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Genetic Association and Expression of Myoglobin Gene Related to Mineral Content in IPB-D2 Chickens

P. Liong[1], A. K. Alfiah[1], I. Khaerunnisa[2], S. Darwati[3], R. Afnan[3], A. B. Dharmayanthi[4], M. F. Al-Habib[5], & C. Sumantri[3*]

[1]Graduate Program in Animal Production and Technology, Faculty of Animal Science, IPB University, Bogor, West Java 16680, Indonesia

[2]Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Bogor, West Java 16911, Indonesia

[3]Department of Animal Production and Technology, Faculty of Animal Science, Bogor Agricultural University, Bogor, West Java 16680, Indonesia

[4]Research Center for Biosystematics and Evolution, National Research and Innovation Agency (BRIN), Bogor, West Java 16911, Indonesia

[5]PT Nutfah Unggul Intimakmur, Boyolali, Central Java 57384, Indonesia

*Corresponding author: ceces@apps.ipb.ac.id

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ABSTRACT

Myoglobin *(MB)* gene encodes the protein of myoglobin, which is a protein found in muscle tissues that plays a crucial role in binding and storing oxygen. This study aimed to analyze the polymorphism of the *MB* gene, examine its relationship with the meat mineral content, and analyze its expression in the liver and thigh muscle tissue of IPB-D2 chicken. A total of 55 IPB-D2 chickens were used in this study. Identification of the gene polymorphism, quantification of the mineral content, and gene expression were performed using the direct sequencing method, the Atomic Absorption Spectroscopy (AAS) method, and the qRT-PCR, respectively. Chicken thigh muscle and liver tissues were used as the source of mRNA in this study. The statistical analysis methods used were the chi-square test to test Hardy-Weinberg equilibrium, and the T-test for mineral association analysis and gene expression analysis. IPB-D2 chicken on average contains 17.81, 0.22, 16.50, and 0.34 mg/kg of Fe, Se, Zn, and Mn, respectively. Two SNPs were found in 5'UTR of the *MB* gene, namely SNP g.17 G>T and SNP g.25 T>C. Genotype TT (g.17 G>T) and genotype CC (g.25 T>C) were significantly (P<0.05) associated with high Fe content in IPB-D2 chicken. The gene expression analysis showed that *MB* mRNA expression in the liver was not statistically different between high Fe (TT/CC) and low Fe (GG/TT and GT/TC) genotype combination. Meanwhile, in the thigh muscle, *MB* mRNA expression in TT/CC genotype combination was significantly higher than GG/TT and GT/TC genotype combination (P<0.05). These results suggest that the identified polymorphisms in the IPB-D2 *MB* gene could serve as references for investigating similar gene in other chicken breeds, especially regarding Fe, Se, Zn, and Mn mineral content.

Keywords: IPB-D2 chicken, *MB* expression, mineral content, myoglobin, polymorphism

ABSTRAK

Gen mioglobin (*MB*) mengkode protein mioglobin, protein yang ditemukan dalam jaringan otot, berfungsi untuk mengikat dan menyimpan oksigen. Penelitian ini bertujuan untuk menganalisis polimorfisme gen *MB*, mengkaji asosiasinya dengan kandungan mineral daging, dan menganalisis ekspresinya pada jaringan otot hati dan paha ayam IPB-D2. Sebanyak 55 ekor ayam IPB-D2 digunakan dalam penelitian ini. Identifikasi polimorfisme gen, kuantifikasi kandungan mineral, dan ekspresi gen dilakukan masing-masing menggunakan metode *direct sequencing*, metode *Atomic Absorption Spectroscopy* (AAS), dan *qRT-PCR*. Jaringan otot paha dan hati digunakan sebagai sumber mRNA dalam penelitian ini. Metode analisis statistik yang digunakan adalah uji *chi-square* untuk menguji kesetimbangan Hardy-Weinberg, dan uji-*T* untuk analisis asosiasi mineral dan analisis ekspresi gen. Secara rata-rata ayam IPB-D2 mengandung Fe, Se, Zn, dan Mn masing-masing sebesar 17.81, 0.22, 16.50, 0.34 mg/kg. Ditemukan dua SNP pada 5'UTR gen *MB* ayam IPB-D2, yaitu SNP g.17 G>T dan SNP g.25 T>C. Genotipe TT (g.17 G>T) dan genotipe CC (g.25 T>C) berasosiasi nyata (P<0.05) dengan kandungan Fe tinggi pada ayam IPB-D2. Analisis ekspresi gen menunjukkan bahwa ekspresi *MB* mRNA di hati tidak berbeda secara statistik antara kombinasi genotipe Fe tinggi (TT/CC) dan Fe rendah (GG/TT dan GT/TC). Pada jaringan otot paha, ekspresi gen *MB* kombinasi genotipe TT/CC lebih tinggi signifikan (P<0.05) dibandingkan kombinasi genotipe GG/TT dan GT/TC. Hasil ini menunjukan bahwa polimorfisme yang teridentifikasi pada gen *MB* IPB-D2 dapat menjadi referensi untuk menyelidiki gen serupa pada rasa ayam lain, khususnya terkait kandungan mineral Fe, Se, Zn, dan Mn.

