



Genetic Diversity of Jawa-Brebes Cattle based on Reproductive Traits Markers of the Growth Hormone Gene

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ABSTRACT

The mutations of the growth hormone (GH) gene significantly affect the fertility traits of various cows including service per period and calving interval. Identification of GH gene polymorphism is crucial to preserve the genetic resources of Jawa-Brebes cattle. Thus, we identified the single nucleotide and genetic polymorphisms in Jawa-Brebes cattle based on intron 3 to exon 5 of the GH gene. The amplified products (1,135 bp) of the GH gene from 24 heads of Indonesian cows (Jawa-Brebes, Bali, Madura, and Peranakan Ongole [PO]) were sequenced using the 1st BASE sequencing method. Clustal W and phylogenetic analyses to detect single nucleotide polymorphism and relationship to other breeds were used in the study. A total of 13 variable sites with 10 parsimony and 3 singleton sites were detected in the GH gene within Jawa-Brebes cattle, namely 3 sites in intron 3, 6 sites in intron 4, and 4 sites in exon 5. Three Indels were detected in Jawa-Brebes cattle at the 1,540th (*g.1539_1540insT*), 1,549th (*g.1548_1549insG*), and 1,895th (*g.1894_1895insC*) positions. The genetic distance was 0.000 to 0.013 within Jawa-Brebes cattle. Jawa-Brebes cattle were genetically close to Indonesian local cattle (Bali, Madura, and PO cattle). In addition, Jawa-Brebes cattle had a closer relationship with *Bos indicus* than *Bos taurus*. The sequences of intron 3, intron 4, and exon 5 of GH gene were polymorphic. The study provided initial bioinformatics of GH gene sequence that might be used to preserve Jawa-Brebes cattle and as reproduction traits marker-assisted selections in further investigation.

Key words: Growth Hormone, Indonesia, Native cattle, Polymorphism

INTRODUCTION

Growth hormone (GH) is a pleiotropic hormone released by acidophilic cells of the anterior pituitary gland with crucial roles in lifespan regulation (Caputo et al. 2021). It is a primary endocrine modulator of various physiological functions including somatic growth, nutrition, and reproduction in mammals (Vélez and Unniappan 2021). In domesticated animals, GH is a putative genetic marker to identify various physiological processes (Valdez-Torres et al. 2020; Bayraktar and Özdemir 2022; El-Mansy et al. 2023; Gerasimov et al. 2023). Furthermore, GH mutation is associated with milk

production in dairy cows (Bangar and Magotra 2021; Bangar et al. 2022). It also affects the fertility traits of cows (Mullen et al. 2011) including service per period and calving interval (CI) (Anamika et al. 2022).

The GH gene in cattle is mapped in chromosome 19 consisting of four introns and five exons (Vukasinovic et al. 1999). Mutations of the GH gene in intron 3 and exon 5 are the loci studied intensively to evaluate the fertility traits of various cows. Mutations of the GH gene in exon 5 impact the CI, service per conception (S/C) and days open (DO) of Holstein cows (Amiri et al. 2018). Similarly, polymorphisms of the GH gene in exon 5 influence the DO and CI of Jersey cows (Komisarek et al. 2011).

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This mutation is also correlated with milk production in the Modicana cows (Bordonaro et al. 2020) and the prevalence of dystocia in the Holstein cows (Hadi et al. 2015). Furthermore, single nucleotide polymorphisms (SNPs) of the GH in the intron 3 gene influence the pregnancy rate of Holstein cows (Öner et al. 2017). Variants of the GH gene in intron 3 also affect the DO and CI of the Holstein cow (Amiri et al. 2018). Moreover, polymorphisms of the GH gene in intron 3 have effects on milk production traits in Sahiwal cows (Sachan et al. 2021).

