Identification of growth hormone receptor (GHR|AluI) gene polymorphism in grati-madura cattle and pamekasan-madura cattle population

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Abstract. The growth traits were one of the important economic characteristics in beef cattle. One of the growth genes that was suspected as a gene candidate for marker assisted selection (MAS) in Madura cattle was the growth receptor gene (GHR). This study aim was to detect the level of GHR gene polymorphism in Grati-Madura cattle and Pamekasan-Madura cattle populations. A total 86 blood samples of Madura cattle have been collected from the experimental barn of Beef Cattle Research Station and 51 blood samples of Madura cattle from Community Farms in Waru Subdistrict, Pamekasan Madura Regency. Blood samples were isolated using a zymo extraction kit. Detection of GHR growth hormone gene diversity using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with AluI restriction enzymes. Data analysis used Chi-Square test for genotype, allele frequencies and Hardy Weinberg Equilibrium (HWE). The analysis result showed that GHR genes was detected 3 genotypes namely AA, AG and GG, each with an allele frequency of 0.267, 0.733 and 0.559, 0.441, this shows that the population of Grati-Madura cattle and Pamekasan-Madura cattle were polymorphic with PIC values of 0.315 and 0.372 respectively and were in the moderate category. The observed genotype frequencies in Grati Madura population deviated from HWE, while in Pamekasan madura population was not deviated from HWE. The value of Ho, He PIC and Ne were 0.394, 0.392, 0.315, 1.886 and 0.498, 0.493, 0.372, 1.973 respectively. In conclusion, The GHR gene polymorphism in Grati-Madura cattle and Pamekasan-Madura cattle were polymorphic and very informative so that can be used as gene candidate for MAS.

1. Introduction
The GHR|AluI gene was one of the growth hormone gene candidates that have high variation in cattle. Growth hormone receptors (GHR) act as cell surface receptors that help mediate the effects of GH on somatic cell growth. GH affects growth and metabolism by interacting with a specific receptor called growth hormone receptor (GHR) on the surface of target cells [1] so that GHR affects the biological action of GH on targeted cells. The GHR gene is located on chromosome 20 [2]. Ge et al. [3] found that SNPs in exon 10 of the GHR genes coding for amino acid substitution, namely SNP in
the 200th position in the form of Ala/Thr and the 257th position in the form of Ser/Gly. The position of SNP 257 was more studied and detected by the PCR-RFLP method using the AluI restriction enzyme. Several previous studies have reported the relationship between the diversity of GHR | AluI genes with the growth traits of cattle in vivo and characteristics of Piedmontese beef [1], carcass fat in Bos taurus cattle [4], intramuscular fat [5] and muscle composition (intramuscular fat, protein and water content) [6]. Identification of GHR | AluI gene polymorphisms in Indonesian local beef cattle has not been widely reported. Several previous studies reported that the GHR|AluI gene was polymorphic in PO Grati [7], Pasundan [8], and Pesisir cattle but monomorphic in Bali cattle (Bos javanicus) [9].

Madura cattle were also local beef cattle that have high genetic diversity, but have not been widely explored, especially regarding growth genes. Madura cattle besides being developed in the in-situ area, namely the island of Madura, have also been developed outside the island of Madura (ex-situ). Beef Cattle Research Station was one of the ex-situ areas that has also developed Madura cattle from 2014 to the present. The exploration of the genetic diversity of Madura cattle was more focused on the growth trait which was one of the important economic characteristics of beef cattle. The growth trait was a quantitative trait that was controlled by many genes. Some of the genes related to growth traits that widely used in the study of candidate genes and associations in beef cattle were GH, GHR and Pit-1 genes, but only GHR was more informative and potentially as a candidate gene. This study aims to identify the GHR | AluI gene polymorphism in the Grati-Madura and Pamekasan-Madura cattle populations.

