Identification of Growth Hormone Releasing Hormone Gene in Local Buffalo (*Bubalus bubalis*) Using PCR-RFLP

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ABSTRACT

GHRH is a hypothalamic hormone which stimulates growth hormone secretion in the pituitary gland. The objective of this study was to identify polymorphisms Growth Hormone Releasing Hormone (GHRH) gene of Indonesian buffalo's. A total of 320 blood samples from Indonesian buffaloes were used to determine polymorphism using PCR-RFLP method. The polymorphism of GHRH gene that spanned within exon 2 and exon 3 was amplified, and their mutation was detected using endonuclease *Hae*III. In this study, there were three GHRH/*Hae*III genotype (AA genotype 0%, AB genotype 36% and BB genotype 64%) determined by two alleles, A (18%) and B (82%). The frequency of A allele was found 15% for Semarang population, 19% for Mataram population, 2% for Medan population and 40% for Banten population. The frequency of B allele was found 85% for Semarang population, 81% for Mataram population, 98% for Medan population and 60% for Banten population. The observed heterozygosis values were different among populations. The highest heterozygosis ($\hat{h}$) 0,485 for Banten population and the lowest was 0.037 for Medan population and the average heterozygosis for all populations ($\hat{H}$) detected was 0.270. Index fixation value of GHRH gene showed there was not fixed into one gene type ($F_{ski} \neq 0$). The smallest genetic distance value of GHRH gene was found between Semarang and Mataram population (0.001) and the highest between Medan and Banten population (0.202).

Key words: buffallo, GHRH gene, PCR-RFLP

INTRODUCTION

Local buffaloes have great potential to be developed as meat-producing animals because it is easy to adjust, has a relative carcass weights higher than the local cows and always maintained in rural areas (Hasinah and Handiwirawan, 2006). Generally local buffaloes not used to meat-producing livestock, although in terms of body weight potential. Genetic quality improvement is still far behind the buffalo from the other cattle. Improvement by considering the genetic markers can be used as an alternative in conducting the selection. One selection method that is currently developing a method of MAS (Marker Assisted Selection) is selected on the basis of markers DNA controlling economic traits.

Growth Hormone Releasing Hormone (GHRH) is one of the role of growth factors stimulate the synthesis and secretion of Growth Hormone in an additive effect on growth. Therefore, GHRH gene is a genetic marker which can be used as one basis for selecting cattle. This study aims to identify the gene diversity of Growth Hormone Releasing Hormone (GHRH) on the local buffalo in Indonesia.

GHRH is a hypothalamic hormone which stimulates growth hormone secretion in the pituitary gland. GHRH stimulates both synthesis and secretion of pituitary growth hormone (GH) binds to specific receptors on somatotrophs (Frohman et al., 1992). Growth-hormone-releasing hormone *(GHRH)*, also known as growth-hormone-releasing factor (GRF or GHRF) or somatocrinin, is a 44-amino acid peptide hormone produced in the arcuate nucleus of the hypothalamus (Connor et al., 2005). Other studies showed that somatotropin, somatoliberin and their synthetic equivalents increased milk production in both dairy cows (Bonneau dan Laarveld, 1999) and in meat cows (Achtung et al., 2001) as well as improved cattle growth rate thereby reducing the time necessary to reach the slaughter weight. Cheong et al. (2006) suggest that polymorphism in GHRH might be one of the important genetic factors that influence carcass yield in Korean native cattle (Hanwoo). Bovine

GHRH gene was linked to CSSM30 on chromosome 13 (Barendse et al., 1994) consists of five exons separated by four introns (Zhou et al., 2000).

Moody et al. (1995) reported the existence of GHRH gene diversity in cattle by PCR-RFLP method using GHRH forward primer 5'-GTA AGG ATG GCT CTG CCA GGT3 'and GHRH reverse 5'-TGC ATG ATG CTG TCC CTC TGG A-3' and restriction enzymes *Hae*III which produces two alleles of 317, 83, 55 bp (allele A) and 196, 121, 83, 55 bp (allele B). Polymorphism sites for GHRH / *Hae*III in cattle Polish Black and White by Dybus and Grzesiak (2006) it covers a part of exon 2, the entire intron 2, and a part of exon 3; the analysed polymorphic site is located in intron 2 (AF242855 – GenBank 2000).

