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Research Article

Identification of the *Myostatin* Gene (MSTN/HAEIII) Exon-3 Polymorphism in Pitalah Ducks in West Sumatra, Indonesia

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ABSTRACT

Introduction: Pitalah ducks are one of Indonesia's local genetic resources that can be utilized to develop quality livestock products. The present study aimed to identify the diversity of the *Myostatin* gene (MSTN/HaeIII) exon-3 in Pitalah ducks, a local duck breed from West Sumatra, Indonesia.
Materials and methods: The present study utilized 40 samples of DNA from the blood of Pitalah ducks (11 males and 29 females, with an average body weight of 1,450 g and an average age of 12 months) that were intensively reared at the Andalas University animal husbandry unit in Indonesia. DNA from blood samples was isolated using the genomic DNA Extraction Kit. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used as the method. The isolated DNA was then amplified using a primer that produced a 438 bp fragment of the MSTN gene. The amplification product was restricted using the HaeIII enzyme, which recognizes the cutting site at GG↓CC.
Results: Based on the findings of 40 samples, 65% of homozygous truncated genotypes (+/+), 35% heterozygous genotypes (+/-), and no homozygous untruncated genotypes (-/-) were found. Based on the current results, two genotypes were identified. The current findings indicated genetic diversity, as the frequency of each allele exceeds 1%. The allele frequency (+) was 0.825, the allele (-) was 0.175, and the observed heterozygosity was greater than the expected heterozygosity.
Conclusion: The allele frequency of the MSTN/HaeIII exon-3 gene in Pitalah ducks is polymorphic (diverse) within the Pitalah duck population. Genotype frequencies of the MSTN/HaeIII exon-3 gene are in Hardy-Weinberg equilibrium.

1. Introduction

One of the growing poultry businesses in Indonesia is duck farming. The duck population in Indonesia has continued to grow year by year, reaching 58,351,458 heads in 2022[1]. Many farmers in Indonesia choose to breed local ducks because local ducks are a source of Indonesian livestock germplasm, easy to cultivate, and the seeds are easily and rapidly accessible. West Sumatra has four types of local ducks that are the genetic resources of Indonesian local livestock and must always be preserved. One of the four types of ducks is the Pitalah duck. But unfortunately, ducks in West Sumatra have not been selected as broiler ducks[2]. Suhaemi and Febriani[3] stated that Pitalah ducks have a higher economic value than Bayang ducks as broilers. Suhaemi[4] also reported that overall, the production potential of Pitalah ducks is superior to Kamang ducks and Bayang ducks. Pitalah ducks have potential as broilers and layers, while Kamang ducks have potential as broilers, and Bayang ducks are superior for average egg weight.

The development of Pitalah ducks can be achieved by enhancing their genetic quality through the application of selection and genetic engineering techniques. One selection that can be done is molecular selection, which does not need a long time and can produce livestock with high economic value, and can be passed on to their offspring. Molecular selection is done by evaluating the nucleotide sequence profile of genes in DNA that can affect livestock productivity

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in livestock improvement. Characterization and identification of local duck population diversity is important to do in the identification of genetic resources and their development. One gene that plays a role in growth is the *myostatin* (MSTN) gene.

The MSTN gene is part of the growth subgene (Transforming Growth Factor/TGF-β) and is useful in regulating the growth of muscle mass[5]. Mardiah et al.[6] stated that the MSTN is an economically important gene that functions as a regulator of growth. The MSTN gene is located on the seventh chromosome, composed of one promoter, three exons, and two introns with a gene length of 6,649 bp and produces 375-376 amino acids[7]. The MSTN gene is predominantly expressed in skeletal muscle[8]. *Myostatin* functions as a negative regulator of muscle growth in poultry and other animals. Disrupting this negative regulator plays a key role in promoting muscle development in chickens[9]. Identification and characterization of MSTN gene diversity can be done by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

The MSTN gene in kampong and broiler chicken populations is polymorphic[10]. Nisa et al.[11] stated that there is diversity in the MSTN gene part of exon 3 in the local duck population. MSTN gene in Kerinci ducks is polymorphic[12]. Mutations of the MSTN gene also affect the performance of Pekin ducks. Seventeen SNPs have been detected in the fowl MSTN gene, indicating that this gene is highly polymorphic[13]. Until now, the linkage of the MSTN gene diversity of the Pitalah duck has never been done. The present study aimed to demonstrate the diversity of the MSTN exon-3 gene in Pitalah ducks, which revealed the identification and diversity of the MSTN gene, without establishing a direct link to muscle growth.

