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Growth Hormone Gene Polymorphism and Its Association with Partial Cumulative Milk Yields of Holstein Friesian Dairy Cattle

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ABSTRACT

Growth hormone gene (GH gene) plays an important role in regulating body growth and in developing mammary gland, similar with its interaction to specific receptors. The GH gene has been considered as one of candidate gene associated with selection on lactation trait and milk production. This study was aimed to determine genetic polymorphism of the GH-*AluI* gene and to associate its genotype variants on various 15-d partial cumulative milk yields in Holstein Friesian (HF) dairy cows. A number of 370 blood samples were collected from six HF populations, respectively from small dairy farmer under the supervision of the North Bandung Milk Cooperation (NBMC) in Cilumber (98) and Pasir Kemis village (96), Dairy Cattle Breeding and Improvement Station (Cikole DCBIS) Cikole (88), Lembang Artificial Insemination Center (Lembang AIC) (17), Singosari Artificial Insemination Center (Singosari AIC (32), and Cipelang Livestock Embryo Center (Cipelang LEC) (40). A polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method was used to identify variant genotypes of the GH gene using *Alu*I restriction enzyme. Genotyping results produced only two genotypes, i.e. LL and LV genotypes, without VV genotype. Frequency of the former was dominant, whilst that was low for the latter (89% vs. 11%); leading to the frequency of L allele was very high (94%) compared to that of V allele (6%). No significant association between variant genotypes (LL and LV) and various 15-d partial cumulative milk yields.

Key words: growth hormone gene, milk yield, Holstein Friesian

ABSTRAK

Gen hormon pertumbuhan (gen growth hormone atau GH) berperan penting dalam mengatur proses pertumbuhan dan perkembangan kelenjar *mammae*, serta dalam interaksinya terhadap reseptor spesifik. Gen GH telah dipertimbangkan sebagai gen kandidat dari kegiatan seleksi sifat laktasi dan produksi susu. Studi ini bertujuan untuk mempelajari polimorfisme gen GH-*AluI* dan asosiasi keragaman genotipe-nya terhadap produksi susu kumulatif parsial pada setiap interval 15 hari dari sapi perah Friesian Holstein (FH). Sejumlah 370 sampel darah dikoleksi dari enam populasi sapi FH, berurutan meliputi peternakan sapi perah binaan Koperasi Susu Bandung Utara (KPSBU) di Pasir Kemis (96 sampel) dan Cilumber (98 sampel), Balai Pengembangan dan Pembibitan Sapi Perah (BPPT-SP) Cikole (88 sampel), BIB Lembang (17 sampel), BET Cipelang (40 sampel), dan BBIB Singosari (32 sampel). *Polymerase chain reactions - restriction fragment length polymorphism* (PCR-RFLP) digunakan untuk mengidentifikasi keragaman genotipe dari gen GH, menggunakan enzim restriksi *Alu*I. Hasil *genotyping* menghasilkan hanya dua tipe genotipe, yaitu genotipe LL dan LV, tanpa genotipe VV. Frekuensi genotipe LL adalah dominan (89%), sebaliknya frekuensi genotipe LV rendah (11%), sehingga diperoleh frekuensi alel L sangat tinggi (94%), sebaliknya rendah untuk alel V (6 %). Tidak ditemukan adanya hubungan yang nyata antara keragaman genotipe (LL dan LV) dengan produksi susu kumulatif parsial.

Kata kunci: gen hormon pertumbuhan, produksi susu, Friesian Holstein

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INTRODUCTION

Indonesian dairy cattle population is currently around 597,129 heads (DGLAH, 2011). Almost all of those dairy cattle, as producers of fresh milk in the country, are Holstein Friesian (HF) of *Bos taurus* dairy cattle. Raising dairy cattle is mostly concentrated in Java Island. Therefore, the highest volumes of producing fresh milk are from East Java, Central Java and West Java, for respectively 268,042; 100,350; and 536,458 tons. However, the capacity of milk production of dairy cattle should be increased, as the milking ability of HF dairy cows has just met around 35% of the national milk demand. Attempts to make genetic improvement will increase milk yield of dairy cattle permanently, because genetic superiority of milk production will be passed from parent to offspring. One effort that can be done to improve genetic ability of HF cows in producing high milk yield is through a selection method.

