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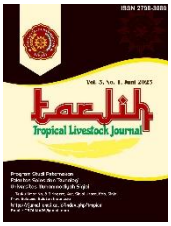

Identification of Single-Nucleotide Polymorphism, Linkage Disequilibrium, and Restriction Enzyme Analysis of the Growth Hormone Gene in Kampung Super Chicken

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ABSTRACT

This study aimed to investigate single-nucleotide polymorphisms (SNPs) and haplotype diversity within the growth hormone (GH) gene of Kampung Super chickens, a hybrid breed derived from KUB males and commercial layer hens. In this study, thirty-eight blood samples of Kampung Super chicken were collected and analyzed. A total of 11 SNPs were identified within intronic regions of the GH gene. Despite their non-coding location, these variants may influence gene regulation through mechanisms such as alternative splicing, mRNA transport, or chromatin accessibility. Linkage disequilibrium (LD) analysis revealed several strong associations among SNP pairs, indicating the presence of conserved haplotype blocks potentially under stabilizing selection. In silico restriction enzyme analysis showed that five SNPs displayed three genotypic classes, and four of them could be effectively genotyped using PCR-RFLP, while one SNP (g.3018G/A) lacked suitable restriction sites. The SNP g.3094C/T exhibited high diagnostic potential using multiple enzymes (StuI, MspI, XmaI), while g.3129A/T was distinguishable using TaqI, although the adjacent SNP overlap limited interpretability. Two SNPs (g.3261C/T and g.3268G/A) were digestible by enzymes with multiple cut sites, complicating gel-based resolution. These findings provide a foundation for future functional and association studies and support the use of GH gene polymorphisms in marker-assisted selection strategies for growth-related traits in Kampung Super chicken.

INTRODUCTION

Chickens are a vital source of accessible and affordable animal protein, particularly for rural and low-income communities (Alders *et al*., 2018). In Indonesia, one of the emerging poultry types with significant production potential is the Kampung Super chicken, a crossbred line developed by mating male KUB (Kampung Unggul Balitbangtan) chickens

with commercial laying hens (Rahayu *et al*., 2021; Nuriliani *et al*., 2022). This hybrid was specifically designed to combine the adaptive and meat quality traits of KUB chickens with the high egg-laying capacity of layer breeds. Given its potential for both meat and egg production, understanding the genetic factors influencing growth in this population is critical for optimizing breeding strategies.

A key gene regulating growth, metabolism, and development in chickens is the growth hormone (GH) gene, which has been widely studied for its association with economically important traits such as growth rate, carcass quality, and reproductive performance (Anh *et al*., 2015; Ghelghachi *et al*., 2013). Nie *et al*. (2005) reported 13 polymorphic sites within intron 4 of the GH gene, suggesting a high level of genetic variability that could be exploited for selection programs. Moreover, variations in the GH gene have been linked not only to growth performance but also to comb morphology, a secondary sexual trait with physiological and economic significance. Comb type is influenced by hormonal regulation, including growth hormone levels, which affect cellular proliferation and vascularization in the comb tissue. The study of Qi *et al.* (2025) reported that higher GH levels are positively associated with larger combs. As such, genetic variation in the GH gene may indirectly influence comb development, which is often correlated with growth rate, health status, and sexual maturity in chickens.

Despite the economic value of Kampung Super chickens, there is currently no published information on the single-nucleotide polymorphisms (SNPs) and haplotype diversity of the GH gene in this population. Understanding the genetic variation within this gene could provide important insights into growth-related traits and help develop marker-assisted selection tools tailored to this hybrid. Therefore, the purpose of this study was to identify the SNPs and evaluate the haplotype diversity of the GH gene in Kampung Super chickens. The findings are expected to contribute foundational genetic data for future improvement programs targeting growth performance and other production traits in this economically important breed.

MATERIALS AND METHODS

Blood Sample Collection and DNA Isolation

Blood samples were collected from thirty-eight Kampung Super chickens, a crossbreed of male KUB and layer hens, by drawing blood from the pectoralis vein into EDTA-containing vacutainer tubes. All chickens were raised under uniform management conditions in Gunung Kidul Regency, Yogyakarta Province. The samples were transported to the laboratory and stored at -20°C until further processing. Genomic DNA was extracted using the gSYNC DNA Extraction Kit (Geneaid, New Taipei City, Taiwan).

