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Association between GH (g.1456_1457insT), GHRH (g.4474 C>A), and Pit-1 (g.244G>A) Polymorphisms and Lactation Traits in Holstein Friesian Cattle

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ABSTRACT

Lactation traits are controlled by many genes, among others, potentially by growth genes. This research was conducted to study genetic polymorphisms of GH, GHRH, and Pit-1 genes and associations of GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes with milk yield and quality in Holstein Friesian (HF) cattle. Genotyping was conducted for HF dairy cows raised by small farmers from North Lembang (SF-NL) (98 heads) and South Lembang (SF-SL) (95 heads), and also from Cikole Dairy Cattle Station (CDCS) (82 heads) in Lembang, West Java, Indonesia. Progeny tested of HF bulls (17 heads) from LAIC (Lembang Artificial Insemination Center) in West Java and from SAIC (Singosari AIC) (32 heads) in East Java were also genotyped. Effects of genotypes on test day milk yield, fat content, and SNF content were analyzed by General Linear Models. The GHRH g.4474 C>A SNP and Pit-1 g.244 G>A SNP generated high frequencies of C allele to A allele, while the two allelic frequencies of the GH g.1456_1457 InsT varied. Compared to the AA and AC genotypes, the CC genotype of the GH gene resulted higher test day milk yield ($p<0.01$), fat content ($p<0.05$), and SNF content ($p<0.05$). Further the CC genotype of the GHRH gene yielded higher milk yield ($p<0.05$), while the GG genotype of the Pit-1 gene resulted higher fat content ($p<0.05$). Therefore the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP are potential to be used as molecular markers for selection on milk yield and quality in domestic HF cattle.

Keywords: Holstein Friesian; growth gene; association; lactation trait

INTRODUCTION

National fresh milk demands are mostly supplied by Holstein Friesian (HF) dairy cattle. HF cows have the ability to produce high milk in temperate climates, but their milk productions decrease in the tropics, due to the influences of genetic and environmental interaction (Anggraeni, 2012; Petrini *et al*., 2016). Selection is required to select dairy cattle with the optimal milk production under local management and tropical climate. Milk qualities providing mainly fat and Solid Non-Fat (SNF) contents are also the determining factors for the selling prices of domestic fresh milk. Breeding programs for the improvement of milk production and milk components are the main concerns in dairy production system and market (Ardicli *et al*., 2019; Hoseinzadeh *et al*., 2015). Selection techniques at the DNA level and quantitative trait locus (QTL) can help to accelerate quantitative selections through the identification of major genes controlling the economic traits. Milk yield and its quality in dairy cattle are quantitative traits and controlled by many genes which are potentially influenced by the growth genes (Ahmadi *et al*., 2015; Ahmadzadeh *et al*., 2019; Bayram *et al*., 2017; Szatkowska *et al*., 2009).

The growth genes providing GH, GHRH, and Pit1 genes influence biological functions of the livestock, such as growth, development, proliferation of udder cells, and lactogenesis (Ahmadi *et al*., 2015; Ahmadzadeh *et al*., 2019; Bayram *et al*., 2017; Kiyici *et al*., 2019; Sami *et al*., 2011; Szatkowska *et al*., 2009). Bovine Growth Hormone Releasing Hormone (bGHRH) gene consists of five exons and four introns and locates in the chromosome number 13 (BTA-13). The GHRH gene affects growth, productivity, milk production, and milk quality in dairy cattle (Szatkowska *et al*., 2009). The AA cows of the GHRH|H*ae*III locus or GHRH g.4474 C>A SNP produced higher milk than the AB (AC) and BB (CC) ones. Further, Pituitary-Specific Positive Transcription Factor 1 (Pit1) gene or POU1F1 gene or Growth Hormone Factor 1 (GHF1) gene consists of six exons. This gene codes a polypeptide chain of 291 amino acids (~33kD) and locates in the bovine chromosome 1 (BTA1) (Huai *et al*., 2011; Ma *et al*., 2017). The Pit-1 g.244 G>A SNP of the A allele, to the B (G) allele, was associated with higher milk production, protein percentage, and fat percentage (Aytekin & Boztepe., 2013). Further, higher milk production was found in the

BB (GG) cows compared to the AA and AB (AG) cows in Holstein cattle (Ahmadi *et al*., 2015).

Investigation on the effects of the growth genes on milk yield and quality of domestic HF cattle was still limited. The information on variant genotypes of the growth genes on lactation traits could be useful to be used as a Marker Assisted Selection (MAS) of milk yield and quality in dairy cattle. Therefore, this study was conducted to identify GH g.1456_1457 InsT, GHRH g.4474C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms and associations of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes with milk yield and quality of HF cows kept under two different managements, under small dairy farmers and a dairy cattle station, in West Java Province, Indonesia.

MATERIALS AND METHODS

Location and Sample Collection

This research was conducted for Holstein Friesian (HF) cows that were kept at small farmers in North Lembang (SF-NL) and South Lembang (SF-SL) as well as from a dairy cattle station at Cikole Dairy Cattle Station (CDCS) in Lembang Subdistrict, West Bandung District, West Java Province, Indonesia. CDCS was located at a high altitude of 1200 m asl in Cikole Village, Lembang Subdistrict, West Java, Indonesia. SF-NL and SF-SL were also located in Lembang Subdistrict, so the climate in these two small farmer locations were approximately similar to that in the CDCS. Average air temperature was around 19.3°C (13.8-24.6°C), relative humidity by 80.5%, and rainfall by 2,393 mm/year.