2. Materials and Methods

2.1. Ethical approval

Animal experiments were conducted following the Republic of Indonesia Law No. 18 of 2009 (section 66), which addressed animal keeping, raising, killing, and proper treatment and care.

2.2. Time and location

The study was conducted at the Livestock Biotechnology Laboratory, Faculty of Animal Husbandry and Biomedical Laboratory, Faculty of Medicine, University of Andalas, Indonesia, from March to April 2024.

2.3. Sampling

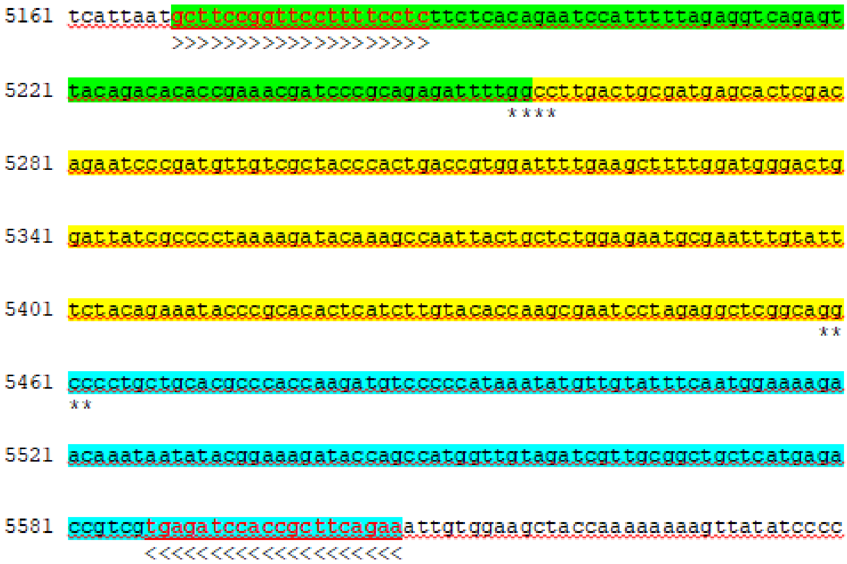
A total of 40 Pitalah duck blood samples (11 males and 29 females with an average body weight of 1450 g and age of 12 months) were obtained by taking blood using a disposable syringe from the brachial vein, under the wing. The blood was taken from Pitalah ducks that were intensively reared at the UPT Faculty of Animal Husbandry, Andalas University, Indonesia. Before sampling, the blood collection site was cleaned and disinfected, and a sterile syringe was used. A total of ± 1 ml of chicken blood was taken by a disposable syringe 20G in the area under the wing, precisely the brachial vein. Blood was drawn carefully to prevent clotting and to maintain sample quality, then accommodated in a vacutainer tube containing EDTA, which was then stored at -20°C.

2.4. DNA isolation

The DNA isolation was performed using a DNA Extraction Kit (Intron G-SpinTM Total DNA Extraction Kit, produced by iNtRON Biotechnology, South Korea). A total of 100 µl of Pitalah duck blood sample, along with 100 µl of CL buffer solution, was put into a 2 ml tube. A total of 20 µl of proteinase K and 5 µl of RNase were added to the sample and homogenized. Subsequently, 200 µl of BL buffer was added and mixed thoroughly. The mixture was then incubated for two minutes at room temperature, followed by 10 minutes at 56°C. After incubation, it was centrifuged briefly to remove condensation. Next, 200 µl of absolute ethanol was added, mixed, and centrifuged. The resulting mixture was transferred into a spin column centrifuge for one minute, then the column was placed into a new two ml collection tube. Following this, 200 µl of Western analysis (WA) buffer was added to the spin column, which was then centrifuged for one minute at 13000 rpm. The flow-through was discarded. Then, 200 µl of WB buffer was added, centrifuged, and the flow-through was discarded again. The spin column was transferred to a new tube and centrifuged for three minutes at 13,000 rpm to remove residual ethanol. Finally, the spin column was placed into a 1.5 ml tube, and 30 µl of capillary electrophoresis (CE) buffer was added. The sample was incubated for one minute to complete the process. The process continued with centrifugation at 1300 rpm for one minute, and 20 µl of CE buffer was again incubated for one minute and centrifuged for one minute at 13000 rpm.

