Avian Beta Defensin 2 (AvBD2) Gene Polymorphism Identification in IPB-D1 Chicken

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ABSTRAK

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Gen Avian Beta Defensin 2 (AvBD2) yang berada di kromosom 3 berperan penting dalam sistem imunitas tubuh ayam dengan menghambat perkembangan mikroorganisme seperti bakteri yang mengifeksi jaringan tubuh. Defensin dihasilkan melalui sel epitel segera setelah adanya cidera jaringan atau infeksi, yang kemudian memproses pematangan sel dendritik untuk memulai respon imun di kelenjar getah bening. Tujuan penelitian ini adalah mengidentifikasi polimorfisme gen AvBD2 pada ayam IPB-D1. Metode PCR dan direct-DNA sequencing digunakan untuk mengidentifikasi keragaman intron 1, ekson 2, dan intron 2 gen AVDB2 pada 47 ekor ayam IPB-D1. Perhitungan frekuensi genotipe, frekuensi alel, dan heterozigositas dilakukan untuk mendapatkan informasi polimorfisme gen AvBD2. Sebanyak 10 mutasi titik ditemukan pada gen AvBD2 yang tersebar di intron 1 (g.4843T>A, g.4853G>A, dan g.4859T>C), ekson 2 (g.4881A>G, g.4889G>A, dan g.5002C>T), dan intron 2 (g.5075C>T, g.5111T>G, g.5116G>T, dan g.5177G>T). Seluruh SNP bersifat polimorfik. Mutasi g.5002C>T menyebabkan perubahan asam amino Ala menjadi Val yang berpotensi menjadi kandidat penciri ketahanan penyakit pada ayam IPB-D1.

Kata Kunci: Gen AvBD2, IPB-D1 chicken, Keragaman

ABSTRACT

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Avian Beta Defensin 2 (AvBD2) gene, located in chromosome 3, plays an important role in the immune system of the chicken by inhibiting the development of microorganisms such as bacteria that infect body tissues. Defensins are produced through epithelial cells immediately after tissue injury or infection, which then processes the maturation of dendritic cells to initiate an immune response in the lymph nodes. The purpose of this study was to discover the polymorphism of the AvBD2 gene in IPB-D1 chickens. PCR and direct-DNA sequencing methods were used to identify the polymorphisms of intron 1, exon 2, and intron 2 AvDB2 genes in 47 chickens. Genotype and allele frequency, and heterozygosity calculations were carried out to obtain information of the AvBD2 gene polymorphism. A total of 10 single nucleotide polymorphisms were found in the AvBD2 gene located in intron 1 (g.4843T>A, g.4853G>A, and g.4859T>C), exon 2 (g.4881A>G, g.4889G>A, and g.5002C>T), and intron 2 (g.5075C>T, g.5111T>G, g.5116G>T, and g.5177G>T). All SNPs are polymorphic. The g.5002C>T mutation causes changes in the amino acid Ala to Val which has the potential to be a candidate for characterizing disease resistance in IPB-D1 chickens.

Key Words: AvBD2 gene, IPB-D1 chicken, Polymorphism

INTRODUCTION

Indonesia's native chicken population in 2020 is estimated to reach 308 million chickens, or around 8.8% of the total national chicken population (DGAHP 2020). Application of extensive traditional rearing system shows the low application of biosecurity and sanitation. The lack of attention of local chicken breeders

breeders to good rearing management and the application of biosecurity affect the health of local chickens. This condition causes local chicken production to be not optimal. Availability of local chicken breeds that are resistant to disease is very important so that breeders could get disease-resistant chickens maintained in suboptimal biosecurity and sanitation conditions. One of the efforts to provide these breeds is through genetic selection of local chickens that have a good immune system.