HF cows in this study were in lactation lengths of 1-5 mo. and lactation periods of 1-4 coming from SF-NL by 98 heads, SF-SL by 95 heads, and CDCS by 82 heads. HF bulls as the sources of frozen semen of AI mating were also sampled to study possible genetic contributions of the GH, GHRH, and Pit-1 polymorphisms of these bulls to the observed HF cows. Therefore, HF bulls were observed from Lembang Artificial Insemination Center (LAIC) by 17 heads in Lembang Subdistrict, West Bandung District, West Java, Indonesia and from Singosari Artificial Insemination Center (SAIC) by 32 heads in Singosari Subdistrict, Malang District, East Java, Indonesia.

Blood Collection

Blood samples were taken through a jugular vein using a venoject needle and a vacutainer tube containing heparin anticoagulant. The blood sample was then added with absolute ethanol by a ratio of 1: 9 and stored at a room temperature (Anggraeni *et al*., 2017).

DNA Extraction

DNA extraction was conducted by following phenol-chloroform standard (Sambrook & Russell. 2001) consisting of four stages, namely blood sample preparation, protein degradation, organic matter degradation, and DNA precipitation. Sample preparation: 200 µL of blood sample was inserted into a 1.5 mL tube, added with 1,000 µL of distilled water, and centrifuged at the speed of 8,000 rpm for 5 minutes. Protein degradation: sample was added with 350 µL of 1xSTE, 40 µL of SDS 10%, and 10 µL of proteinase K5 (mg/mL) then incubated at 55°C for two hours. Degradation of organic matter: the solution was added by 400 µL of phenol, 400 µL of chloroform isoamyl alcohol (24:1), and 40 µL of NaCl, then gently shaken at room temperature for one hour. Precipitation of DNA: 400 µL of supernatant was added with 40 µL of NaCl 5 M and 800 µL of absolute ethanol, homogenized, and then frozen overnight.

Amplification

The PCR mixture consisted of 7.5 µL of GoTaq Green Master Mix (Promega, US). One microliter of each primer (10 µM), 1 uL of DNA template (50 ng), and nuclease-free water was added to the final volume of 15 µL. The PCR program consisted of an initial denaturing at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 1 minute. The amplification process was terminated by extension at 72°C for 5 minutes. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was conducted by using 5 µL of PCR products added with 0.9 µL of distillation water, 0.7 µL of enzyme buffer (Buffer Tango for M*sp*I, Buffer R for H*ae*III, and H*inf*I), and 0.4 µL of restriction enzyme (M*sp*I, H*ae*III, and H*inf*I). PCR product for each gene was cut by a specific restriction enzyme. Specific restriction enzymes used to cut each of GH, GHRH and Pit-1 genes were successively M*sp*I, H*ae*III, and H*inf*I. The MspI enzyme recognizes base sites of C*CGG or GH g.1456_1457 InsT (Dybus, 2002), the H*ae*III enzyme recognizes base sites of GG*CC or GHRH g.4474 C>A SNP (Rini *et al*., 2013), and H*inf*I enzyme recognizes base sites of G*ANTC or Pit-1 g.244 G>A SNP (Dybus *et al*., 2004).

Gene locus, primary sequences (forward and reverse), annealing temperature, size of digested product, and size of PCR product are presented in Table 1. The mixture was incubated at 37°C for 16 hours. Then the mixture was electrophoresed on 2% of agarose gel at 100 volts for 30 minutes and the agarose gel was observed for DNA band length by using ultraviolet light.

Milk Yield and Quality

Data of milk production were test day milk yield (L/d) of individual of HF cows. Similarly, data of milk quality consisted of test day milk fat content (%) and solid non fat (SNF) content (%) of HF cows from SF-NL (98 heads), SF-SL (95 heads), and CDCS (82 heads).

Data Analysis

Molecular data of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms were analyzed by the Popgen32 package (Yeh & Boyle, 1997) for genotype and allele frequencies by computing genotype number of each locus of each sub-

Table 1. Information of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms for primary sequences (forward and reverse), annealing temperature, size of digested product, and size of PCR product

Locus	Primary sequence	Annealing temp. (°C)	PCR product size (bp)	Digestion product size (bp)
GH (g.1456_1457 InsT)	F: 5'-CCCGAGACGGGCAAGAATGC-3'	62	329	TT: 329
	R: 5'-TGAGCAGGGGCCGGAACTCA-3'			CT: 329, 224, 105
	Dybus (2002)			CC: 224, 105
GHRH (g.4474 C>A SNP)	F: 5'-TGAAGGCTGCTCTGGATGGT-3 '	60	451	AA: 312, 94, 45
	R: 5'-TGCTTCCTGATGTCCTGGATAA-3'			AC: 312, 194, 118, 94, 45
	Rini *et al.* (2013)			CC: 194, 118, 94, 45
Pit-1 (g.244G>A)	F: 5′-AAACCATCATCTCCCTTCTTCTT-3′	60	611	AA: 611
	R:5′-AATGTACAATGTGCCTTCTTCTG-3′			AG: 244, 367, 611
	Dybus *et al*. (2004)			GG: 244, 367

