LIÈGE université
Gembloux
Agro-Bio Tech

The genetic mechanism underlying the formation of white feathers in Liancheng ducks

Zhen WANG

Promotors:

Prof. Martine SCHROYEN

Prof. Zhengkui ZHOU

2025

COMMUNAUTÉ FRANÇAISE DE BELGIQUE

UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

The genetic mechanism underlying the formation of white feathers in Liancheng ducks

Zhen WANG

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur: Martine SCHROYEN

Co-promoteur. Zhengkui ZHOU

2025

Résumé

Zhen Wang. (2025). Étude des mécanismes génétiques impliqués dans la formation de la couleur unique du plumage chez les canards Liancheng (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 128 pages, 8 tableaux, 21 figures.

Les populations de canards F2 issues des canards Liancheng et Pékin présentent une viande de haute qualité nutritionnelle et un grand gabarit, tout en se distinguant par la couleur du plumage. Notre objectif est de produire rapidement des canards de chair de haute qualité, caractérisés par un plumage blanc et un bec noir, des traits associés à une production de viande de haut qualité. Les méthodes traditionnelles de sélection ne permettent pas d'atteindre cet objectif efficacement, car elles ne produisent pas de manière fiable le phénotype désiré et n'assurent pas l'obtention d'individus hétérozygotes. Cette limitation empêche également la protection de nos recherches par brevet. Cette thèse vise donc à identifier les gènes responsables du phénotype unique du plumage chez les canards Liancheng. Elle poursuit trois objectifs (1) identifier la répartition de la mélanine dans différents tissus chez les canards Liancheng et d'autres races, (2) explorer le mécanisme génétique du plumage blanc chez les canards Liancheng, (3) appliquer les marqueurs moléculaires identifiés dans cette étude pour accélérer la sélection génétique de canards de chair de haute qualité.

Dans un premier temps, 9 canards — colverts (n=3), Liancheng (n=3) et Pékin (n=3) — ont été analysés par chromatographie liquide haute performance (HPLC) et coloration Masson-Fontana afin de révéler les différences de teneur en mélanine dans les plumes. Des tissus pectoraux, cutanés, hépatiques, adipeux, cérébraux, cardiaques, rénaux, pulmonaires et spléniques de canards à plumage noir âgés de 8 semaines ont été prélevés pour identifier les gènes candidats par analyse de leur expression. Les résultats ont montré que la teneur en mélanine dans les plumes, le bec et les palmures des canards Liancheng était significativement plus élevée que chez les canards Pékin (p<0,05). La mélanine dans les follicules plumifères était localisée dans la crête barbiculaire et la matrice des plumes chez les canards à plumage noir. Cette étude a révélé pour la première fois le phénotype unique de la couleur du plumage chez les canards Liancheng, et a mis en évidence des variations dans l'expression des gènes candidats impliqués dans les voies de formation de la mélanine selon les tissus et les stades de développement.

Dans un second temps, 1,29 Gb du génome du canard Liancheng a été assemblé, avec un ‘contig’ N50 de 12,17 Mb et un ‘scaffold’ N50 de 83,98 Mb. Au-delà de l’effet épistatique du gène *MITF*, l’analyse GWAS a permis d’identifier une région génomique de 0,8 Mb contenant le gène *PMEL*. Ce gène code une protéine spécifique aux cellules pigmentaires, essentielle à la formation de feuillets fibrillaires dans les mélanosomes, les organites responsables de la pigmentation. De plus, une analyse par bioluminescence à double luciférase a révélé deux SNP candidats (Chr33:5,303,994A>G ; 5,303,997A>G) susceptibles de modifier la transcription du gène *PMEL*, influençant potentiellement la coloration du plumage chez les canards Liancheng. Notre étude a ainsi permis d’assembler le génome de référence du canard Liancheng et a apporté des preuves solides que le plumage blanc caractéristique de cette race est attribuable au gène *PMEL*, auparavant considéré comme un “gène manquant” chez les canards.

Enfin, nous avons utilisé ces deux marqueurs moléculaires SNP pour réaliser une expérience d’hybridation. Dans cette expérience, un mâle de génotype bbrr, à plumage blanc et bec jaune, a été croisé avec 8 femelles *BBrr*, toutes à plumage blanc et bec noir. Les 56 descendants commerciaux Bbrr (génération F1) étaient tous à plumage blanc et bec noir. Après hybridation continue des individus F1, les phénotypes et proportions des 224 descendants F2 correspondaient à nos attentes : 61 canards bbrr à plumage blanc et bec jaune, et 163 canards *B_rr* à plumage blanc et bec noir, selon le ratio mendélien de 1:3. Ensuite, 30 canards Pékin ont été croisés avec 120 canards Liancheng, et 602 échantillons sanguins de canards F2 à plumage blanc et bec jaune ont été collectés. Parmi eux, 153 canards de génotype bbrr ont été sélectionnés avec succès. À l’avenir, ces 153 canards seront croisés avec des canards *BBrr* à plumage blanc et bec noir pour produire des canards *Bbrr* à plumage blanc et bec noir. Nous avons ainsi développé une solution complète pour l’élevage de races de canards de chair de haute qualité.

Mots-clés : canards Liancheng, canards Pékin, couleur du plumage, mélanine, gène *MITF*, gène *PMEL*, sélection moléculaire.

Abstract

Zhen Wang. (2025). Study of the genetic mechanisms involved in the formation of unique feather color in Liancheng ducks (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, Liege University, 128 pages, 8 tables, 21 figures.

The F2 duck populations derived from Liancheng Ducks and Pekin Ducks exhibit high nutritional meat quality and large size bodies, while they are segregated by feather color. Our goal is to rapidly produce high-quality meat ducks characterized by white feathers and black beaks, traits associated with premium meat production. Traditional breeding methods are insufficient for achieving this goal efficiently, as they cannot reliably produce ducks with the desired phenotype nor ensure heterozygous genotypes. This limitation also prevents us from protecting our research through patenting. Therefore, this PhD looks at revealing causative genes for unique plumage phenotype in Liancheng ducks. The purpose is three-fold: (1) to identify the melanin deposition in different tissues of the Liancheng and other ducks, (2) to explore the genetic mechanism of white plumage in Liancheng ducks, (3) to apply the molecular markers explored in this study to expedite the genetic breeding of high-quality meat ducks.

Firstly, 9 ducks, Mallards (n=3), Liancheng (n=3) and Pekin ducks (n=3), were examined by high performance liquid chromatography (HPLC) and Masson-Fontana staining to reveal the difference of feather melanin content. Pectoral, skin, liver, fat, brain, heart, kidney, lung, and spleen tissue of 8-week-old black-feathered ducks were collected to identify candidate genes by analyzing their expression levels. The results showed that the melanin content in feathers, beak, and web of Liancheng ducks were higher than that in Pekin ducks ($p<0.05$). Melanin within hair follicle was located in the barb ridge and hair matrix of black feathered ducks. This study firstly revealed the unique feather color phenotype of Liancheng ducks, and highlighted variations in the expression of candidate genes involved in the melanin formation pathways across different tissues and developmental stages.

Secondly, 1.29 Gb of the Liancheng duck genome was assembled involving a contig N50 of 12.17 Mb and a scaffold N50 of 83.98 Mb. Beyond the epistatic effect of the *MITF* gene, GWAS analysis pinpointed a 0.8Mb genomic region encompassing the *PMEL* gene. This gene encoded a protein specific to pigment cells and was essential for the formation of fibrillar sheets within melanosomes, the organelles responsible for pigmentation. Additionally, Dual-luciferase reporter analysis revealed two candidate SNPs (Chr33:5,303,994A>G; 5,303,997A>G) that might alter *PMEL* transcription, potentially influencing plumage coloration in Liancheng ducks. Our study assembled the reference genome of Liancheng ducks, and presented compelling evidence that the white plumage characteristic of this breed was attributable to the *PMEL* gene, previously regarded as a “missing gene” in ducks.

At last, we used the two molecular SNP markers to carry out a hybridization experiment. In this experiment, first, 1 *bbrr* genotype male, that was white-feathered

and had a yellow beak, was crossbred with 8 *BBrr* genotype females, all white-feathered and with black beaks. All 56 *Bbrr* commercial descendants (F1 generation) were white-feathered, with black beaks. After continued hybridization of the F1 individuals, the phenotypes and proportions of the 224 F2 offspring were in line with our expectations. Specifically, this included 61 *bbrr* genotype ducks with white feathers and yellow beaks and 163 *B_rr* genotype ducks with white feathers and black beaks, adhering to the Mendelian ratio of 1:3. Then, 30 Pekin ducks were crossbred with 120 Liancheng ducks, 602 F2 blood samples of the white-feathered yellow beak ducks from the F2 population were collected and 153 *bbrr* genotype ducks with white feathers and yellow beaks were successfully selected. In the future, these 153 ducks will be crossbred with *BBrr* white-feathered black beak ducks to produce *Bbrr* white-feathered black beak ducks. We thus have successfully developed a comprehensive system solution for the breeding of high-quality duck breeds.

In summary, this thesis sheds light on the complex mechanisms of underlying the identification of causative genes for the unique plumage phenotype in Liancheng ducks and offers valuable insights and direction for future research and breeding programs aimed at understanding and influencing avian plumage coloration.

Keywords: Liancheng ducks, Pekin ducks, feather color, melanin, *MITF* gene, *PMEL* gene, molecular breeding.

Acknowledgements

It’s my great honor to take this opportunity to express my special thanks to a variety of people who have helped me a lot during the past four years.

To begin with, I want to express my earnest gratitude to my supervisor and cosupervisor: Prof. Martine Schroyen and Prof. Zhengkui Zhou. I deeply appreciate their continuous support and valuable suggestions for my Ph.D. study and life. Their precious guidance helped me in the realization of this work successfully.

I owe a great appreciation to my thesis committee and jury members (Prof. Nicolas Gengler, Prof. Nadine Buys, Prof. Keith Durkin, Prof. Carole Charlier, Prof. Ludivine Lassois). Thanks for taking your valuable time to read, evaluate and give valuable suggestions to my research and thesis.

I’m very indebted to Dr. Zhanbao Guo and Prof. Shuisheng Hou in China and for the help on the experimental design and molecular breeding work. I am also very grateful to all the employees of Shandong Rongda Animal Husbandry Co., Ltd. for their support and help in my breeding work.

I would also like to thank my cooperators, Dr. Hongfei Liu, Dr. Dapeng Liu, Dr. Simeng Yu, Dr. Hehe Tang, Dr. He Zhang, Dr. Qiming Mou, Dr. Bo Zhang, Dr. Junting Cao, Tong Liu, thanks for your hard work and help during my research work. I am also very grateful to the staffs of the State Key Laboratory of Animal Biotech Breeding, Institute of Animal Science, Chinese Academy of Agricultural Sciences, such as Prof. Wei Huang, Qi Zhang, Dr. Ming Xie, Dr. Jing Tang, Dr. Yunsheng Zhang, Dr. Jian Hu, Dr. Suyun Liang, Dr. Wentao Cai, Dr. Zhen Liu for all the help given.

Besides, special thanks to all the members from Precision Livestock and Nutrition Unit of Gembloux Agro-Bio tech. Thank you all for welcoming me, for your support and kindness. I express my gratitude for the enriching experiences I have had during my time in Beijing and Gembloux. Additionally, I wish to thank China Scholarship Council (CSC) for the financial support to study in Belgium. Importantly, I am deeply moved by my friends Yexun Zhou, Ying Zhao, Kai Wang, Lin Cheng, Yuqian Xu, Jing Wang, Yuqing Ren, Chi Ren, Hui Han. Thank you all for your help during the happy life in Belgium.

My most affectionate thanks go to my family. Particularly, thanks for the patience, care and support from my husband Xiaofei Li over the past years. His constant encouragement and unconditional tolerance and considerate companion are the most powerful emotional anchors for me to complete my study. I also express my gratefulness to my beloved brothers and sister and parents for their unhesitating support. Last but not least, my heartfelt thanks to all the people who helped me during my Ph.D. research and life.

Zhen WANG

August 2025 in Gembloux, Belgium

Tables of Contents

List of Figures

List of Tables

List of Abbreviations

α-MSH	α-melanin stimulation hormone
ASIP	Agouti signaling protein
BF	Black-feathered and black beak ducks
CBD103	Beta defensin 103B
cDNA	complementary DNA
CNV	Copy number variation
CPM	Read counts per million
ddNTP	dideoxy nucleotides
DEG	Differentially expressed gene
EDNRB2	Endothelin receptor B2
EDTA	Ethylenediamine tetra acetic acid
ERK	Extracellular signal-regulatedkinase
GATK	Genome Analysis Toolkit
GWAS	Genome-Wide Association Studies
GY	Grey-feathered and black beak ducks
HIC	High-through chromosome conformation capture
HIFI	High fidelity reads
Indel	Insertion and deletion
HPLC	High performance liquid chromatography
KIT	Stem cell factor receptor
KITL	KIT ligand
LC	Liancheng ducks
LD	Linkage Disequilibrium
LYST	Lysosomal trafficking regulator
MAS	Marker-assisted selection
MC1R	Melanocortin-1 receptor
MD	Mallards
MITF	Microphthalmia-associated transcription factor
MLPH	Melanophilin
mRNA	message RNA
MYO5A	Myosin VA
NCC	Neural crest cells
OCA2	Oculocutaneous albinism II
PCA	Principal component analysis
PDCA	Pyrrole-2,3-dicarboxylic acid
PKA	Protein kinase A
PK	Pekin ducks
PMEL	Promelanosome protein
POMC	Propiomelanocortin

PT	Putain ducks
PTCA	Pyrrole-2,3,5-tricarboxylic acid
RAB27A	member RAS oncogene family
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SLC36A1	Solute carrier family 36 member 1
SLC45A2	Solute carrier family 45 member 2
SOX10	SRY-box transcription factor 10
STX17	Syntaxin 17
TE	Tris·Cl, EDTA Buffer
Tris	Trihydroxymethylaminomethane
TRPM1	Transient receptor potential cation channel subfamily M member 1
TYR	Tyrosinase
TYRP1	Tyrosinase related protein 1
WB	White-feathered and black beak ducks
WY	White-feathered and yellow beak ducks

Chapter I

General introduction

1.1 Formation mechanism of fur and feather color in vertebrates

For hundreds of years, the rich fur color of vertebrates has attracted human attention, but it is only in the past 100 years that the formation mechanism of fur color has been gradually clarified and has become a model system for studying the formation of developmental biology schemata. Birds are the most colorful group of vertebrates (Kelsh et al., 2009; Roulin et al., 2013), and feather color plays an important role in camouflage, attracting the opposite sex, body temperature regulation and group communication. For example, individuals with lighter feathers can reflect more radiant heat than those with darker feathers, so they are more adaptable to hot and bright climates (Hubbard et al., 2010; Emaresi et al., 2013; Kaelin and Barsh, 2013). The variation of feather color is mainly manifested by the difference of hue (such as red, white, black, etc.), the depth of color (such as dilution), and the difference of feather pattern (such as stripes, patches, irregular polygons, etc.) (Baxter et al., 2004; Schmutz and Berryere, 2007). Feather color is mainly related to the content, distribution and proportion of pigments. The main pigments in bird feathers are melanin (eumelanin, pheomelanin) and carotenoids. Eumelanin deposits to form black and brown, pheomelanin deposits to form reddish-brown. Yellow and red are mainly produced by carotenoids obtained from food, and red can also be obtained from other rare pigments. As such, there are the porphyrin pigments in owls or the iron oxide pigments in bearded vultures. In addition, the special structure of feathers can also affect feather color (Roulin et al., 2013). A more in-depth exploration of these different pigments will follow in section 1.2.

In recent years, research related to feather color is mainly based on the study of melanin coloring, that is, the feather color phenotype formed by the interaction of eumelanin and pheomelanin (Emaresi et al., 2013). In vertebrates, neural crest cells (NCCs) differentiate to form melanocytes, whose characteristic organelles, called melanosomes, can synthesize melanin. NCCs have the potential of migration and multidirectional differentiation (Figure 1-1) (Gunnarsson et al., 2011; Hirobe, 2011; Kelsh et al., 2009; Mills and Patterson, 2009). We will describe this more in detail in section 1.3. The NCCs in chicken embryos need to specialize into melanoblasts to migrate into the dorsolateral pathway, and the specialization can alter the cell's response to signals from the dorsolateral pathway. Chondroitin sulfate proteoglycan, peptide nuleic acid (PNA) binding molecules, spinal proteins, receptor tyrosine kinases subfamily (ephrins), slits and other molecules inhibiting NCCs migration have been found in the dorsolateral region (Harris et al., 2008; Kelsh et al., 2009). After melanoblasts enter the dorsolateral pathway, some inhibitory molecules are still present in the dorsolateral pathway. Research using mouse and zebrafish model animals has identified numerous molecules involved in the pathway to melanin cell development.

To date, more than 300 genes causing hair color variation have been identified in mammals (mainly in mice and rats), but the regulatory pathways of pigment genes

remain unclear (Kaelin and Barsh, 2013). Genes that affect the differentiation, migration, proliferation, survival, morphology, structure and function of melanocytes can affect the pigment phenotype, and abnormalities of molecules (structural proteins, enzymes, transcription factors, receptors and growth factors, etc.) involved in any of the aforementioned processes may lead to feather color variation (Fontanesi et al., 2010; Hirobe, 2011; Hoekstra, 2006; Roulin et al., 2013; Santschi et al., 1998). Pigment-related genes mainly function in the following areas: (1) cell development: the formation of NCCs, and the specialization, migration, proliferation and differentiation of melanoblasts; (2) The composition of melanosomes; (3) Regulation of the synthesis of eumelanin melanosome and pheomelanin melanosome; (4) Synthesis and transport of melanosome components; (5) Melanosome transport to melanocyte dendrites; and (6) Transport of Melanosomes to keratinocytes.

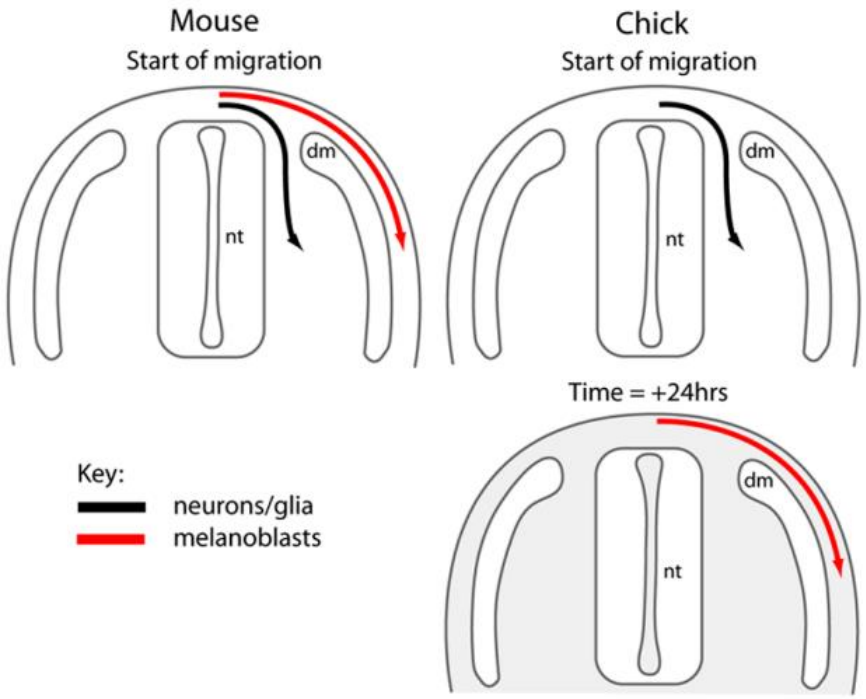

Figure 1-1. Schematic of neural crest cell (NCC) migration in transverse sections (Kelsh et al., 2009). Significant differences are observed in timing and pathways of neural crest cell migration when comparing mouse, and chicken during early embryogenesis. nt: neural tube; dm: dermomyotome.

1.2 The type of pigments that determine the feather color of ducks

1.2.1 Carotenoid

Carotenoids can be widely detected in birds' feathers and beak skin. The normal state of carotenoids will reflect red and yellow light to show a red or yellow phenotype, and some radical groups can also show a purple phenotype after being replaced (LaFountain et al., 2010). Unlike melanin production, birds cannot synthesize carotenoids. Similar to the production of melanin, carotenoids need a precursor substance, phytoene synthase. However, animals lack the enzymes that can catalyze this substance, so they must obtain carotenoids from plants. The most obvious example comes from American goldfinches, which have been shown to produce plumage free of pigmentation during moulting when carotenoids are reduced in their diet. Without a restricted supply of carotenoids, the feathers show a normal bright yellow color (McGraw et al., 2005). Other birds whose feather color is regulated by carotenoids include rosefinches (Carpodacus) and flamingos (Phoenicopteridae) (Fox et al., 1962; Toomey et al., 2012).

Because birds cannot synthesize carotenoids themselves, the carotenoids in their bodies and feathers can only be ingested from food. In addition, the pattern of deposition in feathers is completely different from that of melanin (Attie et al., 2002). It is important to note that for many birds, as is the case for chickens, the carotenoid coloring regions are not evenly distributed in the body and they do not exhibit carotenoids in their feathers as chicks, but they begin to exhibit carotenoid phenotypes after adult molting. As already mentioned, different from various feather colors and variants caused by melanin, carotenoids cannot be synthesized by the animals themselves, and the mechanism of action of genes related to their deposition process is not perfect, so it is difficult to locate specific genes. McGraw et al. (2003) speculated that the reason for this phenomenon may be the spatiotemporal selective absorption of carotenoids by hair follicle tissues. There may exist molecular interactions between carotenoids, and different carotenoids compete with binding proteins, resulting in the lack of lutein in some locations of extraction, and these binding proteins may prevent carotenoids from entering cells at specific times (McGraw et al., 2003).

1.2.2 Structural color

Compared with pigment-based colors, structural colors were discovered late and research in birds has lagged behind. Everything in the world is essentially the same color but it absorbs and reflects light of different wavelengths. Structural color is different from pigment-based color principle in that the color displayed by the pigment is a single color reflected by a single substance to present a single color. Structural color arises from a macroscopic, ordered arrangement that produces a spectrum of colors

through the interaction of light. This includes phenomena such as diffraction, scattering, and interference of light waves at varying wavelengths. For example, changes in the color of mirror feathers on the wings of wild ducks are the result of structural colors. From different angles, different light sources to observe it, the color of the mirror feathers are different, from purple, blue to green (Stavenga et al., 2017). However, the mechanism behind the rich variety of feather colors in birds remains partly unexplained. It is currently widely believed that the blue color of bird feathers mainly comes from nanoscale structures on the feathers. These tiny structures scatter and interfere with light, especially the short-wavelength blue light, making the feathers appear blue. This color is not caused by pigments but is an optical effect produced by physical structures. The green color of bird feathers results from a combination of blue structural color and yellow pigments. Nanostructures in the feathers scatter and interfere with light to produce the blue color, while yellow carotenoid pigments absorb some wavelengths. Together, they create the perception of green.

1.2.3 Melanin

Melanin is made up of eumelanin, which acts on skin or hair to make it appear black, and pheomelanin, which appears brown and yellow. The relative amounts of eumelanin and pheomelanin determine the animal hair/feather phenotype. The melanin synthesis process is very complicated. *TYR*, *TYRP-1* and *TYRP-2* enzymes play a major role in the synthesis of melanin. Melanin synthesis usually goes through the following two steps. First, *TYR* catalyzes tyrosine to produce L-dihydroxyphenylalanine (L-DOPA), and then obtains the catalytic product dopaquinone (DQ) under the action of *TYR*, or directly generates DQ through oxidation reaction. This is the rate-limiting step in the melanin synthesis process, and it is also a critical step. DQ enters the second stage of the reaction as a substrate for the synthesis of melanin, as a precursor substance for the synthesis of eumelanin and pheomelanin. Already in 1966, a study on the pigment division of poultry feathers found that the difference in eumelanin content would cause the difference in the coloration area of poultry main wing feathers (Somes et al., 1966). In quail, changes in pheomelanin were found to affect the pigmentation of feathers, determining whether they appear darker or lighter during embryonic development (Shiojiri et al., 1999). In cattle, the difference between black-haired and yellow-haired individuals is also caused by the different amounts of pheomelanin and eumelanin (Renieri et al., 1993). In both mammals and birds, the analysis of the content of melanin in their hair or feathers revealed a high concentration of pheomelanin in red and yellow hair (Ito et al., 2003). It can be seen that eumelanin and pheomelanin play a decisive role in determining the type, light and shade, as well as coloring surface and volume of the animal's coat or feather color. When the content of pheomelanin is high, the animal's coat color (feather color) presents a yellow / brown phenotype, while when the content of eumelanin is high, the animal's coat color (feather color) generally presents a dark brown or black phenotype. Studies on Liancheng duck feather pigments revealed the exclusive presence of eumelanin.

1.2.4 The practical applications and economic significance of white-feathered ducks

Although the factors that affect feather color of birds include carotenoids, structural colors and melanin, the genetic understanding of the latter two factors is still relatively scarce. Carotenoids and structural colors often determine some special, gorgeous feather color types, but the basic feather color is still determined by melanin. The importance of white-feathered however extends beyond aesthetics, with practical applications and economic advantages that can be summarized as follows:

- **LC ducks have higher meat nutritional value compared to other duck breeds**
  Liancheng Duck is a representative breed of white-feathered meat ducks, renowned for its exceptional nutritional profile. Its meat is rich in 18 amino acids and 10 microelements, with notably low cholesterol levels. Compared to common duck breeds, it contains 2.5 times more iron and zinc. White-feathered meat ducks with the appearance traits of Liancheng Ducks, combined with significantly improved meat nutritional value and a body size close to that of Pekin ducks, are well received by the market and recognized as high-quality meat ducks.
- **Enhanced market competitiveness and sales**
  White feathers make processed meat ducks look cleaner and neater, which is visually more appealing. Consumers are more willing to buy these products, boosting sales and increasing income for farmers and sellers.
- **Reduced processing costs and improved efficiency**
  White feathers are easier to remove mechanically, reducing the difficulty and time spent on feather cleaning during processing. This lowers labor and processing costs while enabling faster production lines and overall higher efficiency.
- **Increased product quality and added value**
  White feathers prevent color inconsistencies, resulting in a more uniform and attractive final duck product. This reduces price discounts due to poor appearance, thus raising market value and consumer acceptance.
- **Suitable for large-scale and standardized production**
  White-feathered ducks are easier to raise and process under standardized, industrialized conditions. This facilitates large-scale production with consistent quality, boosting the economic benefits of the industry.

In summary, white-feathered meat ducks provide significant advantages in product quality, processing efficiency, market sales, and brand building, effectively enhancing the economic benefits of breeding and marketing and representing a key development trend in the modern meat duck industry. In this study, the white-feathered meat ducks with the appearance traits of Liancheng Ducks and significantly improved meat nutritional value, with body size close to that of Pekin ducks were welcomed by market and referred to as high-quality meat ducks.

1.3 Melanin deposition process and regulatory genes

1.3.1 Melanin deposition process

Melanin is the most basic and common pigment that determines the feather color of birds. Whether melanin can be successfully deposited into the skin or hair and feathers depends mainly on three stages (Figure 1-2). The first stage is the development of melanocytes. Melanin is synthesized in the melanosomes, lysosomal organelles within melanocytes. Melanocytes originated from NCCs. Melanocytes of the head are differentiated from facial NCCs, while melanocytes of the trunk and limbs are differentiated from trunk NCCs (Bronner et al., 2012). NCCs originate from the neuroepithelium, and first enter a migration stagnation zone after which they follow different migration routes to reach the exact location of the embryo (Wehrle-Haller et al., 2001). The two migration routes are the dorsolateral and ventral routes (Lin et al., 2007), and the NCC first differentiates into melanoblasts before migration begins. When melanocytes migrate to designated locations along the dorsolateral pathway, they continue to differentiate into melanocytes and produce melanin (Faas et al., 1998). This is where one enters the second stage, the production of melanin. Melanin begins to be produced in the melanosome, and its precursor DQ is catalyzed by tyrosinase. Two things can happen at this time. It can happen that, with sufficient tyrosinase activity, DQ continues to be catalyzed to form chromium dopaquinone and then eumelanin. However, when the activity of real tyrosinase is insufficient, the DQ generated will combine with cysteine to produce cysteinyldopa, which is the precursor of pheomelanin (Kobayashi et al., 1994). After the synthesis of melanin in the melanosome, preparation for deposition begins leading to the final stage of melanin deposition. At this stage, melanin cells are transported outside the melanocytes and enter the keratinocytes to produce color, which is what causes the skin or feather coloring. However, the specific transport mechanism is still unclear. The smooth transport of melanosomes outside melanocytes is very important, and the loss or loss of this function can lead to lighter feather color, such as the lilac feather color of chickens (Vaez et al., 2008).

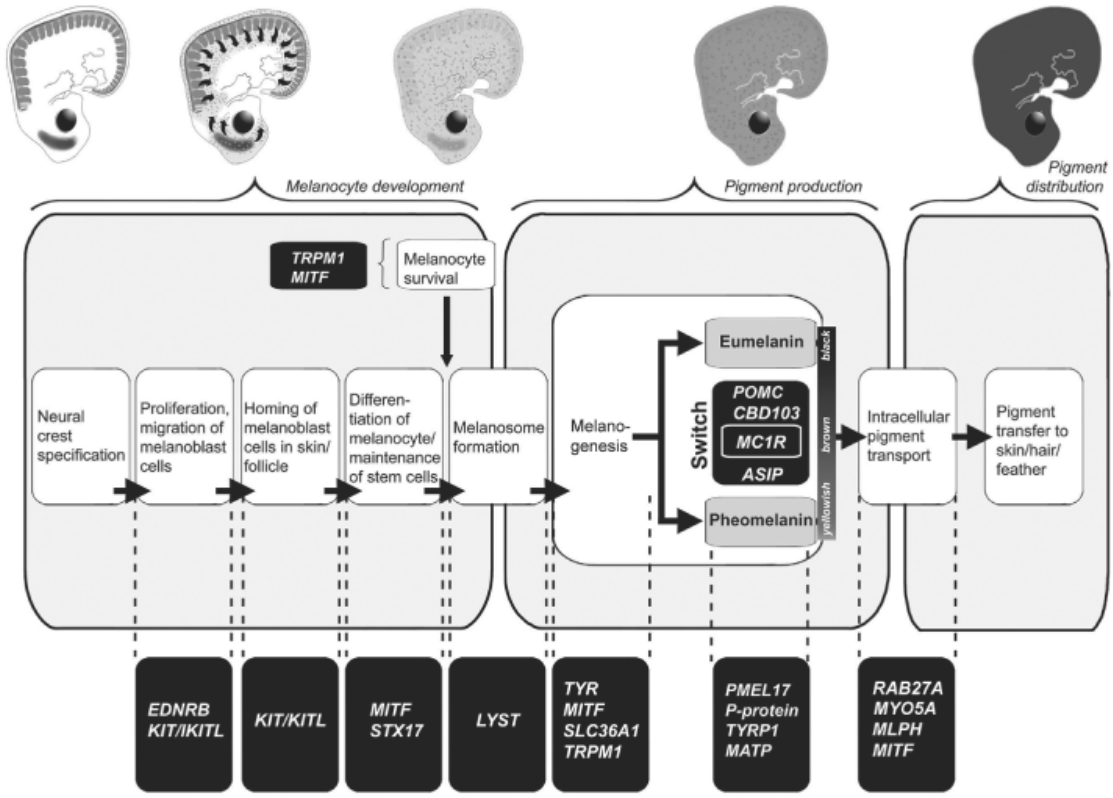

Figure 1-2. Progression of pigment cell development, pigment synthesis, intracellular pigment transport and pigment transfer (Cieslak et al., 2011). The pigmentation progress is subdivided into three major stages: (I) melanocyte development; (II) pigment production; and (III) pigment distribution. The white boxes show stages of the pigmentation process, the black boxes show the active coat-colour-associated genes. Gene names as follows *ASIP, agouti signalling protein; CBD103, beta defensin 103B; EDNRB, endothelin receptor type B; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; KITL, KIT ligand; LYST, lysosomal trafficking regulator; MATP(SLC45A2), solute carrier family 45 member 2; RAB27A, member RAS oncogene family; MC1R, melanocortin 1 receptor; MITF, microphthalmia-associated transcription factor; MLPH, melanophilin; MYO5A, myosin VA; PMEL17 (Premelanosome 17); POMC, propiomelanocortin; P-protein (OCA2), oculocutaneous albinism II; SLC36A1, solute carrier family 36 member 1; STX17, syntaxin 17; TRPM1, transient receptor potential cation channel subfamily M member 1; TYR, tyrosinase; TYRP1, tyrosinase-related protein 1*.

1.3.2 Melanocyte development related genes

The endothelin receptor (EDNR) is a class of G-protein-coupled receptors with 7 transmembrane domains. In addition to the endothelin receptor B (EDNRB), there are two other homomers. However, according to previous studies, only EDNRB plays an important role in influencing animal fur/feather color. *EDNRB* is a necessary participant in the early development of melanocytes to promote the correct migration of melanoblasts (Hosoda et al., 1994; Metallinos et al., 1998). The study of the endothelin B receptor in poultry is not limited to EDNRB, because birds possess a paralogue gene for the endothelin receptor, EDNRB2, which is absent in mammals. Phenotypic

variation caused by an *EDNRB2* mutation can be explained by studying its expression pattern and function. Compared with wild-type skin, the expression of *EDNRB2* in the skin tissue of black bone chickens was found to be significantly increased (Dorshorst et al., 2011). Similar findings were observed in various quail and chicken breeds, where the expression level of *EDNRB2* in the skin of wild-type individuals was significantly higher than that of mutant individuals. Kinoshita et al. (2014) found that *EDNRB2* was associated with tyrosinase-dependent recessive white feather (mo^{w}) and spotted feather (mo) phenotypes. In the study of feather color in Japanese quail, the *EDNRB2* mutation was found to be related to the phenotypes of panda color (s) and spotted white (s^{dw}) feathers, and the *EDNRB2* mutation caused hypopigmentation (Tsudzuki et al., 1993). The expression of *EDNRB2* is essential for melanin deposition, and it mainly inhibits the migration of melanoblasts to other parts of the trunk, resulting in the loss of melanin deposition in parts of the body or even in the whole body.

The *KIT* gene encodes the growth factor receptor of hypertrophic hepatocytes, and the entire gene sequence includes 5 immunoglobulin regions, 1 transmembrane region, 1 near-membrane region, and 1 intracellular protein kinase domain. *KIT* works in conjunction with its ligand stem cell factor (*SCF*), which, similar to the endothelin-B receptor, plays a very important role in the migration and survival of melanoblasts (Yoshida et al., 2001). Therefore, the phenotype caused by mutations in this gene is partially similar to the phenotype caused by *EDNRB* or *EDNRB2* mutations. After phosphorylation of the ligand stem cell factor (SCF), the extracellular signal-regulated kinase (ERK) is activated, and *MITF* is then activated by phosphorylation to play a transcriptional role. At present, it has been found that *KIT* can affect the dominant white character of hair color in mammals such as pigs and horses (Li et al., 2011; Rubin et al., 2012).

The Syntaxin 17 (*STX17*) gene plays an important role in the differentiation of melanocytes by facilitating melanosome maturation and vesicle trafficking, regulating autophagy, and influencing cellular signaling pathways (Sundström et al., 2012). Proper functioning of *STX17* is essential for maintaining efficient melanin synthesis, and any disruption in its activity could potentially lead to pigmentation disorders or other skin-related conditions. Next, the *lysosomal trafficking regulator* (*LYST)* works in melanosome formation (Huizing et al., 2001). Melanosomes are specialized organelles in melanocytes where melanin is synthesized, stored, and transported. The *LYST* gene is crucial for the proper biogenesis of melanosomes, influencing their development from early endosomes to mature, functional melanosomes.