GH Gene Amplification

Amplification of the GH gene was carried out using a specific primer pair based on the method described by Heba *et al*. (2017), targeting a 562 bp DNA fragment. The PCR reaction was prepared in a total volume of 25 µL, consisting of 2 µL of genomic DNA, 9.5 µL of double-distilled water, 12.5 µL of MyTaq™ HS Red Mix (Bioline, UK), and 0.5 µL each of forward and reverse primers. PCR was performed using a SEDI G Thermal Cycler with the following conditions: initial denaturation at 96°C for 3 minutes; 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension step at 72°C for 5 minutes. The success of amplification was checked using 2% gel electrophoresis and visualized in a UV-transilluminator.

Sequencing and Genotyping

The amplified PCR products were submitted to the Central Laboratory of

Universitas Gadjah Mada for sequencing using an automated DNA sequencer to verify the target region. Sequence data were then edited and aligned using BioEdit software to identify single-nucleotide polymorphisms (SNPs) within the GH gene. The alignment also compared to the reference GenBank sequence of chicken GH gene, with accession number AY461843, D10484, KF957979, KF957980, KY176746, and KY176758). The genotyping was performed by manual inspection of the electropherogram. The single peak reflects the homozygous (dominant/recessive) genotype, and the double peak reflects the heterozygous genotype.

Linkage Disequilibrium and Restriction Enzyme Analysis

The genotype data were used to analyze the linkage disequilibrium using the HaploView program. Meanwhile, the sequence template AY461843 and the SNP data were used to analyze the putative restriction enzymes using the NEBcutter V2.0 tool for a further low-cost genotyping method.

RESULT AND DISCUSSION

SNPs Identification

A 562 bp fragment of the GH gene in chicken was successfully amplified (Figure 1). The sequencing results were aligned with six chicken sequences from GenBank (Acc No. AY461843, D10484, KF957979, KF957980, KY176746, and KY176758). Alignment among the samples and GenBank sequences revealed nine polymorphic sites, namely g.3018G/A, g.3029A/G, g.3094T/C, g.3113C/T, g.3127C/T, g.3129A/T, g.3199T/C, g.3261C/T, and g.3268G/A (positions refer to GenBank Acc No. AY461843) as shown in Figure 2. Each SNP was then confirmed by checking its electrophoregram to determine the genotype of each livestock. In the electrophoregram confirmation process, it was found that 2 SNPs were found again, namely SNP g.3172A/G and g.3180A/G, as shown in Figures 3 and 4. Based on electrophoregram observations, as many as 5 SNPs were detected to have 3 genotype types, namely SNP g.3018G/A, g.3094T/C, g.3129A/T, g.3261C/T, and g.3268G/A. While for the other 6 SNPs, only 2 genotype types appeared.

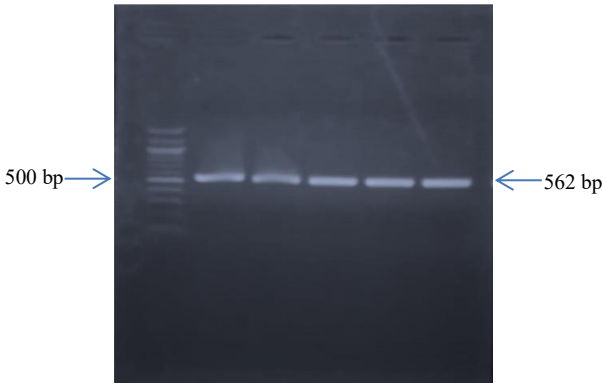

Figure 1. PCR product of the GH gene (562 bp)

All of the identified mutations in this study were located within intronic regions of the growth hormone (GH) gene. While intronic mutations were traditionally regarded as functionally neutral due to their exclusion during mRNA splicing, accumulating evidence suggests otherwise. Nie *et al*. (2005) emphasized that SNPs in non-coding regions, including introns and untranslated regions (UTRs), can exert regulatory functions. These regions may harbor cis-regulatory elements that influence transcriptional activity, mRNA stability, and alternative splicing, ultimately affecting gene expression levels. Further, Jo & Choi (2015) noted that intronic SNPs may also modulate chromatin accessibility, mRNA transport, and even trigger nonsense-mediated decay if aberrant splicing leads to premature stop codons.