population. Study on the effects of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes were separately associated on test day milk yield, fat content, and SNF content using the General Linear Model (GLM) for unbalanced data. Fixed effects were considered for genetic factors of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes and non-genetics factors of lactation length (1-5 mo.) and lactation period (1-4). The statistical model for the GLM analyzed as follow:

$$Y_{ijklmn} = \mu + G_i + M_j + P_k + S_l + T_m + \varepsilon_n$$

where Y_{ijklmn} was individual test day milk, fat content, and solid non fat content, μ was overall means for each trait, G_i was the i^{th} genotype effect, L_j was the j^{th} month of lactation effect (1, 2, 3, 4, 5), P_k was the k^{th} period of lactation effect (1, 2, 3, 4), S_l the l^{th} season of calving effect (1-6, 7-12), T_m was the the m^{th} year of calving effect (2011, 2012, 2013), and ε_n was the n^{th} random residual effect.

Duncan Multiple Range Test was used to investigate mean differences among subclasses. Data analysis was conducted using SAS Program Package (SAS 9.1, 2011).

RESULTS

Genotype and Allele Frequencies

Genotyping of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP successfully fragmented the DNA amplifications for all of the HF cows and HF bulls as targetted in this study. The GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms are presented for the frequencies of genotype in Table 2 and allele in Table 3. Genotyping of the GH g.1456_1457 InsT of the HF cows resulted three genotypes of CC, CT, and TT. HF cows in small farmer of SF-NL location possessed high frequency of the TT genotype (0.510), while the CC and CT genotypes had almost similar frequencies (0.265 and 0.225). Further, HF cows in SF-SL location had the highest frequency of the CC genotype compared to the TT and CT genotypes, i.e., 0.737 vs. 0.021 and 0.242. HF cows in CDCS presented only the CC and CT genotypes with the frequencies of 0.683 and 0.317 respectively. The CC, CT, and TT genotypes of HF cows in SF-NL (0.265, 0.225, and 0.510) were quite similar to those of HF bulls from LAIC (0.000, 0.294, and 0.706) and SAIC (0.063, 0.219, and 0.719). HF bulls from LAIC, as well as HF cows in CDCS, also carried only the TT and CT genotypes.

The GHRH g.4474 C>A SNP produced AA, AC, and CC genotypes for all HF cows. In both SF-NL and SF-SL cows, the frequenciest of the AC cows (0.480 and 0.505) were higher than those of the CC cows (0.459 and 0.411), and the lowest ones were found for the AA genotype (0.061, 0.084). In contrast, CDCS cows showed a slightly higher frequency of the CC genotype (0.561) than the AC genotype (0.402), and the lowest one was found for the AA genotype (0.037). Proportions of the respective AA, AC, and CC genotypes of the HF progeny tested

Table 2. Genotype frequencies of GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms of Holstein Friesian cattle by location

Locations	Gene (Locus)								
	GH (g.1456_1457insT)			GHRH (g.4474 C>A)			Pit-1 (g.244 G>A)		
	CC	CT	TT	AA	AC	CC	AA	AG	GG
SF-NL	0.2653 (26)	0.2245 (22)	0.5102 (50)	0.0612 (4)	0.4796 (49)	0.4592 (45)	0.1429 (17)	0.3980 (37)	0.4592 (44)
SF-SL	0.7368 (65)	0.2421 (28)	0.0211 (2)	0.0842 (8)	0.5053 (48)	0.4105 (39)	0.0737 (9)	0.5263 (48)	0.4000 (38)
CDCS	0.3171 (30)	0.6829 (52)	0.0000 (0)	0.0366 (3)	0.4024 (35)	0.5610 (45)	0.21202 (10)	0.3780 (31)	0.5000 (41)
LAIC	0.0000 (0)	0.2941 (5)	0.7059 (12)	0.0000 (0)	0.4706 (8)	0.5294 (9)	0.1176 (2)	0.1765 (3)	0.7059 (12)
SAIC	0.0625 (2)	0.2187 (7)	0.7188 (23)	0.0625 (2)	0.3438 (11)	0.5937 (19)	0.0625 (4)	0.3750 (6)	0.5625 (22)

Note: SF-NL= Small Famers in North Lembang; SF-SF= Small Famers in South Lembang; CDCS= Cikole Dairy Cattle Station; LAIC= Lembang AI Center; SAIC= Singosari AI Center. (…) = number of animal.

Table 3. Allele frequencies of GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms of Holstein Friesian cattle by location

Locations	Gene (Locus)					
	GH (g.1456_1457insT)		GHRH (g.4474 C>A)		Pit-1 (g.244 G>A)	
	C	T	A	C	A	G
SF-NL	0.3776	0.6224	0.3010	0.6990	0.3418	0.6582
SF-SL	0.8579	0.1421	0.3368	0.6632	0.3368	0.6632
CDCS	0.6585	0.3415	0.2378	0.7622	0.3049	0.6951
LAIC	0.1471	0.8529	0.2353	0.7647	0.2059	0.7941
SAIC	0.1719	0.8281	0.2344	0.7656	0.2500	0.7500

Note: SF-NL= Small Famers in North Lembang; SF-SF= Small Famers in South Lembang; CDCS= Cikole Dairy Cattle Station; LAIC= Lembang AI Center; SAIC= Singosari AI Center.

bulls in LAIC (0.000, 0.471, and 0.529) and SAIC (0.063, 0.344, and 0.594) as two AI centers producing HF frozen semens were nearly the same to that HF cows in CDCS. Nonetheless, HF bulls from LAIC had only the AB and BB genotypes for this locus.

