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Novel Single Nucleotide Polymorphisms in the Sumba Ongole (*Bos indicus*) Growth Hormone Gene

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Abstract. *Agung PP, Putra WPB, Anwar S, Wulandari AS, Zein MSA, Said S, Sudiro A. 2018. Novel Single Nucleotide Polymorphisms in the Sumba Ongole* (Bos indicus*) Growth Hormone Gene. Biodiversitas 19: 676-681.* The Sumba Ongole cattle (*Bos indicus*) is one of the local Indonesian cattle breeds and has excellent potential to gain higher carcass yield compared to other local cattle breeds in Indonesia. The Growth Hormone (GH) gene was a potential target for molecular study due to its importance as growth regulating hormone. This study was aimed to determine Single Nucleotide Polymorphism (SNP) profile of the GH gene in the Sumba Ongole (SO) cattle using PCR and DNA sequencing methods from 34 individual DNA samples. The results from this study showed that there are 28 SNPs identified within the GH gene; Twenty one SNPs were found to occur in the intron region, 4 SNPs in the exon region, and 3 SNPs in the 3' end region. There were 17 SNPs in the SO cattle GH gene were caused by novel mutation. The novel SNP g.1395insC was found only in the SO cattle. Moreover, the frequency of the SNP g.1395insC was the highest among other novel SNPs in the SO cattle GH gene. The moderate PIC value (0.25<PIC<0.50) were found in two novel SNPs i.e. SNP g.1415C>G (intron 4), and SNP g.1526A>G (intron 4). The GH gene in the SO cattle was polymorphic and the 17 novel SNPs need to be further validated.

Keywords: *Bos indicus*, growth hormone, novel SNP, Sumba Ongole

INTRODUCTION

The Sumba Ongole (*Bos indicus*) cattle is one of the local Indonesian cattle breeds. This Ongole cattle breed was imported from India in 1914 (Ministry of Agriculture of the Republic of Indonesia 2014), and is now well-adapted. The cattle have been targeted for breeding programme, and centralized in Sumba Island (East Nusa Tenggara Province, Indonesia). Since then, the Ongole generations resulted from the breeding programme were known as Sumba Ongole (SO) cattle (Hardjosubroto 2004; Sutarno and Setyawan 2006). The SO cattle has excellent potential to gain higher dressing percentage (>50%) compared to other local breeds cattle in Indonesia (Agung et al. 2015).

Growth trait is one of the important parameters in livestock breeding programmes. One of the hormones associated to animal growth is Growth Hormone (GH) which is a protein hormone synthesized and secreted by the anterior pituitary gland (Etherton and Bauman, 1998). The GH is needed for tissue growth, fat metabolism, and normal body growth (Burton et al. 1994). The hormone encoded by the GH gene plays an important role in reproduction traits i.e. superovulation response, ovulation rate, fertility rate and embryo quality (Sumantri et al. 2011) and also affects the average daily gain, carcass yield, and marbling score in livestock (Beauchemin et al. 2006). The important function of the GH gene makes it one of the potential candidate genes for breeding programmes (Beauchemin et al. 2006; Ribeca et al. 2014).

The GH gene polymorphism was intensively studied in many cattle breeds including local Indonesian cattle breeds (Paputungan et al. 2012; Hartatik et al. 2013; Volkandari et al. 2013; Putra et al. 2014; Putra et al. 2016) but limited reports for the Indonesian SO cattle GH gene. Up to present, information about the SO cattle GH gene polymorphism is limited only in the intron region (Agung et al. 2017). This study was conducted to determine Single Nucleotide Polymorphism (SNP) profile of the GH gene in the SO cattle using polymerase chain reaction (PCR) and sequencing methods.

