



## Identification and Characterisation of Alternative Splice Variants of *Hoxb9* and Their Correlation with Melanogenesis in the Black-Boned Chicken

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### ■ Keywords

Homeobox B9 gene; Alternative splicing variant; Melanin; Chicken.



Submitted: 19/September/2018

Approved: 06/January/2019

### ABSTRACT

Homeobox B9 (HOXB9) gene has been demonstrated to be associated with melanogenesis in chicken plumage by high-throughput RNA sequencing. In this study, we cloned and characterised HOXB9 in black-boned chickens. Two alternative splice variants (HOXB9-1 and HOXB9-2) were identified in chicken feather bulbs. Expression analysis of HOXB9 in 11 different chicken tissues by RT-PCR indicated that the two transcripts were only expressed in the kidney, abdominal fat, feather bulbs, skin, and small intestine. No HOXB9-1 or HOXB9-2 transcripts were detected in the breast muscle or the ovary. The two HOXB9 variants were expressed at significantly different levels in black feather bulbs and white feather bulbs ( $p < 0.01$ ), and in black skin compared with white skin ( $p < 0.01$ ). The results indicate that HOXB9-1 and HOXB9-2 may be involved in melanin formation in the plumage and skin. The expression of HOXB9-2 was higher in white than in black muscles ( $p < 0.05$ ), HOXB9-2 may play a role in muscle melanogenesis. Our results suggest that HOXB9, which is partially regulated by alternative splicing, may be involved in the process of melanogenesis in the black-boned chicken.

### INTRODUCTION

Black-boned chickens demonstrate intense pigmentation (hyperpigmentation or fibromelanosis) of their tissues, including the skin, muscle, feathers, claws, gonads, periosteum, and mesentery (Faraco *et al.*, 2001; Dorshorst *et al.*, 2011; Yu *et al.*, 2017). Feather, skin, and muscle pigmentation phenotypes are important economic traits in black-boned chicken production, as chickens with black feathers, skin, and muscles are generally considered to have superior nutritional and medicinal properties (Tian *et al.*, 2007). Melanin-based pigmentation is a major component of chicken feather, skin, and muscle colouration that is often under strong genetic control (Bourgeois *et al.*, 2016). Many signalling pathways and numerous genes are involved in melanin biosynthesis, transport, transfer, and deposition (Cieslak *et al.*, 2011; Zhang J *et al.*, 2015; Zhang XD *et al.*, 2015). To date, more than 150 body colour-associated genes have been found to play well-known roles in pigmentation (Cieslak *et al.*, 2011). Therefore, further exploration of other candidate genes using different techniques may be required to elucidate the complex molecular mechanisms of melanin pigmentation.

In a previous study, we identified the expression of the Homeobox B9 (HOXB9) gene associated with black-boned chicken plumage melanogenesis, based on transcriptome analysis of black and white chicken feather bulbs using high-throughput RNA sequencing (Yu *et al.*, 2018). HOXB9 encodes nuclear protein functions as a transcription



factor involved in cell proliferation and differentiation (Seki *et al.*, 2012). HOXB9 was also identified as a Wnt target gene (Nguyen *et al.*, 2009). Wnt signalling is essential for the complex organogenesis of the skin and its appendages, such as hairs, feathers, and scales (Widelitz, 2008). Moreover, Wnt/ $\beta$ -catenin signalling plays a key role in melanocyte development, proliferation, and differentiation (Yamaguchi, 2004; Yamaguchi *et al.*, 2007; Cho *et al.*, 2009).

In order to further elucidate the role of the HOXB9 gene in melanin formation and deposition, we cloned the HOXB9 gene and identified two alternative splice variants in black-boned chickens, and analysed the relationship between the expression of these alternative splice variants and melanin synthesis in different tissues. Our results provide new insights to further the understanding of the molecular mechanisms of melanogenesis in the black-boned chicken.

