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Novel Single Nucleotide Polymorphisms (SNPs) in Intron 2 and Exon 3 Regions of Leptin Gene in Sumba Ongole Cattle

Research Article

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ABSTRACT

The bovine leptin (LEP) gene was widely used as a candidate gene for molecular selection to improve productivity traits of cattle. This study was carried out to identify single nucleotide polymorphisms (SNPs) in the LEP gene of Sumba Ongole (SO, *Bos indicus*) cows using sequencing method. A total of 31 animals were used in this study for analyses. Research showed that total of 16 SNPs were detected in the LEP gene. Along 2025 bp of LEP gene sequence was analyzed in this study and consisted of intron 2 (1002 bp) and exon 3 (1023 bp). The polymorphic informative content (PIC) value was reached from 0.06 (low) to 0.37 (moderate). Total of 16 SNPs in LEP gene of SO cattle had moderate PIC value (0.25<PIC<0.50) and consisted of twelve SNPs in intron 2 and four SNPs in exon 3. The SNPs with moderate PIC value were detected in intron 2 (g.2325G/T; g.2423A/C; g.2448C/T; g.2456C/G; g.2466C/T; g.2778T/A; g.2857G/A) and exon 3 (g.3260T/C; g.3272T/C; g.3356C/T; g.3468G/A). The SNP of g.3468G/A as the novel SNP in LEP gene of SO cattle that not reported in other breeds of cattle. This SNP was changed the amino acid from glycine (GGG) to arginine (AGG). Two type of mutation were detected in the LEP gene of SO cattle and consisted of transversions (44%) and transitions (56%). It was concluded that the LEP gene in SO cattle was showed polymorphisme and potential for molecular selection in the breeding program through depth research.

KEY WORDS leptin gene, Pasundan cattle, polymorphic informative content, polymorphism, SNPs.

INTRODUCTION

The Sumba Ongole (SO) cattle is included of *Bos indicus* breed that capable to adapt well in Sumba Island of Indonesia. This cattle was decided as one of Indonesian native cattle through decision of Agriculture Ministry of Indonesia No: 427/Kpts/SR.120/3/2014. This cattle was imported from India since 1900 by Dutch colonial government for the drought animals (Hardjosubroto, 1994). Recently, SO cattle was kept by most farmers as the beef cattle or meat production. Previous studies reported that the average of body weight in SO bulls (±2.5 years age) was 353.86-474.08 kg (Said *et al.* 2016a). In addition, the average of dress percentage in SO bulls were 51.42-56.34% (Agung *et al.* 2015). Some of genetic parameters of repeatability, heritability and genetic correlation values of growth trais in SO cattle were high and reveal that growth traits in this cattle can be increased through conventional selection (Said *et al.* 2016b; Putra *et al.* 2018). Despite, growth traits in SO cattle can be performed through molecular selection. Up to present, information about genetic markers in the SO cattle used for molecular selecti-

on is limited. Agung *et al.* (2017) reported that polymorphism in the growth hormone (GH) gene in SO cattle had not association with any growth traits.

Therefore, information about genetic marker candidates need to be explorated. One of the candidate gene that widely use for the molecular selection is leptin (LEP) gene (Putra and Indriastuti, 2017). The LEP gene was located at fourth chromosome (BTA4q32) with length 16.735 bp and consisted of two introns and three exons (Pfister-Ganskow *et al*. 1996). Corva *et al*. (2009) reported that bovine LEP gene (GenBank: U50365) consisted of 34 bp of exon 1, 465 bp of exon 2 (from nuleotide: 877-1342) and 495 bp of exon 3 (from nucleotide: 2961-3456). The LEP is a protein that consisted of 167 amino acids with molecule weight of 16 kDa (Taniguchi *et al.* 2002). In cattle, LEP was synthesized by adipose tissue and involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck *et al.* 1998), milk performance (Liefers *et al.* 2002; Madeja *et al*. 2004) and reproductive traits (Almeida *et al*. 2003; Moussavi *et al.* 2006). Previous studies reported that one single nucleotide polymorphism (SNP) in the intron 2 of bovine LEP gene (GenBank: U50365) were occured at nucleotide position g.2270A/G (Pomp *et al*. 1997; Rasor *et al*. 2002; Oner *et al*. 2017). Morever, this SNP had association with productivity traits of cattle (Oprzadek *et al*. 2003). Despite, previous studies reported that SNP in the exon 3 of bovine LEP gene (GenBank: U50365) were occured at g.2961A/G; g.3100C/T; g.3260T/C; g.3257C/T; g.3272T/C and g.3356 C/T (Shin and Chung, 2007; Jhala *et al*. 2009; Orru *et al*. 2011; Kawaguchi *et al*. 2016). Putra *et al*. (2017) reported that 17 SNPs were confirmed in the 3'flanking region of LEP gene in SO cattle but the effect of these SNPs to productivity traits is not reported. There are no studies that reported the SNPs along intron 2 and exon 3 sequences of LEP gene in SO cattle. This research was carried out to detect the SNPs in intron 2 and exon 3 of LEP gene in population and selectied SO cattle through sequencing method.