Selection is commonly done by selecting superior bulls and cows to be used as sources of genetic material for the next generation. Selection in dairy cows is mainly based on the level of milk production. Milk production is a quantitative trait controlled by many genes and its expression is the accumulation of the factors of genetic, environment, and their interaction. Curently selection can be assisted by using molecular techniques. Selection based on genetic markers for a particular trait makes selection occuring early. Application of the genetic markers into livestock breeding programs can accelerate genetic improvement in livestock.

The GH gene has an important role in growth and development of postnatal longitudinal, growth of mammae and reproduction tissues, as well as metabolisms of protein, lipid, carbohydrate (Akers, 2006). Effects of the GH gene on the growth are observed in several tissues, including bone, muscle, and adiposa. In ruminants, the GH gene contributes to the development of udder glands (Akers, 2006). Growth hormone is an anabolic hormone that is synthesized and secreted by the anterior lobe cells in pituitary somatotrop. In bovine, the GH gene is located on chromosome 19 with a length of about 280 bp, composed by 5 exons and 4 introns. The GH protein consists of 191 amino acids with a molecular weight of 2 kDa (Ayuk & Sheppard, 2006).

The GH gene has been used as a genetic marker for the growth traits in some species such as cattle (Zhou *et al*., 2005; Jakaria *et al*., 2007 and Katoh *et al*., 2008), sheep (Marques *et al*., 2006), and goats (Boutinaud *et al*., 2003). Growth hormone (GH), growth hormone receptor (GHR) and other hormones such as Insulin-Like Growth Factor 1 (IGF1) are widely used as candidate genes of production traits in livestock and subsequently used as a genetic marker for selection. This is because these hormones are regulators of growth and development of the body (Zakizadeh *et al*., 2006). Studies on genetic polymorphism of the GH gene and its relationship to milk production in dairy cattle have been observed in Hungary Holstein Friesian (Balogh *et al*., 2009), Iranian Holstein (Mohammadabadi *et al*., 2010), and Poland Holstein Friesian (Olenski *et al*., 2010). Based on the results of those several studies it was known that the GH gene together with the GHR gene play an important role in regulating the growth of mammary gland and milk production, metabolism, lactation, and body composition (Kovács *et al*., 2006). This research had specific purposes to identify genetic polymorphism of the GH-*Alu*I gene and to association variant genotypes of this gene to various 15-d milk yields in Holstein Friesian (HF) cattle.

MATERIALS AND METHODS

Animals and Milk Yields

Blood samples were collected from HF cattle, male and female, taken from the *vena jugularis*. A total number of 370 HF blood samples were taken from six populations with different management or condition. Blood samples from HF heifers and cows were collected from Cikole Dairy Cattle Breeding and Improvement Station (Cikole DCBIS) located in Lembang, West Java for 88 samples, Cipelang Livestock Embryo Center (Cipelang LEC) for 34 samples, and from North Bandung Milk Cooperation Unit (NBMCU) at small farmers in the two villages of Cilumber (Cilumber NBMCU) for 98 samples and Pasir Kemis (Pasir Kemis NBMCU) for 95 samples. Blood samples from active and non active AI services of HF bulls from Lembang Artificial Insemination Center (Lembang AIC) for 17 samples and Singosari Artificial Insemination Center (Singosari AIC) for 32 samples. The collection of blood samples from these HF bulls was intended to know their genetic potency in transmitting the GH genetic polimorphism to HF cows.

Data of milk yields were collected from 56 HF cows that were genotyped their the GH gene from Cikole DCBIS for production periods of 2008-2010. Data of milk yileds were in the range of I-4 lactation periods. Data of daily milk yields that were recorded weekly were estimated for various partially cumulative milk yields at each 15-d intervals, since the 1st until the 12th partial cummulative milk yields (15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 d).

Primer

Primers used to amplify mutant locus of the GH|*Alu*I gene followed Balogh *et al*. (2009), with a forward primer 5′-CGGACCGTGTCTATGAGAAGCTGAAG-3′ and a reverse primer 5′-GTTCTTGAGCAGCGCGTCGTCA-3′. The amplified product or amplicon had the length of 432 bp.

DNA Sample

DNA samples obtained were from blood and semen. Blood samples used as DNA sources were 353 samples originating from 5 locations, while semen samples as DNA sources were 17 samples from Lembang AIC.