1.3.3 Genes associated with melanin production

The function of *TYR* is to catalyze the transformation of tyrosine into melanin or pheomelanin, and it is a key rate-limiting enzyme in the synthesis of melanin (Gaggioli et al., 2003). Its expression activity determines the rate of melanin production. A mutation in *TYR* will affect the catalytic efficiency of tyrosinase, and thus affect the synthesis of melanin. The chicken *TYR* was determined by linkage analysis to be located on chromosome 1. Chang et al. (2016) found that the albino phenotype of

chickens was caused by the deletion of 6 bases in the coding region of the *TYR* gene (Chang et al., 2006). It is worth noting that in a study of duck feather color and its relationship with *TYR*, Li et al. (2012) found that *TYRP2* was not expressed in duck feather tissues and showed no correlation with duck feather color (Li et al., 2012). However, *TYR* and *TYRP1* were not expressed in duck white bulbs but were highly expressed in black bulbs. The regulation of tyrosinase activity on melanin type is the basis of the formation of basic color of coat/feather color in most animals. As a marker gene for melanin synthesis, the expression of *TYR* can indirectly reflect the content of melanin. *TYRP1*, as a binding protein of *TYR*, has a similar effect on melanin production as *TYR*. In addition, *TYRP1* has also been found to be involved in regulating the proliferation and apoptosis of melanocytes (Wu et al., 2003).

MITF is a member of the basic helix-loop-helix-leucine zipping transcription factor family. These proteins form homologous or heterologous dimers through the zipper domain and then bind to DNA to regulate various growth and development processes, especially the survival, proliferation and differentiation of pigment cells. In the melanin synthesis pathway, *MITF* is not directly involved in the formation of melanin, but mainly activates the melanocyte survival pathway by regulating the expression of specific melanosomal proteins (such as TYR, TYRP1, SILV, TRPM1 and DCT) during the differentiation of melanocytes. Mochii et al. (1998) found in a study in Japanese quail that a mutation in *MITF* caused the silver-white feather phenotype (Mochii et al., 1998). Minvielle et al. (2010) further found that a 2bp deletion in the coding region of *MITF* was significantly correlated with the silver-white feather color. Zhou et al. (2018) found that there was a 6.6Kb insertion between the first and second exon of *MITF* in Pekin ducks, which caused the white feather trait in Pekin ducks (Zhou et al., 2018). This 6.6Kb is the genomic variation is the causative factor for the absence of melanin in the plumage of Pekin ducks. It leads to their white feather phenotype by affecting the expression of the *MITF* transcript.

Melanosome specific protein *PMEL* is an important component of melanosome formation. The amyloid fiber structure in the melanosome observed by electron microscopy in the pre-synthesis stage is synthesized by the PMEL protein, which is also the main structure of the melanosome, and can help the stable deposition of melanin in the body after synthesis (Hurbain et al., 2008; Jiang et al., 2014). In addition, since this protein is the scaffold and basis of the melanosome, this property makes PMEL a specific marker protein for locating melanosomes (Verdy et al., 2012). The chicken *PMEL* gene is located on chromosome 33 and it is considered to be significantly correlated to the dominant white feathers of chickens (Hoashi et al., 2006; Sitaram et al., 2012). Kerje et al. (2004) found that the dominant white plumage of chickens was caused by nine consecutive base insertions in exon 10 of the *PMEL17* gene, but this gene has not been annotated in the reference genome of Pekin ducks.

The *MC1R* gene, full name Melanocortin 1 Receptor gene, also known as the melanocyte-stimulating hormone receptor (*MSHR*) gene, has only one exon (except in goats). The MC1R protein encoded by *MC1R* is a G-protein-coupled receptor. Bioinformatic analyses show that it has 7 transmembrane domains and is the smallest G-protein-coupled receptor with a length of 310~320 amino acids, belonging to one of

the adrenal cortex (MC) receptor families. This family contains five major receptors, namely MC1R, MC2R, MC3R, MC4R and MC5R, which have different expression levels and functions in different tissues. Among them, the gene for *MC1R* is the most widely studied in animal hair color/feather color (Araguas et al., 2018). *MC1R* was first discovered in melanocytes and is mainly expressed in hair follicle and skin melanocytes (Mountjoy et al., 1992). It mainly affects the formation of melanin by regulating downstream genes such as *TYR* and *TYRP1* through the signaling pathway mediated by cAMP.

There are four types of endogenous melanocorticoid ligands: a-MSH, B-MSH, y-MSH and adrenocorticotropin (ACTH). a-MSH is the most important melanin corticosteroid ligand of the MC1R receptor protein (Abdel-Malek et al., 2010). MC1R binds a-MSH with high affinity, and a-MSH acts as an agonist. When it binds to MC1R, it can significantly increase the content of cAMP (Liu-Smith et al., 2015), and the CAMP-mediated signaling pathway is considered to be the main signaling pathway for the activation of MC1R, as well as the main regulator of melanin production. The binding of a-MSH to MC1R is selective, and special mutations can reduce the binding affinity between MC1R and a-MSH ligand or make a-MSH unable to bind to other MC family receptors and specifically activate MC1R. Bednarek et al. (2008) found that the loss of Trp9 in a-MSH makes it inactive for MC3R, MC4R, and MC5R ligands, while it is a fully functional agonist for MC1R. Yang et al. (2017) explored the cause of Try9-deficient a-MSH selective binding and found that the 128th methionine residue in the third transmembrane domain of MC1R protein was mutated to leucine (M128L), which had an important effect on the selective activation of Try9-deficient a-MSH. The selective combination of A-MSH and MC1R suggests that the formation of the final product melanin type is regulated by multiple factors, and it also provides a way to explore the formation mechanism of specific feather color in the future.

As a competitive MC1R inhibitor, the Agouti signal protein (ASIP) effectively prevents the binding of a-MSH to MC1R and inhibits the activity of MC1R (Suzuki et al., 1997). The binding of ASIP and A-MSH to MC1R is mutually exclusive (Ollmann et al., 1998). In addition, ASIP acts as a reverse agonist, reducing the underlying MC1R signal and inhibiting eumelanin synthesis (Wilson et al., 1995). Although the main sequence of ASIP bears no resemblance to ACTH or a-MSH, it binds to MC1R with almost equal affinity. Some studies believe that ASIP regulates the expression of a-MSH by inhibiting the promoter activity of a-MSH. However, pigment synthesis can still be observed in the absence of a-MSH binding to MC1R, which indicates that the regulation of pigment synthesis by ASIP is not entirely carried out by inhibiting the ability of a-MSH to bind MC1R (Graham et al., 2010). How ASIP binds MC1R to produce its effect is still unclear. They also work with *Propiomelanocortin* (*POMC*) and *Beta defensin 103B* (*CBD103*) genes to convert eumelanin and pheomelanin.

Other genes involved in melanin synthesis have been widely studied. The *solute carrier family 36 member 1* (*SLC36A1*) gene plays a significant role in pigment production by facilitating the transport of essential amino acids necessary for melanin synthesis. In addition, studies in black-bone chickens found that the excessive deposition of melanin in black-bone chickens was caused by the overexpression of

EDN3 (Dorshorst et al., 2011). Further research is needed to fully identify and characterize the genes involved in duck feather color determination.

1.3.4 Melanosome transport-related genes

At present, the detailed mechanisms and specific model of melanosome transport are still unclear. However, a complex transporter (Ras complex transporter) composed of Ras oncogene family members (*RAB27A*), myosin (*MYO5A*) and melanophilin (*MLPH*) plays an important role in the transport of melanocytes from melanocytes to in vitro (Hume et al., 2007), because this complex protein acts after the synthesis of melanin, so it has an impact on the phenotypic traits caused by both eumelanin and pheomelanin. Mutations in these genes disrupt the tissue of melanocytes and prevent them from being transported outside the cell to deposit melanin in tissues such as skin, feathers or hair. Therefore, the mutation of this Ras-combined transporter gene mainly leads to light coat/feather color showing a diluted phenotype.

1.4 Application of heterosis in poultry

Heterosis, also known as hybrid vigor, refers to the phenomenon whereby offspring resulting from the crossbreeding of different breeds or lines exhibit superior performance in traits such as growth rate, egg production, disease resistance, and reproductive efficiency, compared to their parental lines. In poultry production, particularly in chickens, ducks, and geese, heterosis is widely exploited and constitutes a fundamental component of modern commercial breeding programs. In poultry, heterosis typically manifests in the following ways:

Table 1-1 The effect of heterosis on poultry traits

Type	Characteristics
Growth performance	Faster growth rate, better feed conversion, heavier body weight
Reproductive traits	Early maturity, high egg production, high fertility rate
Health and Immunity	Stronger disease resistance, better adaptability, higher survival rate
Appearance and Economic traits	Uniform feather color, high dressing percentage, better meat quality

A concrete example of heterosis in meat ducks is demonstrated by the cross between the Pekin duck and the Mallard (Zhou et al., 2018). This study evaluated over 1,000 F_2 hybrids alongside their parental lines (Mallards and Pekin ducks). On average, the body weight of the hybrids increased by approximately 15%, while the feed conversion ratio improved by around 6% compared to the Mallard parent line. Notably, the expression of the major effect gene *IGF2BP1* persisted into post-hatch stages in the hybrids, in

contrast to Mallard ducks where its expression declined following embryonic development. This extended expression was attributed to a natural mutation in a distal enhancer located approximately 100kb upstream of *IGF2BP1*.

In this PhD we describe the development of high-quality ducks derived from Liancheng ducks and Pekin ducks. The resulting progeny exhibited uniform feather coloration as well as superior meat quality and nutritional value compared to purebred Pekin ducks. Simultaneously, these hybrids exhibited a larger body size and faster growth rates compared to Liancheng ducks. These findings highlight the successful application of heterosis in duck breeding. Through systematically designed crosses between different breeds or lines, producers can significantly enhance key traits including productivity, economic efficiency, and disease resistance. In contemporary poultry production systems, including high-efficiency broilers, high-yield layers, and fast-growing ducks or geese, exploiting heterosis is crucial for achieving sustainable industrial-scale success.

1.5 Advances in genome sequencing technology

Since 1977, sequencing technology has made remarkable progress, from the first generation of sequencing technology to the current third generation. Over this period, reading lengths have transitioned from long to short and back to long, reflecting continuous innovation. At present, the second-generation sequencing technologies occupy an absolute advantage in the global sequencing market, holding approximately 70% of the market share. Each technological advancement has greatly promoted the development of genome research, disease medical research, drug development and breeding efforts.

1.5.1 The first-generation sequencing technology

The first generation of DNA sequencing technology, derived from the dideoxy chain termination method proposed by Sanger in 1975 (Slatko et al., 2011), laid the foundation for early genome research, and many major scientific projects, including the Human Genome Project, have adopted this technology (Olson. 1993).

The core methods of this generation of sequencing technology include the dideoxy chain termination method, the chemical degradation method, fluorescent automatic sequencing and hybridization sequencing technology. Among these, the dideoxy chain termination method utilizes characteristic dideoxy nucleotides (ddNTPs), which, due to the lack of 2' and 3' hydroxyl groups, are unable to form new phosphodiester bonds when the DNA strand is prolonged, thus terminating synthesis. By introducing ddNTPs with specific fluorescent labels in the synthesis reaction, and using polyacrylamide gel electrophoresis and autolithography techniques, the DNA base sequence can be accurately inferred.

The chemical degradation method is the process of chemical modification of DNA fragments and subsequent degradation to generate DNA strands of different lengths,

and the length of these strands is estimated by electrophoresis to determine the sequence. Although this method has operational risks and is difficult to automate, it has contributed to the development of our sequencing technology.

Further improvement came with automatic fluorescence sequencing technology, which replaced radiolabeling with fluorescence and incorporated capillary electrophoresis, greatly enhancing the automation and efficiency of sequencing. Lastly, hybridization sequencing, based on DNA hybridization with single-stranded oligonucleotide probes, introduced an innovative approach to sequence analysis.

The first-generation sequencing technology has become an important tool in genome research with a reading length of nearly 1000bp and high accuracy. However, the high cost and low throughput limit its application in large-scale genome sequencing projects. Nevertheless, the development of this generation of technology provided a solid foundation for subsequent innovation and advances in sequencing technology.

1.5.2 The second-generation sequencing technology

As mentioned earlier, second-generation sequencing technologies have emerged as a dominant force in the global sequencing market, currently holding approximately 70% of the market share. Illumina, Roche, and Applied Biosystems (ABI), Complete Genomics and Ion Torrent are all key players in the development and adoption of second-generation sequencing technologies, each contributing unique platforms and innovations. Illumina's Solexa sequencing technology adopted the synthesis side sequencing method, which has the main advantages of being high-throughput and low in cost, although it faces the challenges of short sequence read length and increasing error rate with the increase of sequence length (Hu et al., 2021). Roche's 454 sequencing technique, which assays DNA sequences with optical signals generated by microemulsion PCR amplification and pyrophosphoric acid, offers a long read length, but its accuracy can be challenged when dealing with sequences where the same base occurs consecutively. ABI's SOLID sequencing technology uses four-color fluorescence-labeled oligonucleotides to perform repeated ligand reactions with 99.4% accuracy, making it one of the most accurate methods in second-generation sequencing technology. Complete Genomics uses a similar approach for segmented sequencing, but is also limited by shorter read lengths. Ion Torrent technology uses semiconductor chips to detect pH changes caused by DNA polymerization to determine base types, and although it is low cost and sensitive, its sequencing throughput is small compared to other technologies.

Compared with the first generation, the second-generation sequencing technologies have significantly improved in cost efficiency and processing power, and can complete multiple genetic tests on multiple samples at one time, and even sequence millions of DNA sequences. Its high-throughput capability can not only quickly obtain a large amount of data, but also reflect the abundance of DNA sequences through sequencing times, providing rich quantitative information for research. However, the short read length of this generation of technology (about 30bp to 450bp) limits its ability to handle repetitive sequence regions, and the PCR-dependent process can introduce a single

DNA molecule into a gene cluster composed of the same DNA leading to mismatches, which not only reduces the accuracy of sequencing, but also increases the difficulty of subsequent assembly. Despite these shortcomings, second-generation sequencing technologies remain an indispensable tool in genome research, opening up new paths for life science research.

1.5.3 The third-generation sequencing technology

The third-generation sequencing technology, which marks a major leap in the field of sequencing, mainly relies on the progress of single-molecule sequencing technology (Ambardar et al., 2016). The core advantage of this technique is that it can directly read the sequence of a single DNA molecule, thus avoiding the base substitution errors and false positive results that can be introduced during PCR amplification. With the increase of sequencing depth and the improvement of data correction software, its accuracy was high, showing broad development potential and application prospects.

The third generation of sequencing technology is mainly divided into two categories: single molecule fluorescence sequencing and nanopore sequencing. PacBio's single-molecule real-time sequencing technology (SMRT) and Oxford Nanopore's nanopore single-molecule technology are two of the leading players in this space. PacBio's SMRT technology utilizes zero-mode waveguide (ZMW) technology to capture and identify fluorescent-labeled bases of different colors through the SMRT chip during the process of synthesis and sequencing, allowing high-speed sequence determination at speeds of up to 10 bases per second. Oxford Nanopore's technology, meanwhile, enables sequencing through an entirely different mechanism: as a DNA molecule passes through a nanopore, it causes specific changes in electrical current, which can be analyzed to identify precisely the types of bases that pass through the nanopore.

HiFi sequencing is a single-molecule, real-time sequencing technology (SMRT) that provides incredible single-molecule read accuracy across long reads of tens of kilobases in length or more. HiFi reads are generated by combining information from multiple observations of a single DNA molecule, resulting in over 99% accuracy of individual HiFi reads (Wenger et al., 2019; Chen et al., 2020). Developed by PacBio, this process occurs inside small wells on a special microchip called a SMRT Cell, which contains millions of these tiny wells. HiFi sequencing uses fluorescent light signals to identify DNA bases and modified bases (without bisulfite treatment). As a polymerase enzyme adds new nucleotide bases to a newly replicated strand, it emits tiny flashes of light.

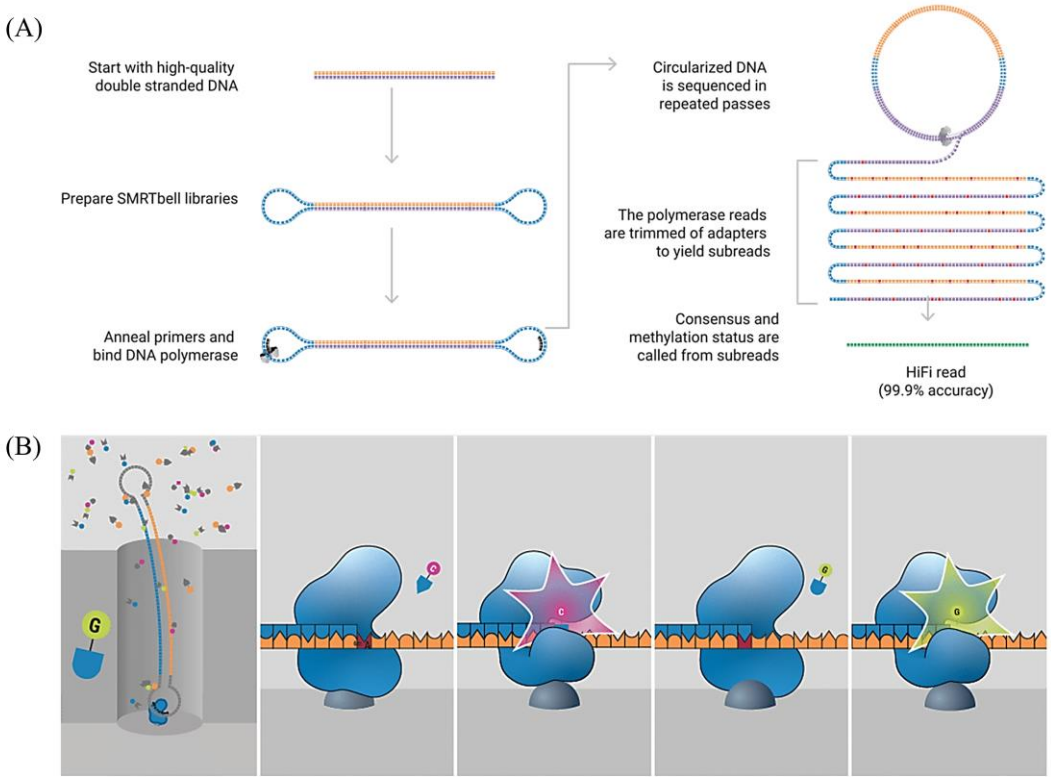

Figure 1-3. The working principle of HIFI sequencing. (A) Attach a linker to both ends of a pair of high-quality DNA sequences, then add primers and DNA polymerase. In this mode, rolling circle sequencing can be performed, that is, after the pair of DNA sequences are sequenced multiple times, the linker and excess errors are removed to obtain subreads, and finally, after error correction, HiFi reads are obtained. (B) Developed by PacBio, this process occurs inside small wells on a special microchip called a SMRT Cell, which contains millions of these tiny wells. HiFi sequencing uses fluorescent light signals to identify DNA bases and modified bases (without bisulfite treatment). As a polymerase enzyme adds new nucleotide bases to a newly replicated strand, it emits tiny flashes of light. Source from Pacbio website (*https://www.pacb.com/technology/hifi-sequencing/how-it-works/*).

The significant advantage of third-generation sequencing is that it can provide read lengths far ahead of first- and second-generation technology, ranging from 30 kilobases to 100 kilobases, which makes it easy to cross highly repetitive or structurally complex genomic regions, effectively compensating for the shortcomings of second generation sequencing technology in dealing with these regions. In addition, this technique can also directly detect methylation modifications on DNA, providing more abundant and accurate information for the comprehensive resolution and assembly of the genome. Through these advanced properties, the third-generation sequencing technologies not only promote the deepening of genomics research, but also opens up new ways for the decoding of complex genome structures and the study of genetic diseases.

1.5.4 Application of genome sequencing technology in domestic ducks

In the field of genetic evolution in ducks, Huang et al. (2013) published a study in Nature Genetics that marked an important milestone by revealing differences in the immune response of ducks to high and low pathogenic H5N1 virus infections through genome sequencing technology (Huang et al., 2013). By comparing transcriptome data from lung tissue of H5N1 infected ducks with healthy controls, the study found that *β-defensin* and *BTNL* genes play a crucial role in the immune response of ducks. Later, Zhou et al. (2018) used second-generation sequencing technology to resequence the whole genome of 1026 generation F2 individuals and their parents obtained from the hybridization of mallard ducks and Pekin ducks. Through in-depth genomic analysis, they found a 6.6Kb intron insertion in *MITF*, a finding that is believed to be a key factor in the regulation of albino feather color in Peking ducks. In addition, the *IGF2BP1* gene has been proven to be the dominant gene controlling the increase in body weight of Pekin ducks. Continuous expression of *IGF2BP1* was found to increase body weight and feed conversion by 15% and 6%, respectively. These results not only enrich our understanding of the genetic diversity and evolutionary history of domestic ducks, but also provide important molecular markers and theoretical basis for the genetic improvement of economic animals, and demonstrate the great potential of genome sequencing technology in poultry research.

1.6 Genome-wide association study of poultry

A genome-wide association study (GWAS) is a tool to detect the polymorphisms of genetic variation within the whole genome of multiple individuals, to correlate diseases or complex quantitative traits, screen out the genetic variation associated with a trait, and then explore the genes associated with the trait variation. Risch et al. (1996) proposed the theory of GWAS for the first time, and found that association analysis had higher detection efficiency than linkage analysis (Risch et al., 1996). The first article on GWAS was a GWAS performed on macular degeneration (Klein et al., 2005), and a series of GWAS studies on human diseases have been published since then. With the rapid development of high-throughput sequencing technology, the genome sequences of various livestock and poultry species have been gradually completed, and more and more GWAS studies have been applied to the detection of SNP loci related to various livestock and poultry traits and the localization and mining of related genes.

1.6.1 GWAS experiment design and statistical analysis

The reliability of GWAS research outcomes depends on a robust and well-structured experimental design. Key factors include the choice of association analysis method, the selection of study population, the sample size, the method of phenotyping, and the use of an appropriate statistical analysis model. Taking the above factors into consideration, a reasonable experimental design is the prerequisite for obtaining reliable results.

Two experimental designs are used in GWAS studies: association analysis based on unrelated individuals and association analysis based on families. Association analysis based on unrelated individuals mainly includes population-based association, based on random populations. There are two methods: study and case-control, the former is suitable for quantitative traits, and the commonly used analysis methods mainly include linear regression equation, covariance analysis, one-way analysis of variance, etc. The latter is more suitable for studying quality traits, and the test methods include Chi-square test and logistic regression analysis. The family-based association study can effectively avoid the influence of group structure on GWAS results. When conducting GWAS based on related groups, a transmission disequilibrium test (TDT) can be used if there is family structure between groups. The principle of the TDT method is to treat the genetic marker SNP as a fixed effect and the relative relationship as a random effect based on a certain allele (Van Steen et al., 2005).

1.6.2 Group selection

For GWAS, the primary population types for trait mapping are natural populations and artificially created populations. As for natural populations, they are mainly germplasm resource groups. The advantages are wide population sources and rich genetic diversity, while the disadvantages include complex population structure, many rare mutations and obvious loss of genetic information. In view of these drawbacks of natural populations, the use of GWAS to study specific traits usually considers the design of specific populations for follow-up experiments, which can avoid the subgroup differentiation of the study samples, and at the same time can amplify the genotype frequency of some rare variants, so that association analysis can obtain more accurate and significant loci. In addition, the population sample for GWAS should have gene exchange, and the population with frequent gene exchange is more likely to accumulate information such as gene mutation sites, thus improving the accuracy of GWAS results.

Population stratification refers to the phenomenon that subgroups appear due to different allele frequencies in a population. It will cause great interference to the results of association analysis when there are some subgroups with different genetic backgrounds in a population. In the actual analysis, in order to improve the accuracy of the results of association analysis, it is necessary to analyze the population structure of the study population when conducting GWAS, and the correction of population stratification may increase the false positive rate of association results. Q-Q (Quantile-quantile) graphs are an effective method to detect population stratification. A Q-Q figure visualizes the statistical results and calculates the P-value corresponding to each SNP based on the observed values (chi-square statistical test for SNP genotypes and analytical traits), arranged in order from smallest to largest on the Y-axis. The expected statistics calculated under the null hypothesis are arranged along the X-axis. The degree of overlap between the two lines can be used to determine whether the population stratification is effectively controlled in GWAS studies and the loci associated with traits.

1.6.3 Progress of GWAS research on poultry

GWAS was first applied to human diseases and genetic defects. With the rapid development of high-throughput sequencing technology and the reduction of sequencing cost, genome sequencing of many species has been carried out successively. In the context of the continuous supplement of species genome information, the object of GWAS research has gradually been applied from human diseases to economic traits such as growth performance, egg and milk production performance of pigs, cattle, sheep, chickens and other livestock and poultry, providing many materials for the research and breeding of animal traits of livestock and poultry. At present, GWAS has been widely used in the mining of major genes affecting complex quantitative traits.

The application of GWAS in poultry started relatively late, and most of the current work has focused on chickens. The completion of the chicken whole genome genetic map (International Chicken Genome Sequencing Consortium. 2004) has laid a good foundation for the use of GWAS mining in chickens and important economic traits such as growth and development, disease immunity and disease resistance traits in chicken. Abasht and Lamont. (2007) found 39 significant SNP loci that were significantly correlated with abdominal fat traits (Abasht et al., 2007). Deng et al. (2019) performed GWAS on 18 carcass traits of Pekin ducks and found 37 QTLS distributed on 26 chromosomes, and identified 36 candidate genes related to body size and carcass traits. Zhu et al. (2019) conducted GWAS on growth and feeding efficiency traits of Pekin ducks, and obtained 15 QTLS related to measured traits and 12 SNPS significantly related to feed efficiency traits. In terms of feather color traits, Zhou et al. (2018) found that *MITF* was significantly correlated with white feather traits of Pekin ducks in the study of F2 resource population of Peking ducks and mallards. These results provide some reference for improving poultry performance, meat quality and disease resistance of poultry. With the further rapid development of sequencing technology, the results of GWAS research will be more reliable and effective, and there will be more and more applications in the location of related genes for complex quantitative traits.

References

Abasht B, Lamont SJ. Genome-wide association analysis reveals cryptic alleles as an important factor in heterosis for fatness in chicken F2 population. Anim Genet. 2007;38(5):491-8.

Abdel-Malek Z, Scott MC, Suzuki I, Tada A, Im S, Lamoreux L, Ito S, Barsh G, Hearing VJ. The melanocortin-1 receptor is a key regulator of human cutaneous pigmentation. Pigment Cell Res. 2000;13 Suppl 8:156-62.

Ambardar S, Gupta R, Trakroo D, Lal R, Vakhlu J. High Throughput Sequencing: An Overview of Sequencing Chemistry. Indian J Microbiol. 2016;56(4):394-404.

Araguas RM, Sanz N, Viñas J, Vidal O. MC1R polymorphism associated with plumage color variations in Coturnix chinensis. Anim Genet. 2018;49(5):475-477.

Attie AD, Hamon Y, Brooks-Wilson AR, Gray-Keller MP, MacDonald ML, Rigot V,

Tebon A, Zhang LH, Mulligan JD, Singaraja RR, Bitgood JJ, Cook ME, Kastelein JJ, Chimini G, Hayden MR. Identification and functional analysis of a naturally occurring E89K mutation in the ABCA1 gene of the WHAM chicken. J Lipid Res. 2002;43(10):1610-1617.

Baxter LL, Hou L, Loftus SK, Pavan WJ. Spotlight on spotted mice: a review of white spotting mouse mutants and associated human pigmentation disorders. Pigment Cell Res, 2004, 17: 215-224

Bednarek MA, Macneil T, Tang R, Fong TM, Angeles Cabello M, Maroto M, Teran A. Analogs of alpha-melanocyte stimulating hormone with high agonist potency and selectivity at human melanocortin receptor 1b: the role of Trp(9) in molecular recognition. Biopolymers. 2008 May;89(5):401-8.

Bronner ME, LeDouarin NM. Development and evolution of the neural crest: an overview. Dev Biol. 2012;366(1):2-9.

Cieslak M, Reissmann M, Hofreiter M, Ludwig A. Colours of domestication. Biol Rev Camb Philos Soc. 2011;86(4):885-99.

Chang CM, Coville JL, Coquerelle G, Gourichon D, Oulmouden A, Tixier-Boichard M. Complete association between a retroviral insertion in the tyrosinase gene and the recessive white mutation in chickens. BMC Genomics. 2006;7:19.

Chen H, Zeng Y, Yang Y, Huang L, Tang B, Zhang H, Hao F, Liu W, Li Y, Liu Y, Zhang X, Zhang R, Zhang Y, Li Y, Wang K, He H, Wang Z, Fan G, Yang H, Bao A, Shang Z, Chen J, Wang W, Qiu Q. Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. Nat Commun. 2020;11(1):2494.

Deng MT, Zhu F, Yang YZ, Yang FX, Hao JP, Chen SR, Hou ZC. Genome-wide association study reveals novel loci associated with body size and carcass yields in Pekin ducks. BMC Genomics. 2019;20(1):1.

Dorshorst B, Molin AM, Rubin CJ, Johansson AM, Strömstedt L, Pham MH, Chen CF, Hallböök F, Ashwell C, Andersson L. A complex genomic rearrangement involving the endothelin 3 locus causes dermal hyperpigmentation in the chicken. PLoS Genet. 2011;7(12):e1002412.

Emaresi G, Ducrest AL, Bize P, Richter H, Simon C, Roulin A. Pleiotropy in the melanocortin system: expression levels of this system are associated with melanogenesis and pigmentation in the tawny owl (Strix aluco). Mol Ecol, 2013, 22: 4915-30

Faas L, Rovasio RA. Distribution patterns of neural-crest-derived melanocyte precursor cells in the quail embryo. Anat Rec. 1998;251(2):200-6.

Fontanesi L, Scotti E, Colombo M, Beretti F, Forestier L, Dall'Olio S. A composite six bp in-frame deletion in the melanocortin 1 receptor (MC1R) gene is associated with the Japanese brindling coat colour in rabbits (Oryctolagus cuniculus). BMC Genetics, 2010, 11: 59

FOX DL. Metabolic fractionation, storage and display of carotenoid pigments by flamingoes. Comp Biochem Physiol. 1962;6:1-40.

Gaggioli C, Buscà R, Abbe P, Ortonne JP, Ballotti R. Microphthalmia-associated transcription factor (MITF) is required but is not sufficient to induce the expression

of melanogenic genes. Pigment Cell Res. 2003;16(4):374-82.

Graham A, Wakamatsu K, Hunt G, Ito S, Thody AJ. Agouti protein inhibits the production of eumelanin and pheomelanin in the presence and absence of alpha-melanocyte stimulating hormone. Pigment Cell Res. 1997;10(5):298-303.

Gunnarsson U, Kerje S, Bed'hom B, Sahlqvist AS, Ekwall O, Tixier-Boichard M, Kämpe O, Andersson L. The Dark brown plumage color in chickens is caused by an 8.3-kb deletion upstream of SOX10. Pigment Cell Melanoma Res, 2011, 24: 268-74

Harris ML, Hall R, Erickson CA. Directing pathfinding along the dorsolateral path -the role of EDNRB2 and EphB2 in overcoming inhibition. Development, 2008, 135: 4113-4122

Hirobe T. How are proliferation and differentiation of melanocytes regulated? Pigment Cell Melanoma Res, 2011, 24: 462-78

Hoashi T, Muller J, Vieira WD, Rouzaud F, Kikuchi K, Tamaki K, Hearing VJ. The repeat domain of the melanosomal matrix protein PMEL17/GP100 is required for the formation of organellar fibers. J Biol Chem. 2006;281(30):21198-21208.

Hoekstra HE. Genetics, development and evolution of adaptive pigmentation in vertebrates. Heredity, 2006, 97: 22-34

Hosoda K, Hammer RE, Richardson JA, Baynash AG, Cheung JC, Giaid A, Yanagisawa M. Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell. 1994;79(7):1267-1276.

Hu T, Chitnis N, Monos D, Dinh A. Next-generation sequencing technologies: An overview. Hum Immunol. 2021;82(11):801-811.

Huang Y, Li Y, Burt DW, Chen H, Zhang Y, Qian W, Kim H, Gan S, Zhao Y, Li J, Yi K, Feng H, Zhu P, Li B, Liu Q, Fairley S, Magor KE, Du Z, Hu X, Goodman L, Tafer H, Vignal A, Lee T, Kim KW, Sheng Z, An Y, Searle S, Herrero J, Groenen MAM, Crooijmans RPMA, Faraut T, Cai Q, Webster RG, Aldridge JR, Warren WC, Bartschat S, Kehr S, Marz M, Stadler PF, Smith J, Kraus RHS, Zhao Y, Ren L, Fei J, Morisson M, Kaiser P, Griffin DK, Rao M, Pitel F, Wang J, Li N. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. Nat Genet. 2013;45(7):776-783.

Hubbard JK, Uy JA, Hauber ME, Hoekstra HE, Safran RJ. Vertebrate pigmentation: from underlying genes to adaptive function. Trends Genet, 2010, 26: 231-239

Huizing M, Anikster Y, Gahl WA. Hermansky-Pudlak syndrome and Chediak-Higashi syndrome: disorders of vesicle formation and trafficking. Thromb Haemost. 2001;86(1):233-245.

Hume AN, Ushakov DS, Tarafder AK, Ferenczi MA, Seabra MC. Rab27a and MyoVa are the primary Mlph interactors regulating melanosome transport in melanocytes. J Cell Sci. 2007;120(Pt 17):3111-22.

Hurbain I, Geerts WJ, Boudier T, Marco S, Verkleij AJ, Marks MS, Raposo G. Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. Proc Natl Acad Sci U S A. 2008;105(50):19726-31.

International Chicken Genome Sequencing Consortium. Sequence and comparative

analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature. 2004;432(7018):695-716.

Ito S, Wakamatsu K. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. 2003;16(5):523-531.

Jiang Z, Lee JC. Lysophospholipid-containing membranes modulate the fibril formation of the repeat domain of a human functional amyloid, pmel17. J Mol Biol. 2014;426(24):4074-4086.

Kaelin CB, Barsh GS. Genetics of pigmentation in dogs and cats. Annu Rev Anim Biosci, 2013, 1: 125-56

Kelsh RN, Harris ML, Colanesi S, Erickson CA. Stripes and belly-spots - a review of pigment cell morphogenesis in vertebrates. Semin Cell Dev Biol, 2009, 20: 90-104

Kerje S, Sharma P, Gunnarsson U, Kim H, Bagchi S, Fredriksson R, Schütz K, Jensen P, von Heijne G, Okimoto R, Andersson L. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics. 2004;168(3):1507-18.

Kinoshita K, Akiyama T, Mizutani M, Shinomiya A, Ishikawa A, Younis HH, Tsudzuki M, Namikawa T, Matsuda Y. Endothelin receptor B2 (EDNRB2) is responsible for the tyrosinase-independent recessive white (mo(w)) and mottled (mo) plumage phenotypes in the chicken. PLoS One. 2014;9(1):e86361.

Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J. Complement factor H polymorphism in age-related macular degeneration. Science. 2005;308(5720):385-9.

Kobayashi T, Urabe K, Winder A, Jiménez-Cervantes C, Imokawa G, Brewington T, Solano F, García-Borrón JC, Hearing VJ. Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. EMBO J. 1994;13(24):5818-25.

LaFountain AM, Kaligotla S, Cawley S, Riedl KM, Schwartz SJ, Frank HA, Prum RO. Novel methoxy-carotenoids from the burgundy-colored plumage of the Pompadour Cotinga Xipholena punicea. Arch Biochem Biophys. 2010;504(1):142-153.

Li Y, Zhu X, Yang L, Li J, Lian Z, Li N, Deng X. Expression and network analysis of genes related to melanocyte development in the Silky Fowl and White Leghorn embryos. Mol Biol Rep. 2011;38(2):1433-41.

Li S, Wang C, Yu W, Zhao S, Gong Y. Identification of genes related to white and black plumage formation by RNA-Seq from white and black feather bulbs in ducks. PLoS One. 2012;7(5):e36592.