This research results is important as the early information to obtain the genetic marker for productivity traits in SO cattle.

MATERIALS AND METHODS

Animals and DNA extraction

A total of 31 DNA samples of SO female cattle from Sumba Island, West Nusa Tenggara Province of Indonesia were used for analysis in the present study. The animals were kept at the breeding station (PT. Karya Anugerah Rumpin) that located at Rumpin District, Bogor Regency, West Java Province of Indonesia. Amount of 3-5 mL of bloods samples were taken from coccygeal vein using venoject and collected in the vaccutainer tubes containing anticoagulant (EDTA).

The DNA extraction was conducted from bloods sample with Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) following the producers instruction.

Performance test

In the breeding station, total of 11 SO cows were randomly selected to maintain in the performance test from 20/05/2013 to 24/05/2014 (369 days of test). Unfortunately, only three confirmed cows (ID: 1937, ID: 2097 and ID: 2099) that had LEP gene sequences information in the tested animals. Thus, all cows were ranked based on the final weight (FW) at end of the test. The performance in the three confirmed cows compare to unconfirmed cows were performed in the present study as the early information to obtain genetics marker in SO cattle.

Amplification of LEP gene and DNA sequencing

The amplification of LEP genes were performed in Mastercycler® gradient machine (Eppendorf, Germany) with two pairs of primers (Table 1). The primers were designed to amplify two regions of intron 2 and exon 3 based on GenBank: U50365.

Table 1 The primer pair, amplicon and PCR program for amplification of leptin (LEP) gene in Sumba Ongole cattle

Gene	Primer	Region	Amplicon (bp)[1]	PCR program
LEP 3	F: 5'-AGTCATGTCCAACTCTTTGAGAC-3' R: 5'-CCACGTGACCACCATGTTTCCAA-3'	Intron 2	1002	94 ˚C 5 min (94 ˚C 30 s, 60 ˚C 45 s, 72 ˚C 45 s) 35 cycles, 72 ˚C 5 min
LEP 4	F: 5'-TGACTGTGAGGGAGGAGTCTGC-3' R: 5'-GAGCTGGAACAGGGAGGAAGACT-3'	Intron 2 Exon 3	1023	94 ˚C 5 min (94 ˚C 30 s, 55 ˚C 45 s, 72 ˚C 45 s) 35 cycles, 72 ˚C 5 min

[1] Based on GenBank: U50365.

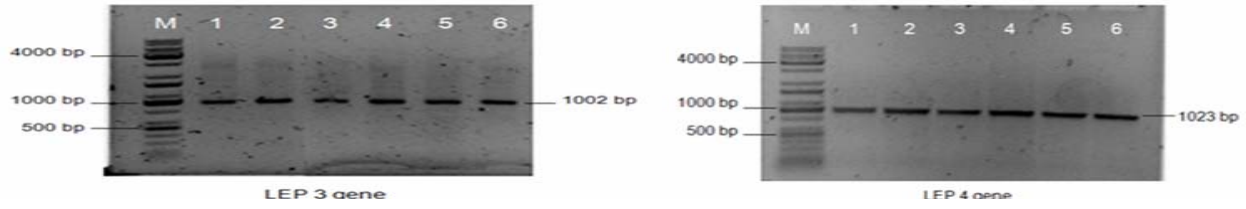

Figure 1 The amplicons of leptin 3 and leptin 4 genes in Sumba Ongole cattle
M: DNA ladder 100 bp and line 1-6: number of sample