MATERIALS AND METHODS

Sample Collection and DNA Isolation

A total of 320 blood sample from Indonesian buffaloes collected from 4 populations; 75 from Semarang (Central Java), 103 from Mataram (West Nusa Tenggara), 65 from Siborong-borong (North Sumatera), and 77 from Banten. DNA isolation was performed using minikit DNA *Genaid*.

PCR-RFLP Analysis

PCR-RFLP method was applied to determine individual genetic variants of the analyzed gene fragment. A 451 bp fragment of the GHRH gene was amplified using a pair of primer Moody et al. (1995) with the following nucleotide sequences: *forward* 5'-GTA AGG ATG CCA GCT CTG GGT3' and GHRH *reverse* 5'-TGC CTG CTC ATG ATG TCC TGG A-3' (2 µl), 2 µl sample DNA, 0.75 unit *Taq polymerase* enzyme, 0.24 mM dNTP, 2 mM $MgCl_2$, 10x *buffer* 2.5 µl and 17.85 µl destilata water. The condition of thermal cycling began with an initial cycle of *pradenaturation* 94°C for 5 min followed by 30 cycles of *denaturation* 94°C for 1 min, *annealing* 60°C for 1 min and *final extention* 72°C for 5 min. Afterwards, the amplification product was digested with endonuclease *Hae*III, which recognizes the sequence GG⏐CC. Then silver staining Tegelstrom (1992) used to visualize the bands.

Statistical Analysis

Alleles frequency for each buffaloes population was calculated in the form type of GHRH gene according to Nei (1987):

$$X_i = \frac{2\,nii + \sum nij}{2n}$$

where:
X_i = allele frequency of -i
n_{ii} = number of individu with genotipe ii
n_{ij} = number of individu with genotipe ij
n = total individu sample

Degree of heterozygosity ($\hat{h}$) is calculated based on allele frequencies at each locus DNA using the formula of Nei (1987):

$$\hat{h} = \frac{2n\,(1 - \sum X_i^2)}{2n - 1}$$

where:
$\hat{H}$ = heterozygosis locus
X_i = allele frequency of GHRH gene type-*i*
N = total of individu sample

Variance of heterozygosity in each population can be calculated by the following formula:

$$V_{sl}(\hat{h}) = \frac{2\,nii + \sum nij}{2n}\left\{2(2n-2)\left(\sum x_i^3 - \left(\sum x_i^2\right)^2\right) + \sum x_i^2 - \left(x_i^2\right)^2\right\}$$

Average heterozygosity ($\hat{H}$) is calculated by the following formula:

$$\hat{H} = \sum_{j=1}^{r} \hat{h}_j / r$$

where:
$\hat{h}_j$= degrees of heterozygosity for the locus of -j
r = number of loci tested
$\hat{H}$ = average heterozygosity

Fixation index in each population derived from the equation:

$$F_{ISki} = \frac{X_{kii} - X^2_{ki}}{X_{ki}\,(1 - X_{ki})}$$

where
X_{kii} = Frekuensi genotipe homozigot alel i pada populasi ke-*k*
X_{ki} = Frekuensi alel i

Genetic distance (D) calculated using the formula:

$$I = \sum_{i=1}^{m} (P_{ix} \times P_{iy}) \Big/ \left[\left(\sum_{i=1}^{m} P_{ix}^2\right)\left(\sum_{i=1}^{m} P_{iy}^2\right)\right]$$

$$D = -\ln I$$

where
D = Genetic distance
P_{ix} = i allele to the population X
P_{iy} = Frequency of allele i in population Y

RESULTS AND DISCUSSION

A 451 bp of GHRH gene fragment was successfully amplified using polymerase chain reaction technique (Figure 1). The polymorphism GHRH gene was found by PCR-RFLP analysis of exon 2 and exon 3 (Figure 2).

