Jurnal Biologi Tropis

Original Research Paper

Genetic Polymorphism of g.3768T>C loci of NRAMP1 gene in Kampung and KUB Chickens

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Article History
Received : April 25th, 2024
Revised : May 01th, 2024
Accepted : May 13th, 2024

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Abstract: Natural Resistance-Associated Macrophage Protein 1 (NRAMP1) is a gene that regulates the body's resistance to illness. The genetic diversity of Kampung and KUB chicken population in Lombok island West Nusa Tenggara province was investigated based on g.3768T>C loci of NRAMP1 gene. Experimental animal consisted of 105 KUB and 67 Kampung chickens. The NRAMP1 gene polymorphisms in both population were identified using PCR-RFLP method. Genetic parameters of g.3768T>C loci were calculated using PopGene32. In this study, the locus g.3768T>C of the NRAMP1gene found three genotypes (TT, CT, CC) with the frequency of the C allele was higher than the T allele, respectively 0.709 and 0.210 in Kampung chicken, and 0.738 and 0.262 in KUB chicken. The mean value of observed heterozygosity (Ho) and expected heterozygosity (He) of g.3768T>C loci in both populations revealed low to medium genetic diversity, with the values 0.222 and 0.400, respectively. The mean value of observed heterozygosity is lower than expected, indicating that the population is heading towards a heterozygosity deficit and proving that the two populations are not in Hardy-Weinberg equilibrium. The mean PIC value of the NRAMP1 gene (g.3768T>C) in both chicken population was 0.319 and classified as fairly informative (0.50 > PIC > 0.25). Meanwhile, the value of the genetic differentiation index (Fst) for Kampung and KUB chicken population were 0.385 and 0.433, indicating that there is differentiation between two chicken population at the observed loci.

Keywords: Fst and chickens, heterozygosity, NRAMP1 gene, polymorphism.

Introduction

Indonesian chickens have high diversity, with different morphological characteristics. Some Indonesian chickens have unique characteristics and are only found and developed in certain areas. For example: Sentul chickens have several varieties of feather colors, developed in Ciamis, West Java (Sartika and Iskandar 2007); Pelung chickens have a uniquely beautiful voice, developed in Cianjur West Java (Iskandar and Susanti 2007); and Merawang chickens have a dominant Columbian feather color, developed in Merawang, Bangka Belitung Islands (Hasnelly et al. 2006). Apart from that, there are Indonesian chickens that do not have special characteristics and are spread across various regions of Indonesia, namely Kampong chickens. Indonesian chicken productivity, such as growth and egg production, is still relatively low (Nataamijaya, 2016). On the other hand, Indonesian chickens have advantages such as being able to adapt very well to tropical environmental temperatures, utilizing low-quality feed sources, and being relatively resistant to several infections/diseases.

Disease is one of the factors that has a negative impact on animal husbandry. The resistance to many diseases varies between poultry breeds and varieties. This is mostly determined by genetic factors or the existence of specific alleles in livestock (Hu *et al*., 2011). Several studies have revealed that genetic variables play a significant role in the

management of poultry resistance and susceptibility to diseases. The natural resistance-associated macrophage protein 1 (NRAMP1) gene has been extensively implicated in several poultry types. The *NRAMP1* gene has been identified as a functional candidate gene that has disease resistance activity in a number of animal species and in human populations (Desmond *et al*. 2019).

Chicken resistance to infectious agents is a phenotypic trait, which is an expression of genetic factors and is influenced by the environment. The genetic component is a group of genes that control the body's immune system against infection. Natural Resistance-Associated Macrophage Protein 1 (NRAMP1) is a gene that regulates the body's resistance to illness. Several intercellular infection pathogenes are influenced by the NRAMP1 gene (Irda *et al.,* 2021). He *et al.* (2013) found a strong correlation between chicken resistance to *S. enteritidis* and a mutation at the NRAMP1 gene's g.24101991A>T location. Several studies have shown that the NRAMP1 gene is associated with tuberculosis in human populations (Li *et al.*, 2022); Salmonella disease in rats (Janiszewski *et al.,* 2021); Tuberculosis disease in cows (Yuan *et al.,* 2021); The nature of pig immunity (Vashchenko *et al.*, 2022); Salmonellosis and virus infections in goats and sheep (Worku, 2017).