MATERIALS AND METHODS

Blood samples and DNA extraction

A total of 34 cattle from the Sumba Island, Indonesia were included in this study. Blood samples (3-5 mL) were taken from coccygeal vein using Venoject and collected in Vacutainer tubes containing an anticoagulant. DNA was extracted from blood samples using the Genomic DNA Mini kit (Geneaid Biotech Ltd., Taiwan) following the manufacturer's protocol. Ethical Clearance Committee of the Indonesian Institute of Sciences, Jakarta, Indonesia has approved all procedures related to the used of animals in this study (Register No. 9879/WK/HK/XI/2015).

PCR amplification and DNA sequencing

The PCR reaction was performed in a Mastercycler® gradient (Eppendorf, Hamburg, Germany) with three pairs of primers (Table 1). The primers were designed from GH gene sequence available in the GenBank (NCBI) database with accession number EF592534. The expected size of the full-length GH gene in this study was 2061 bp. It should be noted that the GH1 primer in this study was not designed to amplify the GH gene from the first nucleotide due to high content of guanine-cytosine.

The PCR reagents composed of 12.5 µL KAPA2G Fast Ready Mix PCR Kit 1X (Kapa Biosystems, Cape Town, South Africa), 1 µL forward and reverse primers (0.2 µM final concentration each), 2 µL DNA samples (5-50 ng>µL), and ddH_2O up to 25 µL final volume. The optimum condition for PCR programme was as follows: initial denaturation at 94°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing 58°C to 64°C (depends on primers) for 45 seconds, extension at 72°C for 45 seconds; and terminated by a final extension at 72°C for 5 minutes. The PCR products were visualized in 1% agarose gel (w/v) and stained with SyBr®. The DNA sequencing was performed for all PCR products using the ABI Prims 3100-Avant Genetic Analyzer in the 1st BASE Laboratory, Malaysia. The SO cattle GH gene sequences were aligned and compared to the sequences that were available in the GenBank for *Bos taurus* (M57764) and *Bos indicus* (EF592534) using MEGA ver 6.0 programmes (Tamura et al. 2013). The position of the sequence in this study was relative to *Bos indicus* GH gene sequence (EF592534).

Statistical analysis

Statistical analysis for the GH gene sequence consisted of allele frequency, expected heterozogosity (He), observed heterozogosity (Ho) effective allele number (ne), and the polymorphism information content (PIC) based on Nei and Kumar (2000) with the following statistical model: χii = (nii/N) for genotype frequency and χi = (2nii + Σnij)/ (2N) for allele frequency, where : χii = frequency of iith genotype; χi = frequency of ith allele; nii = number of individuals with ii genotype; nij = number of individuals with ij genotype; N = number of samples.

RESULTS AND DISCUSSION

The GH gene in the SO cattle was successfully amplified using three pairs of primers. The results indicated that amplification fragment had good specificity, which could proceed directly to sequencing analysis. The size of the PCR product was in accordance with the target size (Table 1), i.e.681 bp, 1072 bp, and 571 bp (Figure 1). The PCR products of the exon 2 to exon 5 (Figure 1.B) indicated the same size as reported by Anwar et al. (2015), i.e., 1072 base pair (bp) approximately.

Table 1. Primers used for the GH gene amplification

Primer ID	Sequence (5' - 3')	Product size (bp)	Target region in the GH gene sequence	Annealing temperature (°C)
GH1_Forward	ATTAGCACAGGCTGCCAGTGG	681	5' Flanking region - part of Exon 3	59
GH1_Reverse	CTCCTCAGTTTCCTCCCACTG			
GH2_Forward*	CAAAGAGTTTGTAAGCTCCC	1072	Part of Exon 2 - part of Exon 5.	58
GH2_Reverse*	GTCATAGGTCTGCTTGAGGA			
GH3_Forward	AGCCTTGACCCAGGGGAAACC	571	Part of Intron 4 - 3' end region.	64
GH3_Reverse	ACCTGGGTACCCATAGAGCC			

Note: *based on Anwar et al. (2015)