The Pit-1 g.244 G>A SNP for all HF cattle observed also generated AA, AG, and GG genotypes. A general pattern revealed that the AG and GG cows gained higher frequencies, whereas the AA cows were at the lowest frequency. Cows in SF-NL and CDCS as well as the HF bulls from the two AI centers were higher for the GG genotype (0.400-0.706) than the AG genotype (0.177-0.433). Even the HF bulls in LAIC reached the highest for the GG genotype over the AG one, i.e., 0.706 vs. 0.177. In contrast, HF cows in SF-SL had lower frequency of the GG genotype than the AG one, i.e., 0.400 vs. 0.526. Yet the Pit-1 g.244 G>A SNP generated very low of the AA genotype either in HF cows (0.073-0.143) or in HF bulls (0.063-0.118).

As the GH g.1456_1457 InsT resulted CC, CT, and TT genotypes, this generated two types of C and T alleles. HF cows in SF-NL and HF bulls from the two AI Centers delivered the T allele at very high frequencies compared to the C allele, i.e., 0.622-0.853 vs. 0.147-0.378. In contrast, HF cows in SF-SL and CDCS had very high the C allele compared to the T allele, i.e., 0.659-0.858 vs. 0.142-0.342. While the GHRH g.4474 C>A SNP generated very high the C allele (0.663-0.766) compared to the A allele (0.234-0.337) for all HF animals. The same condition was found for the Pit-1 g.244 G>A SNP with very high the G allele (0.658-0.794) compared to the A allele (0.206-0.342).

Associations of the Growth Genes with Milk Yield and Quality

Results of the association of variant genotypes of each of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP on lactation traits providing milk yield are presented in Table 4, milk fat content in Table 5, and solid non fat (SNF) content in Table 6.

Table 4 shows a very significant association ($p<0.01$) of the GH (g.1456_1457 InsT) genotypes on test day milk yield in HF cows from SF-SL, of which the CC cows yielded the higher milk (17.39±0.57 L/d) compared to the CT cows (12.59±0.76 L/d) and the TT cows (11.40±0.04 L/d). The same results were observed for all locations. A significant effect ($p<0.05$) of the GHRH (g.4474 C>A) genotypes on milk yield was observed for HF cows from SF-SL, but this result was different from those found in SF-NL and CDCS. In SF-SL, the CC cows (11.82±0.58 lt./d) and the AC cows (11.46±0.55 L/d) yielded more milk than the AA cows (9.61±1.53 L/d). However, there was no effect of the Pit-1 (g.244 G>A) genotypes on milk yield of all HF cows observed.

Table 4. Association of GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes on test day milk yield (L/d)

Gene (locus)	Genotype	Locations							
		SF-NL*		SF-SL**		CDCS		All Locations**	
		N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
GH (g.1456_1457 InsT)	CC	26	11.54±0.95^{a}	65	17.39±0.57^{c}	30	12.35±0.68	121	14.57±0.44^{c}
	CT	22	9.87±0.85^{a}	28	12.59±0.76^{a}	52	12.41±0.37	102	11.93±0.34^{a}
	TT	50	11.45±0.39^{a}	2	11.40±0.04^{a}	-	-	52	11.58±0.39^{a}
GHRH (g.4474 C>A)	CC	45	11.82±0.58^{b}	45	13.52±0.65^{a}	44	12.18±0.43	134	12.50±0.37^{a}
	AC	49	11.46±0.55^{b}	45	13.99±0.77^{a}	35	12.66±0.58	129	12.97±0.39^{a}
	AA	4	9.61±1.53^{a}	5	13.96±1.38^{a}	3	12.31±0.33	12	12.61±0.91^{a}
Pit-1 (g.244 G>A)	GG	44	11.39±0.12^{a}	38	14.16±0.72^{a}	41	12.46±0.53	123	12.73±0.91^{a}
	AG	37	10.71±0.11^{a}	48	13.75±0.72^{a}	31	12.51±0.43	116	12.62±0.39^{a}
	AA	17	10.78±0.25^{a}	9	14.56±1.62^{a}	10	12.18±1.25	36	12.74±0.37^{a}

Note: SF-NL= Small Famers in North Lembang; SF-SF= Small Famers in South Lembang; CDCS= Cikole Dairy Cattle Station. *= Means in the same column with different superscripts differ significantly ($p<0.05$). **= Means in the same column with different superscripts differ very significantly ($p<0.01$). N= number of animal.