In this paper, we investigated the genetic diversity of 2 native chicken populations (Kampong and KUB chicken) -based on g.3768T>C loci of NRAMP1 gene as DNA markers for the nature of resistance to infection. The results of this study are expected to provide scientific information for designing breeding strategies and conservation plans. According to Li *et al.* (2020), the value of heritability for the nature of infection resistance in chickens is relatively low (0.20), therefore, genetic markers can help increase the effectiveness of selection through procedures known as marker assisted-selection (MAS).

Materials and Methods

Total Samples and DNA Extraction

The research samples were collected at the Agricultural Technology Assessment Center, West Nusa Tenggara, Indonesia, as many as 172 chickens consisting of 105 KUB chickens and 67 village chickens. Blood samples for DNA genotyping were collected on a Venoject tube with K2EDTA_ and preserved at -25°C for several weeks. The isolation of genomic DNA from blood samples was performed using the Wizard Genomic kit following the manufacturer's instructions (Promega, Madison, WI, USA).

PCR-RFLP Amplification

The PCR reaction was performed in a 15µL reaction mixture containing 1 µL of genomic DNA, 0.2 µL of forward and reverse primers, 7.5 µL of MyTaq Red Mix, and 6.1 µL of ddH_2O. Initial denaturation at 95°C for 1 minute, followed by 35 cycles at 94°C for 10 seconds, 60°C for 15 seconds, 72°C for 15 seconds, and final extension at 72°C for 1 minute. Primer sequences for NRAMP1 gene amplification were F: CAATGAGACGGTGTCTGTGG (forward) and R: CCCAGAAGAAATCTCTCTGC (reverse). The NRAMP1 gene polymorphisms in chickens were validated using the RFLP method. About 5 µL of DNA amplification product was digested in 0.7 µL buffer, 1 µL ddH_2O, and 0.3 µL SacI restriction enzymes for 4 hours at 37°C. The NRAMP1 gene fragments were separated using 2.5% agarose gel electrophoresis and then visualized using an AlphaImager EP system.

Data analysis

SNP position determination was corrected to Ensembl's DNA sequence using the MEGA-X program (ENSCHIG00000024611). PopGene software (Version 32) was used to determine effective numbers of alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), Shannon's Information Index (I), fixation index (Fis), and differentiation index (Fst) for data as depicted in Tables 1 and 2.

Results dnd Discussion

Polymorphism of The NRAMP1 Gene

An SNP was discovered at position g.3768 T>C in the NRAMP1 gene. SNP g.3768 T>C was found in three chicken genotypes: CC, TC, and TT (Figure. 1). Genotypes CC had 258 and 163 bp; TC had 163, 258, and 421; and TT had 421. The genotype distribution does not become consistent with Hardy Weinberg's law at the

significance level of 0.05. Table 1 displays the genotypes, allele frequencies, and Genetic diversity measures in Indonesian chicken populations across exon 11 of the NRAMP1 gene.

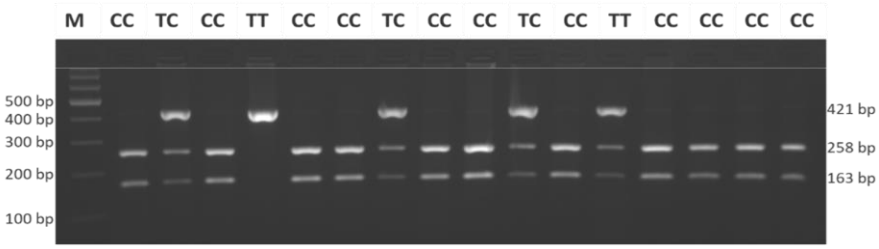

Figure 1. SacI restriction enzyme-based NRAMP1 PCR-RFLP genotyping. M= 100-bp ladder size standard; genotypes CC, CT, and TT