DNA Extraction

DNAs was extracted from blood and semen. Extraction procedure followed the phenol-chloroform

method that was modified by Andreas *et al*. (2010), with the following procedures:

Sample preparation. Semen sample was added by alcohol 400 µl, whereas blood sample was added by alcohol 200 µl, then inserted into a 1.5 ml tube. Alcohol was then eliminated from the sample by adding distilled water until 1000 µl, and left in room temperature for 20 min. Then it was precipitated by centrifugation at a speed of 8000 rpm for 5 min.

Protein degradation. Sample was cleared from alcohol by adding 200 µl 1x STE (sodium tris EDTA), 40 µl sodium dosesil sulfate 10%, and 20 µl proteinase K (5 mg/ml). The mixture was incubated overnight at 55 °C temperature.

Organic material degradation. After incubating, sample was added by 400 µl phenol solution, 400 µl choloform: isoamyl alcohol (24:1), and 40 µl 5M NaCl. Then, the mixture was shaken at room temperature for one hour.

DNA precipitation. Samples was centrifuged at a speed of 5000 rpm for 10 min to separate water phase over phenol phase. The water phase was transferred in a new tube with the volume measured. DNA molecules were deposited by adding a 2x volume of alcohol absolute and 0.1 x volume of 5M NaCl. Then the mixture was incubated at a temperature -20 °C during the night. Subsequent DNA precipitation was centrifugated at a speed of 12000 rpm for 10 minutes. The obtained DNA precipitation was washed by 70% alcohol, then reprecipitated. The precipitated DNAs was cleaned from alcohol by adding 100 µl TE (Tris EDTA). The DNA samples were then stored at -20 °C and ready for use.

Amplification of the GH Gene

Amplification of fragments of the GH gene was done by using PCR (polymerase chain reaction) method. Reagents used for the amplification of the targetted fragment were a 2 µl sample DNA, each primer 25 pmol, 200 µM dNTPs mixture, 1 mM MgCl2, and 0.5 units of DreamTaq™ DNA Polymerase and 1x buffer (Fermentas) in total solution 25 µl. Amplification was by in vitro within GeneAmp® PCR System 9700 (Applied Biosystems™). It was done with the condition of pradenaturation at 94 °C for 5 min, 35 cycles consisting of denaturation at 94 °C for 45 s, annealing primers at 62 °C for 45 s and extention of new DNA at 72 °C for 1 min, and the final extention at 72 °C for 5 min.

Genotyping by RFLP Method

Determination of genotypes of each individual cattle was done by using restriction fragment length polymorphism (RFLP) using AluI enzyme as a restriction enzyme. Visualization was conducted on 2% agarose gel with 0.5 x TBE buffer (tris borate EDTA) at 100 V for 40 min. Gel was stained with an ethidium bromide, and visualized in UV transuliminator, *alpha innotech alpha imager*.

Data Analysis

Genotype frequency represents the ratio of a genotype to total population. Allele frequency is a ratio of an allele to the overall allele at a locus in the population. Mathematic models for genotype and allele frequencies (Nei & Kumar, 2000) were as follows:

$$x_{ii} = (n_{ii}/N) \times 100\%$$
$$x_i = (2n_{ii} + n_{ij}) / 2N$$

x_{ii} = iith genotype frequency
x_i = ith allele frequency
n_{ii} = number of individual with ii genotype
n_{ij} = number of individual with ij genotype
N = total number of individuals

Degree of heterozigosity both observed (h_o) and expected (h_e) were calculated with formula as follows:

$$h_o = 1 - x_i^2$$
$$h_e = 2n (1 - \sum x_i^2) / (2n - 1)$$

h_o = observation heterozigosity
h_e = expected heterozigosity
x_i = ith allele frequency
n = total number of individuals

For study of the associations was analyzed by the General Linear Model (GLM) with one factor. Parameter observed was various partially 15-d cumulative milk yields from 56 heads of the genotyped HF cows from the Cikole DCBIS. Mathematic model (Matjik & Sumertajaya, 2006) was represented as follows:

$$\gamma_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

γ_{ij} = a certain partial cumulative milk yield
μ = average
α_i = additive effect from ith genotype
ε_{ij} = observed error

RESULTS AND DISCUSSION

Amplification of the Growth Hormone Gene

The amplified fragments were visualized on a 1.5% agarose gel (Figure 1). The amplified product (amplicon) of the GH gene had a fragment length of 432 bp, including 55 bp of 4th exon, 4th intron and 99 bp of 5th exon (Balogh *et al*., 2009). One of key factor in determining the success of amplification is annealing temperature. Annelaling temperature is a temperature allowing the primers attaching on DNA templates during a PCR process. The annealing temperature 60 °C for 1 min in this study was accordance with those of some previous studies (Balog *et al*., 2009; Mohammadabadi *et al*., 2010, and Andreas *et al*., 2010).