## MATERIAL AND METHODS

### Ethics statement

All experiments involving animals were approved by the Animal Care and Use Committee of Leshan Normal University, China. The procedures were performed in accordance with the approved guidelines and regulations of the regional Animal Ethics Committee.

### Birds and sample collection

Eighteen 52-week-old female Muchuan black-boned chickens reared in the breeding farm of Muchuan County Black Phoenix Black-boned Chicken Industry Co. Ltd. were evaluated in this study. All chickens were reared under the same conditions. Birds were fed and

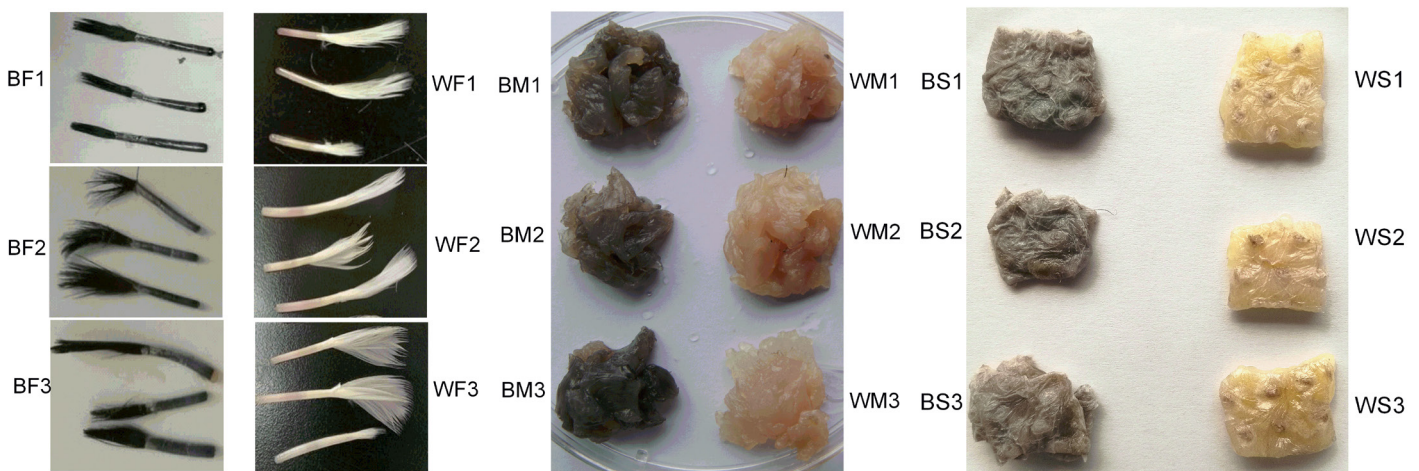
managed by the farm employees according to the poultry company's practices.

Birds were killed by exsanguination for the collection of skin, feather bulbs (black), kidney, small intestine, abdominal fat, liver, gizzard, breast muscle, heart, comb, and ovary tissues. These tissues were frozen in liquid nitrogen immediately after collection and stored at  $-80^{\circ}\text{C}$  prior to RNA extraction.

Feather bulbs were collected from three chickens with black (BF group: BF1, BF2, and BF3) or white (WF group: WF1, WF2, and WF3) dorsal plumage; muscle samples were obtained from three chickens with black (BM group: BM1, BM2, and BM3) or white (WM group: WM1, WM2, and WM3) breast muscle; and skin samples were obtained from three chickens with black (BS group: BS1, BS2, and BS3) or white (WS group: WS1, WS2, and WS3) skin (Figure 1).