Total of 24 µL of PCR reagents containing of 9 µL of KAPA2G Robust HotStart Ready Mix (Kapa Biosystems, South Africa); 1.8 µL of primer forward and reverse; 9.4 µL of ddH_2O and 2 µL of DNA template. The polymerase chain reaction (PCR) program in the LEP gene of SO cattle were presented in Tabel 1. The visualization of amplification product were performed in 1% of Agarose gel (Vivantis, Malaysia) and stained with SyBr®. The DNA sequencing analysis was performed for all PCR products and managed by commercial laboratory service (1st BASE Laboratory, Malaysia) using the sequencing machine of ABI Prisms 3100-Avant Genetic Analyzer. Furthermore, DNA sequence of LEP 3 and LEP 4 genes were aligned and compared to the sequence reference (GenBank: U50365) using MEGA 6.0 (Tamura *et al.* 2013) and BioEdit (Hall, 1999) programs.

Statistical analysis

The statistical analysis in the LEP gene sequences were consisted of genotype frequency, allele frequency, observed heterozigosity (H_o), expected heterozigosity (H_e), number of effective allele (n_e), polymorphic informative content (PIC) and Chi-square value (χ^2) according to Nei and Kumar (2000). Therefore, the genetic diversity data (H_o; H_e,; n_e; PIC and χ^2) were calculated with CONVERT (Glaubitz, 2004), CERVUS (Kalinowski *et al.* 2007) and POPGENE (Yeh and Boyle, 1997) programs.

RESULTS AND DISCUSSION

Two PCR product of bovine LEP gene along 1002 bp (LEP 3 gene) and 1023 bp (LEP 4 gene) were successfully to amplified for the sequencing analysis (Figure 1). Total of 16 SNPs in the LEP gene were confirmed in the present study and consisted of 10 SNPs in LEP 3 gene and 6 SNPs in LEP 4 gene (Table 2).

According to the Table 2, the PIC values were ranged from 0.06 (low) to 0.37 (moderate). Seven SNPs in the intron 2 (g.2325G/T; g.2423A/C; g.2448C/T; g.2456C/G; g.2466C/T; g.2778T/A; g.2857G/A) had moderate PIC value (0.25≤PIC≤0.50). Meanwhile, moderate PIC value in the exon 3 were occured in all SNPs (g.3260T/C; g.3272T/C; g.3356C/T; g.3468G/A). Previous studies reported that SNP g.2270A/G in the inron 2 was occured in many cattle breeds i.e. Angus × Nellore (Almeida *et al.* 2003), Friesian Holstein (Oprzadek *et al.* 2003).

However, this SNP was not detected in the animals studied and showed AA genotype in all samples (monomorphic).

The A allele in the SNP g.2270A/G is considered as the favourable allele for weight at first calving trait in crossbred cattle (Almeida *et al.* 2003). Despite, the SNP g.2270A/G was not associated with service per conception in Friesian Holstein heifers (Oner *et al.* 2017), but had significant association with some productive traits in Black-and-White cattle (Oprzadek *et al.* 2003). The monomorphism in SNP g.2270A/G. in SO cattle can be caused by selection, migration and close breeding system (Falconer and Mackay, 1996).

Orru *et al.* (2011) reported that six SNPs were detected in the exon 3 of bovine LEP gene i.e. g.3100C/T; g.3157A/G; g.3257C/T; g.3260T/C; g.3272T/C and g.3356C/T. Corva *et al.* (2009) reported that six SNPs in the exon 3 of LEP gene had amino acid changed of alanine/valine (g.3100C/T); asparagine/Serine (g.3157A/G); glycine/glycine (g.3257C/T); valine/valine (g.3260T/C); alanine/alanine (g.3272T/C) and proline/proline (g.3356C/T). The SNP g.3100C/T and g.3157A/G were widely used as molecular because this SNP had amino acid changes (Matteis *et al.* 2012).