Lin JY, Fisher DE. Melanocyte biology and skin pigmentation. Nature. 2007;445(7130):843-50.

Liu-Smith F, Poe C, Farmer PJ, Meyskens FL Jr. Amyloids, melanins and oxidative stress in melanomagenesis. Exp Dermatol. 2015;24(3):171-4.

McGraw KJ, Beebee MD, Hill GE, Parker RS. Lutein-based plumage coloration in songbirds is a consequence of selective pigment incorporation into feathers. Comp Biochem Physiol B Biochem Mol Biol. 2003;135(4):689-696.

McGRAW KJ, Hill GE, Parker RS. The physiological costs of being colourful: nutritional control of carotenoid utilization in the American goldfinch, Carduelis tristis. Animal Behaviour 2005;69(3): 653-60.

Metallinos DL, Bowling AT, Rine J. A missense mutation in the endothelin-B receptor gene is associated with Lethal White Foal Syndrome: an equine version of Hirschsprung disease. Mamm Genome. 1998;9(6):426-31.

Mills MG, Patterson LB. Not just black and white: pigment pattern development and evolution in vertebrates. Semin Cell Dev Biol, 2009, 20: 72-81

Minvielle F, Bed'hom B, Coville JL, Ito S, Inoue-Murayama M, Gourichon D. The "silver" Japanese quail and the MITF gene: causal mutation, associated traits and homology with the "blue" chicken plumage. BMC Genet. 2010;11:15.

Mochii M, Ono T, Matsubara Y, Eguchi G. Spontaneous transdifferentiation of quail pigmented epithelial cell is accompanied by a mutation in the Mitf gene. Dev Biol. 1998;196(2):145-59.

Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. Science. 1992;257(5074):1248-51.

Ollmann MM, Lamoreux ML, Wilson BD, Barsh GS. Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes Dev. 1998;12(3):316-30.

Olson MV. The human genome project. Proc Natl Acad Sci U S A. 1993;90(10):4338-44.

Renieri C, Ceccarelli P, Gargiulo AM, Lauvergne JJ, Monacelli G. Chemical and electron microscopic studies of cattle (Bos taurus) with four types of phenotypic pigmentation. Pigment Cell Res. 1993;6(3):165-170.

Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science. 1996;273(5281):1516-7.

Roulin A, Ducrest AL. Genetics of colouration in birds. Semin Cell Dev Biol, 2013, 24: 594-608

Rubin CJ, Megens HJ, Martinez Barrio A, Maqbool K, Sayyab S, Schwochow D, Wang C, Carlborg Ö, Jern P, Jørgensen CB, Archibald AL, Fredholm M, Groenen MA, Andersson L. Strong signatures of selection in the domestic pig genome. Proc Natl Acad Sci U S A. 2012;109(48):19529-19536.

Santschi EM, Purdy AK, Valberg SJ, Vrotsos PD, Kaese H, Mickelson JR. Endothelin receptor B polymorphism associated with lethal white foal syndrome in horses. Mamm Genome, 1998, 9: 306-309

Schmutz SM, Berryere TG. Genes affecting coat colour and pattern in domestic dogs: a review. Anim Genet, 2007, 38: 539-549

Shiojiri N, Niwa T, Wakamatsu K, Ito S, Nakamura A. Chemical analysis of melanin pigments in feather germs of Japanese quail Bh (black at hatch) mutants. Pigment Cell Res. 1999;12(4):259-265.

Sitaram A, Marks MS. Mechanisms of protein delivery to melanosomes in pigment cells. Physiology. 2012;27(2):85-99.

Slatko BE, Kieleczawa J, Ju J, Gardner AF, Hendrickson CL, Ausubel FM. "First generation" automated DNA sequencing technology. Curr Protoc Mol Biol. 2011;Chapter 7:Unit7.2.

Somes RG, Smyth JR. Feather eumelanin distribution variations in buff orpington, new hampshire and rhode island red breeds of fowl. Poultry Science 1966; 45(1): 40-49.

Stavenga DG, van der Kooi CJ, Wilts BD. Structural coloured feathers of mallards act by simple multilayer photonics. J R Soc Interface. 2017;14(133):20170407.

Sundström E, Komisarczuk AZ, Jiang L, et al. Identification of a melanocyte-specific, microphthalmia-associated transcription factor-dependent regulatory element in the intronic duplication causing hair greying and melanoma in horses. Pigment Cell Melanoma Res. 2012;25(1):28-36.

Suzuki I, Tada A, Ollmann MM, Barsh GS, Im S, Lamoreux ML, Hearing VJ, Nordlund JJ, Abdel-Malek ZA. Agouti signaling protein inhibits melanogenesis and the response of human melanocytes to alpha-melanotropin. J Invest Dermatol. 1997;108(6):838-42.

Toomey MB, McGraw KJ. Mate choice for a male carotenoid-based ornament is linked to female dietary carotenoid intake and accumulation. BMC Evol Biol. 2012;12:3.

Tsudzuki M, Nakane Y, Wakasugi N, Mizutani M. Allelism of panda and dotted white plumage genes in Japanese quail. J Hered. 1993;84(3):225-9.

Vaez M, Follett SA, Bed'hom B, Gourichon D, Tixier-Boichard M, Burke T. A single point-mutation within the melanophilin gene causes the lavender plumage colour dilution phenotype in the chicken. BMC Genet. 2008;9:7.

Van Steen K, Lange C. PBAT: a comprehensive software package for genome-wide association analysis of complex family-based studies. Hum Genomics. 2005;2(1):67-9.

Verdy C, Branka JE, Mekideche N. Melanosome transfer evaluation by quantitative measurement of Pmel 17 in human normal melanocyte-keratinocyte co-cultures: effect of an Alaria esculenta extract. J Cosmet Sci. 2012;63(3):197-203.

Wehrle-Haller B, Meller M, Weston JA. Analysis of melanocyte precursors in Nf1 mutants reveals that MGF/KIT signaling promotes directed cell migration independent of its function in cell survival. Dev Biol. 2001;232(2):471-83.

Wenger AM, Peluso P, Rowell WJ, Chang PC, Hall RJ, Concepcion GT, Ebler J, Fungtammasan A, Kolesnikov A, Olson ND, Töpfer A, Alonge M, Mahmoud M, Qian Y, Chin CS, Phillippy AM, Schatz MC, Myers G, DePristo MA, Ruan J, Marschall T, Sedlazeck FJ, Zook JM, Li H, Koren S, Carroll A, Rank DR, Hunkapiller MW. Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. Nat Biotechnol. 2019;37(10):1155-1162.

Wilson BD, Ollmann MM, Kang L, Stoffel M, Bell GI, Barsh GS. Structure and function of ASP, the human homolog of the mouse agouti gene. Hum Mol Genet. 1995;4(2):223-30.

Wu H, Park HY. Protein kinase C-beta-mediated complex formation between tyrosinase and TRP-1. Biochem Biophys Res Commun. 2003;311(4):948-53.

Yang Y, Chen M, Ventro G, Harmon CM. Key amino acid residue in Melanocortin-1 receptor (melanocyte α-MSH receptor) for ligand selectivity. Mol Cell Endocrinol. 2017;454:69-76.

Yoshida H, Kunisada T, Grimm T, Nishimura EK, Nishioka E, Nishikawa SI. Review:

melanocyte migration and survival controlled by SCF/c-kit expression. J Investig Dermatol Symp Proc. 2001;6(1):1-5.

Zhou Z, Li M, Cheng H, Fan W, Yuan Z, Gao Q, Xu Y, Guo Z, Zhang Y, Hu J, Liu H, Liu D, Chen W, Zheng Z, Jiang Y, Wen Z, Liu Y, Chen H, Xie M, Zhang Q, Huang W, Wang W, Hou S, Jiang Y. An intercross population study reveals genes associated with body size and plumage color in ducks. Nat Commun. 2018;9(1):2648.

Zhu F, Cheng SR, Yang YZ, Hao JP, Yang FX, Hou ZC. Genome-Wide Association Study of Growth and Feeding Traits in Pekin Ducks. Front Genet. 2019;10:702.

Chapter II

Objectives, research overview and thesis outline

2.1 Objectives

In this PhD study, our overall objective was to determine the melanin content of feathers, beak, and webbed feet in Liancheng ducks, and to reveal the genetic mechanism behind the unique plumage phenotype formation in Liancheng ducks. The sub-objectives of this study are outlined below, aiming to address key questions related to melanin distribution, genomic characterization, and molecular breeding in Liancheng ducks：

(1) **To quantify and compare melanin content in feathers, beak, and webbed feet in Liancheng ducks**, in relation to white-feathered Pekin ducks and black-feathered Mallards, to better understand pigmentation differences across breeds.

(2) **To construct the first high-quality reference genome for Liancheng duck**, using HiFi technology, followed by genome assembly and annotation, in order to identify the genetic mechanism of white plumage formation, including causative genes and variations.

(3) **To identify molecular breeding markers associated with white plumage coloration and meat quality traits**, enabling rapid genetic selection of high-quality meat ducks and providing valuable insights for future breeding programs and avian pigmentation research.

2.2 Research overview and Experimental design

Three experiments were performed in this thesis. In the first experiment, we wanted to focus on the unique feather color phenotype of Liancheng ducks. The determination of melanin content in duck feathers, hair follicles, and other tissues is represented. The second experiment involved the constructed resource groups in Figure 2-1 and assembling a high-quality genome for the Liancheng duck, providing compelling evidence that the white plumage characteristic of this breed is linked to the causative gene. The technical route of the research concerning the genetic mechanism performed in this thesis is represented in Figure 2-2. The third experiment, which entails breeding high quality commercial meat ducks, is shown in Figure 2-3. Part of this work is ongoing.

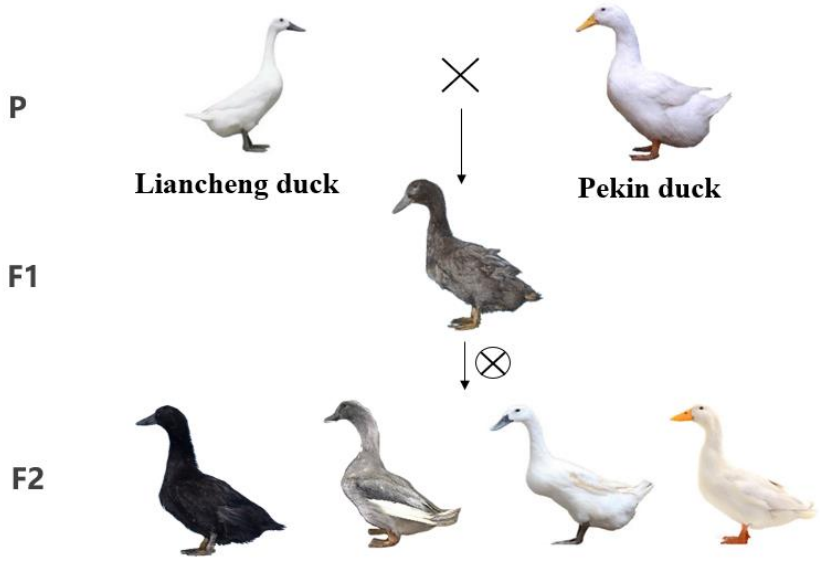

Figure 2-1: The constructed resource groups from Liancheng duck and Pekin duck crosses in this thesis.

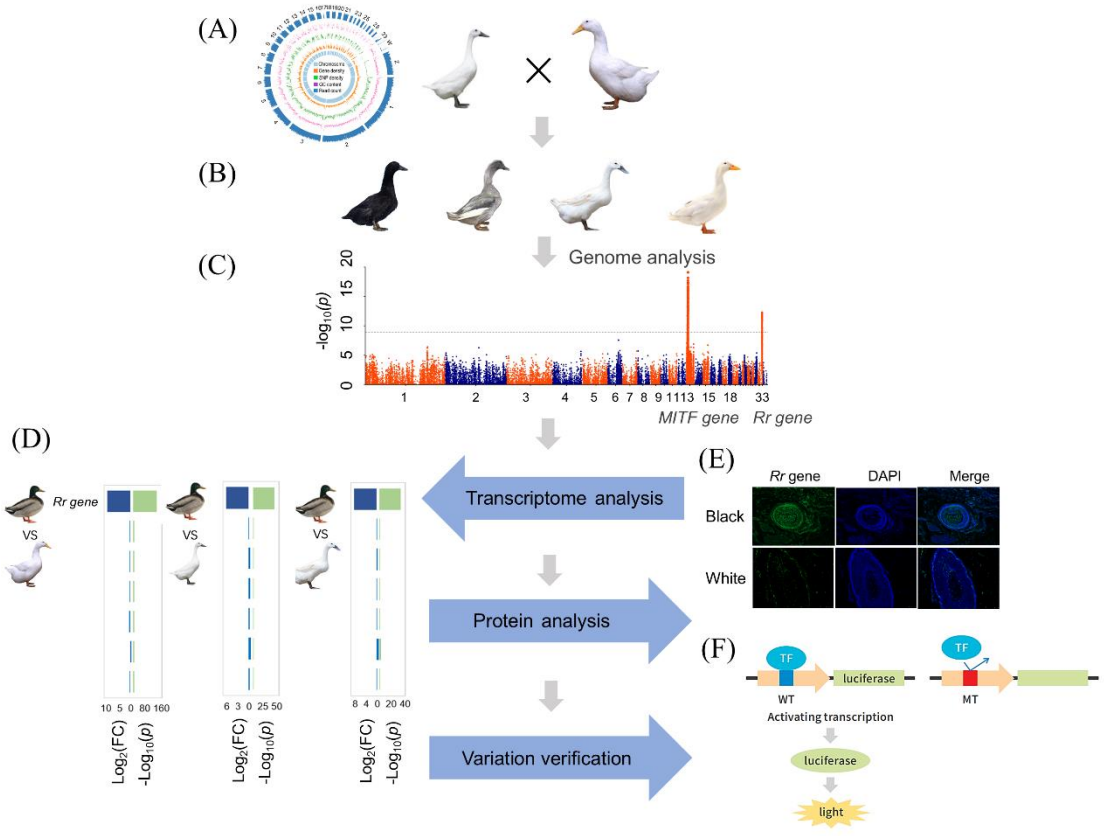

Figure 2-2: The genetic mechanism behind the phenotype shown in Liancheng ducks. The technical route of the genetic mechanism analyses in this thesis is shown in this figure and is further detailed in Chapter IV of this PhD thesis. (A) The first high-quality Liancheng duck genome sequenced by HiFi technology, assembled and annotated. (B) The resource groups from Liancheng duck and Pekin duck crosses. (C) A GWAS analysis showed two signals

affecting the white plumage trait in Liancheng ducks. (D) Gene expression of GWAS candidate region genes, including a causative gene in 1-week-old feather follicle samples of white and black-feathered ducks, with three replicates per sample. Log_2(FC) values were used to analyze gene expression differences and shown in blue, and -Log_{10}(*p*) values are shown in green. (E) Immunofluorescence results show the causative gene in feather bulb specimens of black- and white-feathered ducks, Black for Mallards and White for Liancheng ducks. (F) Functional analysis of the candidate variation controlling the white plumage phenotype of Liancheng duck.

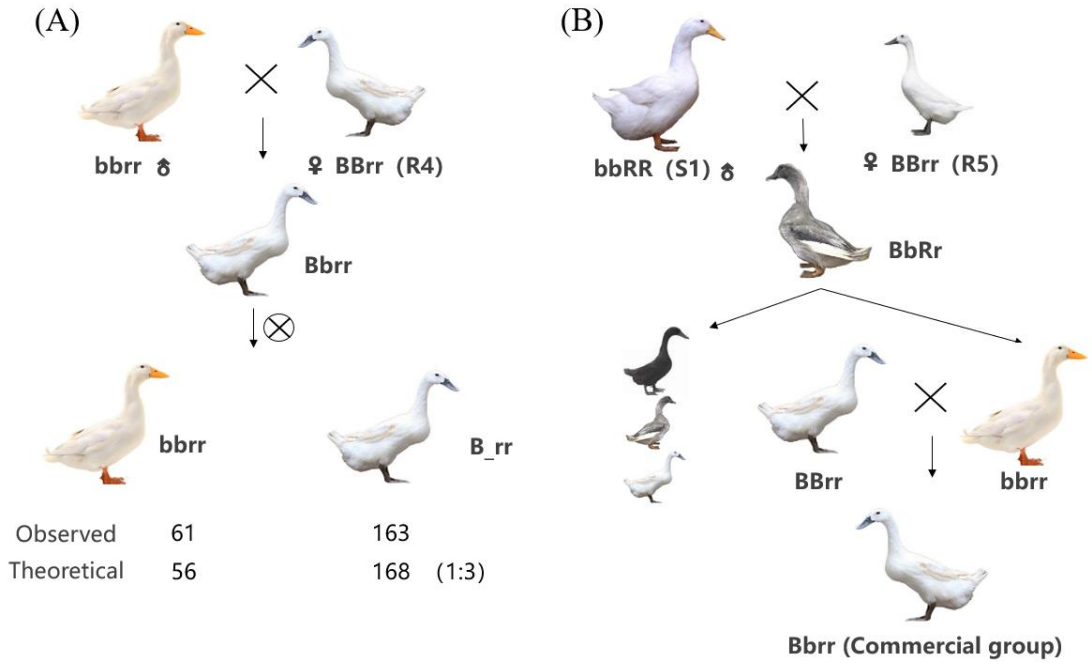

Figure 2-3: The experiment 3 showed (A) Cross breeding results between 1 *bbrr* genotype duck and 8 *BBrr* genotype ducks and (B) the breeding process of commercial *Bbrr* genotypes for high quality meat ducks. Part of the work is ongoing.

2.3 Thesis outline

Chapter I is a general introduction.

Chapter II are the objectives, research overview and the thesis outline.

Chapter III shows the determination of melanin content in various parts in Liancheng ducks (n=3), black-feathered Mallards (n=3), and Pekin ducks (n=3). For this purpose, high performance liquid chromatography (HPLC) and Masson-Fontana staining was used. The results showed that the melanin content of feathers, beak, and webbed feet in Liancheng ducks, Pekin ducks and Mallards. Melanin within hair follicles will be explored in the barb ridge and the hair matrix of black-feathered Mallard ducks. This study will reveal the unique feather color phenotype of Liancheng ducks.

This chapter is published with the following reference: *Wang Z, Guo Z, Mou Q, Liu H, Liu D, Tang H, Hou S, Schroyen M, Zhou Z. Unique feather color characteristics and transcriptome analysis of hair follicles in Liancheng White ducks. Poult Sci. 2024, 103(7):103794, doi: 10.1016/j.psj.2024.103794*.

Chapter IV focuses on exploring the genetic mechanism of white plumage formation of Liancheng ducks. To this extent, the Liancheng duck genome is *de novo* assembled. Beyond the epistatic effect of the *MITF* gene, GWAS analysis was used to pinpoint a specific genomic region encompassing the causative gene. Additionally, Dual Luciferase Reporter Technology revealed candidate variations that might alter causative gene transcription, potentially influencing plumage coloration in Liancheng ducks. Overall, these findings offer significant insights and direction for future studies and breeding programs aimed at understanding and manipulating avian plumage coloration.

This chapter is published with the following reference: *Wang Z, Guo Z, Liu H, Liu T, Liu D, Yu S, Tang D, Zhang H, Mou Q, Zhang B, Cao J, Schroyen M, Hou S, Zhou Z. A high-quality assembly revealing the PMEL gene for the unique plumage phenotype in Liancheng ducks. GigaScience 2025, 14:giae114, doi: 10.1093/gigascience/giae114*.

Chapter V is the application of the earlier found molecular markers to the actual production process. The molecular markers discovered in Chapter IV were applied to the rapid genetic selection and breeding process of Liancheng white-feathered and black-beak ducks in a practical production setting. Firstly, 1 *bbrr* genotype male white-feathered and yellow beak ducks will be crossbred with 8 *BBrr* genotype white-feathered and black beak ducks. All F1 *Bbrr* commercial descendants were white-feathered and black beak, and the genotypes were heterozygous. After F1 ducks continue hybridization, the phenotype and proportion of F2 ducks were in line with our expectations. Then, all blood samples of white-feathered yellow beak ducks from the F2 populations were collected and these *bbrr* genotypes with white-feathered yellow beak ducks were successfully selected. These *bbrr* ducks were crossbred with enough *BBrr* white-feathered black beak ducks, so as to produce *Bbrr* white-feathered black beak ducks.

This chapter is published with the following patents:

Molecular markers related to phenotypic traits of white feathered and black beak ducks and their breeding application in high quality ducks. Zhou Z, Hou S, Guo Z, Wang Z. No:202211228357.6 (Chinese Patent).

A cross-breeding method for effective protection of local high quality duck breeds. Zhou Z, Guo Z, Hou S, Wang Z. No:202310542366.0 (Chinese Patent).

Chapter VI is the general discussion and perspectives.

Chapter III

Determination of melanin content in different parts of Liancheng ducks with unique feather color phenotype

The aim of this chapter was to determine (1) the content of melanin in feathers of Liancheng ducks, Mallards and Pekin ducks, and (2) the content and distribution of melanin in hair follicles of Liancheng ducks, Mallards and Pekin ducks, and (3) the expression levels of candidate genes related to melanin synthesis in skin tissues of black-feathered Mallards at different periods and the expression levels of multiple tissues.

This chapter is adapted from the published article:

Wang Z, Guo Z, Mou Q, Liu H, Liu D, Tang H, Hou S, Schroyen M, Zhou Z. Unique feather color characteristics and transcriptome analysis of hair follicles in Liancheng White ducks. Poult Sci. 2024, 103(7):103794, doi: 10.1016/j.psj.2024.103794.

Author contribution: Zhen Wang conducted animal experiments, sample collection, sampling testing, melanin detection, RNA-seq data analyses, data visualization, manuscript drafting, editing and refinement.

3.1 Abstract

Avian feather color is a fascinating trait, and the genetic mechanism of duck plumage formation is still in the preliminary stage. In this study, 9 ducks from Mallards (n=3), Liancheng (n=3) and Pekin ducks (n=3) were used by high performance liquid chromatography (HPLC) and Masson-Fontana staining to reveal the difference of feather melanin content. RNA-seq from hair follicle tissues (1- and 8-week-old) of Liancheng ducks (n=5) and Pekin ducks (n=7) were used to analyze the candidate genes for the feather melanin synthesis. Pectorale, skin, liver, fat, brain, heart, kidney, lung, spleen of a black-feathered Mallards were collected for candidate gene expression. The results showed that the contents of feathers, beak, web melanin in Liancheng ducks were higher than in Pekin ducks ($p< 0.05$). Melanin within hair follicles was located in the barb ridge and hair matrix of black feather duck, also we found that *TYRP1*, *TYR*, *SOX10* genes were differentially expressed between Liancheng and Pekin ducks ($p<0.05$), and these genes were mainly expressed showed in duck skin tissues. This study revealed the unique feather color phenotype of Liancheng ducks, and the expression difference of melanin formation pathway candidate genes in different tissues and stages.

Key words: Liancheng ducks, RNA-seq, feather color, melanin, poultry

3.2 Introduction

White feather is an important economic trait in ducks, and it can be processed into down jackets, down comforters and other products. After the duck down is washed, extracted and graded, the raw material formed is the main material of a down jacket, and the market demand is large (Gong et al. 2010). For these products, white feather ducks are favored by consumers. The variations of feather color in birds are related to the distribution, content and proportion of pigments (Liu et al., 2023a). Melanin is the main pigment, which mainly affects the color of feathers and the depth of skin color. Melanin production is associated with mutations in related genes, and the amount and distribution of melanin in ducks varies breeds (Zhou et al., 2018).

Mallards are the ancestors of many different types of ducks. After human domestication, domestic ducks have gradually developed a variety of different breeds and types. These duck breeds, although domesticated, still retain many of the characteristics of mallard ducks, but there are also significant differences, such as feather color, and body size (Zhou et al., 2018; Feng et al., 2021). As a typical local breed, the Liancheng duck has white feathers but black beak and webbed feet, while the Pekin duck has white feathers and yellow beak and webbed feet. Both Pekin duck and Liancheng duck represent different aspects of Chinese food culture and are the treasures of Chinese duck breeds. Their feather color has attracted many researchers to conduct in-depth research, but no one has carried out a specific study on the comparison of melanin content between them.

Duck is a good model to study the plumage color of birds, but there are only a few studies describing feather color. Researchers found *MC1R* are related to black feather in ducks, and single nucleotide polymorphism mutations in the *MC1R* regulatory region are found to be associated with duck melanism (Liu et al., 2023a). Some studies have shown that the dark spots in duck feathers are caused by the *EDNRB2* gene (Xi et al., 2021). The *MITF-M* gene is also considered responsible for white feathers (Zhang et al. 2018; Zhou et al. 2018). Although there is already more research performed on duck feather color compared to that of many birds, the research on duck feather color is still in the primary stage, and there are still many genetic mechanisms of duck feather color formation that deserve to be explored.

The main mechanism of High Performance Liquid Chromatography (HPLC) is to oxidize eumelanin to pyrrole Pyrrole-2,3-dicarboxylic acid (PDCA) and Pyrrole-2,3,5-tricarboxylic acid (PTCA), respectively, and then perform liquid phase detection and quantitative conversion. Ultraviolet spectrophotometry can only achieve rough quantification of melanin content. There are problems of low separation degree and interference of similar substances in capillary electrophoresis. Thin layer chromatography (TLC) is a complicated and time-consuming process with low recovery rate. The HPLC method is simple, rapid and accurate to calculate the content of melanin. Masson staining is the most classic method of connective tissue melanin staining (Kwon-Chung et al., 1981), which are used for qualitative detection of skin tissue melanin in this study. At the same time, with the help of advanced sequencing

techniques such as RNA-seq, more and more candidate genes related to feather color in birds can be revealed. RNA-seq results can be used to study the molecular mechanism of feather color formation (Domyan et al., 2019; Du et al., 2023), help us understand the biological process of feather formation of different colors, and provide more useful reference information for poultry breeding. More duck breeds should be investigated to reveal the gene expression profiles associated with duck plumage color. Liancheng duck has a small amount of melanin in its feathers at birth, while Pekin duck has no melanin in its feathers. Therefore, it is worth exploring whether there are differences in feather melanin between adult Liancheng ducks and Pekin ducks, and what the function would be of potential differentially expressed genes (DEGs). In this study, we hope to analyze the formation principle of the unique feather color phenotype of Liancheng duck, a rare local breed in China, so as to provide a theoretical basis for the study of bird feather color and contribute to research related to breeding of white feather in poultry and subtle phenotypic differences in biodiversity.

3.3 Materials and methods

3.3.1 Animal experiment and sample collection

All procedures used for this animal study fully complied with the guidelines for the care and use of experimental animals established by the Chinese Academy of Agricultural Sciences (IAS2022-103). The ducks were randomly selected from a duck farm and were half male and half female, all healthy, and fed in similar conditions. All the duck samples in the study met the requirements of animal ethics. Hair follicle and skin tissue for polyA mRNA extraction and subsequent RNA-seq analyses were collected from ducks (1-week-old, 8-week-old), including 5 Liancheng ducks, and 7 Pekin ducks. 3 Mallards, 3 Liancheng ducks and 3 Pekin ducks were used for the determination of melanin for HPLC (Figure 3-1). Pectorale, skin, liver, fat, brain, heart, kidney, lung, spleen of an 8-week-old black-feathered Mallard were collected for RNA -seq analysis, while skin tissues of 4 samples of black-feathered Mallards at 1 day, 2 weeks, 4 weeks, and 8 weeks after birth were collected for time-course RNA-seq analysis. The sample size for each period is one. All samples of three breeds of ducks were immediately frozen in liquid nitrogen and stored at -80°C. Fresh skin tissue from each of the three breeds of ducks were soaked in 4% paraformaldehyde liquid and fixed for subsequent analysis.

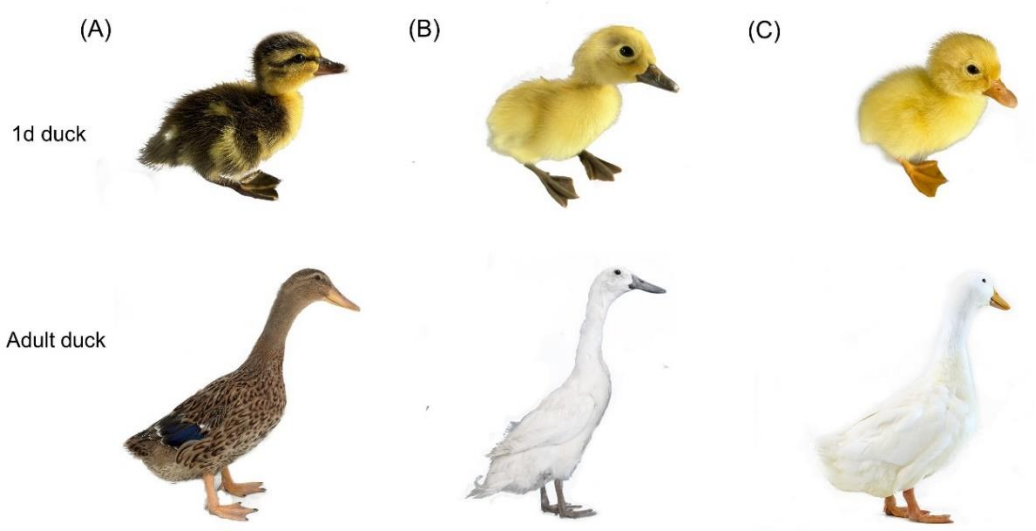

Figure 3-1. 1-day-old and adult periods of Mallards, Liancheng ducks and Pekin ducks. The plumage of Liancheng ducks and Pekin ducks change from light yellow to white after birth.

3.3.2 Determination of melanin content by high performance liquid chromatography

Melanin can be oxidized and hydrolyzed to PDCA and PTCA in the alkaline environment of hydrogen peroxide (Bi et al., 2023). 0.1g/ml were used to make quantitative standard curves. 0.01g plumage tissues of 6-week-old of Liancheng ducks, and Pekin ducks were accurately weighed into centrifugal tubes, and 0.5ml of 30% hydrogen peroxide and 0.5ml of 2M ammonia were added. The hydrolysate was gently mixed and hydrolyzed at 30^{o}C for 12 h. First, 0.4ml of 11.3% ammonium sulfite aqueous solution was added, placed for 5min, then 0.4ml of 4M acetic acid was added, and 3000g of supernatant was collected by centrifugation. Then, the supernatant was added to the solid phase extraction column for adsorption. Third, 3ml aqueous methanol was added for leaching, then 1ml methanol solution containing 10% triethylamine was added twice for elution. The eluted liquid was dried by vacuum drying or nitrogen blowing. The dried liquid was redissolved in 1ml methanol solution containing 0.5% formic acid. Finally, the filtrate was transferred to a 2ml sample vial for HPLC detection (Wang et al., 2014). In this study, ultra-high pressure liquid phase (ACQUITY UPLC system, Waters, USA) was used.The mobile phase is A: water containing 0.1% acetic acid and B: acetonitrile containing 0.1% acetic acid. The chromatography was performed on Agilent ZORBAX C18 column (3.0mmx150mm) with a flow rate of 0.35ml/min and column temperature of 45^{o}C. The acquisition mode of this study is multi-heavy ion detection, and it is negative ion detection, and the ion source voltage and temperature are 5500V and 500 ^{o}C respectively (Wang et al., 2014).

3.3.3 Masson-Fontana staining to determine melanin distribution

Fresh 1×1cm^2 skin tissue from 6-week-old of black-feathered Mallard, Liancheng duck, and Pekin duck were soaked in 4% paraformaldehyde. The samples were placed in the fixing solution of 20 times the sample volume and fixed for more than 24h. Dewaxing and hydration of paraffin sections (Lecia CM1900, Lecia, China) involves the following steps: Firstly, dewaxing in xylene (II) for 10min. Then 70%, 80%, 95% anhydrous ethanol was used to wash for 2min each time, and finally anhydrous ethanol washed for 5min and distilled water washed for 2min. Put it in Fontana ammonia-silver solution, stain it against light for 12-24h or incubate it at 56°C for 30-40min. Soak in distilled water for 5-6 times, 1-2min each time. The sections were treated with hypo solution (sodium thiosulphate) for 1-5min. Treat with tap water for 3-5min. 95% ethanol, anhydrous ethanol dehydration, and xylene transparent, neutral gum seal (Kwon-Chung et al., 1981). Finally, Seal with neutral gum and observe under a Panoramic scanner (3DHISTECH P250 FLASH, 3DHISTECH, Hungary).

3.3.4 RNA extraction, library construction, and sequencing

Using an RNA extraction kit (Vazyme, China), total RNA of the hair follicles of 1- and 8- week old ducks was extracted and converted into cDNA, including 5 Liancheng ducks and 7 Pekin ducks, as well as 4 different periods of Mallard skin tissues and 9 multi-tissues. In total, 25 libraries were finally produced for the RNA-seq experiment. The average output was 6 Gb per library. RNA-seq paired-end reads from each of 25 libraries were mapped against the above-mentioned Pekin duck reference genome using Hisat. The constructed library was sequenced on the Illumina Hiseq X Ten sequencing platform at 150bp both ends (Gai et al., 2023).

3.3.5 Comparative analysis of the sequence of RNA data

According to the Perl script, we will analyze the raw fastq data to filter the raw data in fastq format. This script deletes reads with adapters, low-quality reads, and reads with poly-N. The latest Pekin duck reference genome ZJU1.0 (GCF_015476345.1)) and gene model annotation files (GCF_015476345.1) from the NCBI database. The fragments per kilobase per million values were calculated by Hisat (Zhou et al., 2018). The clean data were then aligned to the reference genome using HTSeq (Liu et al. 2022), and the counts per million mapped sequence read (CPM) for each gene were calculated. Differential expression for each gene in different samples were calculated using edgeR version 3.20.9 software package (Gai et al., 2023; Liu et al., 2023b).

3.4 Results

3.4.1 Plumage melanin content and melanin distribution in hair follicle of ducks

The head, back, abdomen, wing, tail plumages of Mallards, Liancheng ducks and Pekin ducks were determined by HPLC method. Meanwhile, tissue samples from the beak and web tissues of Liancheng ducks and Pekin ducks were collected for the determination of melanin content. The results showed that the content of feather melanin in Liancheng ducks was significantly higher than Pekin ducks (p<0.01) (Figure 3-2), but its melanin content were lower than that of Mallards. The melanin contents of Liancheng ducks in the beak and web were very significantly higher than Pekin ducks (p<0.001). Masson-Fontana staining showed the melanin was located in the barb ridge and hair matrix of black-feathered Mallards, and there was almost no obvious melanin distribution in the hair follicles of Liancheng and Pekin ducks (Figure 3-3). The results showed that there was a small amount of melanin in Liancheng duck feathers, and the head feathers had the highest amount of melanin in all feathers.

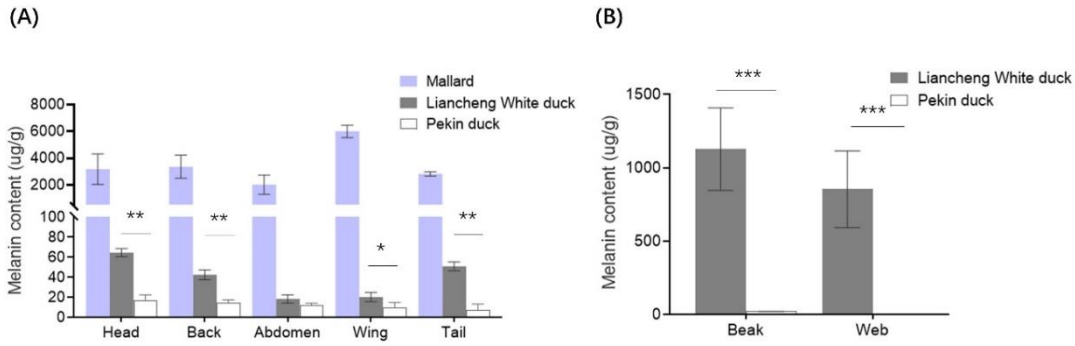

Figure 3-2. Determination of melanin content in duck tissues. (A) Melanin content in head, back, abdomen, wing, tail feathers of Mallards (n=3), Liancheng (n=3) and Pekin ducks (n=3). (B) Content of melanin in beak and web tissues of Liancheng (n=3) and Pekin ducks (n=3). Data are presented as mean ± SD (n=3).