2.5. DNA amplification

The isolated DNA sample was put into the PCR tube as much as 1-2 µl and 18-19 µl premix solution. The premix solution is composed of 10 µl I-Max II, 2 µl forward-reverse primer mixture, and 6-7 µl nuclease-free water. The primer was designed using Primer[3] by the NCBI reference sequence NC_051778.1. Forward primer is a primer located at the front end of the DNA target and serves to mark the front end of the DNA strand to be duplicated; in other words, forward primer will go from the 5’ end to the 3’ end. Meanwhile, the reverse primer is located at the back end of the DNA target[14]. This mixture was incubated in a PCR machine for amplification. The amplification process began with an initial denaturation step at 95°C for three minutes. The second stage consisted of 35 cycles, with each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for one minute. The last stage was final elongation at 72°C for five minutes. The annealing temperature was obtained from the temperature optimization process using eight temperatures. So that from this optimization, the optimal temperature was 54°C. The primers and cut points of the HaeIII enzyme are presented in Figure 1.



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5161 tcattaatgcttccggttccttttcctcttctcacagaatccatttttagaggtcagagt
             >>>>>>>>>>>>>>>>>>>>

5221 tacagacacaccgaaacgatcccgcagagattttggccttgactgcgatgagcactcgac
                                       ****

5281 agaatcccgatgttgtcgctacccactgaccgtggattttgaagcttttggatgggactg

5341 gattatcgcccctaaaagatacaaagccaattactgctctggagaatgcgaatttgtatt

5401 tctacagaaatacccgcacactcatcttgtacaccaagcgaatcctagaggctcggcagg
                                                               **

5461 cccctgctgcacgcccaccaagatgtcccccataaatatgttgtatttcaatggaaaaga
     **

5521 acaaataatatacggaaagataccagccatggttgtagatcgttgcggctgctcatgaga

5581 ccgtcgtgagatccaccgcttcagaaattgtggaagctaccaaaaaaagttatatcccc
           <<<<<<<<<<<<<<<<<<<<
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Figure 1. Position of primer attachment (>>>> = forward and <<< = reverse) on *myostatin* gene sequence with HaeIII cutting site on GG|CC

2.6. Electrophoresis

DNA amplification results were visualized through electrophoresis with agarose gel 1.5%. The gel was made of 1.5 g agarose and 100 ml of 0.5xTBE heated in a microwave at medium-high temperature for two minutes. The gel was printed on a printing tray and left to set. A total of 5 µl of amplification product was taken and then electrophoresed at a voltage of 100 V for 60 minutes, namely until the DNA fragments finished migrating in the gel. Electrophoresis uses a 50 bp ladder. The results of DNA electrophoresis can be seen with the help of UV light[15].

2.7. Genotyping

Genotyping was carried out using the RFLP technique[16]. The materials used in this genotyping process were 5 µl amplification product, 1 µl HaeIII enzyme, 2 µl buffer R, and 12 µl nuclease-free water. The selection of the HaeIII restriction enzyme in the present study was based on the fact that HaeIII was very easy to obtain commercially because it is a common enzyme used to detect mutations in DNA fragments. The HaeIII enzyme has a blunt-end cutting type, which is a type of cutting that produces sequences with blunt ends because the cutting is symmetrical. The HaeIII enzyme, in cutting the MSTN exon-3 gene, was incubated at 37°C for 60 minutes on agarose gel 2%. The electrophoresed DNA samples were observed under UV light. DNA fragments that appeared from the electrophoresis results were compared with the marker to determine the length of the fragment and its genotype.