2. Materials and Methods
2.1. Animal and DNA Samples
A total 86 blood samples of Grati-Madura cattle have been collected from the experimental barn of Beef Cattle Research Station (BCRS) and 51 blood samples of Pamekasan-Madura cattle from community farms in Waru Subdistrict, Pamekasan Madura Regency (CFWP). Blood samples were collected from the jugular vein into 3-mL vaccutainer tubes containing K3E EDTA as anticoagulant. DNA isolation was performed using DNA extraction kit merck Zymo from whole blood samples and then stored at -20°C for further use.

2.2. PCR amplification and PCR-RFLP
The specific fragments containing SNPs of the GHR gene were amplified using primer pairs designed by [1]. The primer information used was given in Table 1. PCR reaction was performed in a total volume of 20 µL. 3 µl DNA containing of approximately (10-100) ng/µL of DNA, 0.2 µl of each primers, 10 µL PCR mix of MyTaqTM HS Red Mix (Bioline, USA), and 6.2 µl ddH2O to a final volume of 20 µl. The PCR conditions were pre-denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing for 15 s at 53.8°C, extension at 72°C for 10 s, and a final extension at 72°C for 5 min. PCR products were electrophoresed on 1.5% agarose gels, stained with GelRed®10,000X in water (Biotium, USA) and visualized under a G-BOX Gel Documetation System (Syngene, UK). The PCR products of GHR were digested with AluI restriction enzyme (New England Biolabs, USA). The digested fragments were electrophorezed on 2% agarose gels, stained with GelRed®10,000X in water (Biotium, USA) and visualized under a G-BOX Gel Documetation System (Infinity VX2).

Table 1. The primers were used to amplify specific fragments of the GHR gene in Madura cattle.

| Gene | GenBank Accession number | Amplicon Size (bp) | Location of Polymorphic Site | Primer Sequences (5' - '3) |
|------|------------------------|------------------|-----------------------------|--------------------------|
| GHR  | AF140284.1             | 342              | Exon 10                     | F: 5' - GCT AAC TTC ATC GTG GAC AAC-3' |
|      |                        |                  |                             | R: 5' - CTA TGG CAT GAT TTT GTT CAG-3' |
2.3. Data Analysis

2.3.1. Allele frequency.
Individual genotypes were determined based on differences in the number and size of RFLP bands. The genotype and allele frequencies were calculated according to the formula Nei & Kumar [10] with the following statistical model formula:

\[ x_i = \frac{(2N_{ii} + \sum_{j \neq i} N_{ij})}{2N} \]

where:
- \( N_{ii} \) = number of individual with ii genotype
- \( N_{ij} \) = number of individual with ij genotype
- \( N \) = number of sample

2.3.2. H-W Equilibrium.
Hardy-Weinberg (H-W) equilibrium was calculated according to the formula Kaps and Lamberson[11]with the following formula model:

\[ X^2 = \sum \frac{(o-e)^2}{e} \]

where:
- \( X^2 \) = Chi-square test
- \( o \) = number of observed in category i
- \( e \) = number of expected in category i

2.3.3. Heterozigosity.
Data of the heterozigosity in the Grati-Madura and Pamekasan-Madura cattle were calculated according to Weir[12] formula and the Polymorphism Information Content (PIC) value of GH, GHR and Pit1 gene were calculated according to [13] formula.

3. Results and Discussion

3.1. GHR[AluI Gene Fragment Amplification
The amplification of GHR | AluI gene fragments in this study was successfully amplified with annealing temperature of 53.8 °C showing that the GHR gene at exon 10 position with a PCR product length of 342 bp (Figure 1). The restriction results from GHR | AluI gene fragments in Grati-Madura cattle have two genotypes, namely the AG and GG while Pamekasan-Madura cattle have three genotypes, namely the AA, AG and GG. The AA genotype have three fragment (91 bp, 101 bp and 50 bp), the AG genotype have four fragment (191 bp, 151 bp, 101 bp and 50 bp) and the GG genotype have two fragment 191 bp and 151 bp Figure 2.