Identification of the GH Gene Polymorphism

The *Alu*I enzyme as a restriction enzyme cut the recoqnized site of AC|GT bases. There are three *Alu*I restriction sites that produce fragment lengths of 20, 51,

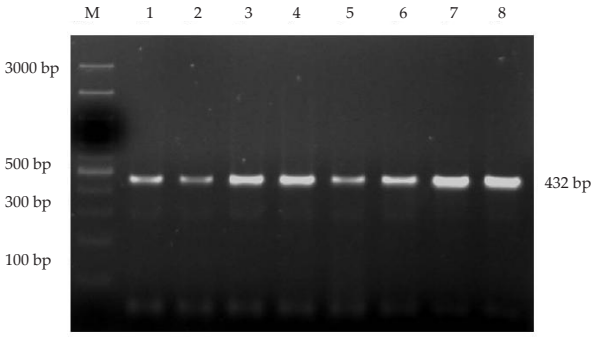

Figure 1. Visualization of the amplified GH gene fragment on 1.5% agarose gel. M= Marker 100 base pairs (bp), 1-8= number of sample.

96 and 265 bp, known as leucine (L) allele, and fragment lengths of 20, 147, and 265 bp, known as valine (V) allele. Genetic variation between L and V alleles was due to mutation at 1758 base resulting the changing base from C to G (Figure 2). A homozygous LL genotype is identified for having four fragment lengths of 20, 51, 96, and 265 bp. A homozygous VV genotype is with the fragment lengths of 20, 147, and 265 bp. A heterozygous LV genotype is then identified by fragment lengths of 20, 51, 96, 147, and 265 bp (Balogh *et al*., 2009).

Genotyping the GH|*Alu*I gene resulted two genotypes, i.e. LL and LV (Figure 3). VV genotype was not found for all samples observed. Visualization of the genotypes of the GH|*Alu*I gene was presented in Figure 3. These results were in line with the study by Pereira *et al*. (2005) by obtaining two genotypes (LL and LV) in Brazilian Canchim cattle. Another study by Curi *et al*. (2006) did not find the VV genotype in Brazilian Zebu cattle and its crosses. However, the results of current study were not similar to that study by Dybus et al. (2002) that identified LL, LV, and VV genotypes of the GH|*Alu*I gene in Polish Black and White cattle. The differences could be caused by breed of cattle, breeding system, and samples genotyped.

Genetic Diversity of GH|*Alu*I Gene within Holstein Friesian

Frequencies of genotypes and alleles of the GH|*Alu*I gene from all of HF cattle observed were presented in Table 1. Genotyping results on the GH|*Alu*I gene showed that frequencies of the L allele were higher than those of the V allele. Frequencies of the L allele of HF cattle observed from all locations ranged from 0.92 to 0.98. Higher frequencies of the L allele in the observed HF cattle resulted higher frequencies of the LL genotype contrasted to those for the LV genotype. No existence of the VV genotype in all of HF cows observed both in breeding station and small farmers could be influenced by HF bulls used for AI services. All of active AI-HF bulls came from the two national AICs (Lembang AIC and Singosari AIC). Genotyping of all HF bulls from both AICs proved that no bull having the VV genotype, whereas the V allele frequencies of those HF bulls were low.