2.8. Data analysis

Genotype frequency was calculated with the following formula[17].

$$x_{ii=}\frac{n_{ii}}{N}$$

Allele frequency was calculated using the formula according to Nei and Kumar[17] as follows.

$$xi = \frac{(2nii + \sum j \neq i\ nij)}{2N}$$

In population genetics, allele and genotype frequencies are essential for assessing genetic diversity. The frequency of the i-th allele is denoted by x_i, while the frequency of the i-th genotype is represented as x_{ii}. The number of individuals possessing the homozygous genotype ii is referred to as n_{ii}, whereas n_{ij} denotes the number of individuals with the heterozygous genotype ij. These variables are fundamental for calculating genetic parameters and evaluating the structure and variability within a population.

Heterozygosity can be calculated using the formula.

$$Ho = \sum_{k}^{s} Wk \sum_{i\neq j}^{q} Xkij \;; He = 1 - \sum_{k}^{s} |Wk \sum_{i}^{q} X_{ki}^{2}$$

Genetic diversity within a population can be evaluated using several key parameters. Observed heterozygosity (Ho) represents the proportion of individuals in a population that are heterozygous at a given locus, while expected heterozygosity (He) refers to the proportion expected under Hardy-Weinberg equilibrium. Another important measure is the effective population size (Wk), which reflects the number of individuals in a population who contribute offspring to the next generation, thus influencing genetic variation over time. Additionally, X^{k}_{ij} denotes the frequency of genotype ij in population k, which is crucial for estimating genetic structure and variation across populations.

To determine whether the allele frequency and genotype frequency of the MSTN gene are in balance, p2 + 2pq + q2 was tested by chi-square. The Hardy-Weinberg law balance test was carried out as follows.

$$X^2 = \sum_{i=1}^{k} \frac{(Oi - Ei)^2}{Ei}$$

The chi-square test (X^2) is commonly used in genetics to evaluate whether observed genotype frequencies deviate significantly from expected frequencies under Hardy-Weinberg equilibrium. In this context, O represents the observed number of individuals with the i-th genotype, while E denotes the expected number of individuals with that genotype based on theoretical predictions. By comparing these values, the chi-square test helps determine if a population is in genetic equilibrium or if evolutionary forces such as selection, mutation, or genetic drift may be acting on it.

3. Results and Discussion

3.1. DNA isolation

DNA extraction aimed to obtain high-quality DNA suitable for further analysis, which is of high purity. DNA purification is a process to separate DNA from cell lysates (proteins, carbohydrates, lipids) and other contaminants[18]. The quality testing process of DNA isolation results using the Nanodrop 2000 Spectrophotometer method. The results obtained in testing the quality of the DNA isolation results of 40 Pitalah duck samples indicated variations in the amount of concentration and purity of the DNA. Of the 40 samples, the DNA concentration ranged from 7.1-288.2 ng/µl, and DNA purity between 1.12-1.87.

The difference in DNA concentration would result in differences in band thickness, which occurs due to different physical treatments and the ability of the extraction buffer to break down cells[19]. A high concentration of DNA isolation results will produce a band that looks qualitatively bright and thick.

However, according to Muladno[20], DNA isolates are pure if they have purity values ranging from 1.8-2.0. This indicated that there are some contaminated DNA samples. If the ratio is < 1.8, it indicates that the DNA still contains protein/phenol and has too little DNA compared to other protein substances[21].

3.2. Amplification of the Myostatin gene

The results of the amplification of the MSTN exon-3 gene obtained PCR products along 438 bp were visualized with a UV transilluminator on a 1.5% agarose gel for 60 minutes with a voltage of 100 volts, which can be seen in Figure 2.