![Figure 1. GHR | AluI gene PCR products (342 bp) used a 50 bp marker.](image)
Figure 2. Genotype visualization of GHR gene restriction result using the AluI enzyme, 50 bp marker and 2% agarose.

3.2. GHR | AluI Gene Diversity

The results of genotype and allele frequencies analysis of the GHR | AluI gene in Madura cattle can be seen in Table 2.

Table 2. Genetic diversity of the GHR | AluI gene in Madura cattle at different locations.

| Gene | Location | n  | Genotype Frequency | Alle Frequency | He  | Ho  | X²   | PIC  | Ne   |
|------|----------|----|--------------------|----------------|-----|-----|------|------|------|
|      |          |    | AA     | AG            | GG  | A   | G    | He  | Ho  | X²   | PIC  | Ne   |
| GH   | IBCRS    | 86 | 0.000  | 0.535         | 0.465| 0.267| 0.733| 0.392| 0.394| 11.462| 0.315| 1.644|
| R    | CFWP     | 51 | 0.294  | 0.529         | 0.176| 0.559| 0.441| 0.493| 0.498| 0.227 | 0.372| 1.973|

Where: X² table (P<0.05): 3.841

Based on Table 2, it can be seen that the GHR | AluI gene in Grati-Madura cattle was found in three genotypes, namely AA, AG and GG with each genotype frequencies of 0.000, 0.535 and 0.465 and the allele frequencies of A = 0.267 and G = 0.733 while the GHR | AluI in Pamekasan- Madura cattle were also found in three genotypes with frequencies 0.294, 0.529 and 0.176, respectively and allele frequencies A = 0.559 and G = 0.441. Based on the results of this study, the genetic diversity of Madura cattle at each location are polymorphic. This was in accordance with Falconer and Mackay [14] that a locus to be polymorphic if one of the allele frequencies was less than 0.99. Based on Table 2, it can be seen that Grati-Madura and Pamekasan- Madura cattle have highest frequencies in AG genotype, while the highest allele frequencies was G alleles in Grati-Madura cattle and A allele in Pamekasan Madura cattle.

GHR gene polymorphisms was also reported polymorphic in several other local beef cattle (Table 3) such as polymorphic in Grati PO [7] and Pasundan cattle [8] but monomorphic in Bali cattle [9] with highest frequencies in the A allele. In Bos taurus cattle, GHR | AluI gene polymorphisms were also reported to be polymorphic with the highest frequencies in the A allele, including Holstein[15], Simmental [16] and FH Indonesia cattle [17].
Heterozygosity

Estimation of heterozygosity value was important in determining genetic variability in a population. Genetic diversity can also be seen from other indicators such as expectant heterozygosity (He), observed heterozygosity (Ho), PIC and Ne. Marson et al.[23] stated that the estimated Ho and He values were calculated to estimate the genetic diversity in the population in order to make it easier to select genetic sources for future generations. The results of the genetic diversity analysis of the GHR | AluI genes in the Madura cattle population in each location were presented in Table 2. The results of statistical analysis showed that the Madura cattle in the Grati population have He and Ho values of 0.392 and 0.394, respectively, while the Madura cattle in Pamekasan have the values of He and Ho of 0.493 and 0.472, respectively. Mulliadi and Arifin [24] stated that the high value of heterozygosity in a livestock population can also be caused by inbreeding mating. Madura and Pamekasan cattle were local beef cattle that were formed from crossing. Based on its history, Madura cattle was the result of crosses between Banteng and Bos indicus [25,26].