GHRH gene segments were amplified in this study located in a part of exon 2, intron 2 and part of exon 3; the analysed polymorphic site is located in intron 2. Fragment length GHRH gene amplification results in cattle according to Moody et al. (1995) was 455 bp, according to the target in the yak Ou et al. (2002) was located at 450 bp in exon 2, intron 2 and exon 3. Franco et al. (2005) reported the GHRH gene fragment length amplification results in pigs is 455 bp located in exon 3.

As a result of digestion with restriction enzyme *Hae*III, three genotypes were identified in this study, their restriction fragments being as follows: 312, 94 and 45 bp- $GHRH^{A}$ $GHRH^{A}$ genotype; 312, 194, 118, 94 and 45 bp- $GHRH^{A}$ $GHRH^{B}$ genotype; and 194, 118, 94 and 45 bp- $GHRH^{B}GHRH^{B}$ genotype. DNA bands with size of 45 bp cannot be displayed because the concentration of acrylamide with 6% less appropriate is used to separate long DNA with less than 60 bp (Muladno, 2002).

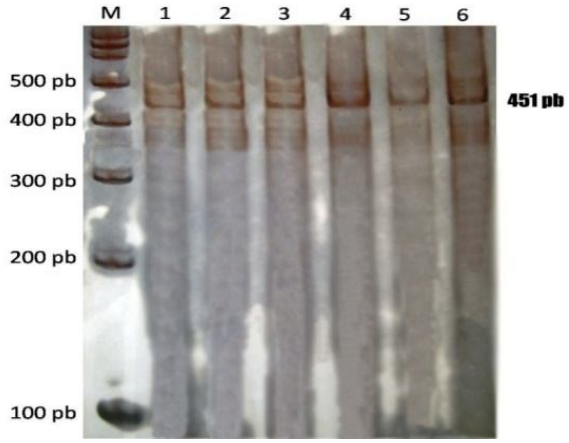

Figure 1. Results GHRH Gene Amplification Using PCR Method at 6% Poliacrilamida Gel on The GHRH Gene Sequences in Cow Cattle (Gen Bank Access No. AF242855) (Zhou et al., 2000).

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4321 cctgtctgtc atttcccagg taccagcaca ggggtgaagg atgctgctct gggtgttctt
                                        Primer Forward ──►
4381 cctcgtgacc ctcaccctca gcagcggctc ccacggttcc ctgccttccc agcctctcag
4441 gtaagcagtt ctgagaagag aagcaagaga gg|cctttga ggatgcgact cgagctggtc
4501 cccagctggg tcctcaggca gcctcccttg ctcatctctg ggagggtggc agactgagcc
4561 ccagagaggt caccacccag ccctggttcc agccctctct ggggacgagc agggcaagag
4621 gcgacagaaa gacctcacag agaccaagtg agcacagtcc cctgg|cctc ccaccccacc
4681 ctttgacctc tgactccttc tactaggatt ccacggtacg cagatgccat cttcactaac
4741 agctaccgga aggttctgg|ccagctgtct gcccgcaagc tactccagga tatcatgaac
                                        ◄── Primer Reverse
4801 aggcagcagg ggtgagccgg cgttctcgtg acttctccct gcaccctcgg ttcatcatga
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Figure 2. Primary Attachment Position and The Site *Hae*III Restriction Enzyme Based

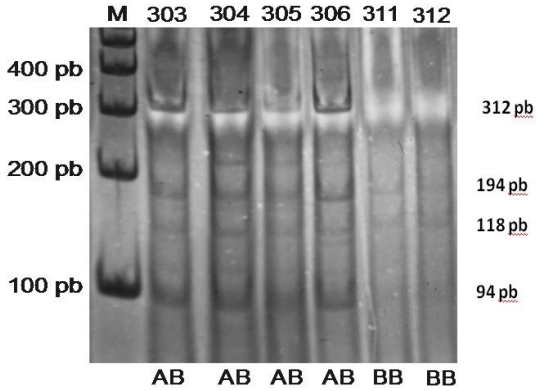

Figure 3. GHRH Gene Fragment Used Pcr-Rflp Method with *Hae*III Enzyme.