### RNA extraction and reverse transcription

Total RNA from each sample was isolated using Invitrogen TRIzol reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. Reverse transcription was performed using the ProtoScript First Strand cDNA Synthesis Kit (NEB, Beijing, China), with the reaction system consisting of 1  $\mu\text{g}$  total RNA, 2  $\mu\text{L}$  d(T)23 VN (50  $\mu\text{M}$ ), 10  $\mu\text{L}$  M-Mulv reaction mix, 2  $\mu\text{L}$  M-Mulv enzyme mix, and diethylpyrocarbonate (DEPC)-treated water to reach a final reaction volume of 20  $\mu\text{L}$ . The reactions were incubated at  $42^{\circ}\text{C}$  for 1 h and terminated by heating at  $80^{\circ}\text{C}$  for 5 min. Reaction products were diluted to 100  $\mu\text{L}$  with DEPC-treated water for PCR, and stored at  $-20^{\circ}\text{C}$ .



**Figure 1** – Phenotypic characterisation. (a) Feather bulb colours of the two types of feather bulbs: black feather bulbs (BF) and white feather bulbs (WF). (b) Meat colour of the two types of breast muscle: black breast muscle (BM) and white breast muscle (WM). (c) Skin colour of the two types of skin: black skin (BS) and white skin (WS).



### Molecular cloning of chicken HOXB9 and its alternative splice variants

Primer H (Table 1) was designed to amplify the coding sequence of chicken HOXB9 using the cDNA sequence retrieved from the NCBI database (XM\_015299445.1). The PCR reaction, performed using 2× EasyPruf PCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions, generated a fragment of about 750 bp. The PCR products were verified on 1.5% agarose gels at constant 120 v for 30min, and the target bands were excised under UV light and purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The purified products were subcloned in the pEASY-T3 vector (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The positive clones with different lengths of chicken HOXB9 cDNA sequence were sequenced by TsingKe Biological Technology (Chengdu, China). Finally, two alternative splice variants of chicken HOXB9 were identified by sequencing.

**Table 1** – Primers used in this study.

Name	Sequence(5'-3')	Annealing temperature	Product length
H1	F: ATGTCCATTTCTGGGACTCTTAGC R: TCACTCCTTGCCCTGCTCTCT	60°C	744/822bp
HS-1	F: GATCTGTAAAGGCAGAGCCG R: CAGCTGGGTTGGTTTGATCTG	60°C	189bp
HS-2	F: CTGCAAGGGAAGGGATCGTT R: GTAGGAAAGAGGACGGCTCG	60°C	138bp
H2 (β-Actin)	F: AGGCGAGATGGTGAAAG R: CACGCTCCTGGAAGATAG	57°C	282bp

### Tissue distribution pattern of HOXB9 gene expression

Quantitative real-time PCR (qRT-PCR) was used to determine the mRNA levels of the two alternative splice variants of HOXB9 in the 11 tissues described above. The volume of each qRT-PCR reaction was 20μL, containing 1 μL reverse transcription reaction mix, 0.4 μL of each primer (10 μmol/L), 10 μL SYBR Green Master Mix (Vazyme, Nanjing, China), 0.4 μL 50× ROX Reference Dye, and 8.2μL ddH<sub>2</sub>O. qRT-PCR was performed using a StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as follows: 95°C for 5min followed by 40 cycles of 0 s at 95°C, 30 s at 60°C, and melt curve analysis from 60°C to 95°C. All reactions were performed in triplicate. To normalise gene expression, the β-actin gene was selected as internal control. Relative gene expression levels were calculated using the comparative Ct (ΔΔCt)

value method. The specific primers (HS-1 and HS-2, Table 1) for the two alternative splice variants (HOXB9-1 and HOXB9-2) were designed based on the unique sequence of the splicing boundary of each variant.

### Expression patterns of the HOXB9 gene in albino and black samples

To investigate the functions of the two alternative splice variants of HOXB9 in melanin biosynthesis in different tissues (feather bulbs, skin, and breast muscle), qRT-PCR was performed to determine the differential expression levels of HOXB9 between group BF and WF, group BM and WM, and group BS and WS. The gene-specific primers used are shown in Table 1. Statistical analyses were performed using Student's *t*-test for independent samples, *p*<0.05 was considered significant.