Kata kunci: Ayam IPB-D2, Mioglobin, ekspresi *MB*, kandungan mineral, polimorfisme *MB*

INTRODUCTION

Myoglobin is an essential protein whose primary function is to bind and carry oxygen to the mitochondria within the muscle cells, allowing the muscles to receive an adequate oxygen supply during periods of physical activity (Prasad *et al.* 2019; Suman and Poulson 2013). Myoglobin belongs to the globin family, heme-containing globular polypeptides with eight α-helices in their protein folds. The heme group absorbs visible light through its double bonds and contains an iron atom in reduced (ferrous/Fe^{2+}) or oxidized (ferric/Fe^{3+}) form (Fernández-Barroso *et al.* 2022). In chickens, myoglobin is responsible for the characteristic red color of their muscle tissue, especially in the dark meat portions such as the legs and thighs (Purslow *et al.* 2019). Myoglobin is not only expressed in skeletal and cardiac muscles but is also expressed in endothelial cells and other tissues such as the brain and liver (Helbo *et al.* 2013).

Minerals in general are responsible for various biochemical reactions and physiological functions in the body. Minerals promote optimum growth, reproduction, and production in livestock. Some minerals such as Iron (Fe), Selenium (Se), Copper (Cu), Zinc (Zn), and Manganese (Mn) function as antioxidants. Depending on the amount required by the livestock, minerals are divided into two main categories: macrominerals and microminerals. The bioavailability of minerals in livestock is influenced by various factors, including species, diet, age, sex, physiological state, and supplements (Trocino *et al.* 2017; Cafferky *et al.* 2019; Prache *et al.* 2022).

IPB-D1 chicken is a local composite breed chicken developed by the Faculty of Animal Husbandry, Bogor Agricultural University. IPB-D1 chicken is a result of crossbreeding between the F1 ♂Pelung x ♀Sentul and the F1 ♂Kampung x ♀Parent Stock, strain Cobb (Sumantri *et al.* 2020). The Indonesian Ministry of Agriculture has officially released IPB-D1 chicken based on decree No.693/KPTS/PK.230/M/9/2019. Benefits, such as heterosis, arise from combining genes from different breeds, which can mask the effects of inferior genes (Buchanan and Northcutt 2000). The superiority of this new crossbreed is fast growth, high-quality meat, and resistance to diseases, especially Newcastle Disease (ND) and Salmonella sp. IPB-D1 chickens reached 1.38 ± 0.09 kg (male) or 1.18 ± 0.12 kg (female) at the age of 12 weeks (Al-Habib *et al.* 2020a). There have been several studies to improve genetic quality in IPB chickens, such as the prolactin gene in IPB-D1 chickens as a marker for egg production traits (Romlah *et al.* 2020) and CD1B gene diversity for disease resistance traits (Al-Habib *et al.* 2020b).

