Buletin Peternakan 49 (1): 28-34, February 2025

ISSTAP
Indonesian Society for Sustainable Tropical Animal Production

Bulletin of Animal Science

ISSN-0126-4400/E-ISSN-2407-876X Accredited: 36a/E/KPT/2016
http://buletinpeternakan.fapet.ugm.ac.id/

Doi: 10.21059/buletinpeternak.v%vi%i.99303

Identification of Gene Diversity of Melanocortin 4 Receptor for Commercial Rabbit Breeds

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Article history
Submitted: 21 August 2024
Accepted: 10 December 2024

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ABSTRACT

Molecular genetic markers are regularly implemented in rabbit management for better breeding by selecting for specific genotypes. This study aimed to examine the diversity of the melanocortin-4 receptor gene in rabbits in Central Java, Indonesia. A total of 35 bucks were used in this study, comprising 10 New Zealand White, 11 Hyla and 14 Hycole. The DNA was extracted by Gene JET Whole Blood Genomic DNA extraction kit. Single Nucleotide Polymorphism (SNP) was detected by Polymerase Chain Reaction (PCR) and DNA sequencing. Hot carcass weight, cold carcass weight, reference weight and cut point 1-5 weight were among the parameters that were measured. This study showed that Melanocortin 4 Receptor (MC4R) genes were polymorphic, containing missense mutations and one SNP at 519G→A SNP 519GA had two alleles, A and G, with three genotypes (AA, GG, and AG). The rabbit population did not achieve Hardy-Weinberg Equilibrium (HWE) and showed a low level of genetic diversity as indicated by the heterozygosity results. The result of this study concluded that the frecuency AG as a genotype was higher than either GG or AA and A had a higher allele frequency than G. We recommended improving the validity of the data across various rabbit populations.

Keywords: *Commercial rabbits, Diversity, MC4R gene, SNP*

Introduction

Rabbits (*Oryctolagus cuniculus*) are utilized not only as potential livestock for producing high-quality meat, skin, and fur but also as pets, ornamental animals, and subjects for research experiments. Due to their ability to adapt, rabbits have been widely distributed throughout various regions (Yusni, 2019). Rabbit meat is acknowledged for its high protein content (approximately 22%) and high levels of unsaturated fat (32.7 – 35.7 mg/100g) (Siddiqui *et al*., 2023). Moreover, it contains low levels of cholesterol (47 mg/100g of loin) and sodium (42 mg/100g), resulting in a viable meat for consumers (Cullere and Zotte, 2018). It contains more vitamin B (Ezema and Eze, 2015). It is regarded as one of the most valuable meat sources for humans (Dalle Zotte *et al*., 2016; Martins *et al*., 2018). Common rabbit breeds used for commercial purposes due to their rapid growth and high productivity are New Zealand White (NZW), Hyla, and Hycole (Setiaji *et al*., 2022).

Molecular genetic markers are commonly applied to rabbit management to boost mating and selection processes by selecting preferred genotypes (Hirose *et al*., 2014). These markers are likely to advance some traits such as milk production, meat quality, growth, and development (Singh *et al*., 2014). Additionally, the markers are capable of being applied to enhance selection accuracy and advance the genetic improvement of important economic traits (Radwan *et al*., 2022). Previous studies have demonstrated a relationship between candidate gene polymorphisms and carcass traits in rabbits. For instance, Sternstein *et al*. (2014) reported that SNPs in the myostatin gene were correlated with body weight in rabbits.

The Melanocortin 4 receptor (MC4R), a member of the G-protein-coupled receptor family, was expressed in the hypothalamus and played a role in regulating food intake, metabolism, and body weight (El-Sabrout, 2017). In rabbit, growth parameters such as body weight were associated with polymorphisms in the MC4R gene (Fontanesi *et al*., 2013). The potential of the MC4R gene as a candidate for production traits has been identified. Our study aimed to examine the diversity of the MC4R gene in rabbits from Central Java, Indonesia.