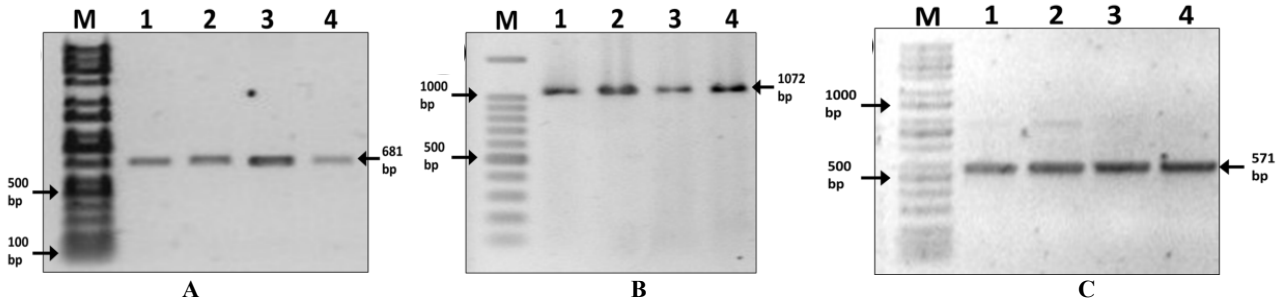

Figure 1. Photograph of gel electrophoresis of the PCR Products. A. 5' flanking region to exon 3; B. exon 2 to exon 5; C. intron 4 to 3' end region; M: 100 bp DNA ladder; 1-4: samples

The DNA sequence alignment analysis revealed that there are 28 SNPs found in the SO cattle GH gene that spread over from intron 1 to the 3' end region (Table 2). Most of the mutations –indels and base substitutions- found in the SO cattle GH gene also occurred in both *Bos indicus* (EF592534) and *Bos taurus* (M57764) cattle. However, there were 17 SNPs observed only in the SO cattle GH gene. These include SNP g.1395insC, g.1415C>G, and g.1526A>G that occurred in low frequency, and SNP g.1395insC with high frequency (1.00). Of these observed SNPs, SNP g.1395insC was caused by novel mutation in intron 4. Its association with GH gene expression is unknown. However, Dybus (2002) and Arango et al. (2014) considered that polymorphism in the intron region was associated with several animal productivity traits.

Table 2. Information of the SNPs in the SO cattle GH gene

Location	Position relative to M57764	Position relative to EF592534	*Bos taurus* (M57764)	*Bos indicus* (EF592534)	SO cattle (This study)	N_{indv}	N_{pop}	Freq
Intron 1	800	298	G	G	G	26	27	0.96
					C	1	27	0.04
Intron 1	904	403	-	Insertion G	Insertion G	27	27	1.00
Intron 1	942	442	C	C	C	23	27	0.85
					T	4	27	0.15
Exon 2	1059	558	C	T	C	27	27	1.00
Intron 2	1279	778	G	G	A	1	31	0.03
					T	1	31	0.03
					G	29	31	0.94
Intron 2	1304	803	C	T	C	31	33	0.94
					T	2	33	0.06
Intron 3	1482	981	G	G	G	32	33	0.97
					A	1	33	0.03
Intron 3	1483	982	G	G	G	32	33	0.97
					A	1	33	0.03
Intron 3	1540	1040	-	Insertion T	Insertion T	33	33	1.00
Intron 3	1547	1047	C	T	C	6	33	0.18
					T	27	33	0.82
Intron 3	1549	1050	-	Insertion G	Insertion G	33	33	1.00
Intron 3	1666	1167	GA	Inversion AG	Inversion AG	33	33	1.00
Intron 3	1692	1193	C	T	T	33	33	1.00
Intron 4	1895	1395	-	-	Insertion C	32	32	1.00
Intron 4	1914	1415	C	C	C	25	32	0.78
					G	7	32	0.22
Intron 4	1930	1431	G	G	G	25	30	0.83
					A	5	30	0.17
Intron 4	1947	1448	T	G	T	2	23	0.09
					G	21	23	0.91
Intron 4	1985	1486	G	G	G	16	17	0.94
					T	1	17	0.06
Intron 4	1988	1489	-	-	Insertion C	1	14	0.07
Intron 4	2025	1526	A	A	A	26	33	0.79
					G	7	33	0.21
Intron 4	2056	1557	G	G	G	29	32	0.91
					A	3	32	0.09
Intron 4	2062	1563	G	G	G	31	32	0.97
					T	1	32	0.03
Exon 5	2141	1642	C	C	C	30	32	0.94
					G	2	32	0.06
Exon 5	2230	1731	C	C	C	30	32	0.94
					T	2	32	0.06
Exon 5	2291	1792	A	C	A	4	32	0.13
					C	28	32	0.87
3' end	2346	1847	C	T	C	32	32	1.00
3' end	2438	1939	G	G	G	27	32	0.84
					A	5	32	0.16
3' end	2537	2038	G	T	G	24	31	0.77
					T	7	31	0.23