IPB-D2 chickens are the result of the 9th generation of IPB-D1 chickens, which were selected with IgY levels above 10 mg/ml in their blood serum. The IPB-D2 chicken is a candidate parent (female lines) expected to have higher ND and *Salmonella sp*. resistance than the IPB-D1 chicken. There are several studies on the genetics of IPB-D2 chicken. Lestari *et al.* (2022) found 4 SNPs of the *DMA* gene in IPB-D2 related to IgY concentration and ND antibody titers. Miraj *et al.* (2022) found 6 SNPs and 2 base deletions of the *BG1* gene related to disease resistance in IPB-D2 chicken. Lestari *et al.* (2023) found 4 SNPs of the *CD4* gene related to the immunocompetence index in IPB-D2 chickens. The ability to resist disease and an excellent immune system cannot be separated from the role of minerals within the animal body (Stefanache *et al.* 2023). The aim of this study was to analyze the polymorphism and expression of *MB* gene related to their association with Fe, Se, Zn, and Mn mineral content in IPB-D2 chickens.

MATERIAL AND METHODS

Animals and Phenotypic Parameters

All research procedures from rearing to slaughter have been approved by the Animal Ethics Commission, National Research and Innovation Agency (Reg. No.: 83/Klirens/X/2021). A total of 55 third-generation (G3) of IPB-D2 chickens from PT. Nutfah Unggul Intimakmur aged 10-12 weeks were used in this study. Phenotypic data of the Fe, Se, Zn, and Mn mineral content were obtained under the IPB iLab analysis service using thigh meat samples. The analysis was conducted using Atomic Absorption Spectrophotometry (AAS), following the Association of Analytical Chemists/AOAC (2015) 968.08 (4.8.02).

Methods

Gene Polymorphism

Two Single Nucleotide Polymorphisms (SNPs) of the *MB* gene were found in this study, namely g.17 G>T and g.25 T>C. The primers were designed using Primer3 (https://primer3.ut.ee/) using data obtained from Ensembl (Access No.: ENSGAL00000012541). The amplification target was between the 5' UTR and exon 1 with a polymerase chain reaction (PCR) product of 558 bp. The primers are as follows: Forward: 5'-TGATGCTGACACATGGCTTG-3' and Reverse: 5'- TTCCCCAGATGGTGAGGAC-3'. DNA was extracted from thigh muscle tissue using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions, with the ethanol drying time done overnight. The *MB* gene fragments were amplified using GoTaq Green Master Mix (Promega, USA). The PCR product was observed using a UV Transilluminator (Biorad™, California, USA). PCR products were sequenced by Macrogen (South Korea). The results were processed using FinchTV 1.4.0.

mRNA Expression of *MB* Gene

mRNA was extracted from liver and thigh muscle tissue using RNeasy® Mini Kit (Qiagen) and RNeasy® Fibrous Tissue Mini Kit (Qiagen), respectively. mRNA was then used as a template for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Gene expression levels were determined using RT-PCR on the CFX Connect Real-Time PCR System (Bio-Rad), utilizing the 2x SensiFASTTM SYBR® No-ROX Mix (Bioline). The *MB* primer sequence used was generated using the Primer Designing Tool https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the GenBank sequence (accession number: NM001167752.3), while the primer sequence of β-Actin (Khaerunnisa *et al.* 2023) was

used as the reference gene. The sequence of the *MB* gene is as follows: Forward: 5'-ACCCTGAGACTTTGGATCGC-3'and Reverse: 5'-GGGATTTTGTGCTTCGTGGC-3'. The RT-PCR master mix was made with a total volume of 10 µL with the following composition: 2x Sensifast SYBR No-ROX mix (5 µL), 10 µM forward primer (0.4 µL), 10 µM reverse primer (0.4 µL), cDNA template (2 µL) and nanopure H2O (2.2 µL). A two-step amplification program was used with pre-denaturation at 95 °C for 2 minutes, followed by 40 cycles of denaturation (95 °C for 5 seconds) and annealing (60 °C for 15 seconds). Melting curve analysis was performed from 65-95 °C, at 0.5 °C/cycle. Amplification of target and reference gene was carried out in triplicate.