Allelic and genotypic identification of exon 11 of the NRAMP1 gene using the *RFLP*-SacI technique produces two alleles namely C and T alleles with three genotypes TT, CT, and CC (Figure 2). The distribution of allelic frequency of the C allele was higher than the T allele, respectively 0.709 and 0.210 in Kampung chicken, and 0.738 and 0.262 in KUB chicken, while the frequency distribution of the genotypes TT, CT, and CC were respectively: 0.164; 0.254 and 0.582 in Kampung chicken, and 0.152, 0.219 and 0.629 in KUB chicken (Table 1). The Chi-square (χ^2) test showed that the genotype distributions of exon 11 of the NRAMP1 gene were not at Hardy-Weinberg equilibrium (H-WE) in Kampung and KUB chicken (Table 2). The genotype frequencies at polymorphic loci of exon 11 of the NRAMP1 gene showed a highly significant difference ($P<0.01$). This is similar to the exon 11 of the NRAMP1 gene in SenSi-1 Agrinak Chickens, as reported by Ardiyana et al. (2020) where the allelic frequencies distributions between the C and T allele differed, the C allele (0.858) being higher than that of the T allele (0.142).

Table 1. Allele and Genotype Frequencies of the NRAMP1|SacI exon 11 gene

SNP POSİTİON	CHİCKEN	N	Genotype Frequencies			Allele Frequencies	
			TT	TC	CC	T	C
g.3768T>C	Kampung	67	0.164	0.254	0.582	0.210	0.709
rs14616006	KUB	105	0.152	0.219	0.629	0.262	0.738
	Total	**172**					

All chicken populations were dominated by CC genotypes, while the TT genotype was rare among the population studies. Previous studies have shown that the SNP at exon 11 of the NRAMP1 gene is polymorphic in some chicken breeds, such as five local Chinese chickens (He et al., 2013), Malaysian native chickens (Tohidi et al. 2013), Indonesian native chickens (Muhsinin et al. 2016), Kampung Chicken (Hadrawi et al. 2016), and SenSi-1 Agrinak chickens (Ardiyana et al., 2020). This study demonstrated that NRAMP1|SacI exon 11 in chicken populations is polymorphic since each gene fragment has three genotypes (TT, TC, and CC) and two alleles with the C allele were significantly higher than the T allele. It was supposed that breeding selection and management caused the high frequency of the C allele in chicken populations. There is a tendency for breeders to prefer chickens with the C allele over the T allele, resulting in the accumulation of certain genotypes.

Gene frequency is influenced by selection, mutation, population mixing, internal crossing, outer crossing, and genetic drift (Al-Koofee and Mubarak, 2019). The accumulation of the C allele in both chicken populations may also be caused by natural selection, where the C allele shows higher resistance to pathogens, especially salmonella enteritidis. Ardiyana et al. (2020) reported the frequency of the C allele was higher than the T allele in the concentration of

immunoglobulin Y (IgY), antibodies titers against *S enteritidis*, and ND. Some previous research also reported that the C allele of the NRAMP1 gene has a significant association with *Salmonella enteritidis* load in the poultry population (Kramer et al. 2003, Liu et al. 2003, Fanny et al. 2010, Tohidi et al. 2013, and Desmond et al. 2019).

Genetic diversity within the chicken breeds

The genetic diversity of a population is represented by the number of alleles, expected heterozygosity (He), observed heterozygosity (Ho), and Polymorphic Information Content (PIC) values as the fundamental data for individual and population discrimination (Seo et al., 2016). Data in Table 2 indicate a genotypic imbalance in the two chicken populations where the observed heterozygosity was significantly different from the Hardy-Weinberg expectation agreement. In this study, the low level of Ho and He values revealed low genetic diversity in the two chicken populations. The occurrence of heterozygosity deficits in the population is caused by selection pressure at certain loci and the possibility of inbreeding.

By definition, expected heterozygosity represents the probability that a randomly selected individual from a population in Hardy Weinberg equilibrium is heterozygous, whereas observed heterozygosity indicates the effective proportion of heterozygous individuals at each locus (Carco et al., 2018). The results of measuring the heterozygosity value of g.3768T>C loci in all samples are presented in Table 2. The mean value of observed heterozygosity (Ho) and expected heterozygosity (He) of g.3768T>C loci in Kampung and KUB chicken populations revealed low to medium genetic diversity, with the values 0.222 and 0.400, respectively. The expected heterozygosity (He) value is higher than observed heterozygosity values (Ho) for both chicken population. Ho and He values can be used to determine genetic imbalances in a population.