### Sequence analysis of the chicken HOXB9 gene

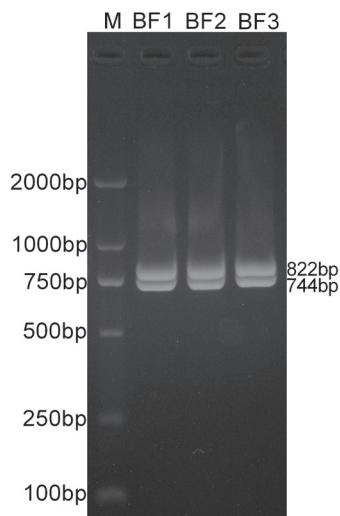
The obtained cDNA sequences were matched using DNAMAN software (version 8.0.8.789). BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) was used to identify homologous sequences in the NCBI database. The HOXB9 gene open reading frame (ORF) was predicted using the ORF Finder at NCBI (<https://www.ncbi.nlm.nih.gov/orffinder>). Chromas software (version 2.23; Technelysium, Tewantin, Australia) was employed to examine the sequence chromatograms. Related sequences were obtained from the Ensembl database (<http://www.ensembl.org>) and the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank>).

## RESULTS

### cDNA sequence analysis of the chicken HOXB9 gene

The chicken HOXB9 gene was amplified by RT-PCR using the primer H1. Two PCR products of different lengths were obtained from black feather bulbs (Figure 2), suggesting that the chicken HOXB9 gene may have two alternative splice variants, HOXB9-1 (GenBank accession number: MH048960) and HOXB9-2 (GenBank accession number: MH048961).

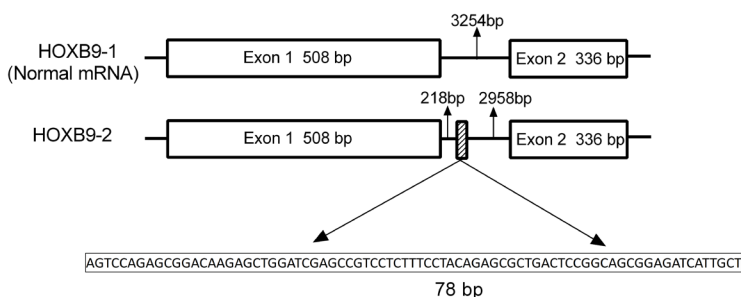
The HOXB9-1 transcript contains an ORF of 741base pairs (bp) and encodes a protein of 247 amino acids (aa). The HOXB9-2 transcript contains an 819-bp ORF, which is generated due to a 78-bp fragment inserted in the HOXB9-1 transcript, and encodes a 273-aa polypeptide.



**Figure 2** – Amplified products of the chicken *HOXB9* gene. Lane M, D2000 DNA Marker.

### Structural analysis of the chicken *HOXB9* gene

*HOXB9-1* has a standard gene structure and includes two exons and one intron, which have nucleotide sizes of 508, 336, and 3,254 bp, respectively. The total length of the *HOXB9-2* coding region is 822 bp, with a 78-bp insert at the 3' end of exon 1, which results in an insert of 26 amino acid residues (Figure 3) compared with the normal *HOXB9* gene. The sequence matching and alignment analysis revealed that the 78-bp insert was a partial sequence of the intron; thus, the type of alternative splicing is the partial retention of an intron in the mature transcript (Figure 3).

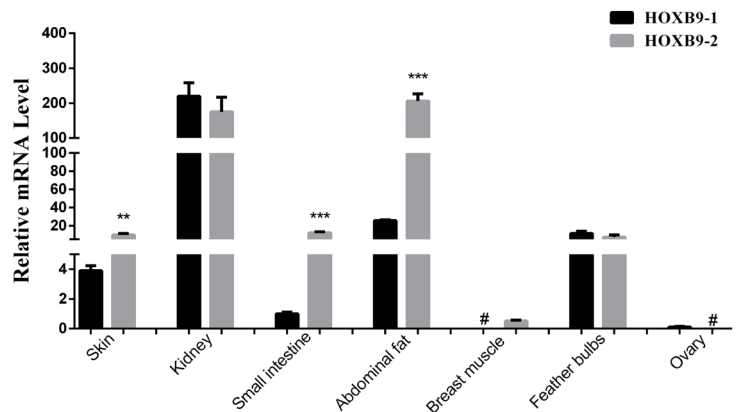


**Figure 3** – Schematic diagrams of the structures of alternative splice variants of the *HOXB9* gene. Exons are indicated by rectangles, whereas introns are indicated by black lines. The box containing diagonal lines indicates that the variant includes the partial sequence of an intron.