Statistical Analysis

Genotype data from sequencing results were used for calculating allele and genotype frequencies, and for assessing Hardy-Weinberg Equilibrium (HWE) (Nei and Kumar 2000). Statistical analysis of the association between each SNP and mineral content was performed using the T-test, followed by the Duncan's Multiple Range Test (DMRT) post-test in SPSS v26.0. Similarly, the *MB* gene expression levels among different genotype combinations were compared using the T-test, followed by the DMRT post-test. The relative quantification of target gene mRNA was determined using the comparative CT method, based on the difference in cycle threshold (ΔCT) values between the target and reference genes. The obtained data were analyzed in terms of mean ± standard deviation. Differences between means were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Meat Mineral Content of IPB-D2 Chicken

Microminerals, also known as trace elements, are minerals required by the body in smaller amounts compared to macrominerals. Despite their small required quantities, microminerals are vital for many physiological processes and play crucial roles in maintaining health and wellness (Stefanache *et al.* 2023). This research focused on several trace elements with antioxidant properties, including Fe (iron), Zn (zinc), Se (selenium), and Mn (manganese). Table 1 descriptively presents the phenotypic data for the mineral content in IPB-D2 chickens. On average, the contents of Fe, Se, Zn, and Mn were found to be 17.81, 0.22, 16.50, and 0.34 mg/kg, respectively. When compared to broiler chickens (Benamirouche *et al.* 2019, Safitry *et al.* 2022, Yang *et al.* 2016), IPB-D2 chickens exhibit higher levels of Fe and Zn. The high content of antioxidant minerals in IPB-D2 chickens contributes to their ability to resist diseases, particularly those caused by oxidative damage. Table 1 also shows high standard deviations and Coefficient of Variation number indicate significant diversity in the mineral content among the studied IPB-D2 chickens.

Iron (Fe) is an essential trace element used in the body to form molecules, such as hemoglobin and myoglobin (Camaschella 2019). It functions as an oxygen carrier from the lungs to tissues via red blood cell hemoglobin, an oxygen-storing protein in the muscle, a transport medium

Table 1. Descriptive statistics of mineral content in IPB-D2 chicken thigh

Mineral	n	Mean	SD	CV
Fe	53	17.81	6.25	0.35
Se	55	0.22	0.05	0.25
Zn	55	16.50	5.70	0.35
Mn	42	0.34	0.17	0.49

Note: n= Number of samples; SD=Standard deviation; CV=Coefficient of variation. The result is expressed in mg/kg.

for intracellular electrons, and an integrated part of key enzyme systems in various tissues such as catalase (Gupta 2014). Within catalase, iron plays a critical role in enabling the enzyme to catalyze the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). This reaction is crucial as it protects cells from oxidative stress caused by the accumulation of hydrogen peroxide (Weiss *et al.* 2005). Fe deficiency can cause anemia which can reduce the body's immunity to inflammation and can cause reproduction problems (McCann *et al.* 2007).

Manganese (Mn) plays a pivotal role in numerous physiological processes, ranging from bone development and digestion to reproductive health, energy production, immune response, and neurological function (Chen *et al.* 2018). A critical function of Mn involves its role in antioxidant defense, particularly through enzymes like manganese superoxide dismutase (MnSOD). MnSOD plays a vital role in neutralizing harmful reactive oxygen species, thereby protecting cells from oxidative damage (Candas and Li 2014).

Zinc (Zn) plays a critical role in a wide range of physiological processes, including bone formation, immune cell function, and tissue growth (Bagherani and Smoller 2016). It is also crucial to the structure and function of superoxide dismutase (SOD), an antioxidant enzyme that protects the body from free radicals such as superoxide (Weiss *et al*. 2005). Zn influences the NFκB signaling pathway, a critical regulator in various biological processes such as apoptosis, innate and adaptive immune responses, and inflammatory reactions (Jarosz *et al.* 2017).

Selenium (Se) is essential in detoxifying harmful substances and protecting cells from heavy metal toxicity such as arsenic, cadmium, and mercury (Brown and Arthur 2001). Selenium forms a significant structural component of several enzymes, including glutathione peroxidase, thioredoxin reductase, and deiodinases, as well as proteins containing selenium, known as selenoproteins (Stefanache *et al.* 2023). Glutathione peroxidase catalyzes the reduction of peroxides, including hydrogen peroxide (H_2O_2) and lipid peroxides, to water (in the case of H_2O_2) and alcohol from lipid peroxides (Weiss *et al.* 2005).