Jawa-Brebes cattle, an Indonesian native breed, has distinctive reproductive traits (Fig. 1) (Hartanto et al. 2023). It is well-documented that the Jawa-Brebes cow has a notably extensive reproductive lifespan with a moderate CI (Budiyanto et al. 2023). Interestingly, the reproductive performance of the Jawa-Brebes cow is normal even with inadequate nutritional feed intake (Panjono et al. 2017). Therefore, Jawa-Brebes cattle is a preserved and protected high-reproductive indigenous cattle with potential meat production (Haq et al. 2020). However, the massive cross-breed mating (Adinata and Affandhy 2017) and there are no genetic markers (Hartanto et al. 2023) threaten the sustainability of Jawa-Brebes cattle. Identification of the diversity of genes related to reproductive characteristics is crucial to preserving Jawa-Brebes cattle.



Fig. 1: Indonesian Native Jawa-Brebes Cattle

Diversity of the GH gene has been observed in Indonesian local cattle (Agung et al. 2018; Hartatik et al. 2020; Dakhlan et al. 2022). However, there is no study conducted to observe the diversity of the GH gene in Jawa-Brebes cattle to the best of our knowledge. Thus, we identified the SNP of the GH gene in intron 3 to exon 5 in Jawa-Brebes cows and performed phylogenetic analysis by comparing the GH gene sequences of Jawa-Brebes cattle, Bali cattle, Madura cattle, and PO cattle with the available GH cattle sequences from Genbank.

MATERIALS AND METHODS

Ethical consent

The Committee of Research Ethics, Veterinary Medicine, Gadjah Mada University, Indonesia approved all procedures in this study (00143/EC-FKH/Int./2021).

Animals, sampling and DNA extraction

Animals in the study were 17 heads of Jawa-Brebes cows, 2 Bali cows (*Bos javanicus*), 2 Madura cows (Indonesian crossbred *Bos javanicus* and Zebu), and 3

Peranakan Ongole (Indonesian crossbred, PO) cows. The Jawa-Brebes cows were taken from Bantarkawung, Brebes Regency, Central Java, Indonesia. A total of 750-800 μ L blood samples were collected from the jugular vein through a syringe and put into an absolute ethanol-contained microtube. Genomic DNA Mini kit (Geneaid Biotech Ltd., Taiwan) was used to extract genome DNA from 200 μ L blood samples following the manufacturer's guidance.

DNA amplification and sequencing

The primers were GH-JBF: 5'-ACTCCATCCAGAACACCCAG-3' and GH-JBR: 5'-TGCCTGCTATTGTCTTCCCA-3'. Primers were designed using an online program, Primer 3 (<https://primer3.ut.ee/>) (Untergasser et al. 2012), based on the *Bos taurus* GH gene sequence (Genbank accession number: M57764.1). The GH gene was amplified using an Infinigen polymerase chain reaction (PCR) machine (Biotech Infinigen, CA, USA). The PCR consisted of 1 μ L of each primer, 3 μ L template DNA, 20 μ L distilled water, and 25 μ L master mix (Bioline, London, UK). The PCR was performed with pre-denaturation at 94 $^{\circ}$ C for 5min, followed by 35cycles at 94 $^{\circ}$ C for 45sec, 54 $^{\circ}$ C for 30sec, and 72 $^{\circ}$ C for 95sec, with a final elongation at 72 $^{\circ}$ C for 5min. The amplified products were detected on 1% agarose gel with 1kbp DNA ladder (Genaid, Taiwan) and, then, sequenced using 1st BASE sequencing by PT. Genetika Science, Indonesia.

Data analysis

All analyses were performed in the MEGA X software (Kumar et al. 2018). GH gene sequences of all samples in the study were aligned and compared with sequences available in GenBank for *Bos taurus* (M57764.1, JQ711182.1, J00008.1), *Bos indicus* (EF592533.1, EF592534.1, JN232516.1), *Bos grunniens* (ON100508.1, AY271297.1), and *Bos frontalis* (DQ491198.1, EF065627.1) using Clustal W (Thompson et al. 1994). Then, the neighbor-joining statistical method (Saitou and Nei 1987) with bootstrap test 1000x repetition (Felsenstein 1985) and Kimura 2-parameter model (Kimura 1980) were used to build the phylogenetic tree based on GH sequences.

