complex genetic context in this region indicates the difficulty of the identification of causal sequence variations in relation to the mutant dermal pigment inhibiting allele at the *Id* locus.

The 3 SNP define a 646-kb region (78,518,678–79,164,223) that spans aldehyde dehydrogenase 7 family, member A1 (*ALDH7A1*), tripartite motif containing 36 (*TRIM36*), protein geranylgeranyltransferase type I, beta subunit (*PGGT1B*), Fem-1 homolog C (*Caenorhabditis elegans*; *FEM1C*), and GRAM domain containing 3 (*GRAMD3*). Three genes, methylthioadenosine phosphorylase (*MTAP*), and cyclin-dependent kinase inhibitor 2A and 2B (*CDKN2A*, *CDKN2B*), are just on the edge of the refined genome regions, which, together with *TRIM36* and *PGGT1B*, are within the *B* locus (Dorshorst and Ashwell, 2009). Mutations in *CDKN2A/B* are associated with sex-linked barring in chickens (Hellstrom et al., 2010), indicating that the region containing *CDKN2A/B* is the probable location of the *B* locus. These results suggest that the *Id* locus is very close to the known causal mutation of the *B* locus. Punnett (1940) reported that the distance between *B* and *Id* was about 10 cM, and Bitgood (1988) measured that genetic distance as 13.7 cM. Therefore, the physical distance should be around 3 to 4 Mb according to the recombination rate of chicken Z chro-