The results from this studi corresponded with the study by Sorensen *et al*. (2002) by obtaining the higher

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1381 cctcagcaga gtcttcacca acagcttggt gtttggcacc tcggaccgtg tctatgagaa
1441 g|ctgaaggac ctggaggaag gcatcctggc cctgatgcgg gtggggatgg cgttgtgggt
1501 cccttccatg ctgggggcca tgcccgccct ctcctggctt agccaggaga atgcacgtgg
1561 gcttggggag acagatccct gctctctccc tctttctagc agtccagcct tgacccaggg
1621 gaaacctttt ccccttttga aacctccttc ctcgcccttc tccaagcctg taggggaggg
1681 tggaaaatgg agcgggcagg agggagctgc tcctgagggc ccttcggcct ctctgtctct
1741 ccctcccttg gcaggagctg gaagatggca ccccccgggc tgggcagatc ctcaagcaga
1801 cctatgacaa atttgacaca aacatgcgca gtgacgacgc gctgctcaag aactacggtc
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Figure 2. Nucleotide of the GH|*Alu*I gene (Genbank access number J00008). Primary position (bold underlined), *Alu*I enzyme cutting sites (bold), and point mutations (bold red) (Balogh *et al*., 2009).

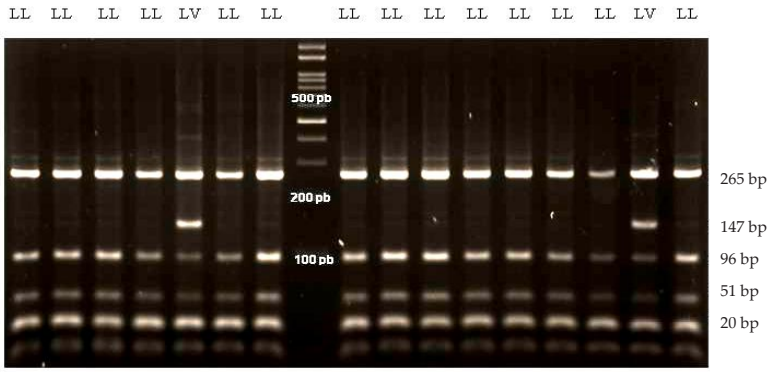

Figure 3. Visualization of the PCR-RFLP GH|*AluI* gene on 2% agarose gel. M: Marker 100 bp.

Table 1. Frequency of genotypes and alleles of the GH|*Alu*I gene

Population	n	Alel		Genotipe		
		L	V	LL	LV	VV
Singosari AIC	32	0.92	0.08	0.84 (27)	0.16 (5)	0.00
Lembang AIC	17	0.97	0.03	0.94 (16)	0.06 (1)	0.00
Cikole DCBIS	88	0.94	0.06	0.88 (77)	0.13 (11)	0.00
Cilumber NBMCU	98	0.93	0.07	0.86 (84)	0.14 (14)	0.00
Pasir Kemis NBMCU	95	0.96	0.04	0.92 (87)	0.08 (8)	0.00
Cipelang LEC	40	0.98	0.02	0.95 (38)	0.05 (2)	0.00
Total	370	0.94	0.06	0.89 (323)	0.11 (47)	0.00

Note: L= leusine, V= valine.

frequenci of the LL genotype (0.85%) than that of the LV genotype (0.15%) of the GH|*Alu*I gene in Danish Holstein cattle. The results of this study, however, contrasted with the study by Grochowska *et al*. (2001) in Polish Friesian cattle that reported LL, LV, and VV genotypes, with the highest frequency for the LL genotype (51%) and the lowest for the VV genotype (13%). Another study by Sabour *et al*. (1997) in Ayrshire, Holstein and Jersey dairy cattle also identified LL, LV and VV genotypes, with the frequencies were 0.29, 0.09, and 0.24 respectively.

Heterozigosity

The degree of heterozigosity represents the mean percentage of heterozygous loci per individual or the mean percentage of heterozygous individuals in a population. Estimation of the heterozygosity degree is important to know genetic variability and to determine the level of polymorphism of alleles. High heterozygosity shows high genetic diversity within a population (Nei & Kumar, 2000).

Predicted degree of heterogozity of the GH|AluI gene was presented in Table 2. heterozygosity of the GH|*Alu*I gene ranged between 0.050-0.156. The highest heterozygosity was found in HF bulls from Singosari AIC, whilst the lowest one was found in HF cows from Cipelang LEC. By comparing the results of observed heterozygosity analysis (H_o) and expected heterozygosity (He) at GH|*Alu*I gene indicated no statistically difference (Table 2). Tambasco *et al*. (2003) stated that if the value of observed heterozygosity (H_o) is much lower compared to that value of expected heterozygosity (H_e), it might indicate a more intense selection or a higher degree of inbreeding.