Polymorphism of *MB* Gene

The MB gene in chickens is located on chromosome 1 and has a gene length of 3.71 kb. This gene consists of three exons and two introns. DNA amplification was performed using the PCR technique, targeting the region between the 5'UTR and exon 1 (Figure 2). This amplification resulted

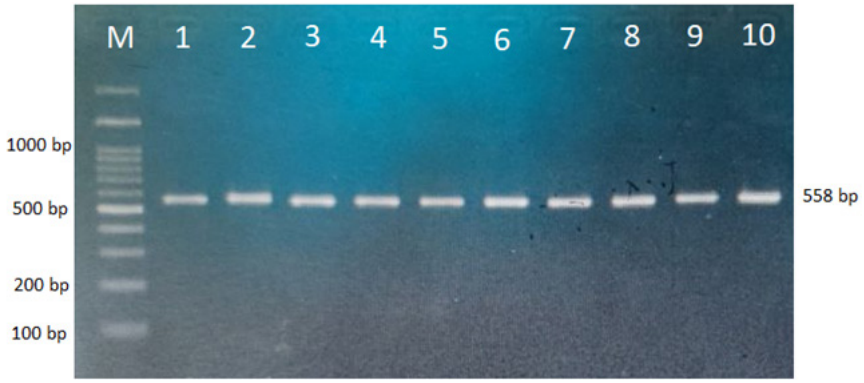

Figure 1. Amplification product of MB gene in IPB-D2 chickens: M= DNA marker 100 bp; 1-10= sample 1-10

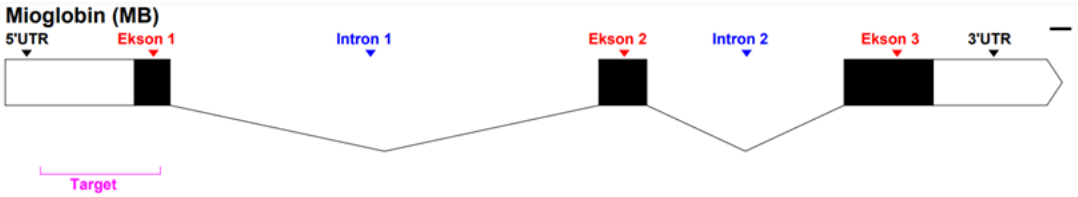

Figure 2. The MB gene structure and target region

in a product 558 bp in length (Figure 1). The sequencing results were evaluated by aligning the sequence with the Ensembl chicken myoglobin DNA sequence (Accession No.: ENSGAL00000012541). Two SNPs were identified in the 5'UTR, namely g.17 G>T and g.25 T>C (Figure 3). SNP g.17 G>T is a transversion mutation, while SNP g.25 T>C is a transition mutation. The two SNPs identified are silent mutations, a type of genetic mutation that does not result in a change to the amino acid sequence of a protein.

SNP g.17 G>T and SNP g.25 T>C in IPB-D2 chickens have two or more alleles with a relative frequency value of more than 1%, categorizing them as polymorphic. SNP g.17 G>T exhibits three genotypes: GG, GT, and TT (Figure 3), with the GG genotype having the highest frequency (Table 2). Similarly, SNP g.25 T>C presents three genotypes: TT, TC, and CC, with the TT genotype being the most frequent. The most prevalent allele for SNP g.17 G>T is the G allele, while for SNP g.25 T>C, it is the T allele. Both SNPs were found to be in Hardy-Weinberg equilibrium, as determined by the Chi-square (X2) test (X2 test < X2 table).