In the context of the GH gene, such regulatory effects can have a direct phenotypic impact, especially on traits associated with growth performance, metabolism, and secondary sexual characteristics like comb development. For instance, SNPs that alter transcription factor binding sites or splicing

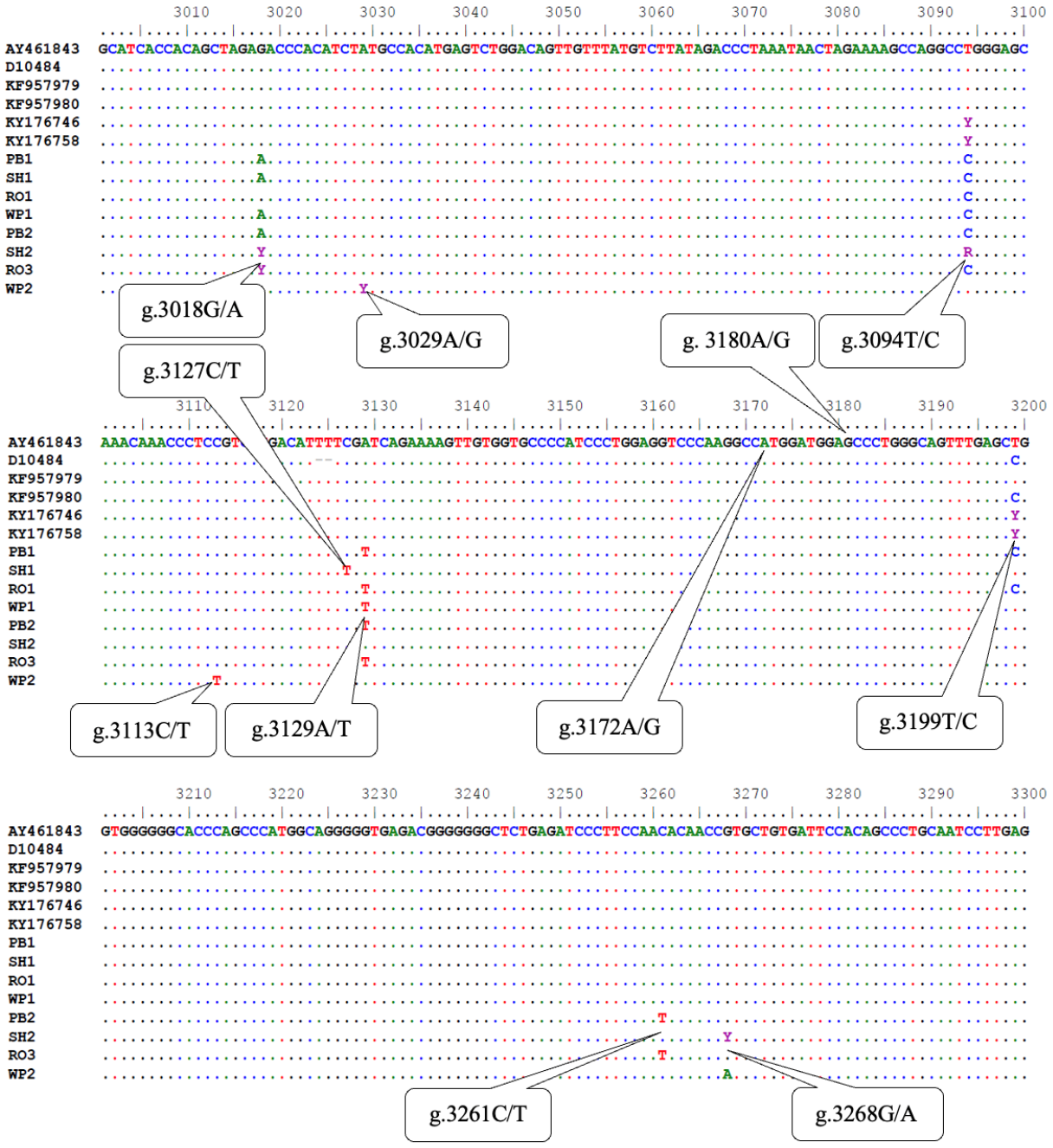

Figure 2. Nine SNPs were found by sequence alignment

enhancers/silencers in introns may lead to differential GH expression, which could manifest as variation in body weight, growth rate, or skeletal development in chickens. While this study did not measure GH mRNA or protein levels directly, the presence of intronic SNPs warrants future investigation into their expression-level consequences using qPCR or RNA-seq.