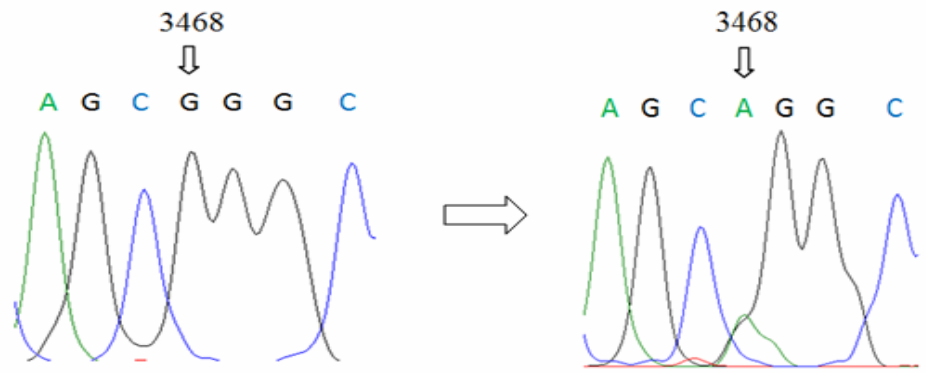

Figure 2 Novel SNP g.3468G/A in the exon 3 of bovine leptin gene in Sumba Ongole cattle was caused amino acid change from glycine (GGG) to arginine (AGG)

Table 2 Detection SNPs in the leptin (LEP) gene of Sumba Ongole cattle based on GenBank: U50365[1]

SNP	Gene	Frequency genotype	N	Frequency allele	H_o	H_e	n_e	PIC	χ^2
	LEP 3	TT (0.85)	23	T (0.93)	0.15	0.14	1.16	0.13	0.17*
g.2310T/A		AT (0.15)	4	A (0.07)					
		AA (0.00)	0						
	LEP 3	GG (0.13)	3	G (0.37)	0.48	0.47	1.87	0.36	0.02*
g.2325G/T		GT (0.48)	11	T (0.63)					
		TT (0.39)	9						
	LEP 3	GG (0.93)	28	G (0.97)	0.07	0.06	1.07	0.06	0.04*
g.2351G/A		GA (0.07)	2	A (0.03)					
		AA (0.00)	0						
	LEP 3	CC (0.83)	24	C (0.92)	0.17	0.16	1.19	0.15	0.26*
g.2361C/T		CT (0.17)	5	T (0.05)					
		TT (0.00)	0						
	LEP 3	AA (0.90)	26	A (0.95)	0.10	0.10	1.11	0.09	0.09*
g.2362A/T		AT (0.10)	3	T (0.05)					
		TT (0.00)	0						
	LEP 3	AA (0.90)	26	A (0.95)	0.10	0.10	1.11	0.09	0.09*
g.2377A/T		AT (0.10)	3	T (0.05)					
		TT (0.00)	0						
	LEP 3	AA (0.39)	11	A (0.68)	0.57	0.44	1.77	0.34	2.70*
g.2423A/C		AC (0.57)	16	C (0.32)					
		CC (0.04)	1						
	LEP 3	CC (0.93)	2	C (0.03)	0.93	0.50	1.99	0.37	22.97
g.2448C/T		CT (0.07)	28	T (0.97)					
		TT (0.00)	0						
	LEP 3	CC (0.42)	11	C (0.63)	0.42	0.46	1.87	0.36	0.20*
g.2456C/G		CG (0.42)	11	G (0.37)					
		GG (0.16)	4						
	LEP 3	CC (0.12)	2	C (0.56)	0.88	0.49	1.97	0.37	16.35
g.2466C/T		CT (0.88)	23	T (0.44)					
		TT (0.00)	0						
	LEP 4	TT (0.05)	1	T (0.35)	0.60	0.45	1.83	0.35	2.69*
g.2778T/A		TA (0.60)	14	A (0.65)					
		AA (0.35)	8						
	LEP 4	GG (0.48)	15	G (0.71)	0.45	0.41	1.70	0.33	0.29*
g.2857G/A		GA (0.45)	14	A (0.29)					
		AA (0.07)	2						
	LEP 4	TT (0.41)	9	T (0.61)	0.41	0.47	1.90	0.36	0.41*
g.3260T/C		TC (0.41)	9	C (0.39)					
		CC (0.18)	4						
	LEP 4	TT (0.71)	15	T (0.81)	0.19	0.31	1.45	0.26	3.07*
g.3272T/C		TC (0.19)	4	C (0.19)					
		CC (0.10)	2						
	LEP 4	CC (0.52)	12	C (0.54)	0.04	0.50	1.99	0.37	19.15
g.3356C/T		CT (0.04)	1	T (0.56)					
		TT (0.44)	10						
	LEP 4	GG (0.76)	13	G (0.82)	0.12	0.29	1.41	0.25	6.02
g.3468G/A		GA (0.12)	2	A (0.18)					
		AA (0.12)	2						