RESULTS

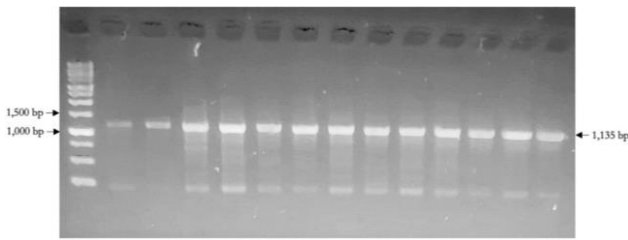
Genetic variation

Fig. 2 shows the PCR analysis successfully amplified a 1,135bp product of the GH gene in the Jawa-Brebes cattle. The amplicon product included a 963bp partial sequence of the GH gene that consisted of 226bp of intron 3, 162bp of exon 4, 273bp of intron 4, and 302bp of exon 5 (Fig. 3). The genetic diversity of Jawa-Brebes cattle based on a partial sequence of the GH gene (intron 3 to exon 5) in this study is presented in Table 1.

A total of 13 variable sites with 10 parsimony and 3 singleton sites were detected in the 963-bp partial sequence of the GH gene in Jawa-Brebes cattle. Nucleotide diversity and haplotype diversity were 0.00383 and 0.8309, respectively. The study found 9 haplotypes from 17 samples of Jawa-Brebes cattle with 6 unique haplotypes and 3 shared haplotypes (Hap 1, Hap 3, and Hap 9). Furthermore, haplotype 3 had 7 members, whereas haplotype 1 and haplotype 9 had 2 members.

Table 1: Genetic diversity of Jawa-Brebes cattle based on partial sequence of growth hormone (GH) gene

Traits	Value
Sample number	17
Number of sites	967
G+C contents	0.616
Invariable (monomorphic) sites	954
Number of variable (polymorphic) sites	13
Singleton variable sites	3
Parsimony informative sites	10
Nucleotide diversity (Pi)	0.00383
Number of haplotypes (h)	9
Haplotype diversity (Hd)	0.8309
Haplotype members:	
Haplotype 1 (Hap 1)	2 (Jawa-Brebes 1, Jawa-Brebes 3)
Haplotype 2 (Hap 2)	1 (Jawa-Brebes 2)
Haplotype 3 (Hap 3)	7 (Jawa-Brebes 6, Jawa-Brebes 8, Jawa-Brebes 9, Jawa-Brebes 11, Jawa-Brebes 15, Jawa-Brebes 17)
Haplotype 4 (Hap 4)	1 (Jawa-Brebes 5)
Haplotype 5 (Hap 5)	1 (Jawa-Brebes 7)
Haplotype 6 (Hap 6)	1 (Jawa-Brebes 10)
Haplotype 7 (Hap 7)	1 (Jawa-Brebes 12)
Haplotype 8 (Hap 8)	1 (Jawa-Brebes 13)
Haplotype 9 (Hap 9)	2 (Jawa-Brebes 14, Jawa-Brebes 16)