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Defensin is part of the immune system that has broad antimicrobial activity (Wilson et al. 2013). Beta defensin is a member of the defensins family found in avian including chickens and has the potential as an alternative to antibiotics due to its role to neutralize various pathogens such as colibacillosis and zoonosis (Derache et al. 2012, Kim & Lillehoj 2019, Zhao et al. 2014). Hong et al. (2012) stated that the pathogens that phagocytes by antimicrobial activity in the AvBD2 gene include *Salmonella enterica* serovar Typhimurium*, C. perfringens*, and *Escherichia coli* found in the digestive tract. Moreover, the AvBD2 gene plays an important role in the level of immunity in chickens (Hong et al. 2012). AvDB2 has been reported to play a role in ileum and cecum mucosal defense in broiler embryos and neonatal chicks (Terada et al. 2018). The AvBD2 gene was isolated from leukocytes, epithelial cells of the skin, digestive tract, and respiratory tract. The role of AvBD2 in resistance to Newcastle Disease virus infection was reported by Liu et al. (2018). Several molecules have also been reported to suppress the action of AvBD2 in defense against NDV, one of which is p38 MAPK (Liu et al. 2018). In addition, localization of AvBD2 and AvBD4 was found in chickens that were vaccinated against ND and Marek's disease (Shimizu et al. 2020).

Defensins are produced immediately after tissue injury or infection. Defensins are released from epithelial cells, including keratinocytes and infiltrating leukocytes. Defensins facilitate the uptake of antigens and processing of immature dendritic cells (iDCs) into mature dendritic cells (mDCs). These mDCs then migrate to the lymph nodes to initiate an immune response (Hazlett & Wu 2011). The chicken AvBD2 gene is located on chromosome 3 and consists of four exons and three introns (Xiao et al. 2004). AvBD2 protein in chickens consists of 22 signal peptides, 6 propieces, and 36 mature peptides, which are equal to other avian such as ostrich, duck, goose, and turkey (Lu et al. 2014).

The polymorphisms of the AvBD2 gene is indicated by the discovery of single nucleotide polymorphysms (SNP) which can be used as genetic markers. Bagnicka et al. (2010) stated that SNPs on Avian beta defensin have a strong correlation to be associated with antibacterial activity against Gram-positive and Gram-negative bacteria. Mommarazi and Habibi (2017) reported that the mutation rate of chicken AvBD2 was 15.4 SNPs/kb, which showed that in every 1000 bp of AvBD2 gene sequence, 15.4 SNPs were found. This shows a high genetic diversity and has a high response to disease resistance (Morammazi & Habibi 2017).

IPB-D1 chicken is Indonesian local chickens that have been released by the Minister of Agriculture of the Republic of Indonesia in 2019 as superior broiler and layer (Sumantri et al. 2020). IPB-D1 chickens are the result of crossing of Pelung x Sentul (male) and Kampung x Cobb strain broilers (female), so they have a blood composition of 75% local chickens (Sumantri et al. 2020). Apart from having superior productivity, IPB-D1 chickens are also developed to have resistance to ND virus (Al-Habib et al. 2020) and Salmonella enteritidis infection (Susanti et al. 2020). Genetic information regarding the diversity of the AVBD2 gene in IPB-D1 chickens has never been reported. The objective of this study is to discover the Single Nucleotide Polymorphism (SNP) of the partial AvBD2 gene in IPB-D1 chicken using direct-DNA sequencing method. Identification of the polymorphism of the AvBD2 gene may be a potential candidate gene for improving genetic quality in IPB-D1 chickens, especially in their immune system.

MATERIALS AND METHODS

Chickens rearing management and blood sample collection

All procedures performed in this study were based on animal research ethics (IPB Animal Care and Use Committee IACUC approval ID: 163-2019). A total of 47 IPB-D1 chickens were used for this study. All chickens were provided by the Animal Genetics and Breeding Division, Faculty of Animal Science, IPB University, Indonesia. Feeding was given twice a day. Chickens were fed 100% commercial feed, 70% commercial feed and 30% rice bran, and 60% commercial feed and 40% rice bran for starter (0-4 weeks), grower (4-12 weeks), and finisher (12-21 weeks), respectively. Clean drinking water is always available. The ND vaccine was administrated to chickens at 3 and 7 days old. At 21 weeks old, blood samples were collected from axillary vein on the wing.