Table 5. Association of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes on test day fat content (%)

Gene (Locus)	Genotype	Locations							
		SF-NL		SF-SL		CDCS		All locations	
		N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
GH (g.1456_1457 InsT)	CC	26	4.28±0.19[b]	65	3.36±0.09[b]	30	3.31±0.08[a]	121	3.41±0.06
	CT	22	3.14±0.14[a]	28	3.86±0.12[b]	52	3.35±0.06[a]	102	3.41±0.06
	TT	50	3.32±0.11[a]	2	2.63±0.05[a]	-	-	52	3.51±0.27
GHRH (g.4474 C>A)	CC	45	3.57±0.13[a]	45	3.12±0.12[a]	44	3.14±0.07[a]	134	3.34±0.06
	AC	49	3.83±0.10[a]	45	3.26±0.09[a]	35	3.14±0.06[a]	129	3.46±0.06
	AA	4	3.348±0.63[a]	5	3.46±0.28[a]	3	3.14±0.53[a]	12	3.54±0.27
Pit-1 (g.244 G>A)	GG	44	3.38±0.12[a]	38	3.31±0.11[a]	41	3.49±0.07[b]	123	3.54±0.06
	AG	37	3.86±0.11[b]	48	3.07±0.11[a]	31	3.22±0.08[a]	116	3.46±0.07
	AA	17	3.50±0.25[a]	9	3.46±0.20[a]	10	3.28±0.13[a]	36	3.33±0.13

Note: SF-NL= Small Famers in North Lembang; SF-SF= Small Famers in South Lembang; CDCS= Cikole Dairy Cattle Station. Means in the same column with different superscripts differ significantly ($p<0.05$). N= number of animal.

Table 6. Association of genetic polymorphisms of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) SNPs on test day solid non fat content (%)

Gene (Locus)	Genotype	Locations							
		SF-NL		SF-SL		CDCS		All locations	
		N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
GH (g.1456_1457 InsT)	CC	26	8.34±0.06	65	8.54±0.05[b]	30	8.33±0.05	121	8.33±0.03[b]
	CT	22	8.44±0.09	28	8.44±0.08[b]	52	8.30±0.03	102	8.30±0.03[b]
	TT	50	8.23±0.09	2	7.02±0.04[a]	-	-	52	8.16±0.08[a]
GHRH (g.4474 C>A)	CC	45	8.13±0.08	45	7.70±0.06[a]	44	8.17±0.04	134	8.17±0.04[a]
	AC	49	8.20±0.07	45	7.55±0.06[a]	35	8.17±0.04	129	8.16±0.03[a]
	AA	4	8.67±0.32	5	8.75±0.21[b]	3	8.44±0.01	12	8.44±0.14[a]
Pit-1 (g.244 G>A	GG	44	8.36±0.06	38	8.05±0.07[a]	41	8.28±0.04	123	8.28±0.03[a]
	AG	37	8.34±0.09	48	7.95±0.06[a]	31	8.26±0.04	116	8.26±0.04[a]
	AA	17	8.31±0.17	9	8.00±0.07[a]	10	8.25±0.11	36	8.25±0.09[a]

Note: SF-NL= Small Famers in North Lembang; SF-SF= Small Famers in South Lembang; CDCS= Cikole Dairy Cattle Station. Means in the same column with different superscripts differ significantly ($p<0.05$). N= number of animal.

Table 5 shows that the GH (g.1456_1457 InsT) genotypes significantly affects ($p<0.05$) the test day fat content in SF-NL and SF-SL, except in CDCS. In SF-NL it was found that the CC cows yielded higher fat content (4.28±0.19%) than the CT (3.14±0.14%) and TT (3.32±0.11%) cows. However, in SF-SL, both the CC (3.36±0.09%) and CT (3.86±0.12%) cows produced higher fat compared to the TT cows (2.63±0.11%). Conversely, the GHRH (g.4474 C>A) genotypes gave no significant effects on milk fat content of all HF cows. Further, the Pit-1 (g.244 G>A) genotypes significantly affected ($p<0.05$) milk fat content of HF cows in SF-NL and CDCS. The heterozygous AG cows (3.86±0.11%) produced higher fat than the homozygous AA and GG cows (3.38±0.12% and 3.50±0.25%), respectively at the first location, whereas the GG cows yielded higher fat (3.49±0.07%) compared to the AG (3.22±0.08%) and AA (3.28±0.13%) cows at the later location.

Table 6 shows that the GH (g.1456_1457 InsT) genotypes significantly affects ($p<0.05$) test day SNF content of HF cows in SF-SL and all locations. The CC (8.540±0.05%) and CT (8.438±0.08%) cows produced higher SNF than the TT cows (7.02±0.04%) in SF-SL. The same condition was found for all locations. However, the GHRH (g.4474 C>A) genotypes significantly affected ($p<0.05$) SNF only for HF cows in SF-SL. These genotypes did not significantly affect SNF in the SF-NL and CDCS locations. SNF content of the AA cows (8.75±0.21%) were higher compared to SNF contents of the CC (7.70±0.06%) and AC cows (7.55±0.06%). In contrast, Pit-1 (g.244 G>A) genotypes expressed no significant effect on SNF content in all HF cows observed.

DISCUSSION

Genetic Polymorphism of the Growth Genes

Genetic polymorphisms of the growth genes providing the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP from PCR-RFLP techniques in this study were reflected by variant genotypes and alleles of the observed HF cows and HF bulls.