**Fig. 2:** Visualization of electrophoresed products using 1% agarose gel with DNA stain and 1 kbp DNA ladder

1261 gcagaaacgg ggggtgtggt ggtggggagg gttccgaata aggcggggag ggaaccgcg
 1321 caccagctta gacctgggtg ggtgtgttct tccccagga gcgcacctac atccccgag
 1381 gacagagata ctccatccag aacaccagc ttgcctctcg ctctctctgaa accatccccg
 1441 cccccacggg caagaatgag gccccagcaga aatcagtgag tggcaacctt ggaccgagga
 1501 gcagggggacc tcttcatcc taagttagct gccccagctc ccgcaaccgc ctggggcgcc
 1561 cttctccccg aggtggcgga gttgtttgga tggcagtgga ggaatgatgtt gggcggtgtt
 1621 ggcaggaggt cctcggcgag agcccgacct tgcagggctg ccccagacc ccgcaacca
 1681 ccgaccacc acctgccagc aggacttga gctgcttcgc atctcactg tctcatcca
 1741 gtggtgctt gggccccgc agttctcag cagagcttc accaacagct tgggtttg
 1801 cactcggac cgtgtctatg agaagctgaa ggacctggag gaaggcatcc tggcctgat
 1861 ccgggtgggg atggcgtttg ggtcccttc catgtggggg ccagcccccc cctctctg
 1921 cttagccagg aqaatgcagc tgggcttggg gagacagatc cctgctctct cctcttct
 1981 agcagtcag ccttgaccga ggggaacct ttccccctt tgaacctcc ttctcgccc
 2041 ttctcaagc ctgtaggga ggtggaaaa tggagcggc aggaggagc tgcctctgag
 2101 ggccttcgg cctctctgtc tctcctccc ttggcaggag ctggaagatg gcaccccc
 2161 gctcggcag atctcaagc agacctatga caaatttgc acaaacatgc gcagtgacga
 2221 ccgctgctc aagaactacg gctgctctc ctgcttccg aaggacctg ataaagcga
 2281 gactactctg aggtcatga agtcccgcc ctccggggag gccagctgtg cttctagt
 2341 ccagccatc tgtgtttgc cctccccgc tgccttctt gacctggaa ggtgccact
 2401 cactgtctt ttcctaataa aatgaggaat ttgcatcga ttgtctgag aggtgtcatt
 2461 ctattctggg ggggtgggtg gggcaggaca gcaaggggga ggaatgggaa gacaatagca
 2521 ggcatgctgg ggaatcggtg ggctctatg gtaccaggt gctgaagaat tgaccgggtt
 2581 cctcctggc cagaagaagc caggcacatc cctctctctg tgacacacc tgtccacgc
 2641 cctggttctt agttccagcc ccaactatag gacactata gctcaggagg gctccgctt
 2701 caatcccacc cqtaaaagta cttggagcgg tctctcctc cctcatcagc ccaccaacc

Fig. 3: Growth hormone (GH) sequence target (intron 3 to exon 5) in Jawa-Brebes cattle (Red box: primer forward and reverse of GH gene, Underline: intron 3 to exon 5)

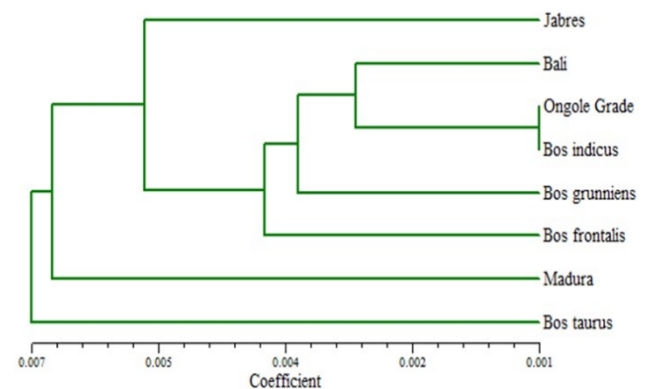
Table 2 displays the polymorphic sites of the GH gene in Jawa-Brebes cattle haplotypes and references to other breeds. The comparison between Jawa-Brebes cattle and various cattle breeds discovered 27 variable sites and 4 Indels. Notably, the sequence of the GH gene in Jawa-Brebes cattle was closely resembling to other indigenous Indonesian cattle, such as Bali, Madura, and PO cattle. Jawa-Brebes cattle displayed a higher degree of similarity with *Bos indicus* in comparison to other species. A distinctive nucleotide alignment, specifically *g.1947 G>T*, was observed in comparison to *Bos taurus*. Furthermore, Indels were

identified at 1,540th and 1,549th positions when contrasted with *Bos taurus*. Additionally, an Indel at the 1,895th site was present in both *Bos taurus* and *Bos indicus*, while an Indel at the 2,067th site was distinct to *Bos grunniens*.