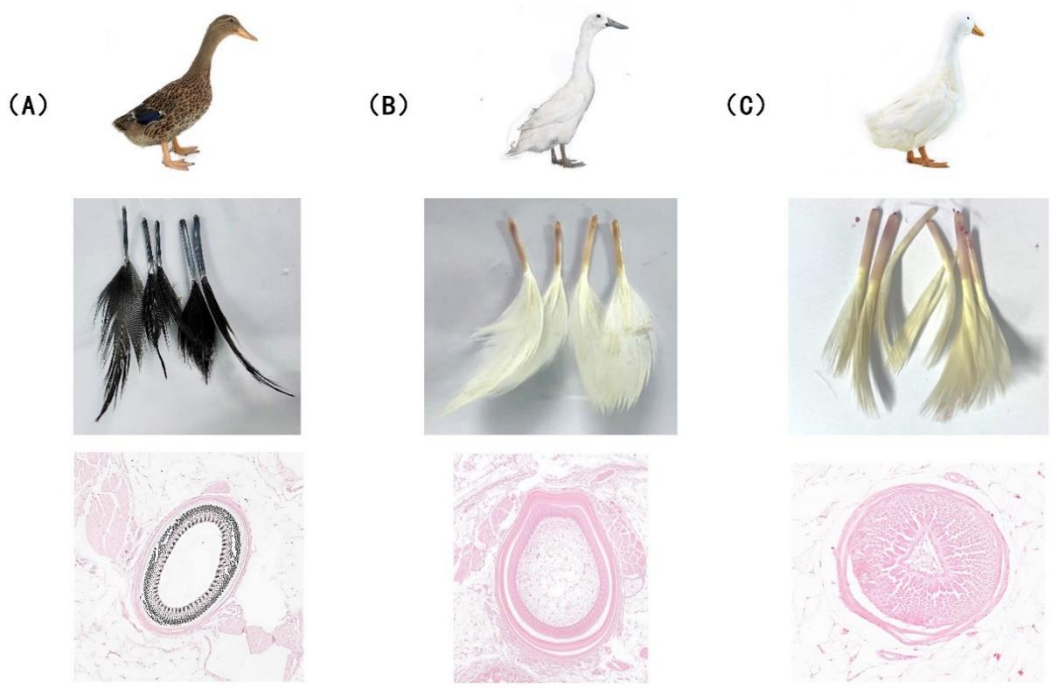

Figure 3-3. Masson-Fontana staining of melanin in the skin tissues of black-feathered Mallard (A), Liancheng duck (B) and Pekin duck (C), and their hair bundles, and hair follicles in the back skin.

3.4.2 Differentially expressed genes in hair follicles of Liancheng and Pekin ducks

After filtering DEGs with an FDR ≤ 0.05 and a | Log2 (fold change) | ≥ 1, 659 DEGs were found between 1-week-old Liancheng and Pekin ducks, while 2093 DEGs were found between 8-week-old Liancheng and Pekin ducks. Among these differential genes, we found genes including *TYRP1*, *TYR* and *SOX10*, that were higher expressed in the hair follicles of Liancheng ducks than in those of Pekin ducks. These genes participate in the melanin synthesis pathway, indicating that the feather color of Liancheng duck and Pekin duck is indeed different in the synthesis of melanin. The RNA-seq results showed that the CPM expression of these candidate genes in hair follicles of Liancheng ducks was higher than that in Pekin ducks (Figure 3-4).

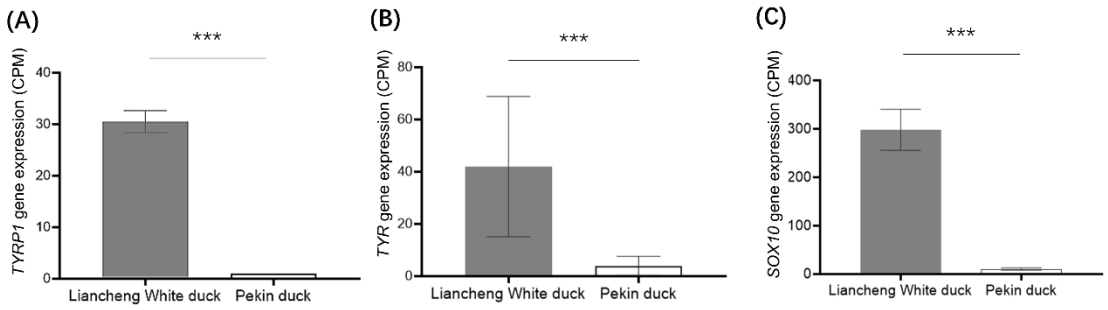

Figure 3-4 Expression levels of candidate genes that are differentially expressed in hair follicles of Liancheng (n=5) and Pekin ducks (n=7). *** p<0.001.

3.4.3 Melanin synthesis candidate gene expression of different tissues and periods in hair follicle of black-feathered Mallards

The expression of differentially expressed *TYRP1*, *TYR*, and *SOX10* genes varied across various tissues. However, these genes demonstrated heightened expression within skin tissues compared to other tissues, such as pectorales, liver, fat, brain, heart, kidney, lung, and spleen (Figure 3-5A). The results showed that *TYRP1*, *TYR*, and *SOX10* were highly expressed in the skin tissue of black-feathered Mallards. Meanwhile, the expression levels of these candidate genes had variational tendency in different growth stages of black-feathered Mallards (Figure 3-5B). The expression levels were higher in hair follicle tissues at the early stage, and reached a peak at 2-week-old, which was consistent with the early molting behavior of ducks. These results indicated that *TYRP1*, *TYR* and *SOX10* genes were highly expressed in duck dark-feathered skin tissues, while their expression levels were generally low in other tissues.

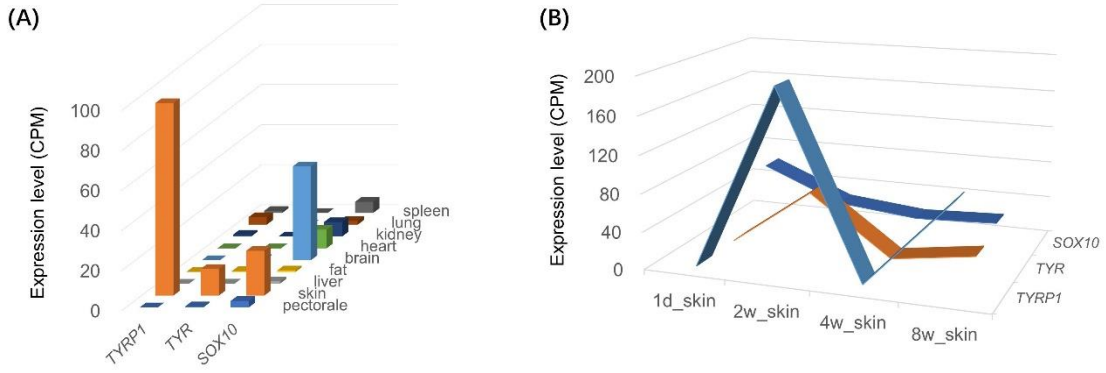

Figure 3-5 Expression levels of melanin synthesis gene *TYRP1*, *TYR*, and *SOX10* in different tissues and different periods of skin tissues of black-feathered Mallards.

3.5 Discussion

Ducks are a good model for studying feather color since they have a variety of feather color patterns (Zhou et al., 2018). Different breeds of ducks have different feather colors and patterns, and these differences are mainly due to the interaction of genetic factors. As a typical local breed, the Liancheng duck has white feathers but black beak and webbed feet, while the Pekin duck has white feathers and yellow beak and web, but the plumage color of both the Liancheng and Pekin duck is white (Figure 3-1), and so far no research has been carried out comparing the feather melanin of Liancheng and Pekin ducks. In this study, we first discovered that the content of feather melanin in Liancheng duck was higher than in Pekin duck (p<0.01) (Figure 3-2). Masson-Fontana staining showed that the melanin was located in the barb ridge and hair matrix of black-feathered Mallards, which is consistent with previous findings (Koch et al., 2019), and there was almost no obvious melanin distribution in the hair follicles of Liancheng ducks and Pekin ducks (Figure 3-3). This result indicated that the feather of Liancheng ducks contained a small amount of melanin, and the white feather trait of it was caused by the low content of melanin. Through RNA-seq analysis, we found that there are differences in the expression of genes related to the synthesis of melanin in the feathers

of Liancheng Duck and Pekin duck, including *TYRP1*, *TYR*, and *SOX10* (Figure 3-4). These genes have been widely reported to be involved in the melanin synthesis pathway (Sultana et al., 2018; Yun et al., 2019).

Melanin is synthesized by melanocytes and is mainly found in the skin, feathers and eyes of birds. In this study, the expression levels of the *TYRP1*, *TYR*, and *SOX10* genes in different tissues of the black-feathered Mallards were analyzed, and it was found that they were mainly highly expressed in the skin tissues (Figure 3-5A). Meanwhile, we found that the expression levels of these genes were peaked around two weeks old of age (Figure 3-5B), indicating that these genes played an important role in the early growth and development of duck feathers. The differences in the dynamic expression of these genes may be related to the molting behavior of ducks (Zhang et al., 2023). The early feather development is more vigorous, and later the melanin remains in the feathers, and the feathers are no longer replaced.

The melanin formation pathway is relatively conserved, which means that the basic processes and molecular mechanisms involved in melanin formation are similar across species and individuals. The melanin formation pathway mainly includes the catalysis of tyrosinase and a series of subsequent oxidation and polymerization reactions. The structure and function of these enzymes are conserved between different species and individuals, thus ensuring the stability and reliability of melanin formation pathways (Cooper. 2017). The melanin synthesis pathway is conservative, and melanocytes in animals produce melanocytes, which lead to the formation of melanin, a process that requires the involvement of multiple functional genes. Eumelanin and phemelanin make up the melanin of mammals, with eumelanin appearing brown or black and phemelanin yellow or red, so eumelanin is the main source of duck feather color. Eumelanin can be decomposed into PDCA and PTCA by oxidation. The formation of eumelanin is closely related to the tyrosine pathway. Tyrosine is converted into 3,4-dihydroxyphenylalanine (dopa) in melanocytes, and then further oxidation, decarboxylation and other reactions are converted into indolequinone, and finally indolequinone is polymerized into eumelanin (D'Mello et al., 2016; Koch et al., 2019). The key enzyme in this process is tyrosinase (TYR), which catalyzes the conversion of tyrosine into melanin. Tyrosine is ubiquitous in the body, but the amount and activity of tyrosinase determines whether melanin is eventually formed (Barriales et al., 2023). In addition, this pathway contains some genes that affect the amount of melanin. The *TYRP1* gene encodes tyrosinase-related protein 1, a putative membrane-bound, coppercontaining enzyme that oxidizes the 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into eumelanin pigment in the last step of melanin synthesis (Choudhury et al., 2024). *SOX10* can promote the synthesis of melanin and proliferation of melanocytes. It activates the *TYR* core enhancer, which helps to regulate the tyrosinase gene in melanocytes. *SOX10* is a molecular marker for ectodermal stem cells and progenitor cells, and it regulates the terminal differentiation of oligodendrocytes in the brain and notochord (Yun et al., 2019). Therefore, this study also found that *SOX10* is highly expressed in the brain. At present, the predominant candidate genes related to duck feather color are *TYR* (Sultana et al., 2018), *TYRP1* (Xi et al., 2020), *DCT* (Sultana

et al., 2018), *MC1R* (Liu et al., 2023a), *EDNRB2* (Guo et al., 2022), and *MITF* (Zhou et al., 2018; Lin et al., 2019; Wang et al., 2022).

TYRP2, a key gene in the tyrosine pathway, was not detected in either feather color. This is consistent with another study in human (Dolinska et al. 2022). The loss of *TYRP2* gene expression in melanin-producing melanocytes of black hair follicles showed eumelanogenesis do not require *TYRP2* expression. Meanwhile, the lack of *TYRP2* in melanin production in duck hair follicles was consistent with previous study, so the function of *TYRP2* in ducks and the mechanism of melanin synthesis in duck hair follicles need further study. Taken together, these results suggest that lack of expression of *TYR* and *TYRP1* leads to inadequate biosynthesis of melanin in white hair follicles, which is the direct cause of duck white feathers.

Microphthalmia-associated transcription factor (MITF) transcription factor regulates the expression of *TYR* and *TYRP1*, and *TYRP1* is also involved in the process of melanin generation, and the MITF binding site "M-box" structure has the common characteristics of tyrosinase gene family (Wang et al., 2023). It regulates the differentiation and function of melanocytes through its interaction with transcription factors such as *MITF*. *TYRP1* can also interact with *TYR* to maintain its stability and regulate its catalytic activity. This study did not distinguish the different spliceosomes of *MITF*, so we did not find difference in the expression of *MITF-M* in the hair follicles of Liancheng ducks and Pekin ducks. In the future, the genetic mechanism of duck feather color formation still needs to be further explored, and we believe that there are still many unanswered questions.

The *Premelanosome* (*PMEL*) gene also is thought to be the causative gene of melanin formation in bird feathers, but was not found in this study. We believe that this may be caused by presently incomplete genome assembly and annotation. In Chapter IV, we assembled a high-quality Liancheng duck reference genome for further analysis.

In conclusion, this study suggested that the content of feather melanin in Liancheng duck was higher than that in Pekin duck, and it is much lower than black-feathered ducks. It indicated that the feathers of Liancheng ducks had a small amount of melanin content, resulting in a unique white feather phenotype. Moreover, *TYRP1*, *TYR*, *SOX10* genes mainly expressed in the skin of ducks. This research showed that we should pay attention to the subtle differences between avian feather coloration and help reveal the genetic mechanism behind different avian feather coloration. In actual production, it also helps breeders to quickly select pure white high-quality down and improve production efficiency. However, the formation of feather color phenotypes in birds is complex. Our research is still superficial, and further in-depth and more systematic research should be carried out.

Acknowledgments

This work was supported by grants from the Innovation Program of Chinese Academy of Agricultural Sciences (CAAS--SCAB-202302), the National Science Fund for Distinguished Young Scholars (32325047), the China Agriculture Research System

of MOF and MARA (CARS-42-05), and the National Key R&D Program of China (2023YFD1300300).

References

Barriales K, Kassem S, Sementa D, et al. Localized and regulated peptide pigment formation inside liquid droplets through confined enzymatic oxidation. Chem Commun (Camb). 2023;59(95):14138-14141.

Bi H, Tranell J, Harper DC, et al. A frame-shift mutation in COMTD1 is associated with impaired pheomelanin pigmentation in chicken. PLoS Genet. 2023;19(4):e1010724.

Cooper CD. Insights from zebrafish on human pigment cell disease and treatment. Dev Dyn. 2017;246(11):889-896.

Choudhury A, Ramakrishnan R, Ghosh D. Structure prediction from spectra amidst dynamical heterogeneity in melanin. Chem Commun (Camb). 2024;60(19):2613-2616.

D'Mello SA, Finlay GJ, Baguley BC, Askarian-Amiri ME. Signaling Pathways in Melanogenesis. Int J Mol Sci. 2016;17(7):1144.

Dolinska MB, Woods T, Osuna I, Sergeev YV. Protein Biochemistry and Molecular Modeling of the Intra-Melanosomal Domain of Human Recombinant Tyrp2 Protein and OCA8-Related Mutant Variants. Int J Mol Sci. 2022;23(3):1305.

Domyan ET, Hardy J, Wright T, et al. SOX10 regulates multiple genes to direct eumelanin versus pheomelanin production in domestic rock pigeon. Pigment Cell Melanoma Res. 2019;32(5):634-642.

Du W, Yang Z, Xiao C, et al. Identification of genes involved in regulating the development of feathered feet in chicken embryo. Poult Sci. 2023;102(9):102837.

Feng P, Zeng T, Yang H, et al. Whole-genome resequencing provides insights into the population structure and domestication signatures of ducks in eastern China. BMC Genomics. 2021;22(1):401.

Gai K, Ge Y, Liu D, et al. Identification of key genes related to intramuscular fat deposition in Beijing-You chicken by mRNA and miRNA transcriptome analysis. Poult Sci. 2023;102(11):103035.

Gong Y, Yang Q, Li S, et al. Grey plumage colouration in the duck is genetically determined by the alleles on two different, interacting loci. Anim Genet. 2010;41(1):105-108.

Guo Q, Jiang Y, Wang Z, et al. Genome-Wide Analysis Identifies Candidate Genes Encoding Feather Color in Ducks. Genes (Basel). 2022;13(7):1249.

Koch SL, Shriver MD, Jablonski NG. Variation in human hair ultrastructure among three biogeographic populations. J Struct Biol. 2019;205(1):60-66.

Kwon-Chung KJ, Hill WB, Bennett JE. New, special stain for histopathological diagnosis of cryptococcosis. J Clin Microbiol. 1981;13(2):383-387.

Liu D, Zhang H, Yang Y, et al. Metabolome-Based Genome-Wide Association Study of Duck Meat Leads to Novel Genetic and Biochemical Insights. Adv Sci (Weinh). 2023b;10(18):e2300148.

Liu H, Xi Y, Tang Q, et al. Genetic fine-mapping reveals single nucleotide polymorphism mutations in the MC1R regulatory region associated with duck melanism. Mol Ecol. 2023a;32(12):3076-3088.

Liu X, Zhao J, Xue L, et al. A comparison of transcriptome analysis methods with reference genome. BMC Genomics. 2022;23(1):232.

Lin R, Lin W, Zhou S, et al. Integrated Analysis of mRNA Expression, CpG Island Methylation, and Polymorphisms in the MITF Gene in Ducks (Anas platyrhynchos). Biomed Res Int. 2019;2019:8512467.

Sultana H, Seo D, Choi NR, et al. Identification of polymorphisms in MITF and DCT genes and their associations with plumage colors in Asian duck breeds. Asian-Australas J Anim Sci. 2018;31(2):180-188.

Wang J, Li Y, Feng C, et al. Peptide OA-VI12 restrains melanogenesis in B16 cells and C57B/6 mouse ear skin via the miR-122-5p/Mitf/Tyr axis. Amino Acids. 2023;55(11):1687-1699.

Wang, H., Zhang, L, Li, Q., et al. 2014. Simultaneous determination of melanin and inosine in muscle of black bone chicken by high performance liquid chromatography. Chinese Poultry, 36:12-16. (in Chinese).

Wang L, Yang L, Yang S, et al. Identification of genes associated with feather color in Liancheng white duck using F_{ST} analysis. Anim Genet. 2022;53(4):518-521.

Xi Y, Liu H, Li L, et al. Transcriptome Reveals Multi Pigmentation Genes Affecting Dorsoventral Pattern in Avian Body. Front Cell Dev Biol. 2020;8:560766.

Xi Y, Xu Q, Huang Q, et al. Genome-wide association analysis reveals that EDNRB2 causes a dose-dependent loss of pigmentation in ducks. BMC Genomics. 2021;22(1):381.

Yun CY, Hong SD, Lee YH, et al. Nuclear Entry of CRTC1 as Druggable Target of Acquired Pigmentary Disorder. Theranostics. 2019;9(3):646-660.

Zhang L, Cai C, Liu X, et al. Multi-Stage Transcriptome Analysis Revealed the Growth Mechanism of Feathers and Hair Follicles during Induction Molting by Fasting in the Late Stage of Egg Laying. Biology. 2023;12(10):1345.

Zhang Z, Jia Y, Almeida P, et al. Whole-genome resequencing reveals signatures of selection and timing of duck domestication. Gigascience. 2018;7(4):giy027.

Zhou Z, Li M, Cheng H, et al. An intercross population study reveals genes associated with body size and plumage color in ducks. Nat Commun. 2018;9(1):2648.

Chapter IV

A high-quality assembly revealing *PMEL* gene for unique plumage phenotype in Liancheng ducks

The aim of this chapter was to (1) construct the F2 and Fn resource groups of Liancheng ducks and Pekin ducks, and to (2) finish a high-quality reference genome of Liancheng duck for the first time, as well as to (3) identify the gene and variations responsible for white feather phenotype formation of Liancheng ducks.

This chapter is adapted from the published article:

Wang Z, Guo Z, Liu H, Liu T, Liu D, Yu S, Tang H, Zhang H, Mou Q, Zhang B, Cao J, Schroyen M, Hou S, Zhou Z. (2025). A high-quality assembly revealing PMEL gene for unique plumage phenotype in Liancheng ducks. *Gigascience*. https://doi.org/10.1093/gigascience/giae114.

Author contribution: Zhen Wang conducted resource population, animal experiments, sample collection, bioinformatic analyses, molecular experiment, data visualization, manuscript drafting, editing and refinement.

4.1 Abstract

Plumage coloration is a distinctive trait in ducks, and the Liancheng duck, characterized by its white plumage and black beak and webbed feet, serves as an excellent subject for such studies. However, academic comprehension of the genetic mechanisms underlying duck plumage coloration remains limited. To this end, the Liancheng duck genome (GCA_039998735.1) was hereby *de novo* assembled using HiFi reads, and F2 segregating populations were generated from Liancheng and Pekin ducks. The aim was to identify the genetic mechanism of white plumage in Liancheng ducks.

In this study, 1.29 Gb Liancheng duck genome was assembled, involving a contig N50 of 12.17 Mb and a scaffold N50 of 83.98 Mb. Beyond the epistatic effect of the *MITF* gene, GWAS analysis pinpointed a 0.8Mb genomic region encompassing the *PMEL* gene. This gene encoded a protein specific to pigment cells and was essential for the formation of fibrillar sheets within melanosomes, the organelles responsible for pigmentation. Additionally, a dual-luciferase reporter vector analysis revealed two candidate SNPs (Chr33:5,303,994A>G; 5,303,997A>G) that might alter *PMEL* transcription, potentially influencing plumage coloration in Liancheng ducks.

Our study has assembled a high-quality genome for the Liancheng duck and has presented compelling evidence that the white plumage characteristic of this breed is attributable to the *PMEL* gene. Overall, these findings offer significant insights and direction for future studies and breeding programs aimed at understanding and manipulating avian plumage coloration.

Key words: duck, genome assembly, plumage color, *PMEL*, melanin

4.2 Introduction

Plumage coloration, a diverse and conspicuous trait among bird species, is a valuable attribute for investigating natural and artificial selection. Melanin, the predominant pigment in avian plumage, occurs as a mix of eumelanin and phaeomelanin across various tissues, leading to the extensive color variation observed in birds (Ito et al., 2003; Zhou et al., 2018). Eumelanin deposition in plumage is responsible for black and brown coloration, constituting the predominant pigment in bird feathers (Land et al., 2000). Extensive research on eumelanin and melanin-related genes has significantly enhanced public understanding of avian plumage coloration, which is a captivating ornamental feature. The duck (*Anas platyrhynchos*) (NCBI: txid8839), widely distributed globally, displays a continuum of plumage colors spanning white to black, possibly as an adaptation to varied ecological environments. Those diverse plumage color patterns make ducks a key animal model for pigmentation studies. Despite advancements in understanding the biological and evolutionary aspects of plumage coloration, the genetic basis of these colors in ducks remains poorly understood.

The Liancheng (LC) duck meat is valued for its nutritional and medicinal benefits, supporting digestion, boosting blood health, and providing anti-inflammatory effects, making it ideal for nourishing soups and overall wellness. Meanwhile, the LC duck possesses a distinctive phenotype marked by white feathers, black beak, and black webbed feet. It is recognized for its significant melanin deposition in the beak and webbed feet, primarily due to the involvement of eumelanin as the main pigment (Wang et al., 2024). The biosynthesis of eumelanin is a complex process that involves three critical steps: Initially, tyrosinase catalyzes the oxidation of tyrosine to dihydroxyphenylalanine (DOPA). Subsequently, an oxidase enzyme converts DOPA into dopaquinone. Ultimately, dopaquinone undergoes a series of cyclic transformations, resulting in the production of pigment and the formation of melanin (Haase et al., 1995). This synthetic pathway holds much significance in eumelanin synthesis, particularly in the pigmentation of skin and feathers in ducks. In this process, *MITF* serves as a key target of various signal transduction pathways, also the main regulator of melanin production. However, the genetic basis of melanin deposition and the specific genes involved in the formation of white feathers in LC ducks remain to be further explored.

The initial draft of the duck assembly was first reported in 2013 (Huang et al., 2013). Refinements to the Pekin (PK) duck genome have achieved a scaffold N50 length of up to 76.3 Mb by 2020, which now serves as a foundational resource for duck genome analysis (Li et al., 2021). Compared to the high quality and variety of the genome assemblies of other birds, especially the one for chickens, necessitate further improvements in duck assembly quality. Previous research on animal breeding and genetics in ducks has primarily focused on meat quality (Liu et al., 2023a), adipose tissue deposition (Zhu et al., 2021), and muscle weight (Yu et al., 2023). However, genetic mechanisms of plumage coloration remain largely unexplored, and no reference genome of the famous LC duck has been published, making an upgraded LC duck

genome necessarily important to provide foundational data for relevant future studies. The accuracy of gene localization greatly depends on the quality of the genome assembly (Zhu et al., 2021). A recent study leveraging the widely utilized duck genome assembly (GCA_003850225.1) has pinpointed a 6.6kb intronic insertion within *MITF*. This insertion is suspected to influence splicing events, consequently resulting in the white feather phenotype observed in ducks (Wang et al., 2024). Additionally, another study has uncovered four new single nucleotide polymorphisms (SNPs) in the *MC1R* regulator region associated with black plumage in ducks (Liu et al., 2023b). This partially explains the mechanism of melanin formation in duck feathers. However, questions regarding feather coloration remain unanswered. Long-read sequencing technology, also known as third-generation sequencing, is capable of generating reads exceeding 10 kb in length. This capability allows it to span highly repetitive genomic regions and resolve assembly gaps previously intractable, thereby enhancing the continuity of genomic assemblies. As an alternative to relying on short-read data polishing, PacBio introduces high-fidelity reads (HiFi reads) which can provide more accurate, continuous, and complete genetic information. It has thus become a key technology for research (Wenger et al., 2019). Meanwhile, advancements in gene chips and genome re-sequencing technologies have made genome-wide association studies (GWAS) powerful tools for identifying genetic variations linked to phenotypes. GWAS analysis has uncovered mutations in the *MuPKS* gene responsible for yellow and blue plumage in parrots (Cooke et al., 2017). Additionally, GWAS has localized a nonsense mutation (W49X) in the *SLC2A11B* gene, which is associated with the white eye trait in pigeons (Si et al., 2021). To this end, the presence of the *PMEL* gene, which was previously considered “missing” gene in ducks, was hereby explored based on a high-quality *de novo* genome by HiFi sequencing. Building on previous findings, this research identified two closely linked SNPs in the regulatory region that might influence *PMEL* transcription, leading to the white plumage observed in LC ducks. Overall, this investigation provides a valuable genome assembly, molecular markers for duck breeding, and offers insights into plumage color patterns in avian species.

4.3 Materials and methods

4.3.1 Ducks and sampling

Herein, all animal procedures adhered to the guidelines for the care and use of experimental animals set by the Chinese Academy of Agricultural Sciences (IAS2022-105). The study was approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences. A blood sample was obtained from a female Liancheng duck for the purpose of conducting a *de novo* genome assembly. A total of 366 parental and intercross population duck plumage color phenotypes were recorded from a previous gradient consanguinity population (Huang et al., 2013). These included 117 PK ducks, 59 LC ducks, 38 white-feathered ducks with yellow beaks (WY), 42 white-feathered ducks with black beaks (WB), 67 gray-feathered ducks with black beaks (GF), and 41

black-feathered ducks with black beaks (BF). All underwent whole-genome resequencing (Supplementary Table S1). Additionally, genome data from 23 black-feathered ducks, comprising 20 Mallards (MD) and 3 Putian (PT) ducks, were used for comparative analysis (Zhou et al., 2018).

For transcriptomics analysis, skin tissues from LC ducks were collected at embryonic stages of 12 days, 15 days, 20 days, 28 days, and one week after birth. Each sample group, except for the four 12-day embryo samples, consisted of three biological replicates. Additionally, tissues from an 8-week-old black-feathered Mallard (MD) duck, including heart, fat, muscle, brain, spleen, lung, liver, kidney, and skin, were collected, with one replicate per tissue (Supplementary Table S2) (Zhou et al., 2018). Feather bulb specimens were also collected from feather follicle from 1-week-old black-feathered MD ducks, LC ducks, PK ducks, and WB ducks, with three biological replicates in each sample group (Li et al., 2012; Lin et al., 2019). A total of 37 samples were used for RNA-seq (Supplementary Table S2). For the immunofluorescence assay, skins containing hair follicles from three 1-week-old MD ducks and three LC ducks were collected for protein analysis.

4.3.2 Genome assembly and gene annotation

A combination of PacBio long-read HiFi sequencing and High-through Chromosome Conformation Capture (Hi-C) technologies was hereby utilized to conduct the *de novo* assembly of the Liancheng duck genome. Long-read and long-HiFi (RRID:SCR_021966) sequencing data (PacBio, Beijing, China) were used for the species assembly (Cheng et al., 2021). Sequencing was performed on the PacBio Sequel II platform, and genome assembly was conducted using the hifiasm (https://github.com/chhylp123/hifiasm) versions (v0.19.3 NOV-2023) for Contig generation. The 3D-DNA pipeline (RRID:SCR_017227) (NOV-2023) was utilized for manual curation and orient the genome sequences based on Hi-C contact map (Durand et al., 2016; Dudchenko et al., 2017; Zheng et al., 2023). Ultimately, the BUSCO was used to evaluate the completeness and quality of Liancheng duck genome assembly (IASCAAS_LianchengWhiteDuck, GCA_039998735.1) (Manni et al., 2021).

Protein-coding genes were predicted via the combination of evidence-based prediction and *de novo* prediction. RNA-seq data were used for evidence-based annotation through Maker2 (Version 2.31.10), i.e., a powerful open-source genome annotation tool. RNA-seq data were aligned against the genome using PASA (Program to Assemble Spliced Alignments) (RRID:SCR_014656) to construct a training model for *Augustus* (http://bioinf.uni-greifswald.de/augustus/), a tool designed to predict genes in eukaryotic genomic sequences. Subsequently, *de novo* annotation of the genome was performed utilizing *Augustus*. *De novo* genome assembly refers to a method of piecing together a complete sequence of a new genome, rather than comparing it based on a known genome. Subsequently, the results of the RNA-seq based annotated and the *de novo* based annotation were integrated on the principle that the evidence results were better than those prediction ones. In the final step, genes were filtered to retain those with less than 50% repeated sequences, encoding proteins longer

than 50 amino acids, and having at least one count of expression. Functional annotation of genes was performed using eggNOG software (RRID:SCR_002456) (Powell et al., 2014).

4.3.3 Whole-genome resequencing

A total of 366 samples, consisting of Pekin ducks, Liancheng ducks, and intercross populations, were hereby selected for resequencing (Supplementary Table S1). The genome data of 23 black-feathered ducks included 20 Mallards, and 3 Putian ducks. DNA-eligible samples were identified for further testing. Libraries were established, involving an average read length of 150 bp for all samples. Subsequently, they were sequenced on an Illumina HiSeq X-Ten platform, and an average raw read sequence coverage of 5x was yielded. This 5x coverage ensured the accuracy of variant calling and genotyping when linking back to the Liancheng duck reference genome (IASCAAS_LianchengWhiteDuck, GCA_039998735.1) (Huang et al., 2013). Following the elimination of read pairs containing adapter sequences, a quality assessment of the raw reads was performed using TRIMMOMATIC (RRID:SCR_011848) (version 0.36) and Cutadapt (Bolger et al., 2014; Grewal et al., 2024). Following that, the high-quality reads were aligned to the Liancheng duck reference genome using the Burrow–Wheeler Aligner (BWA-aln) with parameter 'bwa aln genome.fa sample.fastq > sample.sai, bwa sampe genome.fa sample.sai sample.fastq > sample.sam' (Li et al., 2009). Besides, genetic variants were identified from the sequencing data in this study using Genome Analysis Toolkit (GATK) (McKenna et al., 2010). SNPs underwent filtration based on the following criteria: (i) SNPs with minor allele frequency (MAF) >0.05; (ii) the maximum missing rate per SNP set at <0.7; and (iii) SNPs restricted to possessing only two alleles.

4.3.4 Genome-wide association analysis

The GWAS was conducted utilizing a mixed linear model implemented through the EMMAX program (RRID:SCR_024012) (Kang et al., 2010) with genome-wide SNP data and the plumage color phenotype observed in 366 individuals from the resequencing population. The analytical model adopted the form $y = Xb + Ga + e$, with y representing the phenotypic value (plumage color of each duck), X denoting the matrix corresponding to fixed effects, and b signifying the magnitude of the fixed effects. The fixed effects included sex-related influences. G represents the genetic matrix associated with population kinship, while e stands for the random residual. Furthermore, principal component analysis (PCA) was executed using all SNPs, with the top three components incorporated as fixed effects within the mixed model to adjust for population stratification. A Bonferroni correction threshold of $0.01/N$ ($-\log_{10}P = 8.95$) was established to pinpoint significant sites in the GWAS findings (Liu et al., 2023a; Wang et al., 2023), where N indicates the total number of whole-genome SNPs (8,887,194). In addition, fine-mapped analyses were conducted in 328 ducks via

Identity By Descent (IBD) analysis, for the efficiency of IBD fragments in reflecting the genetic relationship between individuals and detecting trait variation. The correlation between IBD fragments and phenotype was used to identify regions affecting trait variation in the genome (Price et al., 2006; Kang et al., 2010). For this analysis, the filtered SNPs (n= 117) met the standard allele frequency difference (ΔAF) >0.8 between the Liancheng ducks and Pekin ducks. In the candidate region (Chr33:5.1-5.5Mb), four recombination breakpoints were identified across the 36 SNPs, and the segregating individuals were subsequently classified using these four recombinant breakpoints.

4.3.5 Transcriptome sequencing and analysis

Skin tissues from LC ducks were collected at embryonic stages of 12 days, 15 days, 20 days, 28 days, and one week after birth. Additionally, tissues including heart, fat, muscle, brain, spleen, lung, liver, kidney, and skin were collected from an 8-week-old black-feathered MD duck. Feather bulb specimens were also obtained from feather follicles of 1-week-old black-feathered MD ducks, LC ducks, PK ducks, and WB ducks. The total RNA was initially extracted from the above tissues using Trizol reagent (Vazyme, Nanjing, China). The RNase enzyme was inactivated by adding pyrrole diethyl carbonate. Final RNA-seq libraries were prepared for the experiment and sequenced on an Illumina platform using the 150-bp paired-end sequencing module. The effective read length was increased by Illumina sequencing, yielding an average production of 6 Gb per library. Using TopHat (RRID:SCR_013035), RNA-seq paired-end reads from each library were mapped to the aforementioned reference genome of the Liancheng duck. Besides, the expression was calculated by using TopHat, and read counts per million (CPM) values for the genes were obtained running htseq-count in figshare database (10.6084/m9.figshare.27311937) (Huang et al., 2013; Anders et al., 2015).

4.3.6 qPCR analysis on PMEL in feather bulb specimens

cDNA from feather bulb specimens, including those of black-feathered, grey-feathered, Liancheng, and Pekin ducks, was reversely transcribed using HiScript III All-in-one RT SuperMix Perfect for qPCR (Vazyme). The quantitative PCR (qPCR) was conducted in a total volume of 10 μl, which included 5 μl of Tap Pro Universal SYBR qPCR Master Mix (Vazyme), 0.8 μl of forward and reverse primers, 0.5μl of cDNA, and 3.7 μl of distilled water. **β**-actin was selected as the internal reference gene. The primer sequence is shown in Table 4-1. All reactions were run in triplicate. The relative mRNA expression levels were calculated using the normalized relative quantification method, followed by the $2^{-\Delta\Delta CT}$ calculation (Schmittgen et al., 2008).