SNP: single nucleotide polymorphism; N: number of observation; H_o: observed heterozigosity; H_e: expected heterozigosity; n_e: number of effective allele; PIC: polymorphic informative content and χ^2: Chi-square value.
* Under Hardy-Weinberg equilibrium (χ^2<5.991).

Previous study reported that SNP g.3100C/T was not associated with live weight, carcass weight, dressing percentage, backfat thickness and marbling score in Hanwoo cattle (Shin and Chung, 2007) and reproductive traits in Czech Fleckvieh cattle (Jecminkova *et al.* 2018).

Orru *et al.* (2011) reported that C allele in the SNP g.3257C/T can be reduced the total lipids contain in Simmental bulls. Moreover, C allele in the SNP g.3100C/T can be increasing the milk protein concentration and milk fat yield in Friesian Holstein cattle (Giblin *et al.* 2010).

Research showed that SNP of g.3100C/T and g.3257C/T were not occured in the LEP gene of SO cattle. New mutation site in the exon 3 of LEP gene of SO was occured in SNP g.3468G/A (Figure 2). This SNP had moderate PIC value (0.25) and caused the amino acid change from glycine (GGG) to arginine (AGG).

A SNP with moderate PIC value can be used for molecular selection (Bourdon, 2000). In this study, the effect of SNP g.3468G/A to the productive traits of SO cattle can not observed because of no performance recorded. According to the FW in the performance test, each confirmed cow were reached 2nd rank (cow ID: 1937), 8th rank (cow ID: 2097) and 10th rank (cow ID: 2099) as presented in Table 3. The phenotypic characteristic of second best SO cows (ID: 1937) was presented in Figure 3.

According to the confirmed cows LEP gene sequences (Table 4), the second best cows (cow ID: 1937) had three different genotype compare to the other confirmed cows based on SNP g.2325G/T; SNP g.2362A/T and SNP g.2466C/T (intron 2). However, these SNP is important to investigate through depth study with large number of sample and performance records data.

Table 3 The rank of Sumba Ongole cows based on final weight at the end of performance test[1]

Rank	Cattle ID	Date of birth	Age (days)	Sire ID	Dam ID	BW (kg)	WW (kg)	YW (kg)	FW (kg)
1	2093	21/03/2012	842	1999	0744	25	57.42	178.30	395.39
2	1937*	12/06/2012	610	8843	0820	17	81.92	206.72	387.17
3	2098	27/04/2012	756	12075	0742	31	66.93	183.53	366.12
4	2102	04/07/2012	688	8843	0824	30	135.70	210.00	361.95
5	2091	31/01/2012	842	8843	0864	25	84.02	221.21	355.95
6	2101	11/06/2012	711	8843	0823	30	144.15	207.28	311.45
7	2096	21/04/2012	762	8843	0860	28	73.97	188.55	283.61
8	2097*	26/04/2012	757	2024	10277	30	76.38	184.65	280.74
9	2094	10/04/2012	773	1999	0865	32	48.31	176.17	273.95
10	2099*	08/05/2012	745	2024	0908	36	74.25	205.22	272.11
11	2095	12/04/2012	771	2024	0593	30	77.05	221.12	252.23
Average			746.18	-	-	29.33	83.64	198.45	315.35

*Animal with the LEP gene sequences information (confirmed cow).
BW: body weight; WW: weaning weight at 205 days of age; YW: yearling weight at 365 days of age and FW: corrected final weight at the end of test (source: PT. Karya Anugerah Rumpin).