Materials and Methods

Animals

This study was conducted at the Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang. A total of 35 bucks were used in this study, comprising 10 NZW, 11 Hyla, and 14 Hycole with an average body weight of 2.10 ± 0.17 kg

Blood Collection

Blood samples were collected and extracted. Approximately 1 mL of blood was drawn from the marginal vein of each rabbit's ear using a disposable syringe. The blood samples were placed in tubes containing EDTA for DNA extraction and subsequently stored in a cold gel ice box.

DNA Extraction

DNA isolation was performed using a Gene JET Whole Blood Genomic DNA Extraction Kit, following the manufacturer's instructions. The quality was assessed using agarose gel electrophoresis before further analysis.

Primer and PCR

The primers used in this study were as follows: forward (F): 5'-CATGAACTCCACCCACCAC-3' and reverse (R): 5'-CTCATAGCACCCTCCATCAGACTAG-3', based on the MC4R GenBank (ID: 127492371), with a DNA product size of 127 bp (Fontanesi *et al*., 2013). Polymerase Chain Reaction (PCR) was used to amplify fragments of the MC4R gene spanning the 5'-flanking region, exon 1, and the intron 3'-untranslated region (3'UTR). The PCR was performed in a total reaction volume of 50 µL, containing 25 µL PCR kit, 19 µL PCR water (ddH2O), 1 µl each of the forward and reverse primers, and 4 µL of genomic DNA. The PCR protocol included pre-denaturation at 95ºC for 1 min, denaturation at 95ºC for 15 s, annealing at 56.4ºC for 15 s, and extension at 72ºC for 55 s, followed by 35 cycles. The quality of the PCR products was visualized by electrophoresis using 1% agarose and a Gel Documentation System (GDS). All PCR samples were sequenced by Genetika Science to determine the position of SNPs.

Data Analysis

The MEGA 11 (Molecular Evolutionary Genetic Analysis) software was used for genotyping. Genetic and allele frequencies were calculated using the allele frequency prediction formula. The Hardy-Weinberg equilibrium was confirmed using the chi-square test.

Allele Frequency (Nei and Kumar, 2000)

$$\chi_i = \frac{(2N_{ii}+N_{ij})}{(2N)},\ \chi_j = 1-\chi_i \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where:
Xi = allel frequency -i
Xj = allel frequency -j
Nii = number of homozygous individuals -ii (homozigot)
Nij = number of heterozygous individuals -ij (heterozigot)
N = number of individuals observed

Genotype Frequency (Nei and Kumar, 2000)

$$\chi_{ii} = n_{ii} / n \quad \ldots\ldots\ldots\ldots\ldots (2)$$

Where:
χ_{ii} = number of genotype frequency -ii
n_{ii} = number of genotype frequency ii
n = number of samples observed

Hardy-Weinberg Equilibrium (Albakri *et al*., 2022)

$$\chi^2 = \sum (Obs-Exp)^2 / Exp \quad \ldots\ldots\ldots (3)$$

Where:
χ^2 = *chi-square*
Obs = observation value
Exp = expected value

If χ^2 count > χ^2 table, then populations are not in Hardy-Weinberg Equilibrium.
If χ^2 count < χ^2 table, then population is in Hardy-Weinberg Equilibrium.

Observed Heterozygosity (H_o) dan Expected Heterozygosity (H_e) (Nei and Kumar, 2000)

$$Ho = \sum_{i \neq j} \frac{n_{ij}}{N} \quad \ldots\ldots\ldots\ldots\ldots (4)$$

$$He = 1 - \sum_{i=1}^{q} x_i^2 \quad \ldots\ldots\ldots\ldots\ldots (5)$$

Where:
Ho = observed heterozygosity
He = expected heterozygosity
n_{ij} = number of heterozygous individuals
N = number of indiviuals observed
x_i = allele frequency -i
q = number of alleles

The H value ranges from 0 (zero) to 1 (one). If the H value is close to 0, the heterozygosity is categorized as low, but if the H value is close to 1, the heterozygosity is categorized as high.