Table 4-1 The information on the primers used for qPCR.

Name	Primer information	TM (℃)	Purpose
qPCR-*PMEL*	F: GGACGTGTCGCAGATGGAGG R: CGCTCTGGTCCCCGAAATCC	63	Expression analysis
β-actin	F: CAGCACGATGAAAATCAAGATCA R: TTGGTAACAGTCCGGTTTAGA	63	Expression analysis

4.3.7 Immunofluorescence experiment

The skin samples of ducks with black and white feathers were embedded in paraffin, fixed in 4% buffered paraformaldehyde, and sectioned into 5 μm slices. Upon overnight fixation at 4°C, the duration was kept within 24 hours to ensure effective preservation of tissue integrity. Subsequently, the sections were dewaxed and rehydrated to enhance adhesion and facilitate the dewaxing process. Antigen retrieval was performed by incubating the sections in EDTA (Servicebio) at 100°C for 20 minutes. Following retrieval, antigens were fixed with Tris-EDTA, and sections were washed thrice in phosphate-buffered saline (PBS), followed by a further rinse. For immunostaining, sections were incubated with a PMEL antibody (ABclonal) at 4°C for 12 hours post pre-treatment with 3% bovine serum albumin (Solarbio) for 30 minutes. The PMEL antibody, an anti-rabbit species, was hereby utilized for immunodetection. Ultimately, feather follicle tissues displaying diverse plumage colors were counterstained with DAPI.

4.3.8 Causative mutation screening and identification

The candidate regions (Chr33:5.24-5.32Mb) among 117 Pekin ducks, 59 Liancheng ducks, and 152 intercross population ducks (excepting 38 WY ducks) were compared based on the Liancheng duck genome. Among the candidate IBD fragments, only regions featuring consistent genotypes and phenotypes were further investigated as *Rr* candidate regions. To eliminate variations with a lower likelihood of being causally involved, the following three-step procedures were conducted: Firstly, the genotype and phenotype information from the 328 parents and intercross ducks was utilized to exclude SNPs based on the standard $F_{ST} < 0.8$ (LC vs PK duck). Secondly, information of 117 Pekin ducks, 59 Liancheng ducks, 42 WB ducks, 43 BF ducks was applied. Candidate regions were selected based on the highest F_{ST} values shared between Liancheng ducks and other breeds. Thirdly, only genotypes that were completely concordant with the phenotypes from the Mallard and Putian duck populations were retained as candidate causative mutations. Subsequently, all indels within the candidate regions were excluded using the method described above. Ultimately, only two SNP variations that showed genotype-phenotype concordance across multiple duck breeds were considered causative variations for the *Rr* locus.

4.3.9 Structural variation detection

In the GWAS candidate region, all CNV structural variations in the selected populations were analyzed, including Pekin ducks, Liancheng ducks, WB ducks, GF ducks, and BF ducks. Ducks from these different feather groups were randomly selected, and the genotype of all individual CNVs was investigated using CNVcaller (RRID:SCR_015752) software (version 0.11) (Guo et al., 2016). The CNV calling and genotyping procedures were consistent with those previously described (Wang et al., 2017). Log_2 fold change values reflected the ratio of sequencing read depths in the 1,000bp window region to that of Pekin duck reads. Therefore, the distribution of all CNV genotypes in the aforementioned populations was examined. Copy numbers of 1, 0.5, 0, 1.5, and 2 corresponded to normal diploidy, loss of heterozygosity, homozygous loss, heterozygous duplication, and homozygous duplication, respectively. Absolute copy numbers above 2 suggested the presence of complex duplications (Wang et al., 2017).

4.3.10 Luciferase reporter assay

Herein, a total of four haplotypes of candidate variations SNP1 and SNP2, along with their upstream and downstream regions, were cloned into the pGL3-basic and pGL3-promoter vector. The *XhoI* and *KpnI* restriction sites were utilized as insertion points in the pGL3-basic vector for promoter activity analysis, whereas the *BamHI* and *SalI* sites were chosen in the pGL3-promoter vector for enhancer activity assessment. A375 and DEF cells were plated in 48-well plates at a density of $0.5x10^5$ per well and cultured for 24 hours in DMEM (Pricella, China) mixed with 10% FBS (Pricella, China). Subsequently, the cells were transfected using Lipofectamine 8000 (Beyotime), with each well receiving an equal amount of DNA (237.5 ng), encompassing the four sequences that included the SNP1 and SNP2 sites. Meanwhile, 12.5ng of pRL-TK vector was added to each well. Following the manufacturer's protocol, cell lysates were harvested post-lysis, and luciferase activity was measured using the Veritas Microplate Luminometer (Promega). Each sample was assayed in triplicate, with Renilla luciferase activity employed to normalize the firefly luciferase readings (Chen et al., 2020; Liu et al., 2023a).

4.4 Results

4.4.1 A newly assembled high-quality Liancheng duck genome

To facilitate a comprehensive analysis of the white plumage Liancheng duck, a *de novo* genome was constructed utilizing HiFi long-read sequencing data, achieving an 88x genome coverage, and 584.72 Gb of Hi-C data were generated. These datasets were then utilized for *de novo* assembly for Liancheng duck genome with contig N50 of

12.17 Mb. The contigs were further scaffolded, corrected and ordered based on Hi-C contact map with scaffold N50 of 83.98 Mb (IASCAAS_LianchengWhiteDuck, GCA_039998735.1) (Fig. 4-1A). Finally, our *de novo* assembled 1.29 Gb Liancheng duck genome exhibited perfect collinearity with Mallards (GCA_008746955.3) and the Pekin duck reference genome (GCA_015476345.1) demonstrated the high quality of our genome assembly (Fig. 4-1B). The length of scaffold N50 in our Liancheng duck genome assembly was the longest among all previously published duck (*Anas platyrhynchos*) genomes, indicating high continuity of our assembly (Fig. 4-1C, 1D). Benchmarking Universal Single-Copy Orthologs analysis (BUSCO, RRID:SCR_015008) (Liu et al., 2023a) revealed 96.7% universal single-copy orthologues, suggesting high completeness of our genome assembly. Based on the high-quality *de novo* assembled of our Liancheng duck genome, a total of 18,819 genes were predicted.

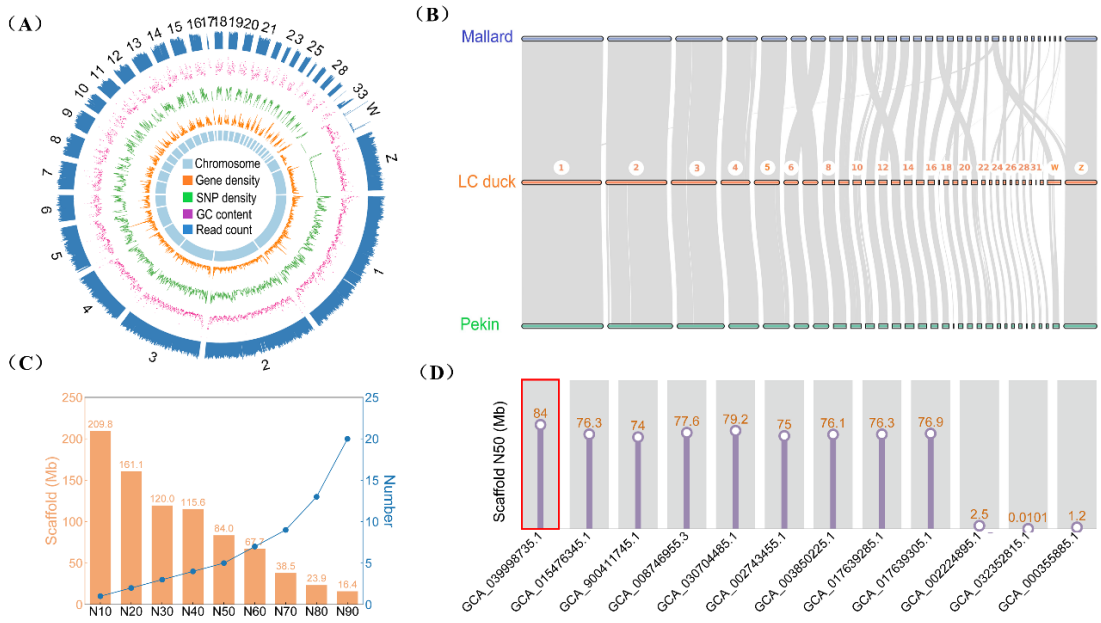

Figure 4-1. Overview of the assembly quality and characteristics of the Liancheng duck genome. (A) Circular diagram illustrating the characteristics of the genome assembly. The tracks from the inner to outer circles represent the following: chromosomes, gene density, SNP density, GC content (%), and read count. The window size of each circle was 200 kb. (B) Genome synteny analysis between the Liancheng duck and Mallard, as well as Pekin duck. Chromosomes 1-33, as well as the two sex chromosomes. (C) Genome statistics for the hifiasm genome assemblies of the Liancheng duck genome in this study. (D) The length of scaffold N50 (Mb) of Liancheng duck in this study (GCA_039998735.1) was compared with all previously published duck (*Anas platyrhynchos*) genomes.

4.4.2 Inheritance of F2 population traits conforming to the law of independent assortment

A crossbreeding study involving 30 Pekin and 120 Liancheng ducks was conducted. All F1 individuals (1,260) displayed a grey plumage color and pattern. In the F2 populations, four phenotypes were observed, namely BF, GF, WB, and WY ducks (Fig. 4-2). The ratio of BF: GF: WB: WY ducks in the F2 population was 235:452:234:360, which closely approximated the theoretical ratio of 3:6:3:4 (Table 4-1 and Fig.4-2). The phenotypic ratio observed followed Mendel's law of independent assortment for two genes. It was hypothesized that the genetic mechanism controlling plumage color in Liancheng ducks was governed by two sites (*Bb* and *Rr* sites), with the allele at the *Rr* site, in interaction with the *Bb* site, determining white plumage in Liancheng ducks (Table 4-2). Within the F2 population, two alleles (*B*, dominant, enabling melanin synthesis, and *b*, recessive, inhibiting melanin synthesis) segregated at the *Bb* locus. The other locus, denoted as *Rr*, possessed two alleles that regulated melanin accumulation in the feather: *R* (dominant, allowing melanin synthesis in the feather) and *r* (recessive, repressing melanin synthesis). The *B* allele at the *Bb* locus displayed an epistatic effect, while the *R* allele at the *Rr* locus demonstrated an incomplete dominance effect. The cross between the Liancheng duck (*BBrr*) and the Pekin duck (*bbRR*) resulted in the production of grey feather ducks (*BbRr*), with the genotypes of BF, GF, WB, and WY being *B_RR*, *B_Rr*, *B_rr*, and *bb_ _*, respectively (Table 4-2, Fig. 4-3). Importantly, no significant difference was observed between the actual and expected numbers within the F2 population (n=1,281, p=0.345), with a squared value of 3.322 ($\chi 2_{0.05(3)}$ =7.81, $\chi 2_{0.01(3)}$ =11.34). The *Bb* locus had previously been identified as the primary gene responsible for white plumage in ducks (Zhou et al., 2018).

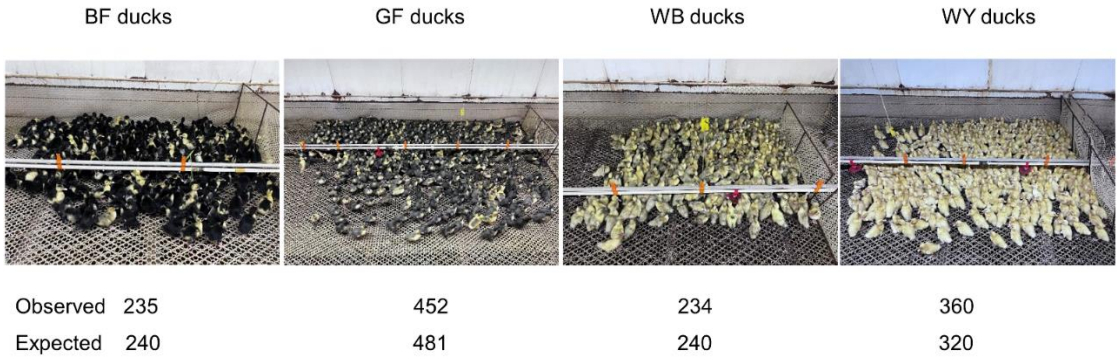

Figure 4-2. Phenotypic characteristics of 1,281 F2 ducks from Liancheng (LC) and Pekin (PK) ducks. BF: Black-feathered ducks with black beaks in F2 population; GF, Grey feathered ducks with black beaks in F1 and F2 population; WB, White feathered ducks with black beaks in F2 populations; WY, White feathered ducks with yellow beaks in F2 population. According to Mendel's law of free combination of two genes (3:6:3:4), the values below the figures represent the number of observed and expected values.

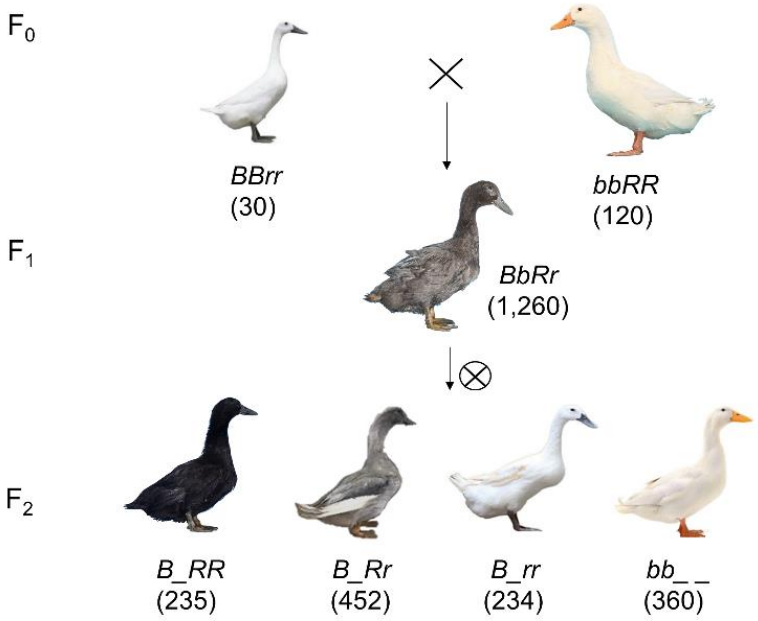

Figure 4-3. The diagram depicted the segregation of plumage colors in the F2 population. The Liancheng duck showed the white-feathered ducks with black beaks (WB), whereas the Pekin duck exhibited the white-feathered ducks with yellow beaks (WY). The F1 generation displayed the gray-feather ducks with black beaks (GF). In the subsequent F2 generations, ducks were observed with phenotypes including black-feathered ducks with black beaks (BF), GF, WB, and WY ducks.

Table 4-2 The number of F2 populations in different phenotypes and the Chi-squared test.

Comparison	BF duck (*B_RR*)	GF duck (*B_Rr*)	WB duck (*B_rr*)	WY duck (*bb_ _*)	Ratios	χ^2 value	*P*-value
Observed number	235	452	234	360	3:5.8:3:4.6	3.322	0.345 (ns)
Expected number	240	481	240	320	3:6:3:4		

Notes: BF represents ducks with black-feathered ducks with black beaks in the F2 population; GF represents ducks with grey-feathered ducks with black beaks in the F2 population; WB represents ducks with white-feathered ducks with black beaks in the F2 population; WY represents ducks with white-feathered ducks with yellow beaks in the F2 population. $\chi^2_{0.05(3)}$ =7.81, $\chi^2_{0.01(3)}$ =11.34; ns, no significant difference.

4.4.3 Genome-wide association analysis for segregating population duck plumage color

The duck samples were re-sequenced with the average depth of 5x. A cohort of 188 ducks from a segregating population derived from Liancheng ducks and Pekin ducks was hereby selected for GWAS analysis. Initially, using the genome of the Liancheng duck as a reference, the present study identified two sites controlling the white feather phenotype on chromosomes 13 and 33 (Fig. 4-4A). This research highlighted a specific gene on chromosome 13 that regulated melanin synthesis in Liancheng ducks. Given that the white plumage phenotype in Liancheng ducks did not show sex-related patterns, cytoplasmic inheritance considerations for white plumage were deemed unnecessary. Subsequently, the *Rr* gene was pinpointed to the 5.24-5.32Mb region of chromosome 33 in Liancheng ducks (Fig. 4-4B), which contained potential candidate genes such as *PMEL*, *RAB5B*, *IKZF4*, *ERBB3*, *PA2G4*, *ZC3H10*, and *ESYT1* (Fig. 4-4C). Within the candidate region (Chr33: 5.24-5.32 Mb), four minimal recombination haplotypes were identified based on the parents and segregating populations from 117 SNPs with an F_{ST} >0.8 (PK vs LC ducks). Only the haplotypes in block 4 (Chr33: 5,303,111-5,304,416, 1,305 bp) located upstream of the *PMEL* gene corresponded to the observed phenotypes (Fig. 4-4D). Additionally, a significantly high peak in the F_{ST} value was observed in the *RR* vs *rr* duck populations within the selected candidate region, while no peak was spotted in *rr* vs *rr* duck populations (Fig. 4-4E).

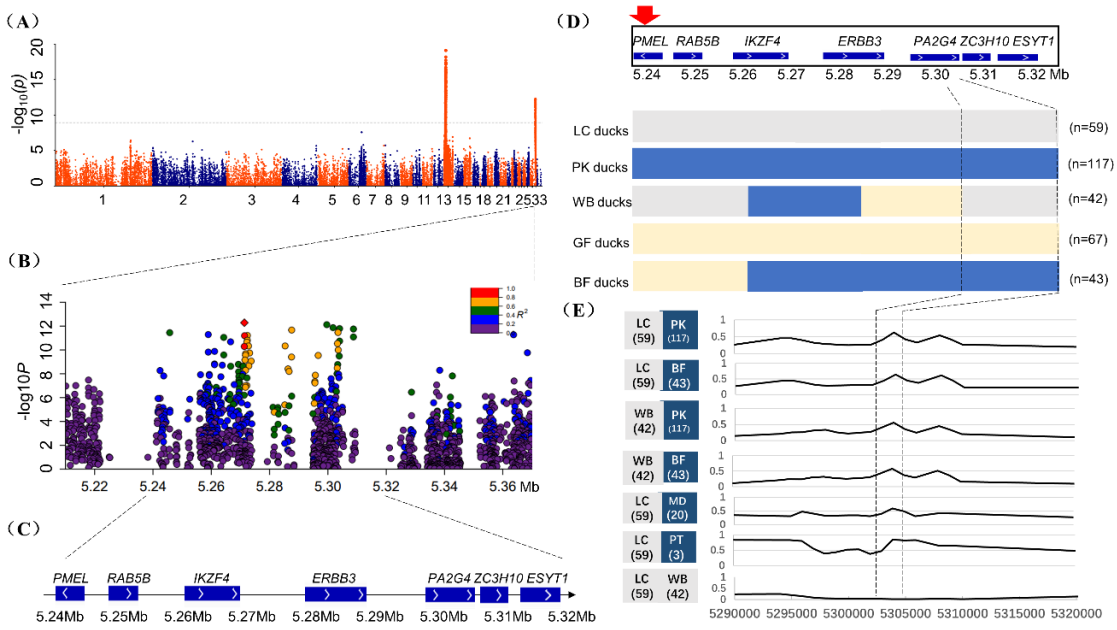

Figure 4-4. Screening for the candidate region associated with the white plumage of Liancheng ducks involved a GWAS on a cohort of 188 ducks from a cross between Pekin ducks and Liancheng ducks. (A) A Manhattan plot showed the genetic effects on plumage color. (B) Locuszoom in the regions on chromosome 33 (5.21-5.37 Mb) linked to white plumage in Liancheng ducks. Genotypic SNPs were identified based on linkage imbalance values compared to the leading SNP in the intercross population duck (Chr33: 5,303,413). (C)

Candidate genes in the region (5.24-5.32 Mb) were identified, with white and black arrows indicating gene orientation and chromosome 33 direction, respectively. (D) Identity by Descent (IBD) analysis used color schemes to refine candidate regions, with blue for Pekin ducks and black-feathered ducks, grey for Liancheng ducks and white-feathered black beak ducks, and yellow for grey plumage ducks (LC vs PK, F_{ST}> 0.8). (E) Genome divergence analysis among six duck breeds, including LC vs PK ducks, BF ducks, Mallards (MD), and Putian (PT) ducks within the candidate region (Chr33: 5.29-5.32 Mb), averaged F_{ST} values in 10kb region in each comparison group.

4.4.4 *PMEL* causes melanin deposition in duck plumages

The region on chromosome 13 was found to encompass the *MITF* gene in the GWAS analysis. Comparison with the Pekin duck genome assembly (GCA_003850225.1) revealed a 6.6 kb insertion in the *MITF* gene, strongly associated with melanin synthesis in ducks (Table 4-3). Following the exclusion of the *MITF* signal, subsequent GWAS analysis identified a single signal on Chromosome 33. Candidate region included *PMEL*, *RAB5B*, *IKZF4*, *ERBB3*, *PA2G4*, *ZC3H10*, and *ESYT1*. Results indicated that only the *PMEL* gene showed significant differential expression (Fig. 4-5A, B), with higher expression levels in black-feathered ducks compared to grey-feathered ducks (-Log10(*p*)>30). RNA-seq results showed no expression of the *PMEL* gene in feather bulb specimens of white-feathered ducks (Fig. 4-5C, D). Other genes within the GWAS candidate range (Chr33: 5.24-5.32Mb) were excluded due to similar gene expression levels in different plumage populations or inconsistent gene expression patterns related to melanin regulation. Furthermore, qPCR results confirmed *PMEL* as the *Rr* gene (Fig. 4-5C). Notably, the *PMEL* gene exhibited an elevated average GC content of 72.4%. Additionally, the *PMEL* gene expression correlated with the plumage color phenotype of Liancheng ducks at various developmental stages, with high expression levels observed in the skin tissue (Fig. 4-5B), further underscoring the importance of establishing a high-quality genome for Liancheng ducks.

Table 4-3 Frequency distribution of 6.6kb insertion within *MITF* gene in duck population.

Breeds	Number	Homozygous insertion	Heterozygous insertion	Wildtype
Pekin ducks	117	117	0	0
Liancheng White ducks	59	0	0	59
White feather yellow beak ducks (F2)	38	38	0	0

Note: This 6.6 kb insertion within *MITF* gene also showed a perfect association with plumage color in duck population, as the color of homozygous individuals was completely white and that of heterozygous individuals or individuals without the insertion.

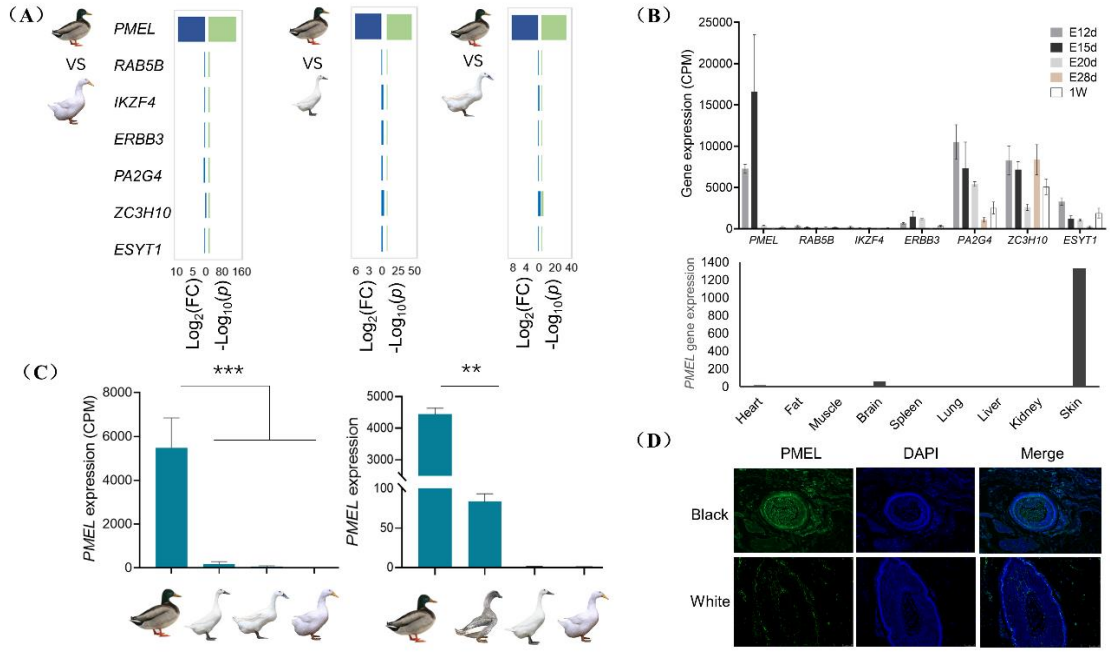

Figure 4-5. Identification of the candidate gene for white plumage in Liancheng ducks (A) Gene expression of seven GWAS candidate region genes (*PMEL*, *RAB5B*, *IKZF4*, *ERBB3*, *PA2G4*, *ZC3H10*, *ESYT1*) in 1-week-old feather follicle samples of white and black-feathered ducks, with three replicates per sample. Log_2(FC) values were used to analyze gene expression differences in blue, and values where -Log_{10}(*p*) values were shown in green. (B) Analysis of the expression levels of the seven candidate genes in skin tissues of Liancheng ducks at different developmental stages. E12d, E15d, E20d, and E28d (also the first day of birth) represent 12, 15, 20, and 28 days of the embryonic period, respectively. Data were shown as mean ± standard error (n=3). (C) CPM and qPCR results of *PMEL* in 1-week-old feather bulb samples of ducks. Data were presented as mean ± standard error (n=3). ** p<0.01; *** p<0.001. (D) Immunofluorescence results showing *PMEL* distribution in feather bulb specimens of black- and white-feathered ducks. Black (Mallards) and White (Liancheng ducks).

4.4.5 *Rr* variation being fine mapped to PMEL upstream regulatory region

The results of the IBD analysis indicated that only the haplotypes in block 4 (Chr33: 5,303,111-5,304,416, 1,305 bp), positioned upstream of the *PMEL* gene, were associated with the observed phenotypes (Fig. 4-4D). Moreover, a pronounced peak was observed in the *RR* vs *rr* duck populations within the candidate region (Fig. 4-4E), further substantiating this segment (Chr33: 5,303,111-5,304,416, 1,305 bp) as the *Rr* locus. Among the identified candidate variations in this region, one copy number variation (CNV) (Fig. 4-6) was initially excluded. Only 12 SNP variants and two Indels were retained after applying a threshold of F_{ST} > 0.8 (PK vs LC ducks) (Table 4-4). It should be noted that all 12 SNP were found in the upstream regulatory region of the *PMEL* gene (Chr33: 5,239,969-5,244,318). Finally, two SNPs (Chr33: 5,303,994A>G; 5,303,997A>G) were hereby identified as the potential causal variants across all duck

breeds (Table 4). Intriguingly, these two SNPs were observed to be in complete linkage disequilibrium.

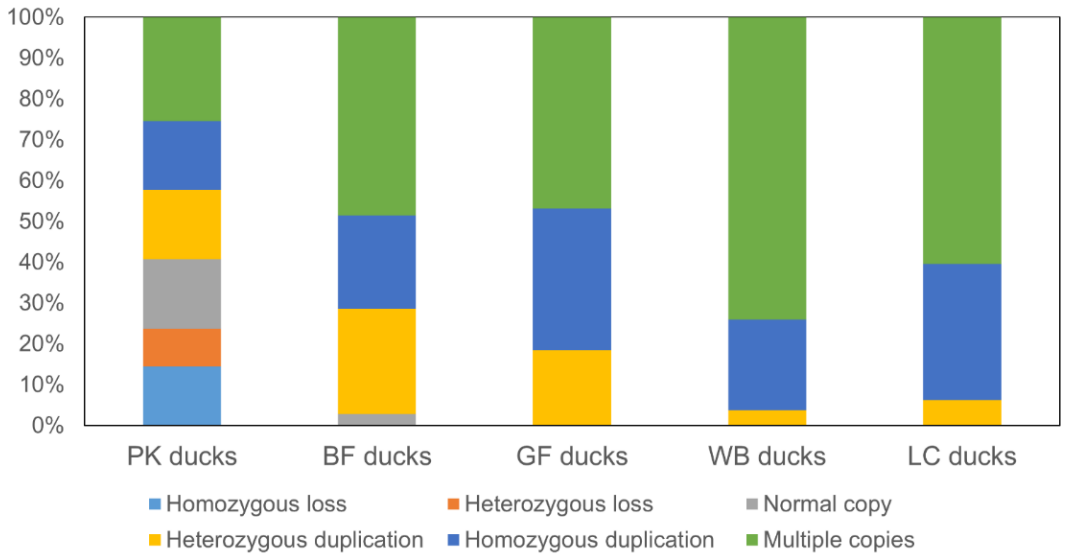

Figure 4-6. Genotypes of candidate CNV variation (Chr33:5,282,001- 5,284,500) in different plumage color populations, including PK ducks (n=31), BF ducks (n=18), GF ducks (n=21), WB ducks (n=12), LC ducks (n=24). When the absolute copy number is 1, it is normal copy number, that is, normal diploid. 0.5 indicates loss of heterozygosity; 0 means homozygous loss; 1.5 indicates heterozygous duplication; 2 represents homozygous duplication; An absolute copy number greater than 2 indicates complex copies.

Table 4-4 Genotypic distribution of SNP candidate variations in different duck breeds based on reference genome Liancheng duck.

Locations	SNPs	LC ducks	Pekin ducks	WB ducks	BF ducks	Mallards	Putian ducks
Chr33		n=59	n=117	n=43	n=43	n=20	n=3
5,303,111	TT	59	1	41	3	0	0
	TC	0	3	2	2	0	0
	CC	0	113	0	38	20	3
5,303,196	CC	59	1	41	2	0	0
	CA	0	3	1	3	1	0
	AA	0	113	1	38	19	3
5,303,277	AA	59	1	41	1	0	0
	AG	0	2	2	2	1	0
	GG	0	114	0	40	19	3
5,303,413	CC	59	2	42	0	0	0
	CT	0	3	1	4	2	0
	TT	0	112	0	39	18	3
5,303,439	AA	59	0	42	1	0	0
	AG	0	4	1	4	1	0
	GG	0	113	0	38	19	3
5,303,461	AA	59	0	42	1	0	0
	AG	0	2	1	4	0	0
	GG	0	115	0	38	20	3
5,303,533	TT	59	2	42	2	0	0
	TC	0	5	1	5	0	0
	CC	0	111	0	36	20	3
5,303,883	CC	59	1	41	2	0	0
	CG	0	2	2	2	0	0
	GG	0	114	0	39	20	3
5,303,994	AA	59	0	40	1	0	0
	AG	0	0	2	2	0	0
	GG	0	117	1	40	20	3
5,303,997	AA	59	0	40	2	0	0
	AG	0	0	2	2	0	0
	GG	0	117	1	39	20	3
5,404,026	AA	59	0	40	2	0	0
	AG	0	1	3	2	1	0
	GG	0	116	0	39	19	3
5,404,416	CC	59	1	41	2	0	0
	CT	0	2	2	2	0	0
	TT	0	114	0	39	20	3
5,303,241-	ins/ins	59	78	40	27	1	3
5,303,245	ins/del	0	36	2	13	5	0
AAGTA/.	del/del	0	3	1	3	14	0
5,304,000-	ins/ins	59	0	40	1	0	0
5,304,007	ins/del	0	0	3	2	0	0
GTCCCCAA/.	del/del	0	117	0	40	20	3

Notes: Genotypes of candidate variations in different breeds. These variations in the candidate region had Fst values greater than 0.8 (Liancheng ducks vs Pekin ducks).

4.4.6 Functional analysis of two candidate SNPs

Considering the location of the two candidate SNPs (SNP1 and SNP2) (Chr33: 5,303,994A>G; 5,303,997A>G) in the noncoding upstream region of the *PMEL* gene, their promoter and enhancer effects were assessed using pGL3 luciferase vectors (Fig. 4-7A, B). Both pGL3 vectors (pGL3-basic-white and pGL3-basic-black) with inserts showed minimal luciferase activity in duck embryo fibroblast (DEF) cells and human melanoma cells (A375) (Fig. 4-7C), indicating no promoter activity at these SNP sites. However, for enhancer activity, those vectors with inserts (pGL3-promoter-white and pGL3-promoter-black) displayed significantly different luciferase activity in DEF cells and A375 cells (ANOVA, $p<0.01$). Notably, Results revealed the higher luciferase activity of both pGL3-promoter-white-1mut and pGL3-promoter-white-2mut compared with pGL3-promoter-white (Fig. 4-7D). This indicated a synergistic enhancement activity by the black alleles of variations SNP1 and SNP2.

Analysis on the JASPAR transcription prediction website (https://jaspar.elixir.no/) revealed that multiple transcription factors might bind differently to sequences surrounding candidate SNPs located at Chr33: 5,303,944-5,304,098. The results suggested that variations in SNP1 and SNP2 could impact the binding of various transcription factors. Furthermore, differential expression of the SOX5 transcription factor was observed in the feather follicles of white- and black-feathered ducks. It was thereby hypothesized through these findings that variations in SNP1 and SNP2 could potentially be key mutations responsible for the white feather phenotype.

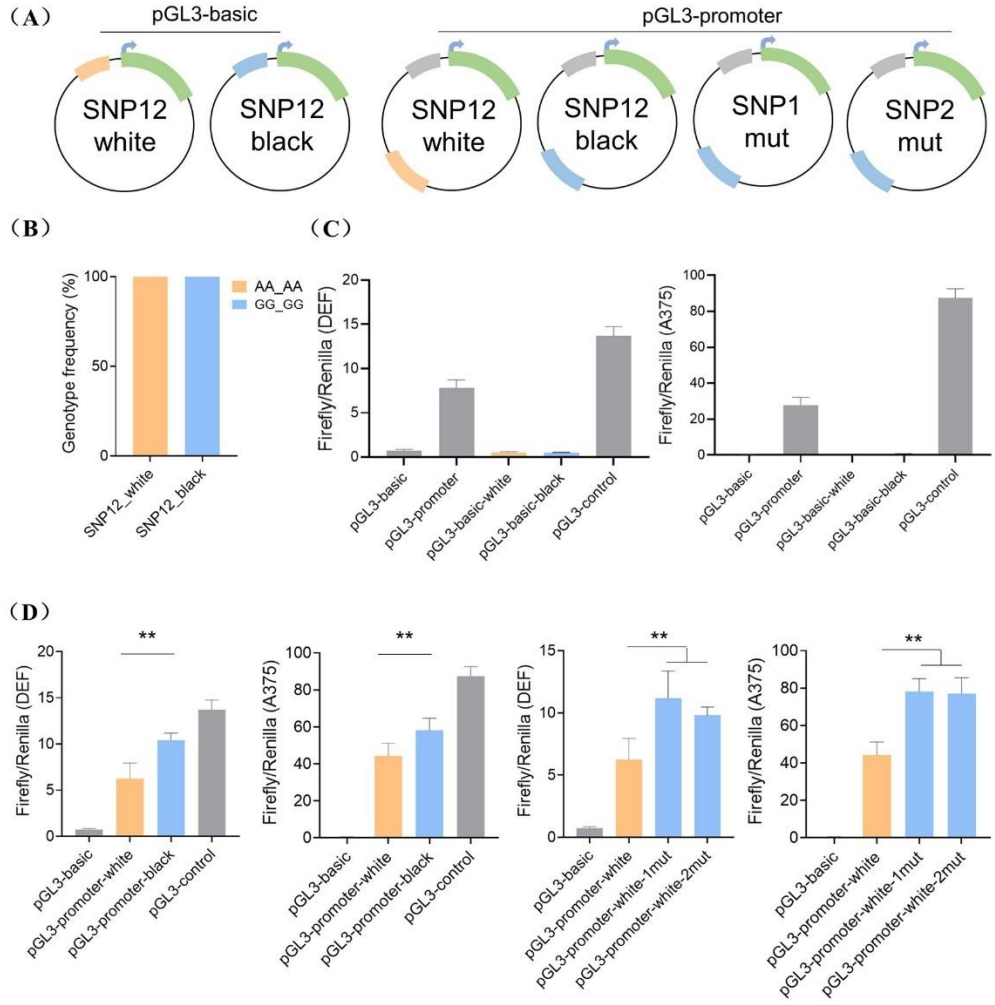

Figure 4-7. Functional analysis of the candidate variation controlling the white plumage phenotype of Liancheng duck. (A) Diagram of six pGL3 vectors for the luciferase reporter gene experiment. Candidate SNP1 and SNP2 (Chr33:5,303,994A>G and 5,303,997A>G) of Liancheng duck and Pekin duck were inserted into empty pGL3-basic and pGL3- promoter vectors. pGL3-basic, pGL3-promoter and pGL3-control were used as negative and positive control. Insertion fragments in pGL3-basic are marked in yellow and blue to verify the promoter activity. The yellow and blue box represents insertion fragment of the pGL3-promoter to verify enhancer activity. Additionally, vectors also contained a green box (luciferase reporter gene), gray box (promoter) and a blue arrow (indicate transcription site). (B) Genotype distribution of SNP1, SNP2 in Liancheng duck (AA_AA, n=59) and Pekin duck (GG_GG, n=117). (C) Validation of promoter activity of homozygous wildtype (AA_AA) and homozygous mutation (GG_GG) in DEF cells and A375 cells. Data are presented as mean ± standard error (n=8), (ANOVA, p>0.05). (D) Enhancer activity of AA_AA, GG_GG, GG_AA, and AA_GG vectors in DEF cells and A375 cells. Data are presented as mean ± standard error (n=8), (ANOVA, ** p<0.01).