Primer designing

Sequence data for primer designing was obtained from the National Center for Biotechnology Information (NCBI) with the Gen Bank access number AY621317.1 (Xiao et al. 2004). The primer sequences in this study is: forward (F): 5'-CCCACAGAGCATCCATGAGG-3' and reverse (R): 5'-TTGCTGTTGTTGCAGGGTTG-3'. These primers produced a 411 bp DNA sequence covering partial intron 1 to intron 2. The primers were designed using the Primer Designing Tool application (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

DNA isolation and amplification

To obtain genomic DNA template, the blood DNA extraction procedure was performed using a Blood/Cell

DNA Mini Kit (Geneaid) according to the manufacturer's instructions. The amplification of AVBD2 gene fragments was carried out using a PCR machine (Gene Amp PCR System 9700, Applied Bio Systems) in a total of 23 μL premix consisting of 2 μL DNA template, 0.3 μL primers, 12.5 μL GoTaq® Green Master Mix (Promega), and 8.2 μL Nuclease-free Water. The PCR conditions used consisted of three stages. The first stage was the pre-denaturation process (95 ºC for 5 minutes). The second stage carried out 35 cycles consisting of denaturation (95 ºC for 10 sec), annealing (62 ºC for 20 sec), and extension (72 ºC for 30 sec). The third stage is the final extension (72 ºC for 5 minutes). PCR products were visualized in 1.5% agarose gel using PeqGreen DNA/RNA Dye (VWR) staining under UV light (Alpha Imager, Alpha Innotech). Direct sequencing was then carried out for each sample using the 1st Base sequencing services in Selangor, Malaysia.

Data analysis

The sequencing results in the form of a chromatogram were then analyzed using the MegaX (Kumar et al. 2018) and BioEdit (Hall et al. 2011) for DNA sequence alignment and for DNA sequence visualization, respectively. Genetic diversity was analyzed by calculating genotype frequency, allele frequency, and heterozygosity values based on Nei & Kumar (2000).

RESULTS AND DISCUSSION

Identification of mutations in partial AVBD2 gene

This study has successfully amplified the 411 bp partial AvBD2 gene in IPB-D1 chickens (Figure 1). A total of 10 SNPs were found in intron 1 (3 SNPs), exon 2 (3 SNPs), and intron 2 (4 SNPs) of the AvBD2 gene (Table 1). One of the mutations in exon 2 was also reported by Morammazi & Habibi (2017) in local Iranian chicken populations, i.e. g.5002C>T. Meanwhile, the other nine mutations have never been reported previously. All mutations found, the types of mutations and amino acid changes can be found in Table 1.

Six out of ten SNPs found were classified as transition mutations, i.e g.4853G>A, g.4859T>C,
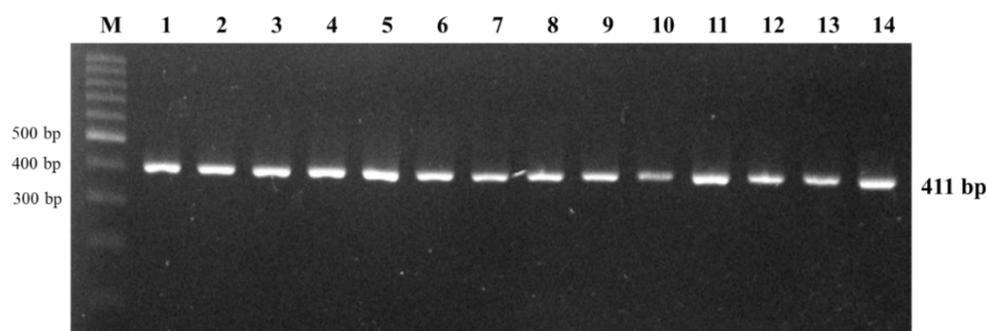
Figure 1. PCR result of partial AvBD2 gene in IPB-D1 chicken. M = DNA ladder 100 bp; 1,2,..14 = sampel