Results and Discussion

DNA Extraction

The electrophoresis results are presented in Figure 1. The quality of the PCR products was assessed using 1% agarose gel electrophoresis and a Gel Documentation System (GDS) to evaluate the DNA extraction outcomes. The DNA was extracted from three rabbit breeds, resulting in samples with thick and clear DNA bands, indicating good quality. These results indicated that thicker bands aligned with higher DNA concentrations. Conversely, thin or smeared DNA bands showed lower DNA quality, possibly due to contamination (Mohamed *et al*., 2020). Various environmental factors, such as temperature and humidity, were found to affect DNA concentration during the pre-analysis phase (Khorwal *et al*., 2024), along with

the duration of blood sample storage. Prasetyo and Sanjaya (2023) showed that degradation possibly occurred during the DNA extraction process due to the presence of nuclease enzymes, which led to damage to nucleic acids, polysaccharide contamination, and secondary metabolites, all of which had an impact on DNA quality.

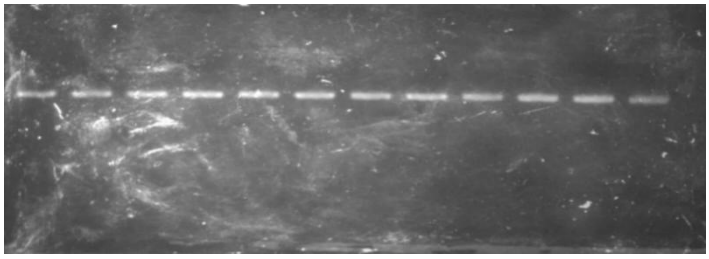

Figure 1. DNA isolation product

DNA Amplification

The PCR technique was carried out to amplify the MC4R gene DNA sequence. DNA fragments exceeding 100 bp are generated, indicating that the DNA products are derived from the entire genome (Figure 2.). The PCR products were visualized using 1% agarose gel electrophoresis and documented with a Gel Documentation System (GDS). As shown in Figure 2, the PCR product appeared as a thick, bright, and clear band. The PCR analysis produced a 1689 bp product, which matched the target length of the MC4R exon 1 gene fragment, which spanned 127 bp from positions 148 to 544. The presence of the thick band suggested that the amplification results were of good quality. In this study, all 35 DNA samples exhibited a single, clear band without any smearing. The PCR products were subsequently sequenced to detect the SNP locations and determine the genotypes of the rabbits.