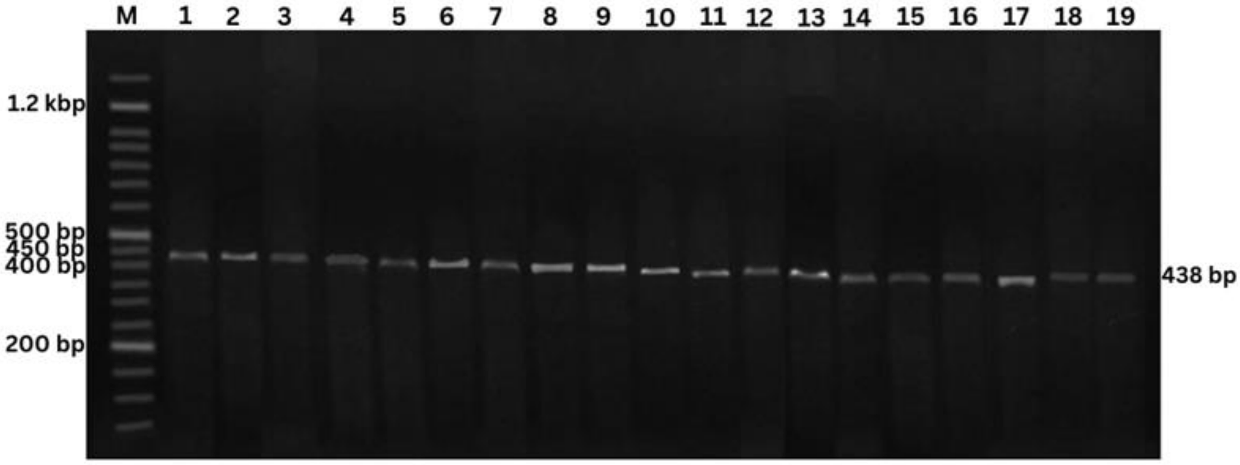

Figure 2. The amplification results of the *Myostatin* gene were visualized using 1.5% agarose gel electrophoresis. Lanes labeled 1 to 19 correspond to individual sample numbers, representing the PCR products obtained from each sample. The lane marked M contains the 50 bp DNA marker, which served as a molecular size reference to estimate the length of the amplified DNA fragments

Based on the PCR results shown in Figure 2, the amplification of the MSTN exon-3 gene in Pitalah ducks using a specific primer pair was successful across all samples. This is demonstrated by the presence of a single band at approximately 438 base pairs in each lane. According to Sritunyalucksana et al.[22], clear, bright, and intact bands without smearing indicate good-quality DNA amplification. However, if the annealing temperature is too low, primers may bind non-specifically to other regions of the DNA, leading to poor-quality or non-specific amplification results.

The annealing temperature greatly affects the process of primer attachment to the DNA template. Too high annealing temperature causes the primer to not stick well to the template, which is characterized by the thinner the band formed, while the low annealing temperature causes the primer to stick to an unspecific attachment site, which will then cause the amplification of unwanted locus fragments[23].

3.3. Genotyping of the Myostatin gene

The results of genotyping the MSTN exon-3 gene in Pitalah ducks using the HaeIII enzyme in Figure 3 indicated two kinds of banding patterns of cutting results. The first pattern was four bands with sizes of 438 bp, 204 bp, 147 bp, and 87 bp. The banding pattern observed indicated that 14 samples exhibit a heterozygous (+/−) genotype, characterized by the presence of both the full-length amplification fragment and additional smaller fragments. The second pattern, identified in 26 samples, demonstrated only three distinct bands at approximately 204 bp, 147 bp, and 87 bp, corresponding to the homozygous truncated (+/+) genotype.

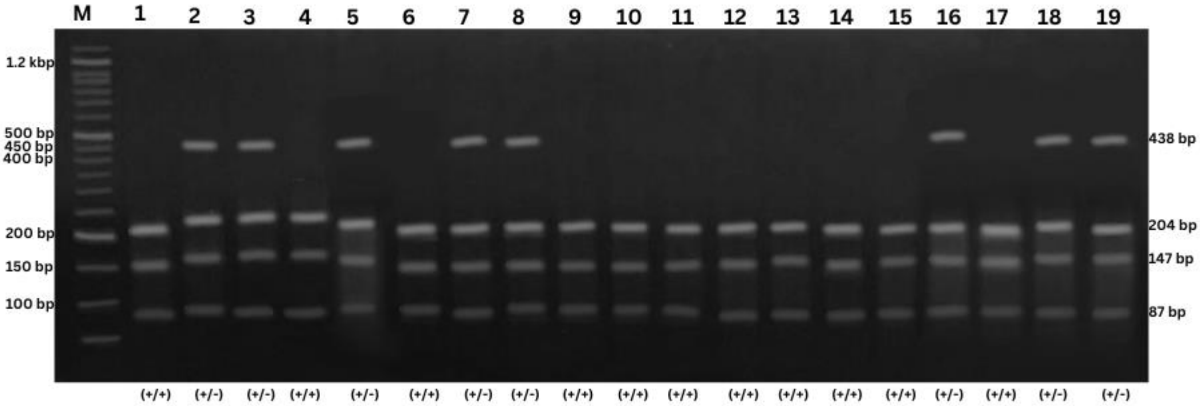

Figure 3. Restriction result of the *Myostatin* /HaeIII gene in Pitalah ducks. Numbers 1 to 19 are sample numbers, M: Marker (50 bp)