4.5 Discussion

The white plumage phenotype is considered a common trait in various avian species, such as chickens (Keeling et al., 2004; Gunnarsson et al., 2011), peafowl (Liu et al., 2022), geese (Xi et al., 2020), and ducks (Wang et al., 2022). The unique white plumage phenotype of Liancheng ducks has been extensively delved into (Gong et al., 2010; Yang et al., 2019; Wang et al., 2022). However, the genetic mechanisms behind remain unclear. The present findings suggested that the in heritance of the plumage color phenotype in Liancheng ducks was likely governed by two autosomal genes, independent of the sex chromosomes (Table 4-1). Despite using the previous Pekin duck reference genome (IASCAAS_PekingDuck_PBH1.5, GCA_003850225.1), three signals were identified through the current GWAS analysis, which was considered to be attributed to an incomplete assembly. To further investigate the white feather phenotype of Liancheng ducks, a high-quality genome was therefore first established for Liancheng ducks (Fig. 4-1). This new reference genome featured a size of 1.29 Gb, with contig and scaffold N50 values of 12.17 and 83.98Mb, respectively. The scaffold N50 length of the Liancheng duck genome was higher than that of other duck genomes (Jiang et al., 2021; Ng et al., 2022; Lavretsky et al., 2023; Hu et al., 2024), representing a more complete and better continuity of the duck genome assembly. However, the chromosomes number identified from this newly genome should still be further improved compared to those of the Muscovy duck and Crested Duck (Xu et al., 2022; Chang et al., 2023). Further pan-genome and functional gene-mining analysis could be conducted in the future (Mueller et al., 2021; Ng et al., 2022; Wang et al., 2023). Taken together, these findings represent the first construction of the Liancheng duck genome, resulting in enhanced genome contiguity compared to previous duck genomes.

MITF is a key regulator of melanin synthesis, controlling the expression of enzymes involved in this process as well as receptors critical to melanocyte function (Dürig et al., 2018; Guo et al., 2022; Pan et al., 2023). This gene produces multiple isoforms through the use of alternative promoters, which share coding exons but feature different amino termini (Karlsson et al., 2007). While *MITF* variants are known to influence melanin regulation, the regulation of these isoforms remains to be explored. Herein, the expression of two *MITF* isoforms, *MITF-B* and *MITF-M*, were discovered in ducks, with only the latter being crucial for melanin synthesis in duck plumage (Lin et al., 2024). *MITF-M* isoforms have also been shown to regulate white coloration in the fur of dogs (Baranowska et al., 2014), llamas (Anello et al., 2019), and mice (Flesher et al., 2020). SNPs, indels, and structural variants were previously found in *MITF* as possible causes of white plumage in ducks (Coding et al., 2019). Two synonymous SNPs (c.114T>G and c.147T>C) and a 14-bp indel (GCTGCAAACAGATG) in intron 7 of duck *MITF* were significantly associated with the black- and white-colored breeds (p<0.001) (Sultana et al., 2018). One variant in the 5'UTR of *MITF* was significantly associated with feather color phenotypes in geese (Ren et al., 2021). A 6.6 kb insertion within the *MITF* gene demonstrated a strong correlation with melanin production in ducks (Zhou et al., 2018), and indicated the on-off role of *MITF* in the melanin

generation pathway of Pekin ducks. *MITF* could promote differentiation-related functions, including regulation of genes involved in pigmentation, such as *PMEL*, *TYR*, *TYRP1*, *DCT*, *MLANA*, *SILV*, and *SLC24A5* (Coding et al., 2019). In the years following the separation of the *MITF* gene, the number of potential target genes increased sharply. Based on GWAS analysis, the role of the *MITF* gene as an epistatic gene controlling melanin synthesis in Liancheng ducks was hereby confirmed, aligning with previous research findings (Yang et al., 2019). Overall, this highlights the significant regulatory role of *MITF* in melanin synthesis in Liancheng ducks and underscores its importance as a key genetic factor in pigmentation.

PMEL, a type I transmembrane transport glycoprotein, is synthesized in the endoplasmic reticulum and plays a crucial role in amyloid fiber formation during stages I and II of melanosome formation in the L-DOPA pathway (Kerje et al., 2004; Batai et al., 2021). Upon synthesis, PMEL is transported to the melanosomes, where it undergoes proteolytic processing to form fibrils (Watt et al., 2011). These fibrils act as a scaffold for the deposition of melanin pigments, catalyzed by enzymes like tyrosinase (Hurbain et al., 2008; Watt et al., 2013). Mutations in the *PMEL* gene can lead to abnormalities in melanosome formation and melanin deposition, impacting plumage coloration in various bird species, including chickens (Keeling et al., 2004; Deng et al., 2024), Junco hyemalis (Abolins-Abols et al., 2018), Japanese quail (Ishishita et al., 2018) and Indian peafowl (Liu et al., 2022). To date, only 21 bird species have annotated the *PMEL* gene among 120 bird genomes. However, the association between the *PMEL* gene and duck plumage color phenotype has not been previously reported.

In this study, the *PMEL* gene was found to be significantly differentially expressed between the feather bulb specimens of white- and black-feathered ducks (ANOVA, $p<0.001$). The immunofluorescence results indicated high expression of the PMEL protein in feather follicle specimens of black and grey plumage ducks, contrasting with low expression in white plumage ducks. Many other studies suggested the possible involvement of the *PMEL* gene in the deposition of feather melanin (Abolins-Abols et al., 2018; Liu et al., 2019; Zheng et al., 2020; Heo et al., 2023). Meanwhile, the *PMEL* gene is also implicated in the formation of white feathers in quail (Yuan et al., 2023) and in the white feathers of chickens at the hatch stage (Hua et al., 2021). Endogenous *PMEL* expression is regulated by *MITF*, with alterations observed in melanoma cells (Falletta et al., 2014). However, the specific interplay between these two genes in determining the plumage color of Liancheng ducks requires further investigation. Overall, this research has annotated the *PMEL* gene in the duck genome for the first time, a gene previously thought to be absent in Pekin ducks. *MITF*, a key regulator of melanin production in ducks, was also identified. The inactivation of *PMEL* in feather bulb specimens led to the distinct white and black feathering characteristic of Liancheng ducks.

In the candidate region identified through GWAS analysis, a total of 12 SNPs, 2 Indel variations, and 1 CNV variation were investigated. Among these variants, only 2 SNPs (Chr33:5,303,994A>G and 5,303,997A>G) were found to be consistently associated with the observed plumage color phenotypes across multiple breeds. Data from promoter activity assays indicated that the SNPs in question were unlikely to serve as

direct *PMEL* promoters for gene expression (Fig. 4-7C). Instead, the present genetic findings indicated that these two linked SNP variations, located in the upstream region of the *PMEL* gene, exhibited functional enhancer activity that might remotely regulate *PMEL* gene expression. The long-range regulation probably modulates *PMEL* gene expression, affecting the black feather pigmentation in ducks, aligning with the melanin phenotype of duck feathers.

Remote regulation elements are thought to engage with target promoters through physical proximity (Soldner et al., 2016), yet the precise functional implications of this proximity are not well defined. In the current research, potential regions encompassing *PMEL* and two candidate SNPs were identified within a single topologically associated domain region, demonstrating this area as a possible part of a genomic region with frequent interactions (Hung et al., 2024). Furthermore, enhancer-promoter interactions might intensify during mammalian development (Chen et al., 2024), potentially accounting for the variation in plumage colors of Liancheng ducks from embryonic to postnatal stages. It was also noted that only SOX5, the predicted transcription factor, displayed varying expression levels in feather bulbs of different plumage colors. Notably, other transcription factors might also contribute to the regulatory mechanism. Moreover, the gray plumage, an intermediate phenotype observed in this study, could be related to a haploinsufficiency effect (Billiard et al., 2021).

Feather phenotype is a complex trait that encompasses a series of interlinked modules (Terrill et al., 2023). Birds exhibit a wide array of elaborate pigmentation patterns, which serve various functions such as attracting mates or providing camouflage or intimidation against predators. Melanin is a crucial pigment in feather coloration, and its role is mediated through the regulated presence, distribution, and differentiation of these melanocytes. Recent studies have reported that the variation of *MITF*, *TYR*, *EDNRB2*, *SLC45A2*, *MC1R* genes and Agouti signaling protein can regulate the production of feather melanin in ducks (Ng et al., 2018; Zhou et al., 2018). However, the role of the *PMEL* gene in feather melanin formation in ducks has not been confirmed. The present study makes the meaningful attempt to elucidate the role of the *PMEL* gene in pigmentation within duck feathers, offering valuable insights into the genetic mechanisms behind plumage coloration and offering practical applications for selective breeding strategies.

Data Availability

The whole-genome sequence data reported in this article have been deposited in the NCBI under accession number No. PRJNA1107839. The resequencing raw data have been deposited in the NCBI SRA under accession No. PRJNA844232. The transcriptomic raw data have been deposited in the NCBI under accession No. PRJNA1109286.

Acknowledgments

This work was supported by grants from the National Science Fund for Distinguished Young Scholars (32325047), the Innovation Program of Chinese Academy of Agricultural Sciences(CAAS--SCAB-202302), the China Agriculture Research System of MOF and MARA (CARS-42-05), and the National Key R&D Program of China (2022YFF1000102). The authors were grateful to Shandong Rongda Agricultural Development Co., Ltd for their help in this sample collection.

Ethical Statement

All animals used in the study were treated following the guidelines for the experimental animals established by the Council of China Animal Welfare. Protocols of the experiments were ap-proved by the Science Research Department of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (CAAS) (Beijing, China).

References

Abolins-Abols M, Kornobis E, Ribeca P, et al. Differential gene regulation underlies variation in melanic plumage coloration in the dark-eyed junco (Junco hyemalis). Mol Ecol. 2018;27(22):4501-4515.

Akdemir KC, Chin L. HiCPlotter integrates genomic data with interaction matrices. Genome Biol. 2015;16(1):198.

Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31(2):166-169.

Anello M, Daverio MS, Silbestro MB, Vidal-Rioja L, Di Rocco F. Characterization and expression analysis of KIT and MITF-M genes in llamas and their relation to white coat color. Anim Genet. 2019;50(2):143-149.

Baranowska Körberg I, Sundström E, Meadows JR, et al. A simple repeat polymorphism in the MITF-M promoter is a key regulator of white spotting in dogs. PLoS One. 2014;9(8):e104363.

Batai K, Cui Z, Arora A, et al. Genetic loci associated with skin pigmentation in African Americans and their effects on vitamin D deficiency. PLoS Genet. 2021;17(2):e1009319.

Billiard S, Castric V, Llaurens V. The integrative biology of genetic dominance. Biol Rev Camb Philos Soc. 2021;96(6):2925-2942.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-2120.

Chang G, Yuan X, Guo Q, et al. The first crested duck genome reveals clues to genetic compensation and crest cushion formation. Genomics Proteomics Bioinformatics. 2023;21(3):483-500.

Chen L, Gu X, Huang X, et al. Two cis-regulatory SNPs upstream of ABCG2 synergistically cause the blue eggshell phenotype in the duck. PLoS Genet. 2020;16(11):e1009119.

Chen Z, Snetkova V, Bower G, et al. Increased enhancer-promoter interactions during developmental enhancer activation in mammals. Nat Genet. 2024;56(4):675-685.

Cheng H, Concepcion GT, Feng X, Zhang H, Li H. Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. Nat Methods. 2021;18(2):170-175.

Coding CR, Arnheiter H. MITF-the first 25 years. Genes Dev. 2019;33(15-16):983-1007.

Cooke TF, Fischer CR, Wu P, et al. Genetic Mapping and Biochemical Basis of Yellow Feather Pigmentation in Budgerigars. Cell. 2017;171(2):427-439.

Deng Y, Qu X, Yao Y, et al. Investigating the impact of pigmentation variation of breast muscle on growth traits, melanin deposition, and gene expression in Xuefeng black-bone chickens. Poult Sci. 2024;103(6):103691.

Dudchenko O, Batra SS, Omer AD, et al. De novo assembly of the Aedes aegypti genome using Hi-C yields chromosome-length scaffolds. Science. 2017;356(6333):92-95.

Durand NC, Shamim MS, Machol I, et al. Juicer Provides a One-Click System for Analyzing Loop-Resolution Hi-C Experiments. Cell Syst. 2016;3(1):95-98.

Dürig N, Letko A, Lepori V, et al. Two MC1R loss-of-function alleles in cream-coloured Australian Cattle Dogs and white Huskies. Anim Genet. 2018;49(4):284-290.

Falletta P, Bagnato P, Bono M, et al. Melanosome-autonomous regulation of size and number: the OA1 receptor sustains PMEL expression. Pigment Cell Melanoma Res. 2014;27(4):565-579.

Flesher JL, Paterson-Coleman EK, Vasudeva P, et al. Delineating the role of MITF isoforms in pigmentation and tissue homeostasis. Pigment Cell Melanoma Res. 2020;33(2):279-292.

Gong Y, Yang Q, Li S, et al. Grey plumage colouration in the duck is genetically determined by the alleles on two different, interacting loci. Anim Genet. 2010;41(1):105-108.

Grewal S, Yang CY, Scholefield D, et al. Chromosome-scale genome assembly of bread wheat's wild relative Triticum timopheevii. Sci Data. 2024;11(1):420.

Guo Q, Jiang Y, Wang Z, et al. Genome-Wide Analysis Identifies Candidate Genes Encoding Feather Color in Ducks. Genes. 2022;13(7):1249.

Guo Y, Gu X, Sheng Z, et al. A Complex Structural Variation on Chromosome 27 Leads to the Ectopic Expression of HOXB8 and the Muffs and Beard Phenotype in Chickens. PLoS Genet. 2016;12(6):e1006071.

Gunnarsson U, Kerje S, Bed'hom B, et al. The Dark brown plumage color in chickens is caused by an 8.3-kb deletion upstream of SOX10. Pigment Cell Melanoma Res. 2011;24(2):268-274.

Haase E, Ito S, Wakamatsu K. Influences of sex, castration, and androgens on the eumelanin and pheomelanin contents of different feathers in wild mallards. Pigment Cell Res. 1995;8(3):164-170.

Heo S, Cho S, Dinh PTN, et al. A genome-wide association study for eumelanin pigmentation in chicken plumage using a computer vision approach. Anim Genet. 2023;54(3):355-362.

Hu J, Song L, Ning M, et al. A new chromosome-scale duck genome shows a major histocompatibility complex with several expanded multigene families. BMC Biol. 2024;22(1):31.

Hua G, Chen J, Wang J, et al. Genetic basis of chicken plumage color in artificial population of complex epistasis. Anim Genet. 2021;52(5):656-666.

Huang Y, Li Y, Burt DW, et al. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. Nat Genet. 2013;45(7):776-783.

Hung TC, Kingsley DM, Boettiger AN. Boundary stacking interactions enable cross-TAD enhancer-promoter communication during limb development. Nat Genet. 2024;56(2):306-314.

Hurbain I, Geerts WJ, Boudier T, et al. Electron tomography of early melanosomes: implications for melanogenesis and the generation of fibrillar amyloid sheets. Proc Natl Acad Sci U S A. 2008;105(50):19726-19731.

Ishishita S, Takahashi M, Yamaguchi K, et al. Nonsense mutation in PMEL is associated with yellowish plumage colour phenotype in Japanese quail. Sci Rep. 2018;8(1):16732.

Ito S, Wakamatsu K. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. 2003;16(5):523-531.

Jiang F, Jiang Y, Wang W, et al. A chromosome-level genome assembly of Cairina moschata and comparative genomic analyses. BMC Genomics. 2021;22(1):581

Kang HM, Sul JH, Service SK, et al. Variance component model to account for sample structure in genome-wide association studies. Nat Genet. 2010;42(4):348-354.

Karlsson EK, Baranowska I, Wade CM, et al. Efficient mapping of mendelian traits in dogs through genome-wide association. Nat Genet. 2007;39(11):1321-1328.

Keeling L, Andersson L, Schütz KE, et al. Chicken genomics: feather-pecking and victim pigmentation. Nature. 2004;431(7009):645-646.

Kerje S, Sharma P, Gunnarsson U, et al. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics. 2004;168(3):1507-1518.

Land EJ, Riley PA. Spontaneous redox reactions of dopaquinone and the balance between the eumelanic and phaeomelanic pathways. Pigment Cell Res. 2000;13(4):273-277.

Lavretsky P, Hernández F, Swale T, et al. Chromosomal-level reference genome of a wild North American mallard (Anas platyrhynchos). G3. 2023;13(10):jkad171.

Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-1760.

Li J, Zhang J, Liu J, et al. A new duck genome reveals conserved and convergently evolved chromosome architectures of birds and mammals. Gigascience. 2021;10(1):giaa142.

Li S, Wang C, Yu W, et al. Identification of genes related to white and black plumage formation by RNA-Seq from white and black feather bulbs in ducks. PLoS One. 2012;7(5):e36592.

Lin R, Lin W, Zhou S, et al. Integrated Analysis of mRNA Expression, CpG Island Methylation, and Polymorphisms in the MITF Gene in Ducks (Anas platyrhynchos). Biomed Res Int. 2019;2019:8512467.

Lin R, Zhao F, Xiong T, et al. Genetic mapping identifies SNP mutations in MITF-M promoter associated with melanin formation in Putian black duck. Poult Sci. 2024;103(1):103191.

Liu D, Zhang H, Yang Y, et al. Metabolome-Based Genome-Wide Association Study of Duck Meat Leads to Novel Genetic and Biochemical Insights. Adv Sci. 2023a;10(18):e2300148.

Liu H, Xi Y, Tang Q, et al. Genetic fine-mapping reveals single nucleotide polymorphism mutations in the MC1R regulatory region associated with duck melanism. Mol Ecol. 2023b;32(12):3076-3088.

Liu S, Chen H, Ouyang J, et al. A high-quality assembly reveals genomic characteristics, phylogenetic status, and causal genes for leucism plumage of Indian peafowl. Gigascience. 2022;11:giac018.

Liu X, Zhou R, Peng Y, et al. Feather follicles transcriptome profiles in Bashang long-tailed chickens with different plumage colors. Genes Genomics. 2019;41(11):1357-1367.

Manni M, Berkeley MR, Seppey M, Zdobnov EM. BUSCO: Assessing Genomic Data Quality and Beyond. Curr Protoc. 2021;1(12):e323.

McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20(9):1297-1303.

Mueller RC, Ellström P, Howe K, et al. A high-quality genome and comparison of short- versus long-read transcriptome of the palaearctic duck Aythya fuligula (tufted duck). Gigascience. 2021;10(12):giab081.

Ng CS, Lai CK, Ke HM, et al. Genome Assembly and Evolutionary Analysis of the Mandarin Duck Aix galericulata Reveal Strong Genome Conservation among Ducks. Genome Biol Evol. 2022;14(6):evac083.

Ng CS, Li WH. Genetic and Molecular Basis of Feather Diversity in Birds. Genome Biol Evol. 2018;10(10):2572-2586.

Pan R, Hua T, Guo Q, et al. Identification of SNPs in MITF associated with beak color of duck. Front Genet. 2023;14:1161396.

Powell S, Forslund K, Szklarczyk D, et al. eggNOG v4.0: nested orthology inference across 3686 organisms. Nucleic Acids Res. 2014;42:D231-D239.

Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006;38(8):904-909.

Ren S, Lyu G, Irwin DM, et al. Pooled sequencing analysis of geese (Anser cygnoides) reveals genomic variations associated with feather color. Front Genet. 2021;12:650013.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-1108.

Servant N, Varoquaux N, Lajoie BR, et al. HiC-Pro: an optimized and flexible pipeline for Hi-C data processing. Genome Biol. 2015;16:259.

Si S, Xu X, Zhuang Y, et al. The genetics and evolution of eye color in domestic pigeons (Columba livia). PLoS Genet. 2021;17(8):e1009770.

Soldner F, Stelzer Y, Shivalila CS, et al. Parkinson-associated risk variant in distal enhancer of α-synuclein modulates target gene expression. Nature. 2016;533(7601):95-99.

Sultana H, Seo D, Choi NR, et al. Identification of polymorphisms in MITF and DCT genes and their associations with plumage colors in Asian duck breeds. Asian-Australas J Anim Sci. 2018;31(2):180-188.

Terrill RS, Shultz AJ. Feather function and the evolution of birds. Biol Rev Camb Philos Soc. 2023;98(2):540-566.

Wang K, Hua G, Li J, et al. Duck pan-genome reveals two transposon insertions caused bodyweight enlarging and white plumage phenotype formation during evolution. Imeta. 2023;3(1):e154.

Wang L, Yang L, Yang S, et al. Identification of genes associated with feather color in Liancheng white duck using F_{ST} analysis. Anim Genet. 2022;53(4):518-521.

Wang X, Zheng Z, Cai Y, et al. CNVcaller: highly efficient and widely applicable software for detecting copy number variations in large populations. Gigascience. 2017;6(12):1-12.

Wang Z, Guo Z, Mou Q, et al. Unique feather color characteristics and transcriptome analysis of hair follicles in Liancheng White ducks. Poult Sci, 2024;103(7):103794.

Watt B, Tenza D, Lemmon MA, et al. Mutations in or near the transmembrane domain alter PMEL amyloid formation from functional to pathogenic. PLoS Genet. 2011;7(9):e1002286.

Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013;26(3):300-315.

Wenger, A.M., Peluso, P., Rowell, W.J. et al. Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. Nat Biotechnol. 2019; 37(10):1155–1162.

Xi Y, Wang L, Liu H, et al. A 14-bp insertion in endothelin receptor B-like (EDNRB2) is associated with white plumage in Chinese geese. BMC Genomics. 2020;21(1):162.

Xu MM, Gu LH, Lv WY, et al. Chromosome-level genome assembly of the Muscovy duck provides insight into fatty liver susceptibility. Genomics. 2022;114(6):110518.

Yang L, Mo C, Shen W, et al. The recessive C locus in the MITF gene plays a key regulatory role in the plumage colour pattern of duck (Anas platyrhynchos). Br Poult Sci. 2019;60(2):105-108.

Yu S, Liu Z, Li M, et al. Resequencing of a Pekin duck breeding population provides insights into the genomic response to short-term artificial selection. Gigascience. 2023;12:giad016.

Yuan Z, Zhang X, Pang Y, et al. Association analysis of PMEL gene expression and single nucleotide polymorphism with plumage color in quail. Anim Biotechnol. 2023;34(9):5001-5010.

Zheng X, Zhang B, Zhang Y, et al. Transcriptome analysis of feather follicles reveals candidate genes and pathways associated with pheomelanin pigmentation in chickens. Sci Rep. 2020;10(1):12088.

Zheng Z, Lai Z, Wu B, et al. The first high-quality chromosome-level genome of the Sipuncula Sipunculus nudus using HiFi and Hi-C data. Sci Data. 2023;10(1):317.

Zhou Z, Li M, Cheng H, et al. An intercross population study reveals genes associated with body size and plumage color in ducks. Nat Commun. 2018;9(1):2648.

Zhu F, Yin ZT, Wang Z, et al. Three chromosome-level duck genome assemblies provide insights into genomic variation during domestication. Nat Commun. 2021;12(1):5932.

Chapter V

Marker assisted selection--Rapid genetic breeding of high-quality meat ducks

The aim of this chapter was to (1) find SNP variants strongly linked to both *MITF* and *PMEL* gene expression, to (2) explore whether the breeding results between 1 *bbrr* genotype duck and 8 *BBrr* genotype ducks were in line with expectation, and to (3) identify the *bbrr* genotype in these F2 collected ducks from Liancheng Ducks and Pekin Ducks, as well as to (4) crossbreed with white-feathered, black-beaked *BBrr* genotype ducks to obtain *Bbrr* genotype ducks with high quality-meat commercial ducks.

At present, the final breeding work has not been completed. After all the breeding work is finished, we plan to write the paper and submit it to a peer-reviewed international journal. However, this chapter is adapted from the published patents:

(1) Molecular markers related to phenotypic traits of white feathered and black beak ducks and their breeding application in high quality ducks. Zhou Z, Hou S, Guo Z, Wang Z. No:202211228357.6 (Chinese Patent).

(2) A cross-breeding method for effective protection of local high quality duck breeds. Zhou Z, Guo Z, Hou S, Wang Z. No:202310542366.0 (Chinese Patent).

Author contribution: Zhen Wang conducted resource population, the design of the experimental plan, sample collection, phenotypic data statistics and recording, Sanger sequencing detection and recording, data visualization, manuscript drafting, editing and refinement.

.

5.1 Abstract

In production practice, the offspring of large-size Pekin ducks (white feather, yellow beak and yellow feet) and high-quality small-size Liancheng ducks (white feather, black beak and feet) are crossbred, and the offspring is moderate in size and good in meat quality, but the feather color is dispersed, and white, gray and black feathered ducks appear. Therefore, the challenge we face is how to efficiently breed individuals with desirable meat quality and large body size and specific phenotypic traits (white feathers and black beaks), a pressing issue that the duck industry needs to address.

In Chapter IV, two leading SNPs identified through GWAS analysis were investigated. SNP1 (Chr13:5,038,595A>G) showed a strong association with the *MITF* gene, while SNP2 (Chr33:5,303,461A>G) was closely linked to the *PMEL* gene. Therefore, we use these two molecular markers to carry out hybridization experiments. In these experiments, firstly, 1 *bbrr* genotype male, that was white-feathered and has a yellow beak, was crossbred with 8 *BBrr* genotype females, all white-feathered and with black beaks. All 56 *Bbrr* commercial descendants (F1 generation) were white-feathered, with black beaks. After continued hybridization of the F1 individuals, the phenotypes and proportions of the 224 offspring were in line with our expectations. Specifically, this included 61 *bbrr* genotype ducks with white feathers and yellow beaks and 163 *B_rr* genotype ducks with white feathers and black beaks, adhering to the Mendelian ratio of 3:1. This further validates the results of Chapter IV. Then, 602 blood samples of the white-feathered yellow beak ducks from the F2 population were collected and 153 *bbrr* genotype ducks with white feathers and yellow beaks were successfully selected. In the future, these 153 ducks will be crossbred with *BBrr* white-feathered black beak ducks to produce *Bbrr* white-feathered black beak ducks. This study focuses on developing a rapid molecular breeding method to produce high-quality meat ducks.
Key words: molecular breeding, SNP, sanger sequencing, crossbreeding, high-quality meat ducks

5.2 Introduction

At present, the meat duck industry is progressively shifting its focus from quantity to quality, and breeding hybrid offspring that exhibits all positive outcomes due to heterosis is an effective measure to promote the development of the meat duck industry. Molecular breeding is revolutionizing the field of animal agriculture by integrating advanced genetic technologies into livestock production. The approach of using molecular markers and genomic information to make informed breeding decisions significantly enhances the efficiency and precision of traditional practices. With the growing global demand for animal products, enhancing productivity and ensuring sustainability in livestock farming have become more crucial than ever. Through the application of molecular breeding techniques, breeders can assess and select for traits like growth rate, disease resistance, and reproductive performance at a much faster pace than conventional breeding methods allow. This leads to accelerated genetic gains, reducing the time required to introduce beneficial traits into breeding populations. For example, in dairy cattle, genomic selection has resulted in notable improvements in milk production and overall herd health (Silpa et al., 2021). Moreover, the integration of modern technologies allows for precise genetic modifications, enabling the enhancement of traits that contribute to animal welfare and environmental sustainability.

Single nucleotide polymorphisms (SNPs), as molecular markers, are widely used in molecular breeding due to their abundant presence in the genome and low detection costs. The principle of using SNPs in molecular breeding mainly involves selecting individuals with desirable genotypes by markers closely linked to target traits, enabling precise selection for these traits. First, SNP loci associated with traits are identified through GWAS or linkage analysis; next, these markers are used to genotype breeding materials to screen animals and plants carrying favorable alleles; finally, these superior genotypes are introduced into target varieties rapidly through hybrid breeding or gene editing techniques. SNP-assisted molecular breeding accelerates the breeding process and enhances breeding accuracy, improving yield, quality, and stress resistance (Yang et al., 2020).

In Chapter IV, two leading SNPs identified through GWAS analysis were used. SNP1 (Chr13:5,038,595A>G) was strongly linked to the *MITF* gene, while SNP2 (Chr33:5,303,461A>G) was strongly linked to the *PMEL* gene. Based on these molecular markers, we developed a rapid molecular breeding method aimed at producing high-quality meat ducks. Both *MITF* and *PMEL* are well-known candidate genes involved in pigmentation pathways, which have been documented to influence phenotypic traits relevant to feather color in ducks. Based on these findings, we developed a rapid molecular breeding strategy using these two molecular markers as selection tools. This marker-assisted selection (MAS) approach enables efficient identification and propagation of advantageous alleles linked to feather color in ducks. The implementation of such molecular markers not only accelerates the breeding process but also enhances the accuracy of selecting superior individuals, thus providing a valuable tool for genetic improvement programs aimed at producing high-quality

meat ducks with white-feathered black beak and feet.

In summary, the application of molecular breeding in livestock is a pioneering step towards achieving more efficient, resilient, and sustainable animal production systems. Continued investment in research and the development of these technologies will be essential for the future of global food security.

5.3 Materials and methods

5.3.1 Ducks and sampling

All blood samples were obtained from the subwing vein. For the F0 generation, 120 Liancheng ducks and 30 Pekin ducks were used. A total of 1260 F1 grey feathered black-beaked ducks were produced and 450 female ducks and 120 male F1 ducks were randomly selected and crossbred in order to produce the F2 generation. In the F2 generation, a total of 602 white-feathered, yellow-beaked ducks were sampled. The study was approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences (CAAS). All animal procedures adhered to the guidelines for the care and use of experimental animals set by the CAAS (IAS2022-105).

5.3.2 DNA extraction

Whole blood was transferred into a sterile centrifuge tube, centrifuged at 4 ℃ for 10 min to retain the precipitation. PBS buffer was added, the precipitation was suspended by vortex oscillation, and centrifuged at 4 ℃ for 10 min to precipitate again. DNA extraction buffer and protease K were added and the total was incubated in a water bath at 37 ℃ overnight, until the cell sediments were completely digested. The next day, 1 mL of Tris saturated phenol was added, and the centrifuge tube was gently shaken on ice for 20 min, after which the top water phase was transferred to another sterilization centrifuge tube with a pipette. A combination of 0.5 mL of saturated phenol and 0.5 mL of chloroform was added, and the total was again shaken on ice for 20 min, after which the top water phase was transferred with a pipette to another 2.0mL sterilization centrifuge tube. Finally, chloroform and pre-cooled anhydrous ethanol were added, the tube was shaken until the DNA precipitated. The concentrations were determined by spectrophotometer. The DNA solution was preserved at -80 ℃ (Kraemer et al., 2019; Tagliaferro et al., 2021).

5.3.3 Primer synthesis and PCR amplification

The upstream and downstream primers for SNP1 within *MITF* (IASCAAS_LianchengWhiteDuck, GCA_039998735.1: Chr13.5,038,595 A>G), and the upstream and downstream primers for SNP2 within *PMEL* (IASCAAS_LianchengWhiteDuck, GCA_039998735.1:Chr33.5,303,461A>G) are

shown in Table 5-1. Both primers and PCR Master Mix were synthesized by the Beijing Qingke Zexi Biotechnology Co., LTD.

In this experiment, the total volume of PCR amplification system was 25 μL, including 12.5 μL PCR Master Mix, 1.6 μL DNA template (20 ng/μL), 1 μL of each, upper and downstream, primer (10 μmol/L), and ddH_2O. PCR amplification conditions were as follows: predenaturing at 95 ℃ for 5 min, denaturing at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 25 s, and this for 32 cycles. A final extension for 10 min was performed at 72 ℃. The PCR products were Sanger sequenced by the Beijing Qingke Zexi Biotechnology Co., LTD to identify the genotypes (Aly et al., 2015; Crossley et al., 2020).

Table 5-1 PCR primers used for detecting two SNP loci of duck genotypes.

Primers	Sequences (5'-3')	Location	Length (bp)
MITF	F: CCTGCTAGACCTGCACAACA	Chr13.5,038,445-	332
	R: TCTCACCTGTGGAAACTGCC	5,038,776	
PMEL	F: AAGGAAAAGCTCAGCCCCTG	Chr33:5,303,291-	337
	R: ATGCCTTTACAGCAGGAGGAG	5,303,627	

5.4 Results

5.4.1 Mating between a *bbrr* male duck and 8 *BBrr* female ducks

One white plumage and yellow-beaked male duck, identified as a *bbrr* genotype, was crossbred with 8 *BBrr* ducks. We found all 56 *Bbrr* descendants of the F1 generation havind white plumage and being black-beaked, and the genotype was heterozygous for the *MITF* gene, namely *Bbrr*. After F1 continued hybridization, the phenotypes and proportions of the 224 offspring were in line with our expectations. It resulted in 61 *bbrr* white-feathered and yellow-beaked ducks and 163 *B_rr* white-feathered and black-beaked ducks. The offspring ratio was around 3:1. This further validates the results of Chapter IV (Wang et al., 2015).