Table 1. Point mutations found in intron 1 to intron 2 of the AvBD2 gene in IPB-D1 chickens along with the amino acid changes occurred

No.	Mutation Position	Location in AvBD2	Mutation Type	Amino Acid (Codon) Change
1.	g.4843T>A	Intron 1	Transversion	-
2.	g.4853G>A	Intron 1	Transition	-
3.	g.4859T>C	Intron 1	Transition	-
4.	g.4881A>G	Exon 2	Transition	-
5.	g.4889G>A	Exon 2	Transition	-
6.	g.5002C>T*	Exon 2	Transition	Ala (GCT) to Val (GTT)
7.	g.5075C>T	Intron 2	Transition	-
8.	g.5111T>G	Intron 2	Transversion	-
9.	g.5116G>T	Intron 2	Transversion	-
10.	g.5177G>T	Intron 2	Transversion	-

*also reported by Morammazi and Habibi (2017)
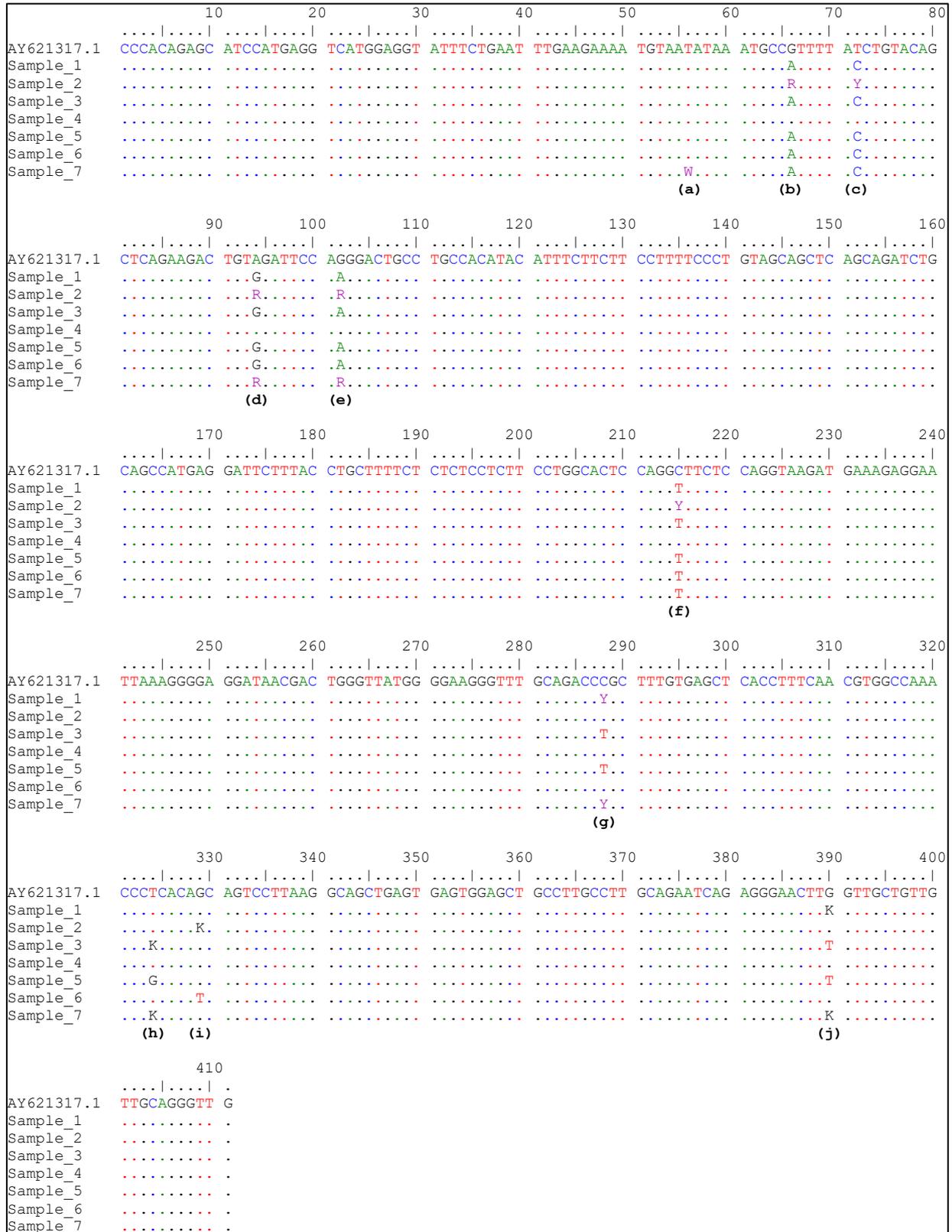
Figure 2. Partial sequence of AvBD2 gene in IPB-D1 chicken including 10 SNPs position, i.e. g.4843T>A (a), g.4853G>A (b), g.4859T>C (c), g.4881A>G (d), g.4889G>A (e), g.5002C>T (f), g.5075C>T (g), g.5111T>G (h), g.5116G>T (i), and g.5177G>T (j)