GH g.1456_1457 InsT Polymorphism

Amplified DNA product of the GH gene produced a fragment length by 327 bp. The length was similar to the findings in Polish cattle (Dybus, 2002) and

Colombian Holstein (Arango *et al*., 2014). The PCR product of the GH g.1456_1457 InsT was located in intron 3 and exon 4 at the base position of 1457. Mutations at the base 104, as cutting sites of the *Msp*I restriction enzyme, was known a C-T mutation as a change from C base to T base (Dybus, 2002). Genotyping the GH g.1456_1457 InsT resulted three genotypes, i.e., CC, CT, and TT. The CC genotype was presented by the only one band (327 bp), the TT genotype was presented by two bands (104 and 223 bp), and the CT genotype was presented by three bands (104, 223, and 327 bp). The GH g.1456_1457 InsT of the HF cattle across locations from this study showed different genotype frequencies. In SF-NL cows, this locus generated the most TT cows (51%), while the CC (26.5%) and CT (22.5%) cows were almost similar. In SF-SL cows, the GH g.1456_1457 InsT presented the most frequent CC cows (73.7%), a lower CT cows (24.2%), and the rarest TT cows (2.1%). In CDCS cows, this locus presented very high of the CC cows than the CT cows, i.e., 68.3% vs. 31.7%. Proportion of the genotypes of the HF cows in SF-NL seemed similar to those of the HF bulls in both SAIC and LAIC, in spite of no TT in LAIC.

The most often frequency of the CC animals in this study were consistent to the result reported by Arango *et al*. (2014) that the highest were CC, followed by CT, then the lowest TT genotypes in Columbian Holstein. Therefore, it was identified a very high frequency of the C allele against the T allele, i.e., 91% vs. 9%. Investigation on the GH|M*sp*I locus or GH g.1456_1457 IinsT locus and GH|A*lu*I locus in Tunisian Holstein by Amiri *et al*. (2018) also confirmed these similar results. The animals delivered common CC genotype (55.5%), moderate CT genotype (29.1%), and low TT genotype (15.4%). These resulted in dominant C allele (70.1%) as compared to T allele (29.9%). Further, the GH|A*lu*I locus generated the highest LL genotype (77.8%), lower LV genotype (18.6%), and the lowest VV genotype (3.7%). These results brought in a very high V allele (87.0%) than L allele (13.0%). By genotyping the GH|A*lu*I locus of the two NZ and Australian imported HF kept at a Dairy Breeding Center in Central Java also reported a very common LL genotype compared to the LV genotype, i.e., 79-8% vs. 16-2%, respectively (Hartatik *et al*., 2015). For the Indonesian local Ongole Grade beef cattle was identified by Hartati *et al*. (2019) that the genotype frequencies of the CC, CT, and TT genotypes were 1.1%, 18.8%, and 80.1%, respectively and the allele frequencies of C and T alleles were 10.5% and 89.5%, respectively.

GHRH g.4474 C>A SNP Polymorphism

Amplified DNA product of the GHRH gene produced the base fragment by 451 bp, located in exon 2, intron 2, and exon 3 of this gene. This fragment fitted to the genome library (GenBank: AF242855) as previously reported by Rini *et al*. (2013). The H*ae*III restriction enzyme recognized the cutting base fragment at the nucleotide sites of 4472, 4666, and 4760 or at the base positions of 118, 312, and 406 of the PCR products. The GHRH|H*ae*III locus or the GHRH g.4474 C>A SNP resulted in three genotypes, i.e., AA genotype by 3 bands (312, 94, and 45 bp), CC genotype by 4 bands (194, 118, 94, and 45 bp), and AC genotype by 5 bands (312, 194, 118, 94, and 45 bp). These three genotypes were consistent to the results reported by previous study (Rini *et al*., 2013).

The GHRH g.4474 C>A SNP of all HF cows yielded AA, AC, and CC genotypes. There was a general pattern that all of the HF cows carried the lowest AA genotype (3.7%-8.4%) compared to the AC genotype (40.2%-50.5%) and the CC genotype (41.1%-56.1%). A similar pattern was also occurred in HF bulls in the two AI Centers. HF bulls in SAIC carried more common CC, lower AC, and very low AA genotypes, i.e., 59.4%, 34.4%, and 6.3%, respectively. Even HF bulls in LAIC generated only the CC and AC genotype, each with 52.9% and 47.1%, respectively. The use of these active AI-mating HF Bulls with the lowest AA genotype could be one possible factor on transmitting the lowest AA cows as the offsprings, instead of the AC and CC cows, in population. The more frequent CC and AC genotypes in the HF cattle across populations causes the high C allele and the low A allele, i.e., 66.3%-76.6% vs. 23.4%-33.7%.

The same results were reported on this genetic polymorphism of HF cattle from the two AI centers and an ET Center providing a very rare of the AA (4.5%) cattle than the AC cattle (37.1%) and CC cattle (58.4%); likewise generating a very low A allele (22.5%) rather than C allele (77.5%) (Rini *et al*., 2013). Some previous studies also confirmed the same results for the lowest AA animals compared to the AB (AC) and BB (CC) animals, such as in Polish Holstein i.e., 7.8% vs. 33.9% and 58.3%; abd Jersey i.e., 3.2% vs. 42.7% and 54.1% (Szatkowska *et al*., 2009). Therefore, all these studies consistently showed a very high of the C allele compared to the A allele, namely 71.9%-75.4% vs. 24.6%-28.1%, respectively.