Association of *MB* Gene and Mineral Content

The results of the association analysis between the *MB* gene and the mineral content are presented in Table 3. Through statistical analysis, it was found that both SNP g.17 G>T and SNP g.25 T>C are significantly associated with the Fe content in IPB-D2 chickens (Table 3). The TT genotype (g.17 G>T) and the CC genotype (g.25 T>C) in IPB-D2 chicken were associated with high Fe content, which was 21.87 ± 2.88 mg/kg and 22.43 ± 2.82 mg/kg, respectively (Table 3). Our result also found that there is no association between the concentration of Zn, Se, and Mn and *MB* gene polymorphism in IPB-D2 chicken.

Genetic variance (breed) has been identified as a significant factor influencing the mineral composition in the muscle of livestock raised under similar conditions (Cabrera *et al.* 2010). Domaradzki *et al.* (2016) in a study reported a significant impact of breed on the mineral levels in young bull beef cattle. Their findings indicated that Polish Holstein-Friesian cattle had lower levels of potassium (K), magnesium (Mg), and calcium (Ca), while demonstrating

Table 2. The number of animals per genotype and allele frequency

No	SNP	n	Genotype Frequency			Allele Frequency		χ^2
1	g.17 G>T	55	**GG**	**GT**	**TT**	**G**	**T**	0.22ns
			0.45 (25)	0.42 (23)	0.13 (7)	0.66	0.34	
2	g. 25 T>C	55	**TT**	**TC**	**CC**	**T**	**C**	0.07ns
			0.47 (26)	0.42 (23)	0.11 (6)	0.68	0.32	

Note: n = number of sample; (…) = number of samples within genotypes; ns = not significant for $\chi 2$ (0.05;1) = 3.84