Table 2. Genotype frequency, allele frequency, and heterozygosityof AvBD2 gene in IPB-D1 chicken

SNP	N	Genotype Frequency			Allele Frequency		Ho	He
g.4843T>A	47	TT 0.936	AT 0.064	AA 0	T 0.968	A 0.032	0.064	0.062
g.4853G>A	47	GG 0.021	AG 0.191	AA 0.787	G 0.117	A 0.883	0.191	0.207
g.4859T>C	47	TT 0.043	TC 0.170	CC 0.787	T 0.128	C 0.872	0.170	0.223
g.4881A>G	47	AA 0.043	AG 0.234	GG 0.723	A 0.160	G 0.840	0.234	0.268
g.4889G>A	47	GG 0.021	GA 0.255	AA 0.723	G 0.149	A 0.851	0.255	0.254
g.5002C>T	47	CC 0.021	CT 0.191	TT 0.787	C 0.117	T 0.883	0.191	0.207
g.5075C>T	47	CC 0.319	CT 0.362	TT 0.319	C 0.500	T 0.500	0.362	0.500
g.5111T>G	47	TT 0.660	TG 0.277	GG 0.064	T 0.798	G 0.202	0.277	0.323
g.5116G>T	47	GG 0.723	GT 0.255	TT 0.021	G 0.851	T 0.149	0.255	0.254
g.5177G>T	47	GG 0.298	GT 0.362	TT 0.340	G 0.479	T 0.521	0.362	0.499

N = total sample; Ho=observed heterozygosity; He=expected heterozygosity

g.4881A>G, g.4889G>A, g.5002C>T, and g.5075C>T. While the g.4843T>A, g.5111T>G, g.5116G>T, and g.5177G>T mutations indicated a transversion mutation. Transition mutations are changes in nucleotide substitution that have the same structure, such as substitution between purine with purine (A>G) or pyrimidine with pyrimidine (C>T). This transition mutation does not change hydrogen bonds, while a transversion mutation is a change in nucleotide substitution that has a different structure, ie. in purine to pyrimidine, or pyrimidine to purine (Luo et al. 2016). Transitions are more conservative in their effects on proteins than transversions that may cause a weaker effect of transitions on structure and function of gene regulation (Stoltzfus & Norris 2016). Figure 2 describes the AvBD2 gene sequence alignment in IPB-D1 chickens.

According to Table 1, there are 3 mutations located in exon 2, i.e g.4881A>G, g.4889G>A, and g.5002C>T. Both g.4881A>G and g.4889G>A located in the non-coding region, which are not translated into amino acids. Meanwhile, the g.5002C>T is located in the coding region. This g.5002C>T mutation also alters the amino acid Alanine to Valine. This missense mutation was also reported by Morammazi & Habibi (2017).