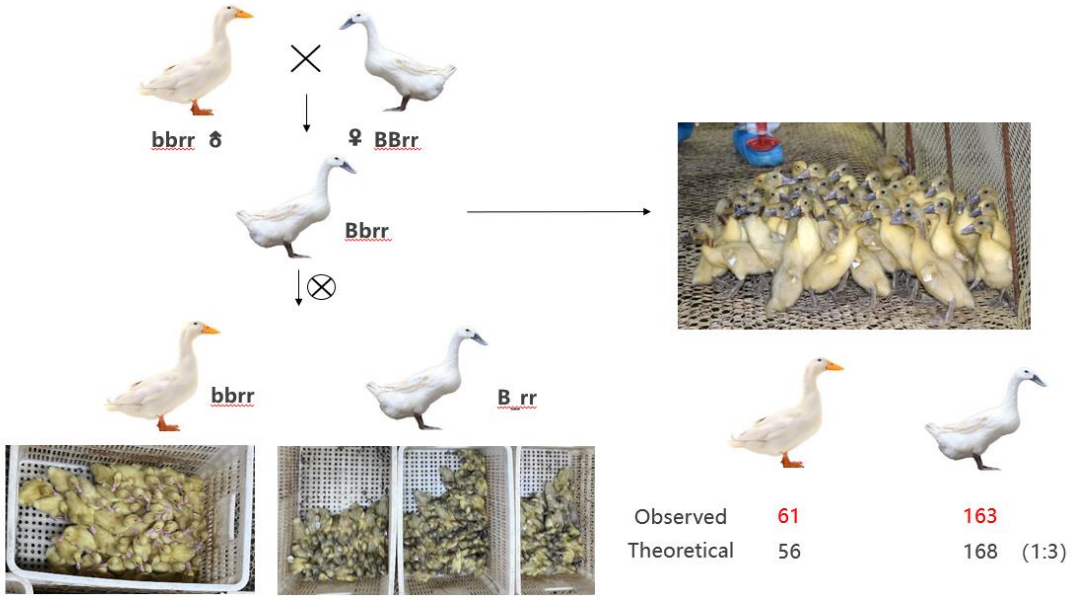

Figure 5-1. Cross breeding results between 1 *bbrr* genotype duck and 8 *BBrr* genotype ducks

5.4.2 Genotyping of 602 white feathered yellow-beaked ducks of the F2 generation

For Liancheng ducks, we randomly sequenced 120 GG_AA (*BBrr*) genotypes, and for Pekin ducks we sequenced 30 AA_GG (*bbRR*) genotypes. This is consistent with our findings. In F2 populations, a total of 602 blood samples of white-feathered yellow-beaked ducks were collected. The Sanger sequencing showed 153 AA_AA (*bbrr*) genotype ducks among these blood samples. The *bbrr* genotype ducks were further selected for crossbreeding in order to obtain the *Bbrr* genotype ducks with white feathers and black beaks. Next, these *bbrr* genotype ducks will be crossbreed with *BBrr* white feathered black-beaked ducks, but this work is still in progress.

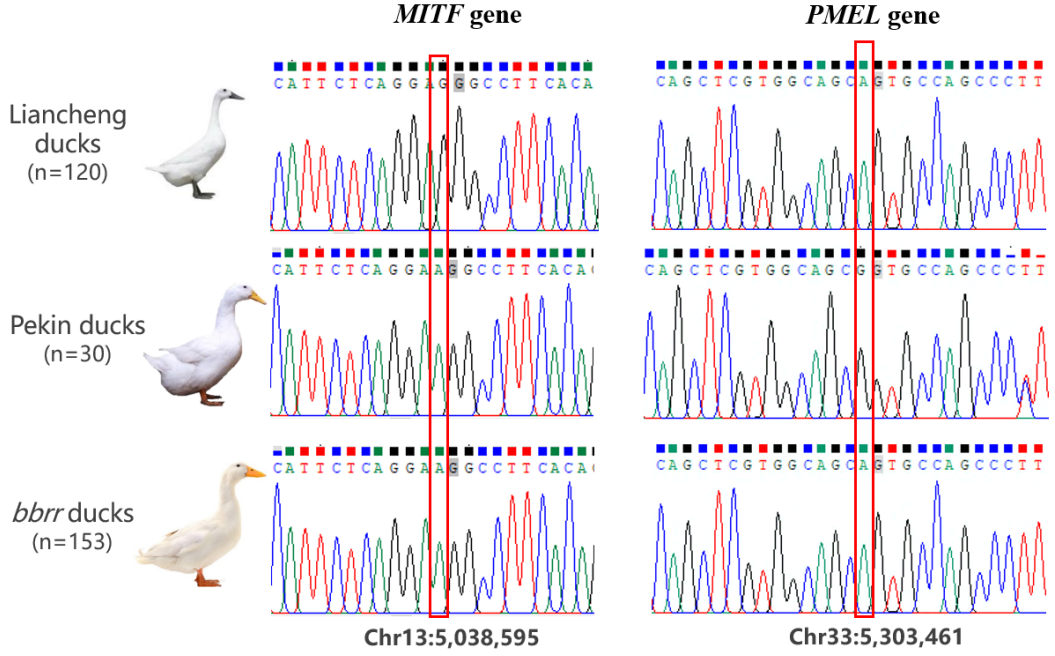

Figure 5-2. Sanger sequencing results of *BBrr*, *bbRR*, and *bbrr* genotype ducks

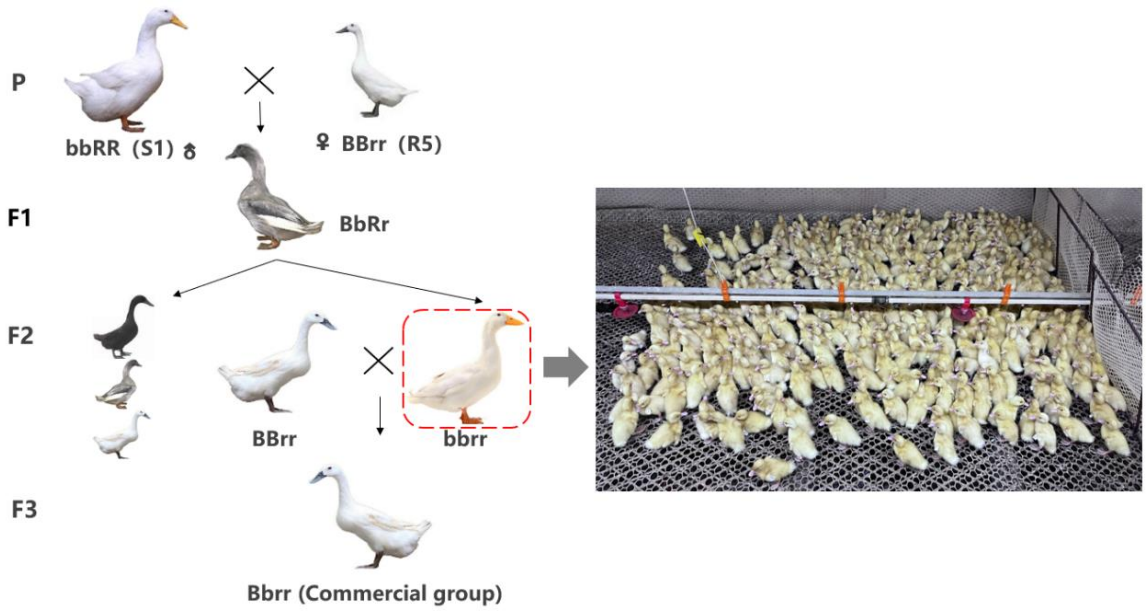

Figure 5-3. The breeding process of commercial *Bbrr* genotype ducks. 153 *bbrr* genotype white-feathered yellow-beaked ducks as parents are obtained, and the next step is to cross with *BBrr* genotype white-feathered black-beaked ducks to obtain the *Bbrr* commercial generation.

5.5 Discussion

In duck production, the white feather phenotype has always been favored by breeders and has an important economic value. Breeding large white-feathered ducks has always been the common goal of producers and researchers. Liancheng duck is highly popular in the market due to its unique white feathers, black beak, and black webbed feet. To determine the genetic mechanism and molecular markers that affect the phenotype formation of Liancheng duck white unique feathers by modern biotechnology is one of the effective means to improve the economic value of ducks.

In this PhD study, as described in the previous chapter, we found that the formation of the unique white feather phenotype of Liancheng duck is not controlled by one gene, but by two genes. Based on this knowledge, we evaluated all potential gene combinations and established a reasonable screening protocol to ensure that the selected lines have the expected genotype and phenotype. Using GWAS analyses, we identified SNP1 (Chr13:5,038,595A>G) and SNP2 (Chr33:5,303,461A>G) as leading SNPs that could be used as markers linked to *MITF* and *PMEL* gene expression. *MITF* is a key regulator of melanin synthesis, controlling the expression of enzymes involved in this process as well as receptors critical to melanocyte function (Dürig et al., 2018; Guo et al., 2022; Pan et al., 2023). The SNP1 genotypes were linked with the on-off role of *MITF* in the melanin generation pathway of ducks. PMEL, a type I transmembrane transport glycoprotein, is synthesized in the endoplasmic reticulum and plays a crucial role in amyloid fiber formation during stages I and II of melanosome formation in the L-DOPA pathway (Kerje et al., 2004; Batai et al., 2021). The SNP2 genotypes were

linked with the expression of *PMEL* in the melanin generation pathway of Liancheng ducks.

The Sanger-sequencing molecular identification method was used for the genotypes of the dominant white feather locus in ducks with or without the dominant white feather locus, and the results were verified. At the same time, the method is simple to operate, and any basic laboratory can meet the requirements of testing (Rodriguez et al., 2023). Compared with the traditional breeding method, the molecular identification method can not only improve the detection speed, but also reduce the labor cost (Chan, 2005). Traditional breeding methods cannot quickly produce high-nutrition meat, large-sized and meat ducks with white-feathered and black beak appearance since traditional breeding cannot guarantee that the genotype will be heterozygous. However, for the molecular identification method one only needs to extract DNA from a blood sample,and then perform a PCR amplification for SNP1 and SNP2 after which you can sequence. This can quickly and accurately identify what alleles the ducks carry for the respective *MITF* and *PMEL* genes.

Utilizing molecular markers associated with genes like *MITF* and *PMEL* allows early and accurate selection of superior individuals, reducing reliance on phenotypic evaluations influenced by environmental factors. MAS accelerates genetic gain and improves trait uniformity, thereby supporting stable production of high-quality meat ducks. Incorporating MAS into breeding programs is essential for sustainable genetic improvement and meets industry demands for consistent product quality. The use of SNPs in molecular breeding holds great potential in modern agriculture, yet it also faces certain challenges. Although SNP markers offer advantages such as high throughput and precision, enabling more efficient genotyping and selection, their effectiveness can be limited by the complexity of the genome and gene-environment interactions (Rockwood-Sluss et al., 1973; Wilker et al., 2009). Future research should focus on integrating multi-omics data to enhance understanding of SNP functions and optimize SNP-based selection strategies for precise regulation of complex traits. Additionally, combining SNP-assisted breeding with advanced technologies like gene editing could further improve breeding efficiency and broaden its application scope (Xu et al., 2014; Zhou et al., 2015), ultimately driving breeding towards greater intelligence and efficiency.

5.6 Conclusion

This research found two SNP variants (SNP1, Chr13:5,038,595A>G; SNP2, Chr33:5,303,461A>G), which were strongly linked to the *MITF* and *PMEL* genes. By sanger sequencing detection, we found the breeding results between 1 *bbrr* genotype duck and 8 *BBrr* genotype ducks were in line with expectation, and we identified the 153 *bbrr* genotype in these F2 ducks. Next they will be crossbred with white-feathered, black-beaked *BBrr* genotype ducks to obtain *Bbrr* genotype ducks with high quality-meat commercial ducks, in order to rapidly produce heterozygous commercially ducks with larger body size and high meat nutrition.

References

Aly SM, Sabri DM. Next generation sequencing (NGS): a golden tool in forensic toolkit. Arch Med Sadowej Kryminol. 2015;65(4):260-71.

Batai K, Cui Z, Arora A, et al. Genetic loci associated with skin pigmentation in African Americans and their effects on vitamin D deficiency. PLoS Genet. 2021;17(2):e1009319.

Chan EY. Advances in sequencing technology. Mutat Res. 2005;573(1-2):13-40.

Crossley BM, Bai J, Glaser A, Maes R, Porter E, Killian ML, Clement T, Toohey-Kurth K. Guidelines for Sanger sequencing and molecular assay monitoring. J Vet Diagn Invest. 2020;32(6):767-775.

Dürig N, Letko A, Lepori V, et al. Two MC1R loss-of-function alleles in cream-coloured Australian Cattle Dogs and white Huskies. Anim Genet. 2018;49(4):284-290.

Guo Q, Jiang Y, Wang Z, et al. Genome-Wide Analysis Identifies Candidate Genes Encoding Feather Color in Ducks. Genes. 2022;13(7):1249.

Kerje S, Sharma P, Gunnarsson U, et al. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics. 2004;168(3):1507-1518.

Kraemer MN, Mencucci MV, Abba M, Lacunza E, Gagliardino JJ. Increasing DNA Isolation Performance from Blood Cells Using a Modified Commercial Low-Cost Reagent. Clin Lab. 2019;65(11).

Pan R, Hua T, Guo Q, et al. Identification of SNPs in MITF associated with beak color of duck. Front Genet. 2023;14:1161396.

Rockwood-Sluss ES, Johnston JS, Heed WB. Allozyme genotype-environment relationships. I. Variation in natural populations of Drosophila pachea. Genetics. 1973;73(1):135-146.

Rodriguez R, Krishnan Y. The chemistry of next-generation sequencing. Nat Biotechnol. 2023;41(12):1709-1715.

Silpa MV, König S, Sejian V, et al. Climate-Resilient Dairy Cattle Production: Applications of Genomic Tools and Statistical Models. Front Vet Sci. 2021;8:625189.

Tagliaferro SS, Zejnelagic A, Farrugia R, Wettinger SB. Comparison of DNA extraction methods for samples from old blood collections. Biotechniques. 2021;70(5):243-250.

Wang Z, Guo Z, Liu H, Liu T, Liu D, Yu S, Tang H, Zhang H, Mou Q, Zhang B, Cao J, Schroyen M, Hou S, Zhou Z. A high-quality assembly revealing the PMEL gene for the unique plumage phenotype in Liancheng ducks. Gigascience. 2025;14:giae114.

Wilker E, Mittleman MA, Litonjua AA, Poon A, Baccarelli A, Suh H, Wright RO, Sparrow D, Vokonas P, Schwartz J. Postural changes in blood pressure associated with interactions between candidate genes for chronic respiratory diseases and exposure to particulate matter. Environ Health Perspect. 2009;117(6):935-940.

Xu R, Li H, Qin R, Wang L, Li L, Wei P, Yang J. Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. Rice (N Y). 2014;7(1):5.

Yang S, Yu W, Wei X, Wang Z, Zhao Y, Zhao X, Tian B, Yuan Y, Zhang X. An extended KASP-SNP resource for molecular breeding in Chinese cabbage (Brassica rapa L. ssp. pekinensis). PLoS One. 2020;15(10):e0240042.

Zhou X, Xin J, Fan N, Zou Q, Huang J, Ouyang Z, Zhao Y, Zhao B, Liu Z, Lai S, Yi X, Guo L, Esteban MA, Zeng Y, Yang H, Lai L. Generation of CRISPR/Cas9-mediated gene-targeted pigs via somatic cell nuclear transfer. Cell Mol Life Sci. 2015;72(6):1175-1184.

Chapter VI

General discussion, conclusion and perspectives

6.1 General discussion

6.1.1 The complex genetic control of feather color traits in ducks

Feather color in ducks is a phenotypically significant trait, influenced by a complex interplay of genetic factors and environmental conditions. Understanding the genetic basis of feather color variability is crucial for both evolutionary biology and poultry science. Multiple loci, genes, and regulatory networks contribute to this trait, making it a defining characteristic of various duck breeds. Ducks exhibit a wide array of feather colors, including shades of white, brown, green, and black, each governed by distinct genetic phenomena. The primary genetic determinants of feather color are the *MITF*, *MC1R* and *ASIP* gene, all of which play important roles in melanin production and distribution (San-Jose et al., 2017). Variations in these genes can lead to diverse outcomes in feather pigmentation, with melanin being responsible for dark colors and pheomelanin contributing to lighter hues (Wang et al., 2022). The *MITF* gene also has been widely reported to control melanin synthesis in ducks (Zhou et al., 2018).

Linkage mapping and GWAS have identified numerous quantitative trait loci (QTLs) associated with feather coloration in ducks (Wang et al., 2024). Except chromosomes 13 and 33, the identification of QTLs on chromosomes 1,2,21 have been reported associated with the presence of specific color patterns in duck breeds (Zhao et al., 2019), such as *MRS2*, *TGFBRAP1*, *STK4*, *CCN5*, *YWHAB* and *LOC113839965* genes. These findings emphasize the polygenic nature of feather color traits where multiple genes collectively influence the phenotype, enhancing the variability within and across breeds. The interaction between epigenetic modifications and genetic predispositions contributes to the intricate regulation of feather coloration, suggesting that environmental factors may also play a role in determining feather color phenotypes (Apopo et al., 2015; Galvan et al., 2017). In the context of evolutionary significance, feather coloration might also play a role in sexual selection. Brightly colored feathers can enhance mate attraction and improve reproductive success, thus creating a selective pressure for certain alleles associated with pigmentation (Nadeau et al., 2007). The interplay between natural and sexual selection processes further complicates the genetic landscape of feather color, necessitating a multifaceted approach to studying its genetics.

Understanding these transcriptomic interactions will be crucial for elucidating the mechanisms underlying feather color variation. In this study, we revealed that the *MITF* gene controls melanin synthesis on the duck's body surface and exhibits epistatic effect. Under the premise that *MITF* plays a role in the synthesis of melanin, the expression of *PMEL* facilitates the deposition of melanin in the hair follicle tissue, resulting in black feather coloration in ducks. Conversely, the inactivation of *PMEL* leads to the characteristic appearance of white feathers and black beaks. This is a typical example of the synergistic action of two genes to control the color of duck feathers, which indirectly proves the complexity of melanin synthesis in duck feathers. It is worth

noting that the pigment on the body surface of Liancheng ducks is only eumelanin, which provides convenience for the study of the genetic mechanism of its pigment formation.

6.1.2 Improved reference genomes of *Anas platyrhynchos*

The study of avian genomics has significantly advanced in recent years, with the duck genome prime focus due to their agricultural importance and unique biological characteristics. The duck reference genome serves not only as a key tool for breeding programs but also plays a critical role in understanding avian biology, evolution, and disease resistance. In this regard, research on the duck reference genome has seen substantial progress, leading to new insights and advancements in the field of genetics. The first complete draft of the Pekin duck genome, a widely bred domestic duck species, was published in 2013, marking a crucial milestone in avian genomics (Huang et al., 2013). With a total genome size of approximately 1.2 billion base pairs, this draft provided initial insights into the genetic underpinnings of important traits such as growth rate and meat quality. Subsequent studies have refined this reference genome using improved sequencing technologies and methodologies, which have considerably increased the accuracy and resolution of the genomic data (Li et al., 2021).

In recent years, advances in genome assembly techniques, particularly the application of long-read sequencing technology, have allowed researchers to enhance the completeness of the duck reference genome. For instance, Zhu et al. (2021) reported the use of third-generation sequencing to produce a more comprehensive assembly of the duck genome. This updated genome sequence proved beneficial for exploring structural variations and complex genomic architectures that were previously unresolved. Furthermore, the development of genomic resources, such as single nucleotide polymorphisms databases and gene expression profile databases, has complemented the reference genome, facilitating breeding efforts. These resources enable researchers to identify genetic markers associated with economically important traits, enhancing selective breeding programs aimed at improving growth performance and disease resistance in domestic duck populations (Sun et al., 2019; Xi et al., 2023). Liu et al. (2023) conducted a GWAS analysis identifying several SNPs linked to meat quality traits, which could facilitate targeted selection in breeding programs.

In this PhD study, the newly assembled Liancheng duck reference genome improves the duck genome information, which plays an important role for advancing genetic information mining in ducks. Our *de novo* assembled 1.29 Gb Liancheng duck genome exhibited perfect collinearity with that of Mallards (GCA_008746955.3) and Pekin ducks (GCA_015476345.1), which demonstrates the high quality of our genome assembly. The length of scaffold N50 in our Liancheng duck genome assembly was the longest among all previously published duck (*Anas platyrhynchos*) genomes, indicating high continuity of our assembly. Based on the high-quality de novo assembly of our Liancheng duck genome, a total of 18,819 genes were predicted. We believe that this is an effective complement to the current duck reference genome. In conclusion, the availability of multiple *Anas platyrhynchos* genome datasets, including that of the

Liancheng duck, has made the duck reference genome reach a significant milestone. This progress is characterized by improved sequencing technologies and a growing diversity of genomic resources. It also is vital for enhancing our understanding of genetic diversity, improving breeding strategies, and addressing the challenges posed by disease and environmental changes in domestic duck populations. The comprehensive genomic and functional dataset generated in this PhD study could be considered for future exploration of additional phenotypes or traits beyond duck feather coloration.

6.1.3 The role of *MITF* in melanin production in duck feathers

Melanin coloration is a crucial phenotype in birds, influencing not only breed identification but also temperature regulation and camouflage. In ducks, feather coloration results from the intricate interplay of multiple genes regulating melanin synthesis, with the *MITF* emerging as a central regulator. *MITF* is a transcription factor that controls the expression of various genes involved in melanocyte development and pigmentation processes (Zhou et al., 2018; Lin et al., 2024). Melanin synthesis in avian species begins with the enzymatic conversion of the amino acid tyrosine into the pigment precursors. The main steps involve the actions of key enzymes, including TYR, TYRP1, and DCT (Leng et al., 2025). These enzymes catalyze the transformation of tyrosine into L-DOPA, eventually producing eumelanin and pheomelanin, the two primary types of melanin found in feathers (Zheng et al., 2020). *MITF* plays a key role in this biosynthetic pathway. It serves as a transcriptional activator that binds to the promoter regions of these critical melanin synthesis genes, thereby modulating their expression levels (Joyjamras et al., 2022). Notably, studies have shown that the depletion of *MITF* leads to a marked reduction in *TYR*, *TYRP1*, and *DCT* expression, resulting in diminished melanin production and altered feather pigmentation (Fang et al., 2002). These findings identified the essentiality of *MITF* in maintaining the appropriate balance of melanin types, critical for the various coloration observed in various duck breeds.

In addition to its direct role in regulating melanin synthesis, *MITF* interacts with various signaling pathways that can influence its activity and stability. The MAPK pathway is one such interaction, where external regulation elements can modulate MITF phosphorylation state, affecting its transcriptional effects (Monmai et al., 2023; Feng et al., 2024). Other signaling molecules, such as *MITF* target genes and other factors, can influence feather pigmentation (Arcila et al., 2025), showing that pigmentation in ducks is not solely a result of pigmentation gene expression, but also of underlying environmental interactions. Through its critical role in the transcription and expression of key melanogenic enzymes, *MITF* directly influences the quality and quantity of feather pigmentation. Genetic variations within *MITF* and its regulatory elements contribute to the wide array of feather colors observed in duck populations, facilitating evolutionary adaptations and selective breeding practices. Through GWAS analysis, the *MITF* gene was confirmed to act as an epistatic regulator gene controlling melanin synthesis in Liancheng ducks, consistent with previous research findings

(Zhou et al., 2018). Future research should continue to explore the molecular mechanisms underlying *MITF* regulation, the interaction with other genes, and its broader implications for avian genetics.

6.1.4 The role of *MITF* and *PMEL* in melanin production in duck feathers

The pigmentation of feathers in ducks is a complex trait influenced by several genetic factors, among which *MITF* and *PMEL* are important. MITF serves as a master regulator of melanocyte development and activity, while PMEL is crucial for the formation of melanin-containing organelles known as melanosomes. Understanding how these two genes function together enhances our knowledge of color variation in duck feathers and the underlying molecular mechanisms of pigmentation.

The *PMEL* gene encodes a protein essential for the formation of melanosomes, where melanin is synthesized and stored in melanocytes. *PMEL* is involved in the structural organization of melanosomes and is critical for the maturation of these organelles. It facilitates the proper stacking of fibrillar structures within the melanosomes, promoting an efficient synthesis of melanin (Watt et al., 2013). Without functional *PMEL*, melanosome development is impaired, leading to reduced melanin accumulation and, consequently, lighter feather coloration. Recent findings indicate that *PMEL* not only plays a structural role but also interacts with *MITF* to regulate the overall pigmentation process in feathers. Specifically, *MITF* can upregulate the expression of *PMEL*, creating a feedback loop that enhances melanin production by stabilizing melanosome operations and promoting melanin aggregation (Falletta et al., 2014). This relationship suggests that both genes work synergistically; while *MITF* initiates the melanin biosynthetic pathway, *PMEL* ensures that melanin is effectively packaged and stored, maximizing pigmentation efficiency.

The interactions between *MITF* and *PMEL* significantly influence the pigmentation phenotypes observed in ducks, not only through their individual functions but also through their regulatory networks. Variations in the *PMEL* gene have been associated with specific color traits in various avian species (Heo et al., 2023). For example, certain alleles of *PMEL* may confer advantages in terms of feather color richness and protection against environmental damage due to UV radiation, which can influence mate selection and survival. Moreover, studies indicate that the expression of *PMEL* can be regulated by various signaling pathways, including those activated by *MITF*. As *PMEL* expression is linked to MITF activity, any genetic changes affecting MITF could have downstream effects on *PMEL* expression, thus amplifying the impact on pigmentation (Falletta et al., 2014). This function emphasizes the complexity of genetic regulation in avian color and the necessity of multi-gene approaches to fully understand these processes.

Endogenous *PMEL* expression is regulated by MITF, with alterations observed in melanoma cells (Du et al., 2003). Overall, this research has annotated the *PMEL* gene in the duck genome for the first time, a gene previously thought to be absent in Pekin ducks. MITF, a key regulator of melanin production in ducks, was also identified. The inactivation of PMEL in feather bulb specimens led to the distinct white and black

feathering characteristic of Liancheng ducks. The interplay between the *MITF* and *PMEL* genes is critical in regulating melanin production and feather coloration in ducks. *MITF* acts as a transcriptional master switch, initiating the melanin biosynthetic pathway, while *PMEL* is essential for melanosome formation and melanin storage. Together, these genes highlight the intricate genetic mechanisms that govern avian pigmentation. Further research into their interactions will not only enhance our understanding of avian biology but may also have practical applications in the breeding of duck breeds with desirable color phenotypes. For example, further research can explore the effect of overexpression or knockout of *MITF* on the expression of *PMEL* genes, and then observe the changes of duck feather color phenotype.

6.1.5 Molecular breeding of white feather trait in ducks

Feather color in poultry is an essential trait with significant economic and aesthetic implications across various species, including chickens, ducks, and peafowl. In China, chicken plumage color is an important economic trait, as most native breeds have characteristic “triple-yellow” appearance (yellow plumage, beak, and claw) and they are preferred over western commercial broilers in the Chinese market (Huang et al., 2020). The primary feathers of ducks also have significant economic value to humans. They can be processed and transformed into products such as badminton balls, feather pens, handcrafts, etc., which are highly valued and cherished by many people (Wang et al., 2024). The dazzling phenotypic characteristics of male Indian peafowl (*Pavo cristatus*) are attractive both to the female of the species and to humans (Liu et al., 2022). The variation in feather color is predominantly attributed to the presence and distribution of melanin pigments, specifically eumelanin and pheomelanin, synthesized by melanocytes. Understanding the genetic basis of feather coloration and its molecular mechanisms has become the key in poultry breeding programs, enabling the development of specific color phenotypes that meet market demands and consumer preferences. Molecular breeding techniques, including Marker-Assisted Selection (MAS) and Genome-Wide Association Studies (GWAS), have transformed the approach to enhancing feather color traits in poultry, allowing breeders to produce lines with desired color phenotypes more efficiently.

QTL mapping studies have identified numerous loci associated with feather color in chicken breeds, revealing that the QTL can contribute to the intensity and type of pigment present in feathers (Huang et al., 2020). For instance, variations in the *TYR* gene can influence melanin production levels, resulting in phenotypic differences in feather coloration. Mutations in this pathway disrupt normal melanin synthesis, leading to lighter or darker feather varieties. Furthermore, the recent identification of various other candidate genes, such as *ASIP* and *SLC45A2*, also plays a role in the pigmentation process, identifying new pathways where genetic selection can be applied (Bovo et al., 2023). The application of molecular breeding techniques has revolutionized the poultry industry, allowing for more rapid and precise improvements in feather color traits. MAS employs genetic markers linked to specific traits to enhance breeding efficiency by allowing early identification of individuals that carry desirable alleles (Yang et al.,

2019), so utilizing SNP markers associated with the *MITF* and *PMEL* allow breeders to select for specific feather colors in Liancheng ducks, as shown in this PhD study.

GWAS have gained popularity in recent years for their ability to map traits associated with specific genetic variations (Sun et al., 2023). By comparing the genomes of birds with contrasting feather colors, researchers have been able to pinpoint specific genes or genomic regions correlated with pigmentation traits. This process enabled the identification of genetic markers that serve as predictors of coloration, facilitating targeted breeding programs that can avoid the traditional time-consuming phenotypic selection methods. Additionally, advances in genomic selection, which utilizes genomic information to estimate breeding values, are increasingly being adopted in poultry breeding. This method allows breeders to evaluate candidates based on genetic potential rather than relying solely on phenotypes. This not only enhances efficiency but also supports maintaining genetic diversity within breeding populations, an essential consideration in breeding programs.

Among poultry species, ducks present unique challenges and opportunities in the context of feather color molecular breeding. The genetic architecture of feather coloration, while complex, showed strong parallels with advancements observed in other avian species. Recent research has begun to find the roles of significant pigmentation genes, particularly *MITF*, *PMEL*, and various pigmentation-related signaling pathways. Findings demonstrating the distinct expression patterns of these genes during different developmental stages yield insights that are crucial for specific breeding strategies (Hua et al., 2021). Research efforts focusing on the development of molecular markers linked specifically to duck feather color phenotypes are underway, driven by the demand for distinct coloration in the market. However, there remains a critical necessity for exploration of QTL mapping and functional studies to establish a comprehensive understanding of how these genetic components interactively influence coloration.

Challenges persist in the form of maintaining genetic variation while achieving trait specificity, as excessive selection pressure can result in inbreeding depression. The future of molecular breeding in ducks will heavily rely on integrating innovative tools, such as CRISPR/Cas9 gene editing, to introduce or modify traits directly. Overall, the journey towards enhancing duck feather color via molecular breeding benefits significantly from continuous genetic exploration and technological advancements. Looking forward, the molecular breeding landscape will persistently evolve, offering exciting opportunities to meet the dynamic needs of the poultry market.

6.1.6 The contribution of our findings to avian biology

This PhD study elucidates the molecular and cellular mechanisms underlying melanin-based coloration in duck feathers, advancing our fundamental understanding of avian feather color regulation. Melanin is the most common pigment in birds, playing crucial roles in camouflage, thermoregulation, and social signaling. By characterizing melanocyte development, migration, and key gene expression patterns, we have clarified the regulatory pathways controlling melanin deposition, thereby deepening

insights into the processes driving feather pigmentation. The findings not only reveal the molecular basis of melanin synthesis but also provide explanations for the ecological and evolutionary diversity of plumage coloration across bird species. In particular, among ducks, melanin regulation reflects adaptive strategies to their environmental conditions and behavioral ecology. Moreover, the molecular regulatory networks identified here offer a valuable model for avian functional genomics, promoting more detailed studies on the genetics of plumage coloration.

In summary, investigations into the melanin formation mechanism in duck feathers expand the biological understanding of avian coloration, enhancing knowledge of avian evolution, ecology, and genetic diversity with significant scientific implications.

6.2 Conclusion

In this PhD thesis, firstly, we investigated the unique feather color phenotype of Liancheng ducks. The results showed that the melanin content of beak, and webbed feet in Liancheng ducks was significantly higher than that of Pekin ducks ($p<0.05$), but there was a very small amount of melanin deposition in the feathers of Liancheng ducks, resulting in the feather color was not pure white. Building upon, the high-quality Liancheng duck genome and GWAS analysis, this PhD study not only confirmed the epistatic effect of the *MITF* gene, but also pinpointed a 0.8Mb genomic region encompassing the *PMEL* gene. This gene encoded a protein specific to pigment cells that was essential for pigmentation in feathers of Liancheng ducks. Finally, we successfully found two molecular markers and used them for rapid breeding of unique feather color phenotypes of Liancheng ducks.

6.3 Perspectives

The identification and characterization of the *PMEL* gene associated with the unique plumage phenotype in Liancheng ducks represent a significant advancement in avian genetics and breeding. This research not only contributes to our understanding of pigment gene regulation in ducks but also offers a valuable genetic marker for selective breeding programs aimed at enhancing desirable traits. Future studies could focus on elucidating the precise mechanisms by which the *PMEL* gene influences plumage coloration and patterning, potentially leading to innovative strategies for controlling other phenotypic traits in poultry.

Furthermore, the molecular breeding of Liancheng Duck presents exciting opportunities to enhance key traits like growth, meat quality, feather coloration. By leveraging genomic technologies, future research can focus on identifying beneficial alleles and employing gene editing techniques, such as CRISPR/Cas9, to refine breeding programs. However, it is essential to consider the ethical implications of genetic modifications and ensure sustainable practices. Collaboration among geneticists, breeders will be crucial for integrating traditional and modern breeding methods, ultimately improving the economic value and preservation of this unique

breed. Ultimately, this research paves the way for not only improving Liancheng duck breeds but also for enhancing the overall genetic resource management of poultry species worldwide.

References

Apopo S, Liu H, Jing L, et al. Identification and profiling of microRNAs associated with white and black plumage pigmentation in the white and black feather bulbs of ducks by RNA sequencing. Anim Genet. 2015;46(6):627-635.

Arcila J, Peña-Villalobos I, Muñoz-Pacheco CB, et al. Urbanization's hidden influence: Linking landscape alterations and feather coloration with pigeon's cholesterol levels. Environ Res. Published online February 12, 2025.

Bovo S, Ribani A, Utzeri VJ, Taurisano V, Bertarini G, Fontanesi L. Whole genome sequencing identifies candidate genes and mutations that can explain diluted and other colour varieties of domestic canaries (Serinus canaria). Anim Genet. 2023;54(4):510-525.

Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, Fisher DE. MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. Am J Pathol. 2003;163(1):333-343.

Falletta P, Bagnato P, Bono M, et al. Melanosome-autonomous regulation of size and number: the OA1 receptor sustains PMEL expression. Pigment Cell Melanoma Res. 2014;27(4):565-579.

Fang D, Tsuji Y, Setaluri V. Selective down-regulation of tyrosinase family gene TYRP1 by inhibition of the activity of melanocyte transcription factor, MITF. Nucleic Acids Res. 2002;30(14):3096-3106.

Feng Y, Xie N, Inoue F, et al. Integrative functional genomic analyses identify genetic variants influencing skin pigmentation in Africans. Nat Genet. 2024;56(2):258-272.

Galvan I, Moraleda V, Otero I, Álvarez E, Inacio Â. Genetic favouring of pheomelanin-based pigmentation limits physiological benefits of coloniality in lesser kestrels Falco naumanni. Mol Ecol. 2017;26(20):5594-5602.

Heo S, Cho S, Dinh PTN, et al. A genome-wide association study for eumelanin pigmentation in chicken plumage using a computer vision approach. Anim Genet. 2023;54(3):355-362.

Hua G, Chen J, Wang J, Li J, Deng X. Genetic basis of chicken plumage color in artificial population of complex epistasis. Anim Genet. 2021;52(5):656-666.

Huang T, Pu Y, Song C, Sheng Z, Hu X. A quantitative trait locus on chromosome 2 was identified that accounts for a substantial proportion of phenotypic variance of the yellow plumage color in chicken. Poult Sci. 2020;99(6):2902-2910.

Huang T, Pu Y, Song C, Sheng Z, Hu X. A quantitative trait locus on chromosome 2 was identified that accounts for a substantial proportion of phenotypic variance of the yellow plumage color in chicken. Poult Sci. 2020;99(6):2902-2910. doi:10.1016/j.psj.2020.01.030

Huang Y, Li Y, Burt DW, et al. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. Nat Genet. 2013;45(7):776-783.

Joyjamras K, Netcharoensirisuk P, Roytrakul S, Chanvorachote P, Chaotham C. Recycled Sericin Hydrolysates Modified by Alcalase® Suppress Melanogenesis in

Human Melanin-Producing Cells via Modulating MITF. Int J Mol Sci. 2022;23(7):3925.

Leng D, Yang M, Miao X, et al. Dynamic changes in the skin transcriptome for the melanin pigmentation in embryonic chickens. Poult Sci. 2025;104(1):104210.

Li J, Zhang J, Liu J, et al. A new duck genome reveals conserved and convergently evolved chromosome architectures of birds and mammals. Gigascience. 2021;10(1):giaa142.