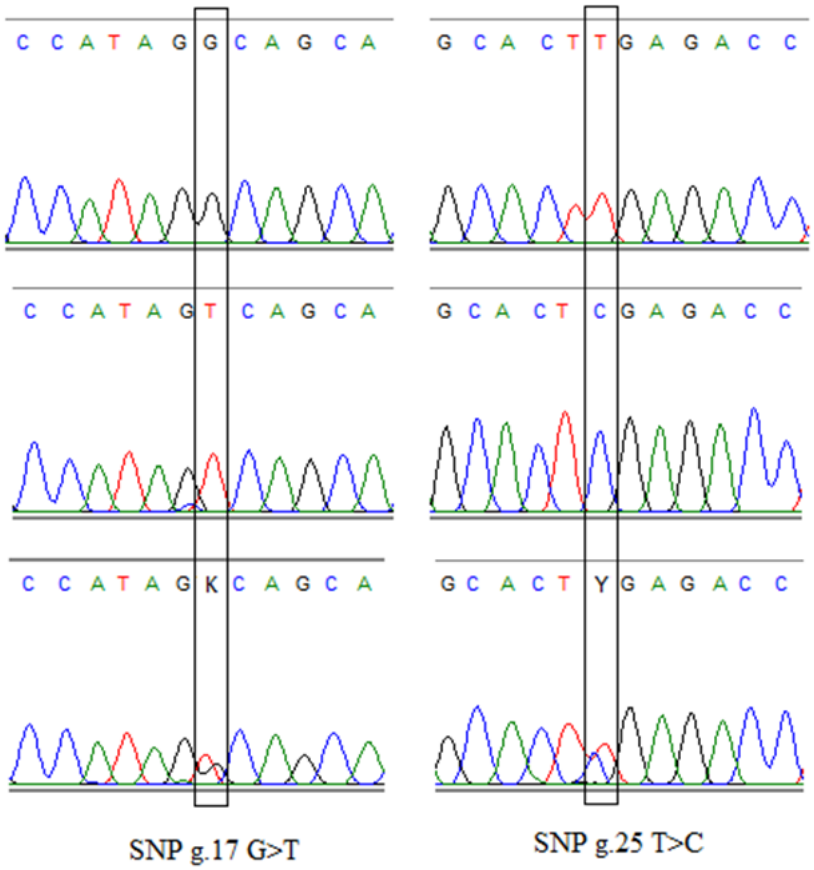

Figure 3. SNPs position in MB gene

Table 3. Myoglobin (MB) gene polymorphisms association with mineral content

Mineral	Genotype/Mineral Content					
	g.17 G>T			g.25 T>C		
	GG	**GT**	**TT**	**TT**	**TC**	**CC**
Fe	16.5±6.77a (24)	17.05±3.97a (20)	21.87±2.88b (6)	16.72±6.54a (25)	16.90±4.20a (20)	22.43±2.82b (5)
Se	0.23±0.06 (25)	0.22±0.05 (23)	0.20±0.04 (6)	0.23±0.06 (26)	0.22±0.05 (23)	0.22±0.06 (6)
Zn	17.12±5.87 (25)	15.99±5.24 (23)	13.61±3.96 (6)	17.32±5.61 (26)	15.74±5.34 (23)	15.82±7.80 (6)
Mn	0.37±0.19 (17)	0.35±0.15 (19)	0.31±0.24 (7)	0.38±0.18 (19)	0.32±0.16 (18)	0.34±0.24 (6)

Note: (…) = number of samples within genotypes. Results are expressed as mg/kg (mean±standard deviation). Different letters in the same row and SNP indicate statistical differences (P<0.05).

higher manganese (Mn) contents compared to other breeds (such as Polish Red cattle, White-Backed cattle, Polish Black-and-White cattle and Simmental cattle). Mineral composition is also influenced by mutations, Safitry *et al.* (2022) stated that the content of the mineral Fe in cemani chicken reached two times higher in homozygous cemani (Fm/Fm) than in heterozygous cemani (Fm/fm^{+}).

In addition to genetic variation, mineral content can be affected by factors such as nutrition, species, sex, age at slaughter, muscle types, physiological status, and the production system of the meat samples (Domaradzki *et al.* 2016). According to a study by Chen *et al.* (2016), the breast muscle of both broiler chickens and spent hens contains higher levels of calcium, sodium, and manganese, but lower levels of zinc, iron, manganese, and selenium compared to the thigh muscle. Kasap *et al.* (2018) revealed that male lamb meat showed higher zinc concentrations, but lower calcium, magnesium, and iron concentrations than female lamb meat. Qwele *et al.* (2013) discovered that *longissimus thoracis et lumborum* muscle of goats fed with a diet supplemented with *Moringa oleifera* leaves had more mineral contents (iron, copper, calcium, zinc) than those fed diets supplemented with sunflower cake (SC) and grass hay (GH).

Expression of *MB* Gene

From the 2 SNPs, 6 genotype combinations were identified in the IPB-D2 chicken samples. The genotype combinations found included: GG/TT(24), GT/TC(21), TT/CC(6), GT/TT(2), TT/TC(1), and GG/TC (1). The three genotype combinations with the highest sample numbers were selected for the gene expression analysis, 3 samples

each. GG/TT and GT/TC genotype combination represent low Fe content, and TT/CC genotype combination represent high Fe content. *MB* gene expression was performed using RT-PCR based on the significant difference results of the association analysis in the IPB-D2 *MB* gene polymorphism with Fe content.

Our result found that *MB* mRNA expression in the liver tissue was not significantly different (P>0.05) between the GG/TT, the GT/TC, and the TT/CC genetic combination (Figure 4). In contras to the liver tissue, the mRNA expression of *MB* in the high Fe content genotype combination (TT/CC) was 2.5 to 3 times higher than in the low Fe genotype combination (GG/TT and GT/TC) (Figure 5). Comparatively, *MB* gene expression in thigh muscle was significantly higher (P<0.05) than in the liver (Figure 6).

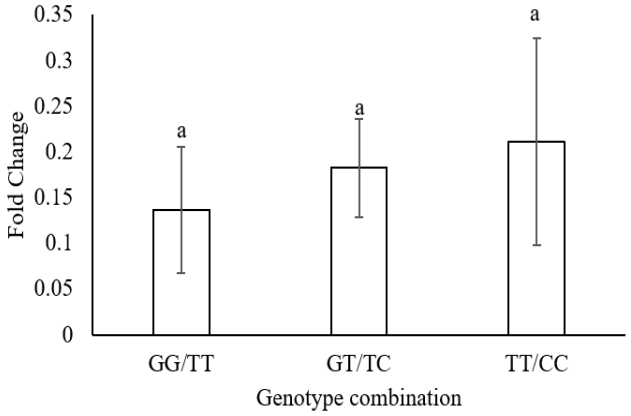

Figure 4. Expression levels of MB in IPB-D2 liver between high Fe (TT/CC) and low Fe (GG/TT and GT/TC) genotype combination. Different letters indicate statistically significant differences (P<0.05)