Based on the heterozygosity values obtained in the GH|*Alu*I gene from all of HF cattle observed from all locations, it could be stated that the GH|*Alu*I gene had a low degree of genetic diversity. Selection in livestock expects high heterozygosity, as the high heterozygosity reflects genetic variation of genes in a population. A higher value of heterozygosity of genes could give a greater opportunity for selection of genes in a population.

Association between the GH Genotypes and Partial Cumulative Milk Yield

Investigation of the association between variant genotypes of the GH|*Alu*I gene on partial cumulative milk yields of HF cows was conducted at Cikole DCBIS in Lembang, West Java. Study on the effects of the LL and LV genotypes on various partially cumulative milk yields at each 15-d interval of HF cows were presented in Table 3. The results generally seemed that the LV cows tended to have a higher milk production than those of the LL cows. These were really evident for cumulative milk yields around 135 d to 180 d of lactation. Statistical analysis however proved that those LL and LV genotypes the GH|*Alu*I gene did not give significantly effects on all of partially cumulative milk yields observed. These results indicated that the examination on the GH gene solely did not provide sufficiently effect on milk production of HF cattle. This was because milk production is one of quantitative traits that are controlled by poly genes. Beside of that, milk production as a quantitative trait is also affected by other factors, both genetic and environment factors. Some environment factors could be possible in affecting dairy cattle milk yields, such as lactation periode (calving age), days open, days dry, calving season, and calving year (Anggraeni, 2012).

Table 2. Heterozigosity observed (*Ho*) and heterozigosity expected (*He*) on GH|*Alu*I gene

Location	n	GH|*Alu*I	
		Ho	*He*
Lembang AIC	17	0.059	0.057
Singosari AIC	32	0.156	0.144
Cikole DCBIS	88	0.125	0.117
Cilumber NBMCU	98	0.143	0.133
Pasir Kemis NBMCU	95	0.084	0.081
Cipelang LEC	40	0.050	0.049

Note: n= sum of sample (head).

Table 3. Associations between the GH gene polymorphism with partial cumulative milk yield (L)

Partial cumulative milk yield (d)	Genotype of the GH|*Alu*I gene		P
	LL	LV	
15	145.6±66.65	147.4±103.7	0.957
30	316.0±136.0	316.2±197.8	0.998
45	464.4±180.1	484.0±272.0	0.818
60	598.9±216.7	654.0±345.0	0.603
75	730.8±236.8	823.0±392.0	0.433
90	861.8±264.0	965.0±450.0	0.435
105	992.9±284.3	1103.0±492.0	0.442
120	1118.0±316.0	1249.0±508.0	0.400
135	1243.5±345.9	1394.0±599.0	0.273
150	1366.0±360.6	1542.0±599.0	0.327
165	1483.0±301.4	1691.0±637.0	0.284
180	1585.0±406.3	1815.0±659.0	0.354

Note: L= leusine, V= valine.

Results of this study however differed from that reported by Grochowska *et al*. (2001) that identified the GH|AluI gene significantly influenced on 305-d milk production. The LL cows were reported producing higher milk yield by 171.7 kg compared to those LV cows. It was also reported that the first cows producing fat content of 8.8% higher than the latter cows. Yardibi *et al*. (2009) also reported that the variant genotypes (LL, LV, and VV) of the GH|*Alu*I gene had positive correlation with percentages of fat content and protein content of milk, but no correlation was found between those variant genotypes with milk production of dairy cattle. Investigation on some other breeds of dairy cattle proved that the LL genotype had higher milk production than the VV genotype (Dario *et al*., 2008; Sadeghi *et al*., 2008).

CONCLUSION

The GH|*Alu*I gen of HF cattle from six populations observed in this study had only two genotypes, i.e. LL and LV genotypes and with two types of L and V alleles. Frequencies of the LL genotype were very high (0.88-0.94), whilst those ferquencies of the LV genotype were very low (0.05 to 0.16). The values of both of observed heterozygosity (H_o) and expected heterozygosity (H_e) of the GH|*Alu*I gene were not significantly different that could be an indication of a closer mating within population. No significant association between varian genotypes of the GH|*Alu*I gene with partially cumulative milk yileds.

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