Lin R, Zhao F, Xiong T, et al. Genetic mapping identifies SNP mutations in MITF-M promoter associated with melanin formation in Putian black duck. Poult Sci. 2024;103(1):103191.

Liu D, Zhang H, Yang Y, et al. Metabolome-Based Genome-Wide Association Study of Duck Meat Leads to Novel Genetic and Biochemical Insights. Adv Sci. 2023;10(18):e2300148.

Liu S, Chen H, Ouyang J, Huang M, Zhang H, Zheng S, Xi S, Tang H, Gao Y, Xiong Y, Cheng D, Chen K, Liu B, Li W, Ren J, Yan X, Mao H. A high-quality assembly reveals genomic characteristics, phylogenetic status, and causal genes for leucism plumage of Indian peafowl. Gigascience. 2022;11:giac018.

Monmai C, Kim JS, Chin JH, Lee S, Baek SH. Inhibitory Effects of Polyphenol- and Flavonoid-Enriched Rice Seed Extract on Melanogenesis in Melan-a Cells via MAPK Signaling-Mediated MITF Downregulation. Int J Mol Sci. 2023;24(14):11841.

Nadeau NJ, Burke T, Mundy NI. Evolution of an avian pigmentation gene correlates with a measure of sexual selection. Proc Biol Sci. 2007;274(1620):1807-1813.

San-Jose LM, Ducrest AL, Ducret V, et al. MC1R variants affect the expression of melanocortin and melanogenic genes and the association between melanocortin genes and coloration. Mol Ecol. 2017;26(1):259-276.

Sun J, Zhu D, Xu J, et al. Rifampin resistance and its fitness cost in Riemerella anatipestifer. BMC Microbiol. 2019;19(1):107.

Sun Y, Wu Q, Lin R, et al. Genome-wide association study for the primary feather color trait in a native Chinese duck. Front Genet. 2023;14:1065033.

Wang H, Twumasi G, Xu Q, et al. Identification of candidate genes associated with primary feathers of tianfu nonghua ducks based on Genome-wide association studies. Poult Sci. 2024;103(9):103985.

Wang JM, Liu HH, Ma SC, et al. Progress on the formation mechanism of sexual dimorphism plumage color in birds. Yi Chuan. 2022;44(6):491-500.

Watt B, van Niel G, Raposo G, Marks MS. PMEL: a pigment cell-specific model for functional amyloid formation. Pigment Cell Melanoma Res. 2013;26(3):300-315.

Xi Y, Wu Q, Zeng Y, et al. Identification of the genetic basis of the duck growth rate in multiple growth stages using genome-wide association analysis. BMC Genomics. 2023;24(1):285.

Yang L, Mo C, Shen W, et al. The recessive C locus in the MITF gene plays a key regulatory role in the plumage colour pattern of duck (Anas platyrhynchos). Br Poult Sci. 2019;60(2):105-108.

Zhang X, Zhu T, Wang L, et al. Genome-Wide Association Study Reveals the Genetic Basis of Duck Plumage Colors. Genes (Basel). 2023;14(4):856. Published 2023 Mar 31.

Zheng X, Zhang B, Zhang Y, et al. Transcriptome analysis of feather follicles reveals candidate genes and pathways associated with pheomelanin pigmentation in chickens. Sci Rep. 2020;10(1):12088.

Zhou Z, Li M, Cheng H, et al. An intercross population study reveals genes associated with body size and plumage color in ducks [published correction appears in Nat Commun. 2018 Sep 25;9(1):3974. doi: 10.1038/s41467-018-06521-6.]. Nat Commun. 2018;9(1):2648.

Zhu F, Yin ZT, Wang Z, et al. Three chromosome-level duck genome assemblies provide insights into genomic variation during domestication. Nat Commun. 2021;12(1):5932

Appendix A-Publications.

1. **Wang, Z**., Guo, Z., Mou, Q., Liu, H., Liu, D., Tang, H., Hou, S., Schroyen, M., & Zhou, Z. (2024). Unique feather color characteristics and transcriptome analysis of hair follicles in Liancheng White ducks. Poultry science, 103(7), 103794.
2. **Wang, Z**., Guo, Z., Liu, H., Liu, T., Liu, D., Yu, S., Tang, H., Zhang, H., Mou, Q., Zhang, B., Cao, J., Schroyen, M., Hou, S., & Zhou, Z. (2025). A high-quality assembly revealing the PMEL gene for the unique plumage phenotype in Liancheng ducks. GigaScience, 14, giae114.
3. Tang, H., Zhang, H., Liu, D., **Wang, Z**., Yu, D., Fan, W., Guo, Z., Huang, W., Hou, S., & Zhou, Z. (2022). Genome-wide association study reveals the genetic determinism of serum biochemical indicators in ducks. BMC genomics, 23(1), 856.
4. Liu, D., Zhang, H., Yang, Y., Liu, T., Guo, Z., Fan, W., **Wang, Z**., Yang, X., Zhang, B., Liu, H., Tang, H., Yu, D., Yu, S., Gai, K., Mou, Q., Cao, J., Hu, J., Tang, J., Hou, S., & Zhou, Z. (2023). Metabolome-Based Genome-Wide Association Study of Duck Meat Leads to Novel Genetic and Biochemical Insights. Advanced science,10(18), e2300148.
5. Tang, H., Zhang, H., Liu, D., Li, S., **Wang, Z**., Yu, D., Guo, Z. B., Hou, S., & Zhou, Z. (2023). Changes in physical architecture and lipids compounds in skeletal muscle from Pekin duck and Liancheng white duck. Poultry science, 102(12), 103106.
6. Molecular markers related to phenotypic traits of white feathered and black beak ducks and their breeding application in high quality ducks. Zhou Z, Hou S, Guo Z, **Wang Z**. No:202211228357.6 (Chinese Patent, Under review).
7. A cross-breeding method for effective protection of local high quality duck breeds. Zhou Z, Guo Z, Hou S, **Wang Z**. No:202310542366.0 (Chinese Patent, Under review).

Appendix B- Duck genome resequencing in Chapter IV.

Supplement Table 1. List of 389 duck genome resequencing used in the study

Library_ID	Sample_name	Biosample or Accession	Sex	Breed	Age	Tissue
1	2005-01218	SAMN31217796	female	Pekin duck	6W	Blood
2	2005-01523	SAMN31217797	male	Pekin duck	6W	Blood
3	2005-03124	SAMN31217798	male	Pekin duck	6W	Blood
4	2005-03223	SAMN31217799	male	Pekin duck	6W	Blood
5	2005-09326	SAMN31217800	male	Pekin duck	6W	Blood
6	2005-09522	SAMN31217801	male	Pekin duck	6W	Blood
7	2005-10425	SAMN31217802	female	Pekin duck	6W	Blood
8	2005-16319	SAMN31217803	female	Pekin duck	6W	Blood
9	2005-17104	SAMN31217804	male	Pekin duck	6W	Blood
10	2005-21327	SAMN31217805	female	Pekin duck	6W	Blood
11	2005-21513	SAMN31217806	male	Pekin duck	6W	Blood
12	2005-26312	SAMN31217807	male	Pekin duck	6W	Blood
13	2005-27224	SAMN31217808	female	Pekin duck	6W	Blood
14	2005-28235	SAMN31217809	female	Pekin duck	6W	Blood
15	2005-30422	SAMN31217810	male	Pekin duck	6W	Blood
16	2005-33304	SAMN31217811	male	Pekin duck	6W	Blood
17	2005-35510	SAMN31217812	female	Pekin duck	6W	Blood
18	2005-38308	SAMN31217813	male	Pekin duck	6W	Blood
19	2005-40302	SAMN31217814	female	Pekin duck	6W	Blood
20	2005-40401	SAMN31217815	male	Pekin duck	6W	Blood
21	2005-40403	SAMN31217816	female	Pekin duck	6W	Blood
22	2005-42105	SAMN31217817	male	Pekin duck	6W	Blood
23	2005-43211	SAMN31217818	female	Pekin duck	6W	Blood
24	2005-47217	SAMN31217819	female	Pekin duck	6W	Blood
25	2005-47308	SAMN31217820	female	Pekin duck	6W	Blood
26	2005-47310	SAMN31217821	female	Pekin duck	6W	Blood
27	2005-48409	SAMN31217822	male	Pekin duck	6W	Blood
28	2005-48503	SAMN31217823	female	Pekin duck	6W	Blood
29	2008-06417	SAMN31217824	male	Pekin duck	6W	Blood
30	2008-10531	SAMN31217825	male	Pekin duck	6W	Blood
31	2008-12454	SAMN31217826	male	Pekin duck	6W	Blood
32	2008-182	SAMN31217827	female	Pekin duck	6W	Blood
33	2008-183	SAMN31217828	female	Pekin duck	6W	Blood
34	2008-184	SAMN31217829	female	Pekin duck	6W	Blood
35	2008-185	SAMN31217830	female	Pekin duck	6W	Blood

36	2008-186	SAMN31217831	female	Pekin duck	6W	Blood
37	2008-187	SAMN31217832	female	Pekin duck	6W	Blood
38	2008-188	SAMN31217833	female	Pekin duck	6W	Blood
39	2008-189	SAMN31217834	female	Pekin duck	6W	Blood
40	2008-190	SAMN31217835	female	Pekin duck	6W	Blood
41	2008-191	SAMN31217836	female	Pekin duck	6W	Blood
42	2008-192	SAMN31217837	female	Pekin duck	6W	Blood
43	2008-194	SAMN31217838	female	Pekin duck	6W	Blood
44	2008-30432	SAMN31217839	female	Pekin duck	6W	Blood
45	2008-34321	SAMN31217840	male	Pekin duck	6W	Blood
46	2008-40321	SAMN31217841	male	Pekin duck	6W	Blood
47	2008-47240	SAMN31217842	male	Pekin duck	6W	Blood
48	2008-48431	SAMN31217843	female	Pekin duck	6W	Blood
49	2008-49577	SAMN31217844	female	Pekin duck	6W	Blood
50	2008-53541	SAMN31217845	male	Pekin duck	6W	Blood
51	2008-5439	SAMN31217846	male	Pekin duck	6W	Blood
52	2008-5541	SAMN31217847	male	Pekin duck	6W	Blood
53	2008-947	SAMN31217848	male	Pekin duck	6W	Blood
54	2008-949	SAMN31217849	male	Pekin duck	6W	Blood
55	2008-950	SAMN31217850	male	Pekin duck	6W	Blood
56	2008-952	SAMN31217851	male	Pekin duck	6W	Blood
57	2008-953	SAMN31217852	male	Pekin duck	6W	Blood
58	2011-082104	SAMN31217853	male	Pekin duck	6W	Blood
59	2011-1103	SAMN31217854	female	Pekin duck	6W	Blood
60	2011-1907	SAMN31217855	female	Pekin duck	6W	Blood
61	2011-1908	SAMN31217856	female	Pekin duck	6W	Blood
62	2011-1909	SAMN31217857	female	Pekin duck	6W	Blood
63	2011-1910	SAMN31217858	female	Pekin duck	6W	Blood
64	2011-1911	SAMN31217859	female	Pekin duck	6W	Blood
65	2011-1912	SAMN31217860	female	Pekin duck	6W	Blood
66	2011-1913	SAMN31217861	male	Pekin duck	6W	Blood
67	2011-1916	SAMN31217862	female	Pekin duck	6W	Blood
68	2011-221103	SAMN31217863	male	Pekin duck	6W	Blood
69	2011-221107	SAMN31217864	male	Pekin duck	6W	Blood
70	2011-272105	SAMN31217865	female	Pekin duck	6W	Blood
71	2011-342103	SAMN31217866	female	Pekin duck	6W	Blood
72	2011-392102	SAMN31217867	male	Pekin duck	6W	Blood
73	2011-392105	SAMN31217868	male	Pekin duck	6W	Blood
74	2011-392109	SAMN31217869	male	Pekin duck	6W	Blood
75	2011-431107	SAMN31217870	male	Pekin duck	6W	Blood
76	2011-432106	SAMN31217871	male	Pekin duck	6W	Blood
77	2011-452101	SAMN31217872	female	Pekin duck	6W	Blood
78	2011-461102	SAMN31217873	male	Pekin duck	6W	Blood
79	2011-461104	SAMN31217874	male	Pekin duck	6W	Blood

80	2011-491110	SAMN31217875	female	Pekin duck	6W	Blood
81	2011-591104	SAMN31217876	male	Pekin duck	6W	Blood
82	2011-651101	SAMN31217877	female	Pekin duck	6W	Blood
83	2011-681107	SAMN31217878	male	Pekin duck	6W	Blood
84	2011-772161	SAMN31217879	female	Pekin duck	6W	Blood
85	2011-852105	SAMN31217880	female	Pekin duck	6W	Blood
86	2011-871108	SAMN31217881	male	Pekin duck	6W	Blood
87	2011-881103	SAMN31217882	male	Pekin duck	6W	Blood
88	2014-0003	SAMN31217883	male	Pekin duck	6W	Blood
89	2014-3347	SAMN31217884	male	Pekin duck	6W	Blood
90	2014-3408	SAMN31217885	male	Pekin duck	6W	Blood
91	2014-3462	SAMN31217886	female	Pekin duck	6W	Blood
92	2014-3518	SAMN31217887	male	Pekin duck	6W	Blood
93	2014-3529	SAMN31217888	male	Pekin duck	6W	Blood
94	2014-3536	SAMN31217889	female	Pekin duck	6W	Blood
95	2014-3540	SAMN31217890	female	Pekin duck	6W	Blood
96	2014-3554	SAMN31217891	male	Pekin duck	6W	Blood
97	2014-3623	SAMN31217892	female	Pekin duck	6W	Blood
98	2014-3648	SAMN31217893	female	Pekin duck	6W	Blood
99	2014-3702	SAMN31217894	female	Pekin duck	6W	Blood
100	2014-3719	SAMN31217895	female	Pekin duck	6W	Blood
101	2014-3724	SAMN31217896	male	Pekin duck	6W	Blood
102	2014-3743	SAMN31217897	female	Pekin duck	6W	Blood
103	2014-3748	SAMN31217898	female	Pekin duck	6W	Blood
104	2014-3775	SAMN31217899	female	Pekin duck	6W	Blood
105	2014-3867	SAMN31217900	female	Pekin duck	6W	Blood
106	2014-3916	SAMN31217901	male	Pekin duck	6W	Blood
107	2014-3973	SAMN31217902	female	Pekin duck	6W	Blood
108	2014-3994	SAMN31217903	female	Pekin duck	6W	Blood
109	2014-4029	SAMN31217904	male	Pekin duck	6W	Blood
110	2014-4035	SAMN31217905	male	Pekin duck	6W	Blood
111	2014-4241	SAMN31217906	male	Pekin duck	6W	Blood
112	2014-4273	SAMN31217907	male	Pekin duck	6W	Blood
113	2014-4352	SAMN31217908	male	Pekin duck	6W	Blood
114	2014-4374	SAMN31217909	female	Pekin duck	6W	Blood
115	2014-4410	SAMN31217910	male	Pekin duck	6W	Blood
116	2014-4461	SAMN31217911	male	Pekin duck	6W	Blood
117	2014-4523	SAMN31217912	female	Pekin duck	6W	Blood
118	R388	SRR19799083	female	Liancheng duck	6W	Blood
119	R389	SRR19799081	female	Liancheng duck	6W	Blood
120	R391	SRR19799080	male	Liancheng duck	6W	Blood
121	R401	SRR19799079	male	Liancheng duck	6W	Blood
122	R404	SRR19799078	female	Liancheng duck	6W	Blood
123	R407	SRR19799077	female	Liancheng duck	6W	Blood

124	R408	SRR19799076	female	Liancheng duck	6W	Blood
125	R410	SRR19799075	female	Liancheng duck	6W	Blood
126	R419	SRR19799074	male	Liancheng duck	6W	Blood
127	R425	SRR19799072	male	Liancheng duck	6W	Blood
128	R427	SRR19799070	male	Liancheng duck	6W	Blood
129	R431	SRR19799069	male	Liancheng duck	6W	Blood
130	R433	SRR19799068	male	Liancheng duck	6W	Blood
131	R435	SRR19799067	male	Liancheng duck	6W	Blood
132	R436	SRR19799066	female	Liancheng duck	6W	Blood
133	R437	SRR19799065	female	Liancheng duck	6W	Blood
134	R444	SRR19799064	male	Liancheng duck	6W	Blood
135	R445	SRR19799063	female	Liancheng duck	6W	Blood
136	R448	SRR19799062	female	Liancheng duck	6W	Blood
137	R449	SRR19799061	male	Liancheng duck	6W	Blood
138	R450	SRR19799059	male	Liancheng duck	6W	Blood
139	R454	SRR19799058	female	Liancheng duck	6W	Blood
140	R458	SRR19799057	female	Liancheng duck	6W	Blood
141	R459	SRR19799056	male	Liancheng duck	6W	Blood
142	R460	SRR19799055	male	Liancheng duck	6W	Blood
143	R461	SRR19799054	male	Liancheng duck	6W	Blood
144	R462	SRR19799260	female	Liancheng duck	6W	Blood
145	R463	SRR19799259	female	Liancheng duck	6W	Blood
146	R466	SRR19799258	male	Liancheng duck	6W	Blood
147	R470	SRR28988868	female	Liancheng duck	6W	Blood
148	R474	SRR28988867	female	Liancheng duck	6W	Blood
149	R475	SRR28988866	female	Liancheng duck	6W	Blood
150	R481	SRR28988865	female	Liancheng duck	6W	Blood
151	R482	SRR28988864	male	Liancheng duck	6W	Blood
152	R483	SRR28988863	male	Liancheng duck	6W	Blood
153	R486	SRR28988862	female	Liancheng duck	6W	Blood
154	R492	SRR28988861	female	Liancheng duck	6W	Blood
155	R493	SRR28988860	female	Liancheng duck	6W	Blood
156	R496	SRR28988859	male	Liancheng duck	6W	Blood
157	R497	SRR28988858	female	Liancheng duck	6W	Blood
158	R498	SRR28988857	female	Liancheng duck	6W	Blood
159	R501	SRR28988856	male	Liancheng duck	6W	Blood
160	R503	SRR28988855	male	Liancheng duck	6W	Blood
161	R504	SRR28988854	male	Liancheng duck	6W	Blood
162	R505	SRR28988853	male	Liancheng duck	6W	Blood
163	R512	SRR28988852	female	Liancheng duck	6W	Blood
164	R513	SRR28988851	male	Liancheng duck	6W	Blood
165	R515	SRR28988850	male	Liancheng duck	6W	Blood
166	R516	SRR28988849	male	Liancheng duck	6W	Blood
167	R518	SRR28988848	male	Liancheng duck	6W	Blood

168	R522	SRR28988847	female	Liancheng duck	6W	Blood
169	R523	SRR28988846	male	Liancheng duck	6W	Blood
170	R524	SRR28988845	male	Liancheng duck	6W	Blood
171	R526	SRR28988844	female	Liancheng duck	6W	Blood
172	R528	SRR28988843	female	Liancheng duck	6W	Blood
173	R535	SRR28988842	female	Liancheng duck	6W	Blood
174	R539	SRR28988841	male	Liancheng duck	6W	Blood
175	R541	SRR28988840	male	Liancheng duck	6W	Blood
176	R542	SRR28988839	male	Liancheng duck	6W	Blood
177	R1000	SRR19799120	female	WB duck	6W	Blood
178	R1001	SRR19799119	female	WB duck	6W	Blood
179	R1002	SRR19799118	male	WB duck	6W	Blood
180	R1004	SRR19799053	male	WB duck	6W	Blood
181	R1006	SRR19799052	male	WB duck	6W	Blood
182	R1007	SRR19799051	female	WB duck	6W	Blood
183	R1008	SRR19799050	female	WB duck	6W	Blood
184	R1009	SRR19799049	male	WB duck	6W	Blood
185	R1010	SRR19799048	male	WB duck	6W	Blood
186	R1012	SRR19799047	male	WB duck	6W	Blood
187	R1016	SRR19799045	female	WB duck	6W	Blood
188	R1017	SRR19799044	female	WB duck	6W	Blood
189	R1018	SRR19799042	male	WB duck	6W	Blood
190	R1020	SRR19799041	female	WB duck	6W	Blood
191	R1106	SRR19799199	female	WB duck	6W	Blood
192	R1110	SRR19799197	female	WB duck	6W	Blood
193	R1111	SRR19799196	female	WB duck	6W	Blood
194	R1112	SRR19799195	female	WB duck	6W	Blood
195	R1113	SRR19799193	male	WB duck	6W	Blood
196	R1115	SRR19799192	male	WB duck	6W	Blood
197	R1116	SRR19799191	female	WB duck	6W	Blood
198	R1120	SRR19799189	male	WB duck	6W	Blood
199	R1127	SRR19799015	female	WB duck	6W	Blood
200	R1130	SRR19799014	male	WB duck	6W	Blood
201	R1290	SRR19799288	female	WB duck	6W	Blood
202	R1292	SRR19799287	male	WB duck	6W	Blood
203	R1300	SRR19799280	female	WB duck	6W	Blood
204	R1301	SRR19799279	female	WB duck	6W	Blood
205	R1305	SRR19799277	male	WB duck	6W	Blood
206	R1306	SRR19799276	female	WB duck	6W	Blood
207	R1311	SRR19799273	male	WB duck	6W	Blood
208	R1313	SRR19799271	male	WB duck	6W	Blood
209	R1317	SRR19799268	female	WB duck	6W	Blood
210	R1319	SRR19799267	female	WB duck	6W	Blood
211	R1320	SRR19799266	female	WB duck	6W	Blood

212	R1323	SRR19799264	female	WB duck	6W	Blood
213	R1324	SRR19799262	male	WB duck	6W	Blood
214	R1325	SRR19799261	female	WB duck	6W	Blood
215	R1327	SRR19798945	male	WB duck	6W	Blood
216	R997	SRR19799123	male	WB duck	6W	Blood
217	R998	SRR19799122	female	WB duck	6W	Blood
218	R999	SRR19799121	female	WB duck	6W	Blood
219	R1074	SRR19799024	male	GF duck	6W	Blood
220	R1075	SRR19799023	male	GF duck	6W	Blood
221	R1076	SRR19799022	female	GF duck	6W	Blood
222	R1077	SRR19799020	female	GF duck	6W	Blood
223	R1079	SRR19799018	female	GF duck	6W	Blood
224	R1081	SRR19799224	female	GF duck	6W	Blood
225	R1082	SRR19799223	male	GF duck	6W	Blood
226	R1083	SRR19799222	female	GF duck	6W	Blood
227	R1084	SRR19799221	female	GF duck	6W	Blood
228	R1085	SRR19799220	female	GF duck	6W	Blood
229	R1086	SRR19799219	female	GF duck	6W	Blood
230	R1087	SRR19799218	male	GF duck	6W	Blood
231	R1088	SRR19799215	male	GF duck	6W	Blood
232	R1089	SRR19799214	male	GF duck	6W	Blood
233	R1091	SRR19799212	male	GF duck	6W	Blood
234	R1092	SRR19799211	female	GF duck	6W	Blood
235	R1093	SRR19799210	male	GF duck	6W	Blood
236	R1094	SRR19799209	male	GF duck	6W	Blood
237	R1095	SRR19799208	male	GF duck	6W	Blood
238	R1096	SRR19799207	male	GF duck	6W	Blood
239	R1098	SRR19799206	male	GF duck	6W	Blood
240	R1099	SRR19799204	female	GF duck	6W	Blood
241	R1101	SRR19799203	female	GF duck	6W	Blood
242	R1102	SRR19799202	male	GF duck	6W	Blood
243	R1103	SRR19799201	male	GF duck	6W	Blood
244	R1162	SRR19798993	male	GF duck	6W	Blood
245	R1227	SRR19798991	male	GF duck	6W	Blood
246	R1228	SRR19798989	female	GF duck	6W	Blood
247	R1231	SRR19798988	female	GF duck	6W	Blood
248	R1232	SRR19798987	female	GF duck	6W	Blood
249	R1234	SRR19798986	male	GF duck	6W	Blood
250	R1236	SRR19798985	female	GF duck	6W	Blood
251	R1240	SRR19798983	female	GF duck	6W	Blood
252	R1241	SRR19798982	male	GF duck	6W	Blood
253	R1242	SRR19798981	male	GF duck	6W	Blood
254	R1243	SRR19798980	female	GF duck	6W	Blood
255	R1244	SRR19798978	male	GF duck	6W	Blood

256	R1246	SRR19798977	male	GF duck	6W	Blood
257	R1247	SRR19798976	female	GF duck	6W	Blood
258	R1250	SRR19798974	male	GF duck	6W	Blood
259	R1282	SRR19799295	male	GF duck	6W	Blood
260	R1283	SRR19799294	male	GF duck	6W	Blood
261	R1284	SRR19799293	female	GF duck	6W	Blood
262	R1286	SRR19799292	male	GF duck	6W	Blood
263	R1287	SRR19799291	female	GF duck	6W	Blood
264	R947	SRR19799232	male	GF duck	6W	Blood
265	R950	SRR19799230	female	GF duck	6W	Blood
266	R952	SRR19799228	male	GF duck	6W	Blood
267	R953	SRR19799227	female	GF duck	6W	Blood
268	R954	SRR19799226	female	GF duck	6W	Blood
269	R965	SRR19799146	female	GF duck	6W	Blood
270	R969	SRR19799142	male	GF duck	6W	Blood
271	R971	SRR19799141	male	GF duck	6W	Blood
272	R973	SRR19799140	female	GF duck	6W	Blood
273	R975	SRR19799137	female	GF duck	6W	Blood
274	R977	SRR19799136	male	GF duck	6W	Blood
275	R979	SRR19799135	female	GF duck	6W	Blood
276	R981	SRR19799134	male	GF duck	6W	Blood
277	R982	SRR19799133	female	GF duck	6W	Blood
278	R983	SRR19799132	female	GF duck	6W	Blood
279	R984	SRR19799131	male	GF duck	6W	Blood
280	R985	SRR19799130	male	GF duck	6W	Blood
281	R986	SRR19799129	female	GF duck	6W	Blood
282	R988	SRR19799127	female	GF duck	6W	Blood
283	R989	SRR19799126	female	GF duck	6W	Blood
284	R990	SRR19799125	male	GF duck	6W	Blood
285	R991	SRR19799124	female	GF duck	6W	Blood
286	R1056	SRR19799039	male	BF duck	6W	Blood
287	R1057	SRR19799038	female	BF duck	6W	Blood
288	R1058	SRR19799037	male	BF duck	6W	Blood
289	R1059	SRR19799036	male	BF duck	6W	Blood
290	R1061	SRR19799035	male	BF duck	6W	Blood
291	R1063	SRR19799033	male	BF duck	6W	Blood
292	R1065	SRR19799031	male	BF duck	6W	Blood
293	R1066	SRR19799030	female	BF duck	6W	Blood
294	R1067	SRR19799029	female	BF duck	6W	Blood
295	R1068	SRR19799028	male	BF duck	6W	Blood
296	R1069	SRR19799027	male	BF duck	6W	Blood
297	R1070	SRR19799026	female	BF duck	6W	Blood
298	R1073	SRR19799025	male	BF duck	6W	Blood
299	R1160	SRR19798994	female	BF duck	6W	Blood

300	R914	SRR19799257	male	BF duck	6W	Blood
301	R915	SRR19799254	female	BF duck	6W	Blood
302	R917	SRR19799253	male	BF duck	6W	Blood
303	R921	SRR19799252	female	BF duck	6W	Blood
304	R922	SRR19799251	male	BF duck	6W	Blood
305	R924	SRR19799250	female	BF duck	6W	Blood
306	R926	SRR19799249	female	BF duck	6W	Blood
307	R928	SRR19799248	male	BF duck	6W	Blood
308	R931	SRR19799247	female	BF duck	6W	Blood
309	R932	SRR19799246	male	BF duck	6W	Blood
310	R933	SRR19799245	female	BF duck	6W	Blood
311	R934	SRR19799243	male	BF duck	6W	Blood
312	R936	SRR19799242	female	BF duck	6W	Blood
313	R938	SRR19799241	male	BF duck	6W	Blood
314	R939	SRR19799240	female	BF duck	6W	Blood
315	R940	SRR19799239	female	BF duck	6W	Blood
316	R941	SRR19799238	male	BF duck	6W	Blood
317	R942	SRR19799237	female	BF duck	6W	Blood
318	R944	SRR19799236	female	BF duck	6W	Blood
319	R945	SRR19799235	female	BF duck	6W	Blood
320	R946	SRR19799234	female	BF duck	6W	Blood
321	R948	SRR19799231	male	BF duck	6W	Blood
322	R951	SRR19799229	male	BF duck	6W	Blood
323	R958	SRR19799225	female	BF duck	6W	Blood
324	R961	SRR19799151	male	BF duck	6W	Blood
325	R963	SRR19799148	female	BF duck	6W	Blood
326	R964	SRR19799147	male	BF duck	6W	Blood
327	R966	SRR19799145	female	BF duck	6W	Blood
328	R968	SRR19799143	male	BF duck	6W	Blood
329	R1026	SRR19799040	female	WY duck	6W	Blood
330	R1131	SRR19799013	female	WY duck	6W	Blood
331	R1133	SRR19799011	female	WY duck	6W	Blood
332	R1134	SRR19799010	male	WY duck	6W	Blood
333	R1137	SRR19799008	female	WY duck	6W	Blood
334	R1138	SRR19799007	male	WY duck	6W	Blood
335	R1139	SRR19799006	female	WY duck	6W	Blood
336	R1143	SRR19799004	male	WY duck	6W	Blood
337	R1144	SRR19799003	female	WY duck	6W	Blood
338	R1150	SRR19799000	female	WY duck	6W	Blood
339	R1152	SRR19798999	male	WY duck	6W	Blood
340	R1154	SRR19798998	female	WY duck	6W	Blood
341	R1155	SRR19798997	male	WY duck	6W	Blood
342	R1158	SRR19798996	female	WY duck	6W	Blood
343	R1159	SRR19798995	male	WY duck	6W	Blood

344	R1253	SRR19798972	female	WY duck	6W	Blood
345	R1254	SRR19798971	female	WY duck	6W	Blood
346	R1255	SRR19798970	male	WY duck	6W	Blood
347	R1258	SRR19798969	female	WY duck	6W	Blood
348	R1259	SRR19798967	male	WY duck	6W	Blood
349	R1260	SRR19798966	male	WY duck	6W	Blood
350	R1263	SRR19798964	female	WY duck	6W	Blood
351	R1264	SRR19798963	male	WY duck	6W	Blood
352	R1265	SRR19798962	male	WY duck	6W	Blood
353	R1266	SRR19798961	female	WY duck	6W	Blood
354	R1268	SRR19798960	female	WY duck	6W	Blood
355	R1269	SRR19798959	male	WY duck	6W	Blood
356	R1270	SRR19798958	female	WY duck	6W	Blood
357	R1271	SRR19798956	female	WY duck	6W	Blood
358	R1272	SRR19798955	male	WY duck	6W	Blood
359	R1273	SRR19798954	male	WY duck	6W	Blood
360	R1274	SRR19798953	female	WY duck	6W	Blood
361	R1275	SRR19798952	female	WY duck	6W	Blood
362	R1276	SRR19798951	female	WY duck	6W	Blood
363	R1277	SRR19798950	female	WY duck	6W	Blood
364	R1278	SRR19798949	male	WY duck	6W	Blood
365	R1279	SRR19798948	male	WY duck	6W	Blood
366	R1280	SRR19798947	female	WY duck	6W	Blood
367	26	SRR7091465	female	Mallard	6W	Blood
368	28	SRR7091426	female	Mallard	6W	Blood
369	29	SRR7091425	female	Mallard	6W	Blood
370	32	SRR7091424	female	Mallard	6W	Blood
371	33	SRR7091423	female	Mallard	6W	Blood
372	34	SRR7091430	female	Mallard	6W	Blood
373	45	SRR7091429	female	Mallard	6W	Blood
374	46	SRR7091428	female	Mallard	6W	Blood
375	49	SRR7091427	female	Mallard	6W	Blood
376	6	SRR7091458	female	Mallard	6W	Blood
377	7	SRR7091457	female	Mallard	6W	Blood
378	70	SRR7091474	female	Mallard	6W	Blood
379	76	SRR7091472	female	Mallard	6W	Blood
380	78	SRR7091475	female	Mallard	6W	Blood
381	80	SRR7091476	female	Mallard	6W	Blood
382	82	SRR7091438	female	Mallard	6W	Blood
383	83	SRR7091437	female	Mallard	6W	Blood
384	84	SRR7091440	female	Mallard	6W	Blood
385	90	SRR7091439	female	Mallard	6W	Blood
386	96	SRR7091441	female	Mallard	6W	Blood
387	PT1	SRR7091456	male	Putian duck	6W	Blood

388	PT2	SRR7091455	female	Putian duck	6W	Blood
389	PT3	SRR7091515	female	Putian duck	6W	Blood

Supplement Table 2. List of 37 ducks for RNA-seq used in the study.

Library_ID	Accession	Sample_name	Sex	Breed	Age	Tissue
1	SRR28981185	LC_E12_S1	male	Liancheng duck	E12d	Skin
2	SRR28981184	LC_E12_S2	female	Liancheng duck	E12d	Skin
3	SRR28981183	LC_E12_S3	male	Liancheng duck	E12d	Skin
4	SRR28981182	LC_E12_S4	female	Liancheng duck	E12d	Skin
5	SRR28981181	LC_E15_S1	male	Liancheng duck	E15d	Skin
6	SRR28981180	LC_E15_S2	female	Liancheng duck	E15d	Skin
7	SRR28981179	LC_E15_S3	male	Liancheng duck	E15d	Skin
8	SRR28981178	LC_E20_S1	female	Liancheng duck	E20d	Skin
9	SRR28981177	LC_E20_S2	male	Liancheng duck	E20d	Skin
10	SRR28981176	LC_E20_S3	female	Liancheng duck	E20d	Skin
11	SRR28981175	LC_E28_S1	male	Liancheng duck	E28d	Skin
12	SRR28981174	LC_E28_S2	female	Liancheng duck	E28d	Skin
13	SRR28981173	LC_E28_S3	male	Liancheng duck	E28d	Skin
14	SRR28981172	LC_1W_S1	female	Liancheng duck	1W	Skin
15	SRR28981171	LC_1W_S2	male	Liancheng duck	1W	Skin
16	SRR28981170	LC_1W_S3	female	Liancheng duck	1W	Skin
17	SRR28981169	LC_1W_F1	male	Liancheng duck	1W	Feather follicle
18	SRR28981168	LC_1W_F2	female	Liancheng duck	1W	Feather follicle
19	SRR28981163	LC_1W_F3	male	Liancheng duck	1W	Feather follicle
20	SRR28981164	PK_1W_F1	female	Pekin duck	1W	Feather follicle
21	SRR28981165	PK_1W_F2	male	Pekin duck	1W	Feather follicle
22	SRR28981166	PK_1W_F3	female	Pekin duck	1W	Feather follicle
23	SRR28981167	White_1W_F1	male	WB duck	1W	Feather follicle
24	SRR28981158	White_1W_F2	female	WB duck	1W	Feather follicle
25	SRR28981159	White_1W_F3	male	WB duck	1W	Feather follicle
26	SRR28981160	Wild_1W_F1	female	Mallard	1W	Feather follicle
27	SRR28981161	Wild_1W_F2	male	Mallard	1W	Feather follicle
28	SRR28981162	Wild_1W_F3	female	Mallard	1W	Feather follicle
29	SRR29069675	Heart	female	Mallard	8W	Heart
30	SRR1295549	Fat	female	Mallard	8W	Fat
31	SRR29069671	Muscle	female	Mallard	8W	Muscle
32	SRR29069677	Brain	female	Mallard	8W	Brain
33	SRR29069669	Spleen	female	Mallard	8W	Spleen
34	SRR29069672	Lung	female	Mallard	8W	Lung
35	SRR29069673	Liver	female	Mallard	8W	Liver
36	SRR29069674	Kidney	female	Mallard	8W	Kidney
37	SRR29069670	Skin	female	Mallard	8W	Skin