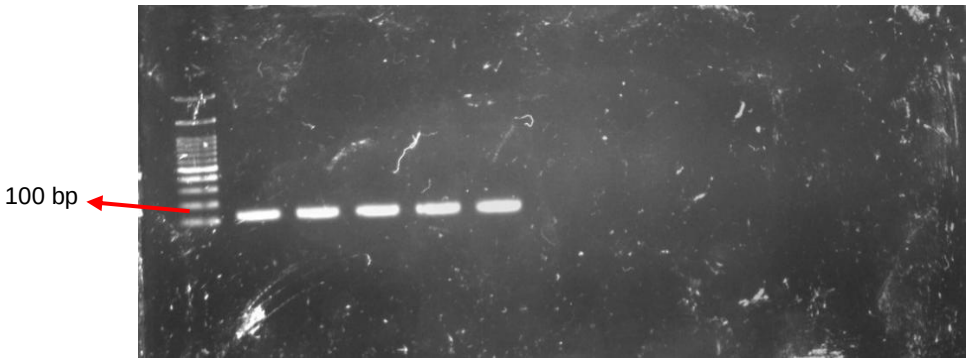

Figure 2. Visualization of PCR product

SNP Identification and Genotyping

The SNP site in the targeted sequence was identified through sequencing, while the homozygous and heterozygous genotypes were determined through the peak patterns. MEGA 11 was conducted to generate the alignment, with the MC4R gene reference sequence of *Oryctolagus cuniculus* (127 bp) from the NCBI database (Gene ID: 127492371) as the outgroup. The results of identification and alignment are displayed in Figure 3. SNP identification revealed three genotypes: AA, GG, and AG. The results indicated that the presence of an SNP (Single Nucleotide Polymorphism) triggered a single mutation characterized by a transition substitution mutation 519G→A. Lonneti *et al*. (2016) stated that SNPs were able to alter specific genetic loci within the DNA sequence, leading to variation among members of species.

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[                    111 111 111 122 222 222 223 333 333 333 444 444 444 455 555 555 556 666 66]
[          123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 45]

#NZW_15    GCA GCT ATG GAC TGC ACA GCA ATG CCA GCG AGT CCC TGG GGA AGG GCT AGT CTG ATG GAG GGT GC
#NZW_14    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#NZW_13    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#NZW_12    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#NZW_11    ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#NZW_9     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#NZW_7     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#NZW_6     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#NZW_5     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#NZW_1     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_14     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HL_12     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HL_11     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_10     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_17     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_16     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_15     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_14     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_12     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_10     ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_9      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_8      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_7      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_6      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_5      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_2      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HC_1      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_3_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A.. ... ... ... ... ... ..
#HL_4_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_5_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_6_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_7_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_8_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HL_9_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
#HC_4_Edit ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..
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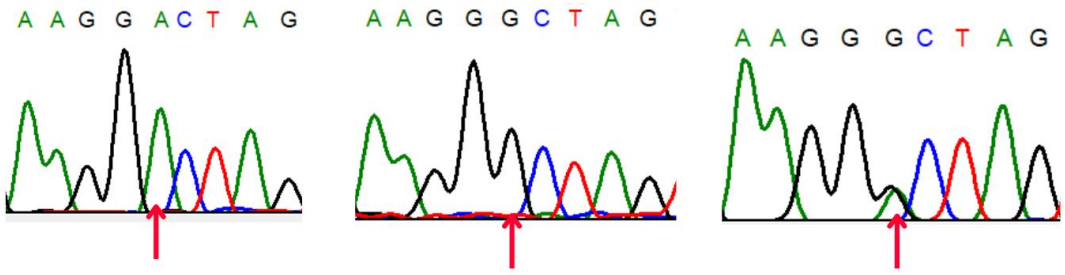
(a)



(b)

Figure 3. (a) SNP position 519G→A; (b) Chromatogram MC4R Gene

Gene Diversity

The SNP 519G→A has two alleles A and G which is AA, GG, and AG genotype (Table 1.). Allele and genotype frequencies are presented in Table 1. The results indicated that among the three rabbit breeds, the AG genotype was the most prominent, accounting for 60%, followed by the AA and GG genotypes at 23% and 17%, respectively. These studies were consistent with previous research by Nahacky *et al*. (2020), which reported that the AG genotype was the most frequent (48%), followed by the AA and GG genotypes (38% and 14%, respectively). The genotype frequencies observed in this study were 0.17 (GG), 0.6 (AG), and 0.23 (AA). The allele frequency for the A genotype was found to be 53%, while the G alelles was 47%, indicating that the A alleles was more dominant in the rabbit population. This observation aligned with the findings of Jiang *et al*. (2008), who reported that among five rabbit breeds (Harbin white rabbit, Tianfu black rabbit, Belgia hare ZIKA rabbit and California rabbit breeds), the A allele was the dominant allele in the MC4R gene. The effect of genotypes on performance traits demonstrated that AG was significantly associated with body weight and feed conversion effiency.