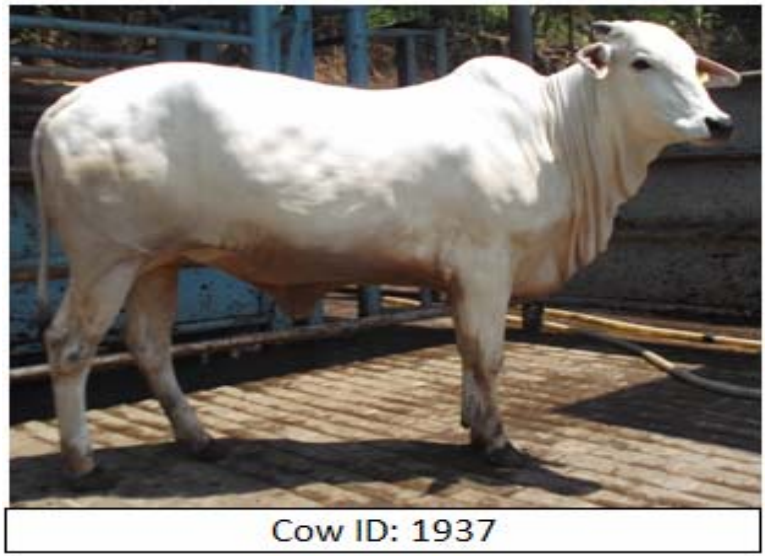

Figure 3 The second best of Sumba Ongole cows (confirmed) based on performance test results

Table 4 Profile of leptin (LEP) gene sequences in the three confirmed cows in the performance test

Gene	Region	SNP	Mutation type	Cattle ID / genotype		
				1937	2097	2099
LEP 3	Intron 2	g.2310T/A	Transversions	TA	TT	TA
LEP 3	Intron 2	g.2325G/T	Transversions	GG	GT	GT
LEP 3	Intron 2	g.2351G/A	Transitions	GG	GG	GG
LEP 3	Intron 2	g.2361C/T	Transitions	CC	CC	CC
LEP 3	Intron 2	g.2362A/T	Transversions	AT	AA	AA
LEP 3	Intron 2	g.2377A/T	Transversions	AA	AA	AA
LEP 3	Intron 2	g.2423A/C	Transversions	AA	AA	AC
LEP 3	Intron 2	g.2448C/T	Transitions	CT	CT	CT
LEP 3	Intron 2	g.2456C/G	Transversions	CG	CG	CG
LEP 3	Intron 2	g.2466C/T	Transitions	CC	CT	CT
LEP 4	Intron 2	g.2778T/A	Transversions	TA	TA	TT
LEP 4	Intron 2	g.2857G/A	Transitions	GA	GA	GG
LEP 4	Exon 3	g.3260T/C	Transitions	TC	TC	TT
LEP 4	Exon 3	g.3272T/C	Transitions	TT	TT	TT
LEP 4	Exon 3	g.3356C/T	Transitions	CC	TT	CC
LEP 4	Exon 3	g.3468G/A	Transitions	GG	GG	GG

CONCLUSION

Research showed that the intron 2 and exon 3 regions of LEP gene in SO cattle are polymorphic. However, the association between SNPs with growth traits of SO cattle is important to analysis through depth research. The genetic diversity of LEP gene in the tested cows can be used as an early information to obtain genetics marker in the future. One SNP in intron 2 (g.2270A/G) and two SNPs in exon 3 (g.3100C/T; and g.3157A/G) of LEP gene were widely used as molecular selection in cattle but both SNPs were not detected in SO cattle. One novel SNP of g.3468G/A was occured in the exon 3 of LEP gene in SO cattle. This SNP was changed the amino acid from glycine (Gly) to arginine (Arg).

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