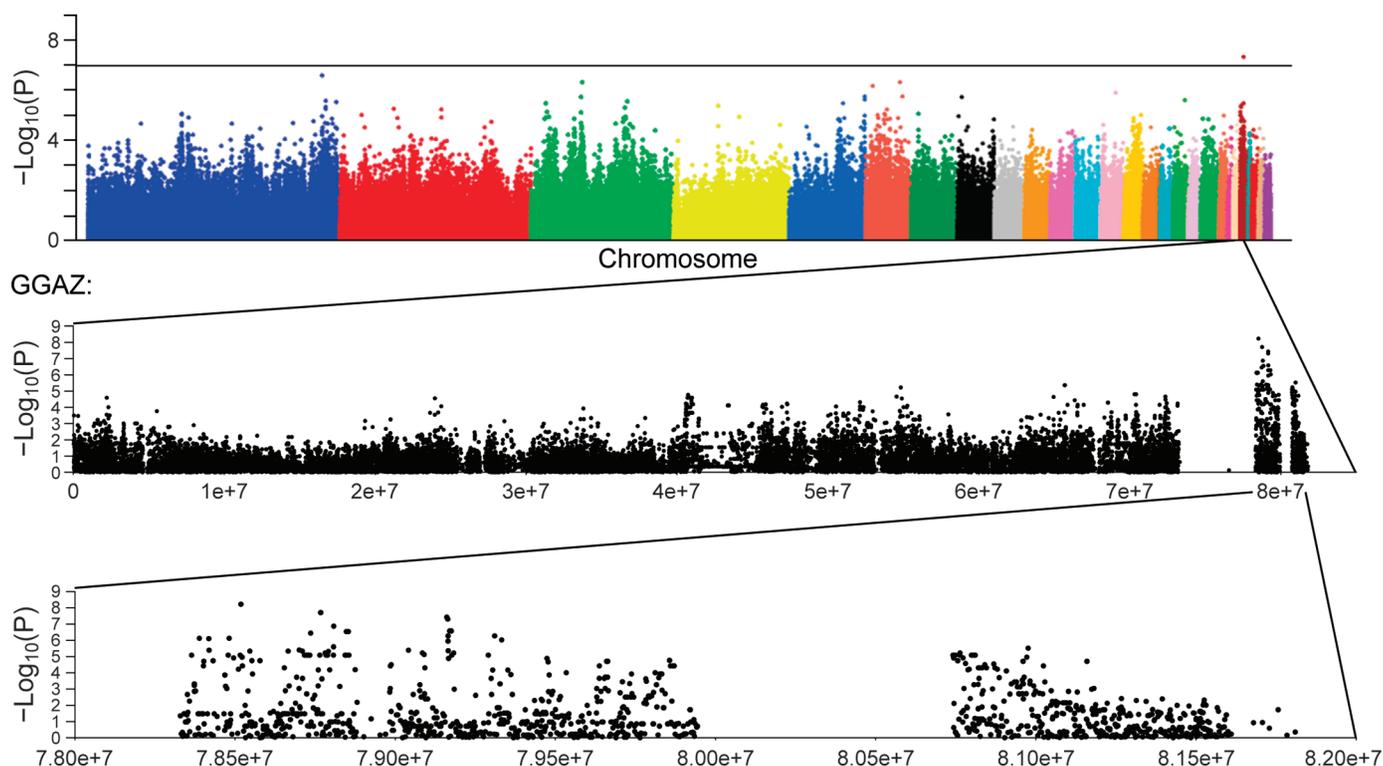


Figure 1. Genome-wide scan for dermal shank pigmentation: $-\log(P\text{-value})$ analysis for association with single nucleotide polymorphisms (SNP). The horizontal lines indicate the genome-wide significance threshold: $-\log(1.1E-07)$ and zoomed view of the distal area of the Z chromosome where the 3 SNP are located.

mosome (Elferink et al., 2010). However, regions near chromosome ends tend to have elevated recombination rates (Jensen-Seaman et al., 2004; Tortereau et al., 2012), and Wahlberg et al. (2007) specifically demonstrated a recombination hot spot at this region in the Z chromosome. A repetitive unit (Z amplicon) containing hundreds of copies of 4 genes is found to constitute 11 Mb of the most distal of the Z chromosome (Bellott et al., 2010), in which the interval associated with chicken dermal shank pigmentation in this study and the region containing the *B* locus, which has been defined as a 355-kb sequence (Dorshorst and Ashwell, 2009), are included. Therefore, the physical distance and genetic distance are not always a simple linear relationship, some genes were found to be much closer to each other than was predicted from their genetic distance (Majewski and Ott, 2000). High-resolution techniques will be necessary to identify the physical distance between *Id* and *B* locus.

A previous study demonstrated that SNP rs14686603 showing significant association with *Id* was located at 78.8 Mb on chromosome Z (galGal4; Dorshorst et al., 2010), and Siwek et al. (2013) reported that the *Id* locus was located at 79.4 Mb on chromosome Z (galGal4), which corresponded well to our results, suggesting that the *Id* locus should be in or near our refined region. Certain genes in this region are involved in melanoma. The *ALDH7A1* gene, which encodes antiquitin, is associated with various forms of stress and disease such as hyperosmotic stress (Brocker et al., 2010), pyridoxine-

dependent epilepsy (Mills et al., 2010; Tlili et al., 2013), and papilloma of the cervix (Prokopczyk et al., 2009). Mice with homozygous disruptions of the *ALDH7A1* gene are reported to have a normal phenotype (Lexicon Genetics Inc., 2005). Rose et al. (2011) reported that in human the expression of *ALDH7A1* was highest in nodular melanoma, then in normal skin, and lowest in superficial spreading melanoma, which might reflect differences in the percentage of initiating melanoma cells. The *MTAP* and *CDKN2A* genes are also involved in human melanoma (Chatzinasiou et al., 2011). Variants in *MTAP* are significantly associated with overall melanoma risk (Kvaskoff et al., 2011). Overexpression of *MTAP* in superficial spreading melanoma results in reduced melanoma cell growth (Rose et al., 2011) and *MTAP* protein expression decreases from benign nevi to malignant melanomas and melanoma metastases, suggesting that downregulation of *MTAP* expression activates melanoma proliferation (Behrmann et al., 2003; Wild et al., 2006). *CDKN2A* is a melanoma susceptibility gene encoding the tumor suppressor p16 protein (Hussussian et al., 1994; Kamb et al., 1994). Mutations or partial loss of function in *CDKN2A* are associated with family melanomas (Milligan et al., 1998; Bishop et al., 2002; Yang et al., 2004), suggesting that *CDKN2A* is involved in melanophore activity.

In summary, this genome-wide association study analysis revealed that 3 SNP located at 78.5 to 79.2 Mb on the Z chromosome were significantly associated with dermal shank pigmentation of chickens, and the *Id*

gene should be in or near our refined interval regions. Further work is needed to confirm the functional significance of these newly identified SNP. High-throughput sequencing techniques may help us to find the causative variations in association with the mutant dermal pigment inhibiting allele at the *Id* locus.

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