N_{indv}: number of individuals. Npop: number of population. Highlighted rows indicate the mutations did not occur in the *Bos taurus* (M57764) or *Bos indicus* (EF592534) cattle

Table 3. Results from statistical analysis of several SNPs found in the SO cattle GH gene

SNP position	Allele frequency			Heterozigosity value		n_e	PIC
				Expected	Observed		
298	G (0.96)	C (0.04)		0.08	0.08	1.08	0.07
442	C (0.85)	T (0.15)		0.26	0.26	1.34	0.22
778	A (0.03)	T (0.03)	G (0.94)	0.11	0.12	1.13	0.11
803	C (0.94)	T (0.06)		0.11	0.11	1.13	0.11
981	G (0.97)	A (0.03)		0.06	0.06	1.06	0.06
982	G (0.97)	A (0.03)		0.06	0.06	1.06	0.06
1047	C (0.18)	T (0.82)		0.30	0.30	1.42	0.25
1415	C (0.78)	G (0.22)		0.34	0.35	1.52	0.28
1431	G (0.83)	A (0.17)		0.28	0.29	1.39	0.24
1448	T (0.09)	G (0.91)		0.16	0.17	1.20	0.15
1486	G (0.94)	T (0.06)		0.11	0.12	1.13	0.11
1526	A (0.79)	G (0.21)		0.33	0.34	1.50	0.28
1557	G (0.91)	A (0.09)		0.16	0.17	1.20	0.15
1563	G (0.97)	T (0.03)		0.06	0.06	1.06	0.06
1642	C (0.94)	G (0.06)		0.11	0.11	1.13	0.11
1731	C (0.94)	T (0.06)		0.11	0.11	1.13	0.11
1792	A (0.13)	C (0.87)		0.23	0.23	1.29	0.20
1939	G (0.84)	A (0.16)		0.27	0.27	1.37	0.23
2038	G (0.77)	T (0.23)		0.35	0.36	1.55	0.29

Note: N_e: effective allele number. PIC: polymorphism information content

The PIC values were ranged between 0.07 to 0.29 (low - moderate). The SNP with high PIC value were not found in this study. The low value of PIC of GH gene in the SO cattle in this study might be caused by the limitation number of sires and selection process may have happened in the SO cattle population (Agung et al. 2017). The moderate PIC values were found only in SNP g.1415C>G, g.1526A>G, and g.2038T>G. It is worth noting that SNP g.1415C>G and SNP g.1526A>G was found only in the SO cattle. The heterozygosity values ranged from 0.08 to 0.35. These low heterozygosity values can be caused by several factors, including null alleles, assortative mating, the Wahlund effect, selection against heterozygotes, inbreeding, or a combination of all these factors (Cervini et al. 2006). The low value of heterozygosity can also indicates that certain breeds are relatively well-conserved (Czerneková et al. 2006; Agung et al. 2017). The low PIC and heterozygosity values in several SNPs in this study were indicated the low level of GH gene diversity in the SO cattle population.