Genetic diversity was identified through the PCR-RFLP analysis of the MSTN/HaeIII gene fragment, as two alleles were detected, including + and −, resulting in two genotypes heterozygous (+/−) and homozygous (+/+). The homozygous (+/+) genotype indicated that both parents contributed the same allele to the offspring, and the heterozygous (+/−) genotype reflected different alleles inherited from each parent, demonstrating genetic variation within the population.

3.4. Genotype frequency and allele frequency

Genotype frequency is the ratio of genotypes to the total population, while allele frequency is the ratio of alleles found at a population locus[17]. Genotype and allele frequencies were obtained based on the results of genotyping observations on sampled individuals, as shown in Table 1 and Figure 4.

Table 1. Genotype and allele frequencies of *Myostatin* /HaeIII gene in Pitalah ducks

Genotype type	Number of Individuals	Genotype Frequency	Number of alleles		Allele frequency	
			+	-	+	-
(+/+)	26	0,65	52	0		
(+/-)	14	0,35	14	14	0,825	0,175
(-/-)	0	0	0	0		
Total	40	1	66	14		

(+/+): Truncated homozygous genotyped individuals, (+/-): Heterozygous genotyped individuals, and (-/-): Homozygous genotyped individuals are not truncated

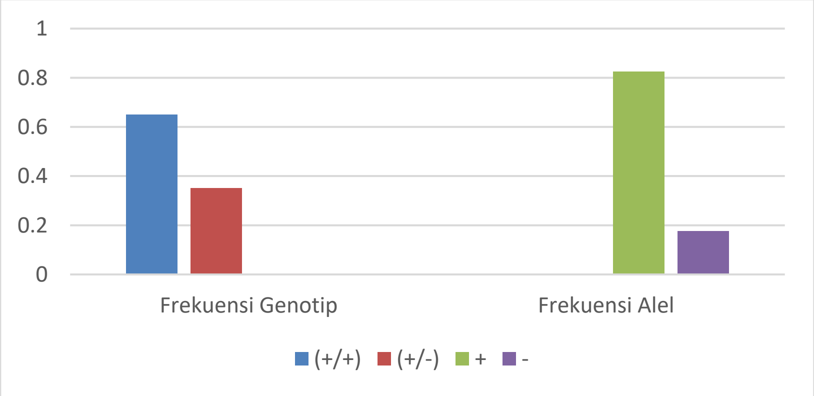

Figure 4. The genotype and allele frequency in Pitalah ducks

Based on the current results, MSTN/HaeIII in Pitalah ducks revealed two genotypes, including the truncated homozygous genotype (+/+) and the heterozygous genotype (+/-). The truncated homozygous genotype (+/+) was the most prevalent at 0.65, while the heterozygous genotype accounted for 0.35. The homozygous untruncated genotype (-/-) was not detected in MSTN/HaeIII in Pitalah ducks.

Based on the data analysis, the MSTN gene in the observed Pitalah duck population is considered diverse (polymorphic), as the allele frequencies are ≤ 99% which aligns with the findings outlined by Nei and Kumar[17] and Allendorf et al.[24], who stated that a gene is classified as polymorphic when no single allele exceeds a frequency of 99%, indicating the presence of genetic variation within the population.

3.5. Heterozygosity

Anggraeni et al.[25] stated that the value of heterozygosity is obtained from the calculation of genotype frequency and allele frequency at each locus. The difference in variation between individuals is shown in the heterozygosity value. The results of the current study obtained heterozygosity values as shown in Table 2.