Pit-1 g.244 G>A SNP Polymorphism

The PCR products of the the Pit-1|H*inf*I locus or the Pit-1 g.244 G>A SNP produced a base fragment by 611 bp that was consistent to the genome library (GenBank: Y 15995 and AM 490263). This fragment length matched to the reference (Dybus *et al*., 2004). A point mutation for the change of G to A base at exon 6 at the base 357 was identified by the HinfI restriction enzyme at the restriction base site of G*ANTC (Dybus *et al*., 2004). This G/A base mutation is called silent mutation for resulting in no change in amino acids. Further, the HinfI restriction enzyme recognized the cutting base fragments at nucleotide base positions of 312, 194, 118, 94, and 45 bp of the PCR products. AA genotype was presented by only one band (611 bp), GG genotype was presented by 2 bands (244 and 367 bp), and AG genotype was presented by 3 bands (244, 367, and 611 bp). However, the rest two fragments, i.e. 94 bp and 45 bp, were out of running. Therefore, A allele was presented by only one fragment (611 bp) and G allele had 2 fragments (244 and 367 bp). The same genotypes of the Pit-1|H*inf*I locus or Pit-1 g.244 G>A SNP were reported in Polish Black-and-White cattle (Dybus *et al*., 2004), Iranian native and

Holstein cattle (Doosti *et al*., 2011), and Iranian Holstein cattle (Ahmadi *et al*., 2015; Hoseinzadeh *et al*., 2015).

HF cows in this study showed many the AG (39.8-52.6%) and GG (40.0-46.3%) cows rather than few the AA cows (7.3%-14.3%). A similar pattern was found for HF Bulls from the two AI centers that presented high the AG (17.7%-37.5%) and GG (56.3%-70.6%) bulls with low the AA (6.3%-11.8%) bulls. As previously described from the genotyping results of the GHRH g.4474 C>A SNP and Pit-1 g.244 G>A SNP polymorphisms also showed the active AI-mating HF Bulls carrying more the AG and GG genotypes and rare the AA genotype. These condition might also be a possible cause of high the AG and GG cows rather than the AA cows as the offsprings across population. More common of the AG and GG animals in the population caused higher the G allele compared to the A allele in HF cattle across population, i.e. 65.8%-79.4% vs. 20.6%-34.2%.

The results found in this study were consistent with the results of some previous studies indicating that *Bos taurus* dairy cattle expressed higher of the AG and GG genotypes with a lower of the AA genotype, and a higher frequency of the G allele compared to the A allele. Proportions of the AA genotype compared to the AG and GG genotypes in domestic HF cattle, Poland Red Holstein cattle, Iranian Holstein cattle were in the ranges of 3.5%-6.0% vs. 29.8%-40.0% and 54.0%-66.7% (Ahmadi *et al*., 2015; Aytekin & Boztepe, 2013; Hoseinzadeh *et al*., 2015; Özdemir, 2012). From these findings, the A allele was in the range of 18.5%-26.0% and the G allele were in the range of 74.0%-81.6%. Therefore, based on all these results, it can be stated that the Pit-1 g.244 G>A SNP of the *Bos taurus* dairy cattle tends to have more of the AG and GG genotypes than the AA genotype.

Association of the Growth Genes Polymorphisms on Milk Yield and Quality

Genes controlling lactation traits in dairy animals are of great concern during breeding programs due to their decisive economic values. Growth genes could be functioned as markers assisted selection (MAS) due to their main roles on growth, development of udder cells, process of lactogenesis, and proliferation of udder cells, and lactation traits (Ardicli *et al*., 2019; Bayram *et al*., 2017; Sami *et al*., 2011; Szatkowska *et al*., 2009). This study examined possible influences of the GH (g.1456_1457 InsT), GHRH (g.4474 C>A), and Pit-1 (g.244 G>A) genotypes on milk yield and milk quality in Holstein Friesian cattle.

Milk Yield

The GH (g.1456_1457 InsT) and GHRH (g.4474 C>A) genotypes significantly affected the test day milk yield of HF cows kept by small farmers in SF-SL. However, genotypes of the GH and GHRH genes did not significantly affect the test day milk yield of HF cows in SF-NL and CDCS. The GH (g.1456_1457 InsT) genotypes in SF-SL indicated that the homozygous CC cows produced higher test day milk than the CT and TT cows, with the differences by 4.8 lt./d and 5.99 lt./d, respectively. There was no significant effect of these genotypes on milk yield in SF-NL and CDCS that was similar to the findings in domestic HF cows by Hartatik *et al*. (2015). It was found that there was no significant effects of the GH|A*lu*I genotypes on milk production, fat and protein contents in the imported NZ and Australian HF cows kept in a dairy breeding center in Central Java.