### Tissue distributions of two *HOXB9* transcripts: *HOXB9-1* and *HOXB9-2*

The mRNA levels of *HOXB9-1* and *HOXB9-2* in 11 tissues were evaluated by qRT-PCR. Neither transcript of *HOXB9* was expressed in the liver, gizzard, heart, or comb tissues. The highest expression levels of *HOXB9-1* and *HOXB9-2* were detected in the kidney, abdominal fat, feather bulbs, skin, and small intestine

tissues (Figure 4). *HOXB9-1* and *HOXB9-2* were weakly expressed in the ovary and breast muscle tissues, respectively. The mRNA level of *HOXB9-2* was significantly higher than that of *HOXB9-1* in skin ( $p < 0.01$ ), small intestine ( $p < 0.001$ ), and abdominal fat ( $p < 0.001$ ). No *HOXB9-1* or *HOXB9-2* transcripts were detected in the breast muscle and ovary, respectively.



**Figure 4** – The mRNA expression profiles of *HOXB9-1* and *HOXB9-2* in various tissues of the black-boned chicken. Expression levels of *HOXB9-1* and *HOXB9-2* within the same tissues were compared using *t*-test, and significant differences are indicated with asterisks (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). # indicates that no *HOXB9* mRNA was detected in the tissue. The mRNA level in small intestine tissue was defined as 1.0.

### Functional characterisation of the two *HOXB9* variants in melanogenesis in different tissues

The qRT-PCR analysis revealed that the mRNA levels of *HOXB9-1* and *HOXB9-2* were higher in the WF group than in the BF group (115.27-fold,  $p < 0.001$ , and 103.60-fold,  $p < 0.01$ ) (Figure 5a). The mRNA levels of *HOXB9-1* and *HOXB9-2* were 22.49- and 67.71-fold higher in the WS group than in the BS group, respectively ( $p < 0.01$ ) (Figure 5c). The mRNA level of *HOXB9-2* was significantly higher than that of *HOXB9-1* in breast muscle ( $p < 0.05$ ) (Figure 5b).

## DISCUSSION

Numerous studies have investigated pigmentation in animals (mammals, birds, and fish). A large number of genes, such as the genes for tyrosinase (TYR) (Chang *et al.*, 2006; Yu *et al.*, 2017), endothelin 3 (EDN3) (Dorshorst *et al.*, 2011), agouti-signalling protein (ASIP) (Voisey & Van 2002; Zhang J *et al.*, 2015), premelanosome protein (PMEL17) (Kerje *et al.*, 2004), and the Rab family (RAB27A) (Hume *et al.*, 2007), has been reported in different signalling pathways involved in melanin synthesis, pigment type switching, melanosome formation, and pigment transport, transfer, and deposition. However, the genetic mechanisms influencing melanin-based colouration



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**Figure 5** – HOXB9 mRNA expression levels in various white and black tissue samples from black-boned chickens. The relative mRNA expression of the two variants were compared between white and black (a) feather bulbs, (b) breast muscle, and (c) skin. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

remain poorly characterised. The melanogenesis in the black-boned chicken provides an excellent system for studying the molecular mechanisms of melanin pigmentation. Our previous study found that the HOXB9 gene was associated with melanin formation in plumage, and was identified as a pigmentation gene in the chicken (Yu *et al.*, 2018). However, the structure of chicken HOXB9 and its role in melanogenesis, have not yet been elucidated.