Nie et al. (2018) mentioned that Valine is one of 3 branched chain amino acids (BCAAs), including leucine (Leu) and isoleucine (Ile). These BCAAs play an important role in the regulation of energy homeostasis, nutrient metabolism, immunity and disease resistance in humans and animals (Nie et al. 2018). The non-synonymous mutation of the AvBD2 gene plays an important role in the antimicrobials found in different muscle tissues (Hellgren et al. 2010). This Alanine to Valine mutation needs to be further studied to understand its effect on disease resistance in chickens.

Avian beta defensin gene family has been reported to have strong association with chicken bacterial resistance. A total of 5 SNPs at the AvBD5 gene were reported to be associated with Salmonella susceptibility in China local chicken (Zhang et al. 2020). Using same chicken population, four SNPs at the AvBD14 gene were also associated with Salmonella susceptibility (Zhang et al. 2020). To our knowledge, no studies has been reported the association between the AvBD2 gene with bacterial resistance in chicken.

Polymorphism Information of the AVBD2 Gene

Table 2 shows a total of 10 SNPs found in this study, and each SNP has 2 alleles and 3 genotypes except g.4843T>A which has 2 genotypes (TT and AT) of 2 alleles (A and T). Individuals with genotype AA were not found at g.4843T>A. All alleles on each SNP had a frequency higher than 0.01 (1%), which indicated that all SNPs in the AVBD2 gene were polymorphic in the IPB-D1 chicken population. In accordance with the explanation of Nei & Kumar (2000) that a population with 2 or more alleles with a frequency value of more than 0.01 or 1% indicates that the gene is polymorphic. The highest allele frequency at g.4843T>A, g.4853G>A, g.4859T>C, g.4881A>G, g.102G> A, g.5002C>T, g.5111T>G, g.5116G>T, and g.5177G>T are the T, A, C, G, A, T, T, G and T alleles, respectively. Whereas at the g.5075C>T, both C and T alleles have the equal frequency (0.5).

Heterozygosity value calculations were carried out to determine how high the level of genetic diversity at a locus based on allele frequencies (Wang et al. 2015). All loci were found to have Ho values lower than 0.5, this indicated that all loci had a low level of diversity (Allendorf et al. 2013). Apart from being an indicator of the level of diversity, the comparison of Ho and He is used to indicate the occurrence of that mentioned by Allendorf et al. (2013). Of the ten loci found, 7 loci had lower Ho values than He, i.e g.4853G>A, g.4859T>C, g.4881A>G, g.5002C>T, g.5075C>T, g.5111T>G, and g.5177G>T. Ho value which is lower than He may indicate the occurrence of inbreeding in the IPB-D1 chicken population.

Previously, it was explained that the mutation of the nucleotide C to T in the missense mutation g.5002C>T in exon 2 is known to change the amino acid Alanine to Valine. Results of the amino acid sequence prediction showed that the T allele will produce the amino acid Valine which plays an important role in immunity and disease resistance. Based on Table 2, the T allele at the g.5002C>T locus has a much higher frequency (0.883) than the C locus (0.117). This indicates that most of the IPB-D1 chicken population tends to have good resistance. However, this proposal needs to be strengthened by further studies.

CONCLUSION

In this study, a total of 10 SNPs were found in the intron 1 to intron 2 of the AvBD2 gene in the IPB-D1 chicken population. All SNPs are polymorphic. A total of 7 SNPs were found in intron 1 (g.4843T>A, g.4853G>A, g.4859T>C) and intron 2 (g.5075C>T, g.5111T>G, g.5116G>T and g.5177G>T). While the other 3 SNPs were found in exon 2 (g.4881A>G, g.4889G>A, g.5002C>T). A missense mutation was found in g.5002C>T which changed the amino acid Ala to Val. This g.5002C>T has the potential as a candidate marker for disease resistance in IPB-D1 chickens.

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