Table 2. Heterozygosity value of *Myostatin* /HaeIII gene in Pitalah ducks

No	Heterozygosity	Value
1	Ho	0.35
2	He	0.29

Ho: Heterozygosity of observation, He: Heterozygosity expectancy

The Ho value is greater than the He value (Ho > He) from the calculation of the heterozygosity value. Based on the study by Khaerunnisa et al.[26], the observed heterozygosity (Ho) in the Sentul, F1 crossbreeds of Kampung × Layer, F1 crossbreeds of Kampung × Cobb broiler, and F2 crossbreeds of Kampung × Cobb broiler chicken populations was higher than the expected heterozygosity (He). This finding suggested that there was no evidence of intensive inbreeding within these populations, indicating a relatively high level of genetic diversity. According to Rell et al.[27], the factors that influence differences in heterozygosity values are mutation rate, migration, population size, selection, and mating patterns. Khaerunnisa et al.[26] stated that the MSTN gene of exon 2 in Indonesian chickens is polymorphic. In the present study, the association of MSTN |BSA gene polymorphisms with chicken carcass characteristics in Indonesian chickens was demonstrated, indicating that MSTN may be an important candidate gene for chicken carcass characteristics.

3.6. Hardy-Weinberg equilibrium

A population can be said to be in Hardy-Weinberg equilibrium if the genotype frequency p2 +2pq+q2 =1, where p^2 represents the frequency of the homozygous dominant genotype, 2pq represents the frequency of the heterozygous genotype, and q^2 represents the frequency of the homozygous recessive genotype. This equation assumes that allele and genotype frequencies remain constant from generation to generation in the absence of evolutionary forces such as selection, mutation, migration, or genetic drift. Hardy-Weinberg equilibrium was closely related to genotype frequency and allele frequency. It was necessary to conduct a chi-square test to determine whether the data obtained in the current study were in Hardy-Weinberg equilibrium or not. Hardy-Weinberg equilibrium was tested with Chi-square (X2)[28]. The results of observations of Hardy-Weinberg equilibrium in the Pitalah duck population are presented in Table 3.

Table 3. Hardy-Weinberg equilibrium chi-square test in the Pitalah duck population

Hardy-Weinberg equilibrium	Genotype frequency (+/+)	(+/-)	(-/-)	Total	X2h	X2t (0.05)
O	26	14	0	40		
E	27.23	11.55	1.22	40	1.80	5.99
(O-E)2/E	0.06	0.52	1.22	1.80		

X2t (0.05) = 5.99, X2h< X2t (0.05) = not significantly different

Table 3 indicated that the results of testing the diversity of the MSTN exon-3 gene in Pitalah ducks were in Hardy-Weinberg equilibrium. Where the calculated X2 value is smaller than the X2 table, it means that the observed genotypes are not significantly different from the expected genotype frequency. The population is said to be in Hardy-Weinberg equilibrium if, statistically, the calculated X2 value is smaller than the X2 table value[24].

5. Conclusion

There are two types of genotypes in the MSTN/HaeIII exon-3 gene of Pitalah ducks, namely truncated homozygotes (+/+) and heterozygotes (+/-). The allele frequency of the MSTN/HaeIII exon-3 gene is polymorphic and varies within the Pitalah duck population. The genotype frequencies of the MSTN/HaeIII exon-3 gene in Pitalah ducks are in Hardy-Weinberg equilibrium. This approach enabled the evaluation of genetic traits related to growth, offering an insight into their potential as broiler-type poultry. Based on the current result, Future studies can explore the associations between genotypes and traits observed in Pitalah ducks, validation tests using sequencing methods, and direct assessments of muscle growth traits in Pitalah ducks, contributing to marker-assisted selection (MAS).

Declarations

Competing interests

The authors have declared no conflict of interest.

Ethical considerations

The author has reviewed all ethical problems, including plagiarism, consent to publish, data fabrication, and falsification.

Authors' contributions

Kusnadidi Subekti was responsible for conceptualization, designing the experiment, fund acquisition, and review. Nada Iswari Samara was responsible for supervision, experiments, sample collection, writing the draft, and editing. Firda Arlina was responsible for statistical analysis and review. All authors read and approved the final edition of the manuscript.

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Availability of data and materials

The data are available with a reasonable request from the corresponding author.

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