The GHRH (g.4474 C>A) genotypes in SL-SF showed that HF cows carrying the C allele, namely the CC and AC cows, produced higher test day milk yield compared to the AA cows, and the differences were by 2.21 lt./d and 1.85 lt./d, respectively. The same result that the AA cows yielded less milk than the AC and CC cows was also reported by Szatkowska *et al*. (2009). However, the Pit-1 (g.244 G>A) genotypes gave no significant difference on daily test milk yield across HF populations. This result did not fit to the other studies pointing the effects of these genotypes on milk production. Higher daily milk yield by 4 kg/d was reported for the BB (GG) cows compared to the AA and AB (AG) cows in Iranian Holstein (Ahmadi *et al*., 2015).

No significant effects of the GH (g.1456_1457 InsT) and GHRH (g.4474 C>A) genotypes on the test day milk yield of HF cows in SF-NL and CDCS and no significant effects of the Pit-1 (g.244 G>A) genotypes on milk yield of all of HF cows could be due to the influences of genetic and nongenetic factors. Differences in lactation months and lactation periods were considered in the GLM analysis, but any non genetic factors had other significant effects. Further, milk production and lactation traits are quantitative traits controlled by many alleles and many genes (Ahmadi *et al*., 2015; Ahmadzadeh *et al*., 2019; Bayram *et al*., 2017; Raschia *et al*., 2018). Therefore, the possible differences in allele frequencies of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP in one or more populations as well as the influences of many other genes could be the cause of no association of the variant genotypes of one or more of these loci on milk production and milk quality.

Milk Quality

The GH (g.1456_1457 InsT) genotypes gave significantly different effects on the test day fat content of HF cows in SF-NL and SF-SL. However, these genotypes did not have significant effect on the test day fat content of HF cows in CDCS. For the first location, the CC cows produced higher fat compared to the CT and TT cows, i.e., by 1.14% and 0.96%, respectively. However, in the later location, the CC and CT cows yielded higher fat compared to the TT cows, i.e., by 0.73% and 1.24%, respectively.

The Pit-1 (g.244 G>A) genotypes proved their effects on test day fat content of HF cows in SF-NL and CDCS. In SF-NL, it was showed that the heterozygous AB cows yielded higher fat compared to both the homozygous genotypes BB and AA, i.e., by 0.48% and 0.36%, respectively. However, in CDCS, it was found that the homozygous GG cows had higher fat compared to the AG and AA cows, i.e., by 0.28% and 0.22%, respectively. Higher milk yield in the GG cows compared to the AG

and AA cows in CDCS fitted to the results reported by Aytekin & Boztepe (2013). Szatkowska *et al*. (2009) reported the existent tendency for the AA cows with a bit more fat content compared to the other two cows (BB/GG and AB/AG cows) in Polish Holstein cattle. These GH (g.1456_1457 InsT) and GHRH (g.4474 C>A) genotypes significantly affected on test day SNF only in HF cows from SF-SL rather than the two other locations. For the GH gene showed higher SNF contents in the CC and CT cows compared to the TT one, i.e. by 1.52% and 1.42%, respectively. For the GHRH gene showed higher SNF in the AA genotypes compared to the AC and CC cows, by 1.20% and 1.05%, respectively. However, the Pit-1 (g.244 G>A) genotypes resulted in the non significant differences in SNF content of all HF cows in this study.

Based on the investigation of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms on lactation traits of HF cows in this study showed that one or more of these loci of the GH, GHRH, and Pit-1 genes caused the differences in milk yield and milk quality, among others, as well as fat and SNF contents in HF cattle. Large effects were proved either in one or more locations that were under semi intensive small farmers, in SF-NL and SF-SL, or intensive in dairy cattle station in CDCS. From all of these results proved that there were significant effects of the GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP polymorphisms on lactation traits. These provided the GH g.1456_1457 InsT was associated with milk production, fat content, and SNF content; the GHRH g.4474 C>A SNP was associated with milk yield and SNF content; and the Pit-1 g.244 G>A SNP was associated with fat content. Compared to the two other genotypes, the CC genotype of the GH gene produced higher test day milk yield ($p<0.01$), fat content ($p<0.05$), and SNF content ($p<0.05$). Further the CC genotype of the GHRH gene yielded higher milk yield ($p<0.05$), while the GG genotype of the Pit-1 gene resulted higher fat content ($p<0.05$). However the CC genotype of the GHRH gene had lower solid non fat content than the two other genotypes. Therefore, these three molecular markers of the GH, GHRH and Pit-1 genes can be possible to be considered as gene-based assisted tools in the dairy breeding program to make genetic improvement of milk yield and milk quality of domestic HF dairy cattle.

CONCLUSION

The GH g.1456_1457 InsT, GHRH g.4474 C>A SNP, and Pit-1 g.244 G>A SNP were in high genetic polymorphisms of the alleles and the genotypes in HF cattle across populations. The CC genotype of the GH gene (g.1456_1457 InsT) was associated with high test day milk yield, fat content, and SNF content. The CC genotype of the GHRH gene (g.4474 C>A SNP) was related to high milk yield, while the GG genotype of the Pit-1 gene (g.244 G>A SNP) was associated to benefit fat content. Therefore these molecular markers of the GH, GHRH, and Pit-1 genes can be potencial to be considered as marker-assisted selection tools to improve milk yield and milk quality in domestic Holstein Friesian cattle.

CONFLICT OF INTEREST

Cece Sumantri serves as editor of the Tropical Animal Science Journal, but has no role in the decision to publish this article. Authors also certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organization related to the material discussed in the manuscript.

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