Comparative analysis reveals that similar GH gene polymorphisms have been reported in various chicken breeds, both local and commercial. For example, the G662A SNP in intron 1 has been detected in Khouzestan native chickens (Alireza *et al.*, 2010), Kadaknath (Thakur *et al.*, 2009), Sentul (Pratama *et al.*, 2023), and KUB chickens (Sidik *et al.*, 2024) using PCR-RFLP. This

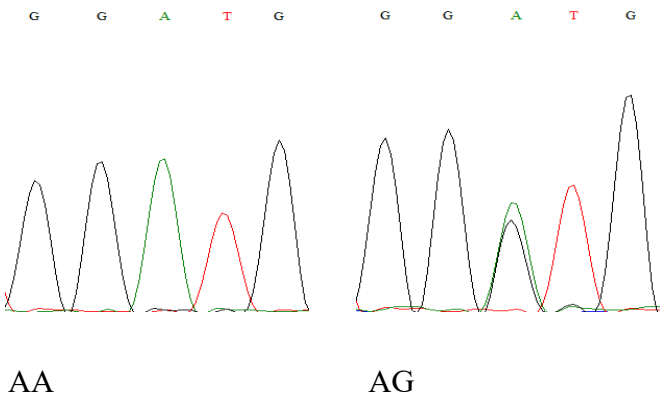

Figure 3. The electropherogram of SNP g.3172A/G

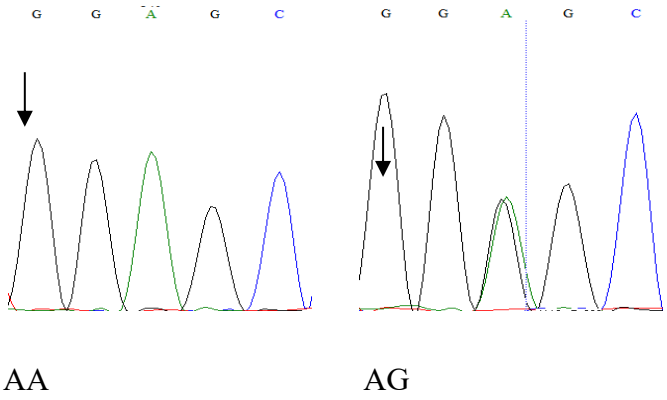

Figure 4. The electropherogram of SNP g.3180A/G

indicates a conserved mutation across diverse genetic backgrounds, including breeds with different growth and adaptability profiles. However, the present study uniquely focuses on the Kampung Super chicken, a hybrid cross of KUB and commercial layer breeds, which has not been previously characterized at the GH gene level. Furthermore, Anh *et al*. (2015) and Al-Khatib & Al-Hassani (2016) identified g.1705G>A in intron 3 in Thai broiler hybrids and commercial broilers, respectively, using EcoRV digestion, while Su et al. (2014) reported multiple SNPs—T185G (5' UTR), G662A (intron 1), T3094C, and C3199T (intron 4)—in Recessive White and Qingyuan Partridge chickens via PCR-LDR. Compared to these breeds, Kampung Super chickens represent a valuable genetic model for studying how GH polymorphisms from both local (KUB) and commercial (layer) parentage may interact.

Linkage Disequilibrium of the GH Gene

The linkage disequilibrium (LD) analysis of 11 single-nucleotide polymorphisms (SNPs) within the growth hormone (GH) gene in Kampung Super chickens revealed varying degrees of genetic association among loci (Figure 5). Several SNP pairs showed strong LD, indicated by high R^2 values, such as g.3113C>T – g.3129A>T (R^2 = 89), and g.3172C>T – g.3180A>T (R^2 = 100), suggesting these loci are likely inherited together and form part of a

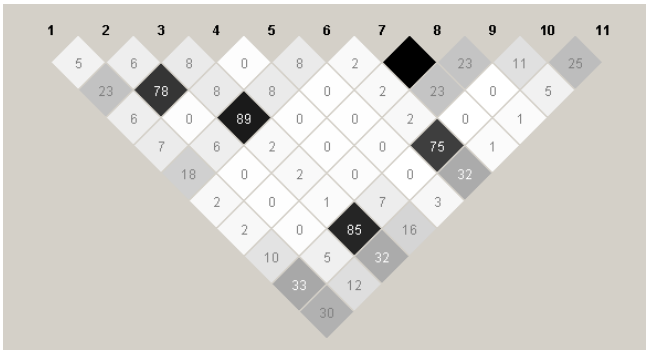

Figure 5. Linkage Disequilibrium (LD) plot based on the r-squared value

conserved haplotype block. These regions of strong LD may be the result of limited historical recombination and potential selection pressure on functionally important regions of the GH gene. Conversely, low R^2 values suggest recombination hotspots or a lack of linkage, indicating that these sites segregate independently in the population. The observed LD structure within the GH gene provides insight into the genetic organization of this locus in Kampung Super chickens, a hybrid resulting from the cross between KUB males and commercial layer hens. The presence of distinct haplotype blocks suggests that certain regions of the GH gene may be under stabilizing selection, potentially influencing key traits such as growth rate, feed efficiency, or hormonal regulation.