Genetic distance and relationship with other cattle breeds

Table 3 displays the genetic distance among individual Jawa-Brebes cattle in this study. The genetic distance was 0.000 to 0.013 within Jawa-Brebes cattle. The most considerable distance was between Jawa-Brebes 5 and Jawa-Brebes 15.

The genetic distance of Jawa-Brebes cattle ranged from 0.0039 to 0.0077 in comparison to other breeds, as shown in Table 4. It showed the closest relationship with Jawa-Brebes cattle was *Bos indicus*, whereas the farthest association with Jawa-Brebes cattle was *Bos taurus*. Fig. 4 presents the kinship relationships of Jawa-Brebes cattle with other breeds.

**Fig. 4:** Phenogram of Jawa-Brebes (Jabres) cattle and other breeds of cattle based on growth hormone (GH) gene

DISCUSSION

The present study discovered 4 single nucleotide polymorphisms (SNPs) of the GH gene in intron 3 (*g.1482 G>A*, *g.1547 C>T*, *g.1577 C>T*, and *g.1694 T>C*) within

in contracted with *Bos taurus*. Agung et al. (2018) found also 2 similar variant nucleotides and 2 Indels, namely *g.1482 G>A*, *g.1547 C>T*, *g.1539_1540insT*, and *g.1548_1549insG*, within Indonesian Sumba Ongole (SO) cattle. Thus, these similarities indicate the close relationships among Indonesian cattle.

Polymorphisms of the GH gene in intron 3 on growth traits have been reported to affect production and reproduction in cattle. Polymorphism of the GH gene at the 1,547th site in intron 3 is associated with body weight gain in the Indonesian Krui (Dakhlan et al. 2022) and Pesisir (Hartatik et al. 2018) cattle. Polymorphism at the 1,547th position of the GH gene has effects on calving interval and days open in Holstein cows (Amiri et al. 2018). Moreover, the pregnancy rate of Holstein cows is associated with a mutation at the 1,547th position of the GH gene 3 (Öner et al. 2017). The *g.1456_1457insT* mutation of the GH gene in intron 3 influences milk production in Holstein cows (Anggraeni et al. 2020). Variation of the GH gene at the 1,547th position (*g.1547 C>T*) in intron 3 was also discovered in Jawa-Brebes cattle. Therefore, the fertility and growth traits of Jawa-Brebes cattle might be attributed to the mutations of the GH gene in intron 3, especially at the 1,547th position (*g.1547 C>T*).

There were 6 variants (*g.1914 C>G*, *g.1930 G>A*, *g.1980 T>C*, *g.1991 C>A*, *g.2009 C>A*, and *g.2056 G>A*) of the GH gene in intron 4 within Jawa-Brebes cattle in the present study. Similarly, the 4 SNPs, namely *g.1914 C>G*, *g.1930 G>A*, *g.1980 T>C*, and *g.2056 G>A*, were detected in Madura cattle. Furthermore, the *g.2009 C>A* mutation was also found in Bali cattle. The finding was similar to the study of Agung et al. (2018) discovered *g.1914 C>G*, *g.1930 G>A*, and *g.2056 G>A* SNPs in the GH gene of SO cattle. Interestingly, these polymorphisms were not discovered in both *Bos indicus* and *Bos taurus*. Moreover, Indel at the 1,895th site (*g.1894_1895insC*) were present in Jawa-Brebes cattle, Madura cattle, Bali cattle, and PO cattle when compared to both *Bos taurus* and *Bos indicus*. Furthermore, the Jawa-Brebes cattle had *g.1947 G>T* SNP in intron 4 when compared to *Bos taurus*. This mutation was also detected in Madura cattle, Bali cattle, PO cattle, and *Bos indicus*. Hence, these shared mutations in intron 4 result in greater similarity in the characteristics of Jawa-Brebes cattle with both Indonesian local cattle and *Bos indicus*, rather than with *Bos taurus*.