Table 1. Alleles Frequency and Genotype MC4R Gene

Parameter	Genotype			Allele		H	χ^2
	GG	AG	AA	A	G		
Observation	0.17 (6)	0.6 (21)	0.23 (8)				
Expectation	7.78	17.44	9.78	0.53	0.47	49.84	1.45
Frequency	0.17	0.60	0.23				

The heterozygosity value of the three rabbit breeds was 49.84%. The observed heterozygosity average (H_o) and expected heterozygosity (H_e) were calculated to assess

genetic diversity within the population, providing valuable information on genetic variability (Edea *et al*., 2015). Nei (1987) stated that the average percentage of heterozygous loci in each individual or population was the most effective method to characterize genetic variation. All outcomes exceeded the critical value of the chi-square table (1.45). Based on the analysis of the MC4R gene, the rabbit population was in Hardy-Weinberg Equilibrium (HWE). According to Allendorf *et al*. (2013) a hardy-weinberg equilibrium state on some population caused by constant allele and genotype frequency from generation to generation that may had an impact from random mating in a wide population. The high chi-square value showed a deviation from HWE, indicating that allele and genotype frequencies in the rabbit population were unstable across generations (Albakri *et al*., 2022).

The MC4R gene had a low diversity value (π) among the three rabbit breeds, resulting in 0.00855. Nei (1987) classified genetic diversity values into three categories: low (0.1-0.4), medium (0.5-0.7), and high (0.8-1.0). One of the factors influencing the low diversity value was inbreeding, as it reduced the diversity of available alleles. Inbreeding harmed phenotypic performance caused by several lethal recessive mutations at homozygous loci (Howard *et al*., 2017). Low genetic diversity could diminish the overall genetic variability within the livestock population. Owour *et al*. (2019) stated that low genetic diversity was detrimental to a population as it might lead to the loss of certain lineages that possessed desirable traits. A potential strategy for mitigation is to establish pedigree records, followed by a deliberate breeding strategy to diversify the specific traits. The SNP are possible to be associated with productive traits that can improve the genetic quality of rabbits in Indonesia.

On site underwent a transitional substitution in the MC4R gene sequence. This variable site, located at the 46th position of the fragment, separated the three rabbit breeds from the comparison species, *Oryctolagus cuniculus*. The 46th site on the fragment had a transitional substitution mutation, resulting in a change in the amino acid A → G (Adenine → Guanine), observed in all three rabbit breeds (NZW, Hyla, and Hycole), particularly in NZW samples 15, 12, 9, 5, 1; HL samples 11, 9, 8, 7, 5, 4; and HC samples 1 and 4. These results revealed that these populations were closely related, such as species within the same genus. Mutations play a significant role in livestock breeding, as they can affect economically valuable traits, such as growth rate. It depends on their specific impact on protein function or regulatory elements (Czerniawska-Piątkowska and Kowalewska-Łuczak, 2020).

Conclusion

The study of the MC4R gene in three rabbit breeds revealed that the AG genotype was the most common, with allele A being dominant. The genetic diversity value was relatively low, indicating potential inbreeding and a deviation from Hardy-Weinberg Equilibrium. The identification of a transitional substitution mutation proved close genetic relationships among the breeds, emphasizing the importance of strategic breeding for better genetic variability.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding statement

This research has been supported by the Universitas Diponegoro (Grant no. 569-82/UN7.D2/PP/VII/2024).

Acknowledgement

The authors thank the World Class University of Universitas Diponegoro for supporting international collaboration through the student exchange program 2024 Batch II.

Author's contribution

Conception: Asep Setiaji, Sutopo; Methodology: Firda Tasya Kamila, Alfa Putri Latifa, Zata Nabilah, Ananda Dwi Agustine; Data Analysis: Firda Tasya Kamila; Validation: Dela Ayu Lestari, Enny Tantini Setiatin; Writing manuscript-original: Firda Tasya Kamila; Writing manuscript-review/revision: Mamat Kamalludin Hamidi, Syaddad Verahry Philco; others: Asep Setiaji.

Ethics approval

The experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Animal and Agricultural Sciences, Universitas Diponegoro (Approval No. 59–01/A-01/KEP-FPP).

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