Based on Table 1, it can be seen that the PIC value in Madura cattle in Grati and Pamekasan populations were 0.315 and 0.372, respectively. The PIC value in this study was in the medium category. This refers to Botstein et al. [13] that there were 3 categories of PIC scores, namely low (≤0.25), moderate (0.25 <PIC <0.5), and high (≥0.5). Based on this classification, the GHR | AluI genes were in the moderate category. A moderate category PIC value indicates that the markers on the GHR gene was very informative and can be associated with growth traits in Madura cattle. The number of effective alleles (Ne) in Grati and Pamekasan Madura cattle were 1,644 and 1,973, respectively, this means that in each population were founded 2 alleles, namely the A and G alleles,

| Breeds                     | n   | Genotype Frequency | Allele Frequency | Referensi               |
|----------------------------|-----|--------------------|------------------|-------------------------|
|                            | AA  | AG     | GG     | A   | G   |                         |
| Holstein Bull              | 400 | 0.635  | 0.250  | 0.115 | 0.760 | 0.240 | Ardici et al. [15]       |
| Simental Bull              | 81  | 0.642  | 0.148  | 0.210 | 0.716 | 0.248 | Ardici et al. [16]       |
| Friesian Holstein(Indonesia)| 370 | 0.580  | 0.340  | 0.080 | 0.750 | 0.250 | Misrianti et al. [17]    |
| Polish-Holstein Friesian Cows| 395 | 0.699  | 0.266  | 0.035 | 0.832 | 0.168 | Olenksi et al. [18]      |
| Polish-Holstein Friesian Bulls| 477 | 0.791  | 0.203  | 0.006 | 0.891 | 0.109 | Olenksi et al. [18]      |
| German-Holstein Sire       | 315 | 0.914  | 0.073  | 0.013 | 0.951 | 0.049 | Hradecká et al. [19]     |
| Grati-PO                   | 186 | 0.661  | 0.253  | 0.086 | 0.788 | 0.212 | Hartati et al. [7]       |
| Pasundan                   | 119 | 0.412  | 0.429  | 0.160 | 0.626 | 0.374 | Putra et al. [8]         |
| Bali                       | 162 | 0.988  | 0.006  | 0.006 | 0.991 | 0.009 | Zulkharnaim et al. [9]   |
| Pesisir                    | 48  | 0.604  | 0.021  | 0.375 | 0.615 | 0.385 | Zulkharnaim et al. [9]   |
| Limousin                   | 21  | 0.238  | 0.095  | 0.667 | 0.286 | 0.714 | Zulkharnaim et al. [9]   |
| Piemontese                 | 213 | 0.240  | 0.500  | 0.260 | 0.420 | 0.580 | Di Stasio et al. [1]     |
| Madrasin                   | 14  | 0.857  | 0.142  | 0.000 | 0.925 | 0.075 | Arganata et al. [20]     |

Chi-square test (χ²) result on the genotype frequency of the GHR | AluI gene showed a difference between the observed and expected ratio or there wasn’t an H-W equilibrium in Grati Madura cattle population, whereas Pamekasan Madura cattle have in H-W equilibrium. Jakaria et al.[21] stated that a population was expressed in the Hardy-Weinberg equilibrium, if the genotype (p2, 2pq and q2) and allele frequencies (p and q) were constant from generation to generation. Factors that can disrupt the equilibrium in a population were mutations, selection, migration and genetic drift [14]. Vasconcellos et al.[22] also stated that the imbalance was also caused by genotypes accumulation, divided populations, mutations, selection, migration and mating in the same group / population (endogamy) that can cause an imbalance in the population.

### 3.3. Heterozygosity

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but they had different allele frequencies. In the Grati Madura cattle population, the highest frequencies were found in the G allele, whereas Madura population in Pamekasan found the A and G alleles with almost the same allele frequencies. Frankham et al. [27] stated that the number of effective alleles will be almost the same as the number of alleles if the allele frequencies were in balance. This was in line with the value of $X^2$ in the Pamekasan Madura cattle population that was in the HWE.

4. Conclusion

In conclusion, The GHR gene polymorphisms in Grati and Pamekasan-madura cattle were polymorphic and very informative so that they can be used as gene candidates for MAS.

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