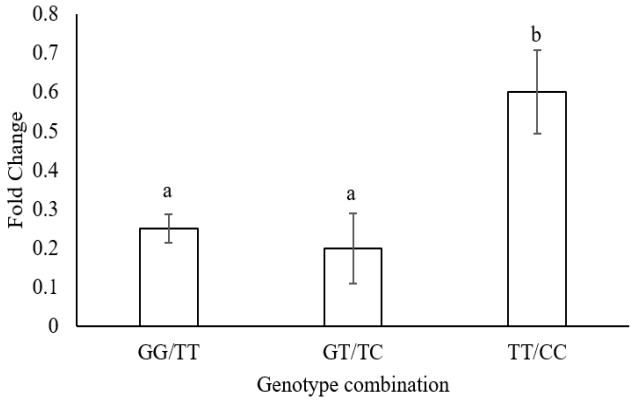

Figure 5. Expression levels of MB in IPB-D2 thigh muscle between high Fe (TT/CC) and low Fe (GG/TT and GT/TC) genotype combination. Different letters indicate statistically significant differences (P<0.05).

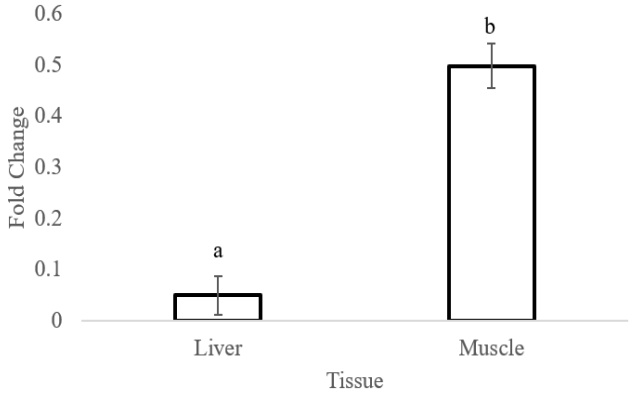

Figure 6. Expression levels of MB in IPB-D2 between liver and thigh muscle tissue. Different letters indicate statistically significant differences (P<0.01)

Muscle tissues that are in continuous motion, such as skeletal and heart muscles, require more oxygen and therefore contain higher levels of myoglobin. A study by Mänttäri *et al.* (2008) demonstrated that short-term exercise induced a significant increase in *MB* content in the *Musculus plantaris* and *Musculus gastrocnemius* muscles. Prasad *et al.* (2019) found that the expression levels of *MB* in muscle tissue varied significantly (P<0.01) among different muscles, breeds, and age groups, but did not show significant differences between sexes. In cases of tissue iron deficiency, the myoglobin concentration in skeletal muscle experiences a significant reduction, typically ranging from 40% to 60% (Beard 2001). The higher expression of the *MB* gene in IPB-D2 chickens with high Fe content (TT/CC genotype combination) suggests that the *MB* gene may play a role in regulating Fe content in the meat of these chickens.

CONCLUSION

The association of polymorphism in the chicken *MB* gene's 5' UTR with iron levels has been discovered for the first time, suggesting the potential of the *MB* gene as a marker for iron content. Current research has found that the TT genotype at SNP g.17 G>T and the CC genotype at SNP g.25 G>T had the potential to be developed as genetic markers in the selection program of high iron IPB-D2 chickens. *MB* gene expression in liver tissue was not significantly different between genotype combinations. Meanwhile, in the thigh muscle, the *MB* mRNA expression of the TT/CC genotype combination was higher compared to the GG/TT and GT/TC genotype combination. *MB* gene expression in thigh muscle tissue was higher compared to the liver tissue. Polymorphisms in the *MB* gene of the IPB-D2 could serve as references for investigating similar gene in other chicken breeds, especially regarding mineral content.

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