The mean value of observed heterozygosity obtained in this study is lower than expected, indicating that the population is heading towards a heterozygosity deficit and proving that the two populations are not in Hardy-Weinberg equilibrium (Table 5). Several factors can contribute towards a heterozygotes deficit. First, the locus is under selection pressure. Second, inbreeding may be common in the population. Third, the Wahlunds effect because the presence of a population substructure (Nei, 1987; Peter et al., 2007) and genotyping errors are likely due to low sample quality (Morin et al., 2009). In our research, this result suggests a divergence from Hardy–Weinberg equilibrium (HWE) and the probability of inbreeding due to strong selection (Barrandeguy and García, 2021).

Table 2. Genetic diversity of Kampung and KUB chicken populations across g.3768T>C of NRAM1 gene

SNP POSİTİON	CHİCKEN	N	Ho	He	PIC	p-value	Chi^2	F*st*
g.3768T>C rs14616006	Kampung	67	0.254	0.413	0.327	0.006	9.939	0.385
	KUB	105	0.219	0.387	0.312	0.000	19.726	0.433
	Total	172						

Ho= observed heterozygosity. He= expected heterozygosity. PIC= polymorphism information content. Chi^2 = Chi-square. F*st* = fixation index

Polymorphism Information Content (PIC) measures the quantity of information per microsatellite and depends on the number of identified alleles and allele frequency (Purwantini and Purwantini, 2010). PIC is the ability of a genetic marker to detect the *polymorphism* among individuals of a population, and often used to measure the informativeness of a genetic marker for linkage studies. In this study, the PIC value of the **NRAMP1 gene** (g.3768T>C) in both Kampung and KUB chicken was 0.327 and 0.312, respectively. According to Serrote *et al.* (2020), a PIC value ≥ 0.50 indicates a very informative locus, a PIC value 0.25 <PIC <0.50 indicates a fairly informative locus, and a PIC value ≤0.25 indicates a low informative locus. Thus, in recent research, the PIC value of **NRAMP1 gene** in both Kampung and KUB chicken populations

was classified as fairly informative (0.50 > PIC > 0.25).

In recent research, we also identified genetic diversity among populations using the F Wright statistic. Genetic differentiation index (Fst) is calculated to predict the proportion of total genetic variation due to inter-population differentiation, and the estimated value of the inter-population genetic differentiation index (Fst) is always positive. Population differentiation is substantial with high Fst. Wu et al. (2008) reported that when there is no differentiation, the Fst value is 0 and when the alleles between populations are quite different, the Fst value is equal to 1. Khan et al. (2021) also reported that the Fst values vary from 0 to 1, with 0 signifying panmictic populations or total genetic material sharing, while 1 implies that population structure explains all genetic variation and that the two populations have no genetic variety or are fixed. Fst<0.05 shows a small genetic difference, 0.05-0.15 moderate genetic difference, 0.15-0.25 high genetic difference, and Fst>0.25 great genetic difference (Khan *et al.*, 2021). Our estimated Fst values for Kampung and KUB chicken population were 0.385 and 0.433. According to the above scale, the entire population exhibited a significant genetic difference (Fst > 0.25).

Conclusion

The exon 11 of NRAMP1|SacI in Kampung and KUB chicken populations is polymorphic since each gene fragment has three genotypes (TT, TC, and CC) and two alleles with the C allele were significantly higher than the T allele. The accumulation of the C allele in both chicken populations was caused by breeder selection, or perhaps also natural selection where the C allele showed higher resistance to pathogens, especially salmonella enteritidis. Base on Genetic differentiation index (Fst), the entire population of Kampung and KUB chicken exhibited a significant genetic difference (Fst > 0.25).

Acknowledgment

This research was funded by (PNBP) Mataram University 2021, with a letter of agreement No: 2871/UN18.L1/PP/2021. Thanks to the Center for the Assessment of Agricultural Technology and CV. Lestari Jaya Farm, West Nusa Tenggara, Indonesia, on the use of the chicken sample. All authors participated equally in this paper's conception, analysis, and completion. The authors of this paper report no conflicts of interest.

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