Restriction Enzyme Identification

Based on the sequence alignment, as 5 SNPs were detected to have 3 genotype types, further restriction site analysis was conducted. In silico restriction enzyme analysis revealed that each SNP could potentially be recognized and differentiated using specific restriction enzymes, providing a basis for genotyping using the PCR-RFLP method (Table 1). Among these, four SNPs (g.3094C/T, g.3129A/T, g.3261C/T, and g.3268G/A) were predicted to be distinguishable using restriction enzymes, while one SNP, g.3018G/A, was not recognized by any of the restriction enzymes tested, and thus cannot be genotyped using standard RFLP-based methods.

The SNP g.3094C/T showed strong potential for PCR-RFLP analysis, with three different enzymes (StuI, MspI, and XmaI) selectively recognizing the C allele, while the T allele lacked recognition sites, resulting in an uncut fragment of 562 bp. Depending on the enzyme used, digestion at this locus produced distinct fragment sizes (e.g., 248/314 bp for StuI, 250/312 bp for MspI, and 249/313 bp for XmaI), allowing for clear genotype discrimination. Similarly, at g.3129A/T, TaqI could differentiate the A allele by cleaving at position 283 bp, generating two fragments of 279 and 283 bp. The T allele, which lacks a recognition site, produced an intact 562 bp fragment. However, the g.3127C/T and g.3129A/T polymorphisms occur within the same recognition site of TaqI (TˇCGA). As a result, digestion with TaqI alone cannot distinguish between changes at these two positions, as both would yield identical fragment sizes.

The SNP g.3018G/A, however, was not recognized by any tested restriction enzyme, rendering it unsuitable for RFLP-based genotyping. Interestingly, g.3261C/T and g.3268G/A were predicted to be digestible by restriction enzymes with more than two recognition sites within the amplified fragment. Due to the complexity of digestion patterns and lack of specificity to a single cut site, these SNPs are less suitable for

Table 1. The possible restriction enzyme(s) for each SNP within the GH gene

Locus	Restriction Enzyme(s)	Allele	Site Number	Cut Position (5' → 3')	Fragment size (bp)
g.3018G/A			Non-recognized		
g.3094C/T	*Stu*I	T	1	248	248, 314
	(AGG▼CCT)	C	-	-	562
	*Msp*I	T	-	-	562
	(C▼CGG)	C	1	250	250, 312
	*Xma*I	T	-	-	562
	(C▼CCGGG)	C	1	249	249, 313
g.3129A/T	*Taq*I	A	1	283	279, 283
	(T▼CGA)	T	-	-	562
g.3261C/T			Multi-site digestion (>2 site)		
g.3268G/A			Multi-site digestion (>2 site)		

conventional RFLP analysis, as their multi-site digestion would produce multiple overlapping bands that are difficult to resolve using agarose gel electrophoresis. According to Taheri *et al*. (2012), the selection of restriction enzymes for genomic analysis should be based on several factors: (1) the size of fragments generated after digestion, (2) the presence and distribution of recognition sites within the target DNA sequence, and (3) the enzyme's sensitivity to DNA methylation.

CONCLUSION

In conclusion, there are eleven SNPs found within intron 4 of the GH gene in Kampung Super chicken. The detection of strong LD between several SNP pairs suggests conserved haplotypes that may influence phenotypic outcomes. In silico restriction enzyme analysis confirmed the feasibility of using specific restriction enzymes for genotyping, particularly g.3094C/T and g.3129A/T. However, the utility of RFLP for SNPs with overlapping or multiple enzyme cut sites was limited. These findings emphasize the relevance of non-coding genetic variation in poultry breeding and provide molecular markers that could be integrated into future marker-assisted selection programs aimed at improving the productivity and adaptability of Kampung Super chickens.

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