In this study, we cloned and characterised HOXB9 in the Muchuan black-boned chicken. Two alternative splice variants were identified in black feather bulbs. Alternative splicing (AS) is a post-transcriptional process that regulates gene expression by increasing protein complexity and modulation of mRNA transcript levels. AS occurs in vertebrate, invertebrate, plant, and fungal genomes (Cheah *et al.*, 2007; Mcguire *et al.*, 2008; Simpson *et al.*, 2010; Mudge *et al.*, 2011). In humans, transcripts from 95% of multi-exon genes undergo AS (Pan *et al.*, 2008), whereas approximately 35% of genes undergo AS in *Arabidopsis* and rice (Simpson *et al.*, 2010). AS events fall into five well-known types of AS patterns, including alternative 5' and 3' splice site selection, intron retention, exon skipping, and mutually exclusive exon splicing (Lu *et al.*, 2015). Our study showed partial intron retention in HOXB9-2.

Many alternatively spliced isoforms are known to have tissue-preferential expression patterns, and differential AS has been demonstrated to play important roles in various biological processes (Thatcher *et al.*, 2014). In this study, we found that the two alternative splicing variants of HOXB9 showed tissue-specific expression patterns, and no expression was found in the liver, muscular stomach, heart, and comb tissues. The higher HOXB9 mRNA level in the skin tissue allows for its functional role in skin development. In addition, the mRNA level of HOXB9-2 is significantly higher than that of HOXB9-1 in skin, small intestine, and abdominal fat tissue. HOXB9-2 may play a specific role in the growth of those tissues in black-boned chickens.

HOXB9-encoded nuclear protein functions as a sequence-specific transcription factor that is involved in the proliferation and differentiation of cancer cells (Hayashida *et al.*, 2009; Seki *et al.*, 2012). However, there have been no reports to date regarding the effects of HOXB9 in the pigmentation of animal skin or coats. Lin *et al.* (2018) reported that HOXB9 can promote melanoma growth and that the protein inhibits cell apoptosis in a mouse xenograft model (Lin *et al.*, 2018). To analyse the potential roles of the two transcripts of HOXB9 in melanin biosynthesis in black-boned chicken skin, the mRNA levels of HOXB9-1 and HOXB9-2 were detected and comparatively analysed in black and white samples. The results showed that HOXB9 was down regulated in black samples, and that the expression of the two transcripts of HOXB9 was significantly different between black and white tissue samples (in feather bulbs and skin). Current evidence suggests that HOXB9-1 and HOXB9-2 may play a role in melanogenesis of the feathers and skin of chickens. These data are also consistent with the RNA sequencing analysis results in our previous study (Yu *et al.*, 2018). Interestingly, HOXB9-1 was undetectable in both black and white breast muscle tissues, whereas the mRNA levels of HOXB9-2 were associated with black and white muscle colours. The results of this study clearly identify HOXB9 as a pigmentation gene that may be involved in melanin biosynthesis in chicken feathers, skin, and muscle tissue. Further studies are needed to characterise the molecular mechanisms of HOXB9 and its two transcripts in melanogenesis in the chicken.

In conclusion, we report the cloning and functional expression of two alternative splicing variants (HOXB9-1 and HOXB9-2) of the *HOXB9* gene. Our findings demonstrate the significant associations of the mRNA levels of the two variants with melanogenesis in the feather bulb and skin. Only HOXB9-2, which may play a potential role in muscle melanogenesis, was significantly differentially expressed between black and white muscles ( $p < 0.05$ ). These findings provide



new insight into our understanding of the biochemical characteristics and physiological role of chicken *HOXB9*.

## ACKNOWLEDGEMENTS

This work was supported by Sichuan Science and Technology Program (2018JY0452). This work was also funded by Scientific Research Plan Projects of Locality and University Cooperation of Leshan Normal University (HX170068), and the Scientific Research Project by Leshan Normal University (Z16020).

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