Moreover, our result found 3 mutations of the GH gene in exon 5 (*g.2230 C>T*, *g.2291 C>A*, and *g.2438 G>A*) within Jawa-Brebes cattle. Similarly, the *g.2230 C>T* and *g.2291 C>A* SNPs were discovered in Madura cattle. Moreover, PO cattle had the *g.2291 G>A* variant. A similar finding was demonstrated by Agung et al. (2018), whereby SO cattle also had 3 mutations in exon 5, namely *g.2230 C>T*, *g.2291 C>A*, and *g.2438 G>A* mutations. The *g.2291 C>A* is also found in Kerana (EF592533) and Butana (EF592534) cattle (*Bos indicus*) (Musa et al. 2013).

Mutations of GH gene in exon 5 affect insulin-like growth factor-1 (IGF-1) and non-esterified fatty acids (NEFA) concentrations in cows (Ruprechter et al. 2011). IGF-1 levels are highly associated with post-partum ovarian activity resumption (Konigsson et al. 2008) and energy balance in cows (Wathes et al. 2021). Furthermore, IGF-1 status impacts pregnancy in the first artificial insemination (Gobikrushanth et al. 2018). Moreover, SNPs

in exon 5 influence DO, service per conception, and CI of cows (Amiri et al. 2018; Komisarek et al. 2011). The polymorphisms in exon 5 also are correlated to the occurrence of dystocia (Hadi et al. 2015) and the production of healthy quality milk in cows (Bordonaro et al. 2020). Thus, we suggest that the polymorphisms of the GH gene in exon 5 were linked to the fertility traits of Jawa-Brebes cattle.

Among individual Jawa-Brebes cattle, Jawa-Brebes 5 (Hap 4) and Jawa-Brebes 15 (Hap 3) had the most considerable genetic distance. The distance was affected by the 9 mutations of the GH gene consisting of 3 mutations in intron 3 (*g.1482 G>A*, *g.1547 C>T*, and *g.1692 T>C*), 4 variants in intron 4 (*g.1914 C>G*, *g.1930 G>A*, *g.1980 T>C*, and *g.2056 G>A*), and 2 polymorphisms in exon 5 (*g.2230 C>T* and *g.2291 C>A*) within Jawa-Brebes 5 and Jawa-Brebes 15. These mutations might influence the difference in reproductive traits within Jawa-Brebes cattle.

The GH gene sequence in Jawa-Brebes cattle was highly similar to Indonesian local cattle (Bali, Madura, and PO cattle) in this study. Moreover, Jawa-Brebes cattle had a closer relationship with *Bos indicus* than *Bos taurus*. The finding confirmed that Jawa-Brebes cattle is a cross-bred Indonesian indigenous cattle between Bali, Madura, and PO cattle from hundred years ago (MARI, 2012). Similar to our finding, Prihandini et al. (2022) discovered that Jawa-Brebes cattle was genetically close to Bali, Madura, and PO cattle based on *HSP70* gene sequences.

Conclusion

The sequences of intron 3, intron 4, and exon 5 of GH gene were polymorphic. The study provided initial bioinformatics of GH gene sequence that might be used to preserve Jawa-Brebes cattle and as reproduction traits marker-assisted selections in further investigation.

Conflict of interests

All authors have no conflict of interest to declare.

Author contributions

AB: first author, designed the study, wrote and revised the manuscript. SH: collected samples, performed laboratory analyses, and reviewed the manuscript. RW and EMNS: supervised and reviewed the manuscript and the revision. AI: Conducted data analysis and reviewed the manuscript. SP: wrote additional parts of the manuscript and reviewed the manuscript.

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