The SNPs found in this study mostly occurs in the intron region of the SO cattle GH gene. There were three mutations that occurred in intron 1, two mutations in intron 2, seven mutations in intron 3, and nine mutations in intron 4. Meanwhile, there were four mutations that occurred in the exon region (one mutation in exon 2 and three mutations in exon 5). Interestingly, three SNPs (g.558T>C, g.1395insC, and g.1847T>C) identified in this study were completely different from *Bos indicus* GH gene (GenBank Accession number EF592534).

Musa et al. (2013) reported a silent mutation of SNP g.558C>T that occurred in exon 2 in the Kenana cattle GH gene, but this was not found in the SO cattle. The SNP g.1047C>T in the intron 3 has been extensively studied in several Indonesian cattle breeds such as the Pesisir cattle (Jakaria et al. 2007), Bali cattle (Jakaria and Noor 2011), Indonesian Ongole-grade cattle (Paputungan et al. 2012), and Aceh cattle (Putra et al. 2014). The occurrence of SNP g.1047C>T was also reported in other countries such as the Indian Zebu cattle (Sodhi et al. 2007), Nellore cattle (Curi et al. 2010), and also Brahman cattle (Hernandez et al. 2016).

The mutation existed in exon 5 at position 1642 was also reported to occur both in the *Bos indicus* and *Bos taurus* cattle (Thomas et al. 2007; Hartatik et al. 2013). The SNP g.1642C>G causes an amino acid change from leucine (Leu) to valine (Val). These SNPs are known as the L or V allele based on the AluI restriction sites (Musa et al. 2013). In the SO cattle, the V allele (recognized with C||G at position 1642) occurred at a low frequency (0.06). The low V allele frequency of SNP g.1642C>G was also reported in Brahman cattle (Thomas et al. 2007) and Indonesian Ongole-grade cattle (Hartatik et al. 2013). Nevertheless, the V allele did not exist Madura cattle (Volkandari et al. 2013) and Pesisir cattle (Putra et al. 2016).

Several association studies reported that the GH gene polymorphism significantly affected productivity traits in beef (Ribeca et al. 2014) and dairy cattle (Mullen et al. 2011). Arango et al. (2014) have further reported that mutation in intron 3 region (g.1047C>T) was associated with weight at the first oestrus and weight at the first calving in Holstein cattle. Dybus (2002) also confirmed that SNP g.1047C>T was associated with milk quality in Polish Black-and-White cattle. In addition, Lee et al. (2013) reported that the SNP g.1642C>G was associated with daily weight gain in the Hanwoo (*Bos taurus*) cattle with moderate PIC value. In contrast, Agung et al. (2017) reported that SNP g.1047C>T was not associated with birth weight, weaning weight, yearling weight, and dressing

percentage in the SO cattle. In addition, it seems the GH gene polymorphisms did not significantly affect the productivity traits in the Indonesian Simmental cattle (Suhada et al. 2016), the Zavot cattle (Akçay et al. 2015), the Indonesian Ongole-grade cattle (Paputungan et al. 2012), and also the Nellore and Nellore x *Bos taurus* cattle (Curi et al. 2010).

The utilization of genetic markers in order to achieve breeding objectives was highly dependent on its polymorphism and its association with productivity parameters (e.g., birth weight, weaning weight, growth rate, dressing percentage). Therefore, validating the genetic markers of growth traits is the initial and crucial step to establish the marker-assisted selection (MAS) programme (Allan et al. 2007). In order to ensure the GH gene utilization in the future, the novel mutations in this study can be used as preliminary data for conducting the comprehensive study and also the association study in larger cattle populations. It can be concluded that the full-length GH gene in the Indonesian SO cattle population was polymorphic based on several specific differences to the *Bos indicus* or *Bos taurus* GH gene. Seventeen SNPs displayed novel mutations and need to be further validated.

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