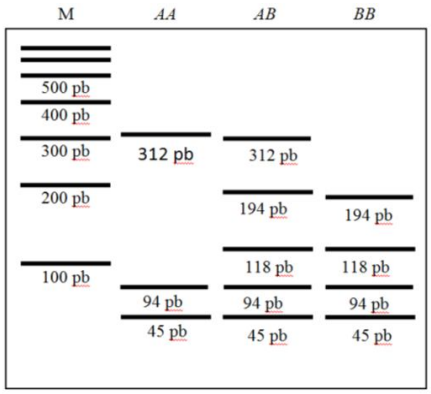

Figure 4. Zymogram of Electrophoretic Pattern Showed Genotipe AA, AB and BB of GHRH Gene.

Table 1. Frequencies of GHRH/*Hae*III genotypes and alleles in local buffaloes based on location

Location	Total Sample (n)	Genotype	Genotype Frequencies	Allele Frequencies
Semarang	61	AA (0)	0.000	A = 0.147
		AB (18)	0.705	B = 0.853
		BB (43)	0.295	
Mataram	86	AA (0)	0.000	A = 0.186
		AB (32)	0.372	B = 0.814
		BB (54)	0.628	
Medan	53	AA (0)	0.000	A = 0.019
		AB (2)	0.038	B = 0.981
		BB (51)	0.962	
Banten	45	AA (0)	0.000	A = 0.400
		AB (36)	0.800	B = 0.600
		BB (9)	0.200	
Total	245	AA (0)	0.000	A = 0.180
		AB (88)	0.360	B = 0.820
		BB (157)	0.640	

Table 2. Heterozygosity values ($\hat{h}$) and average heterozygosity ($\hat{H}$) GHRH gene in local buffaloes

Location	$\hat{h} \pm SE$	$\hat{H}$
Semarang	0.252 ± 0.045	0.270 ± 0.024
Mataram	0.305 ± 0.003	
Medan	0.037 ± 0.026	
Banten	0.485 ± 0.022	
Total	0.461 ± 0.022	

Table 3. Fixation index values of local buffaloes GHRH gene

Location	Allele	F_{Iski}
Semarang	A	-0.175
	B	-3.450
Mataram	A	-0.229
	B	-0.228
Medan	A	-0.019
	B	-0.019
Banten	A	-0.666
	B	-0.666
Total	A	-0.218
	B	-0.197

Table 4. Genetic distance values of GHRH gene in local buffaloes

Location	Location			
	Semarang	Mataram	Medan	Banten
Semarang	0.000			
Mataram	0.001	0.000		
Medan	0.012	0.023	0.000	
Banten	0.089	0.064	0.171	0.000

The results of this study indicate that the GHRH gene in the local buffalo are polymorphic (various) in all populations from four regions in Indonesia. The percentage of successful detection with GHRH gene diversity of PCR-RFLP method in this study for 76.56%, ie from 320 samples can be identified GHRH gene diversity as much as 245 samples. Genotype frequency and allele frequency of the local buffalo GHRH gene is presented in Table 1. Heterozygosity value of the local buffalo in this study ranged from 0.037 to 0.485 (Table 2). Index fixation value of GHRH gene showed there was not fixed into one gene type ($F_{ski} \neq 0$). The results of the analysis of genetic distances based on GHRH gene between the four local buffalo population in Indonesia is presented in Table 4.

Genetic distance can be used to create a dendogram (tree phylogeny) that can be used to show the relationship between population kinship. According to Nei (1987) kinship between populations can be identified by using the simplest method of average - the average genetic distance UPGMA (Unweighted Pair-Group Method with Arithmetic mean). Kinship relations between the four local buffalo populations are shown in Figure 5.

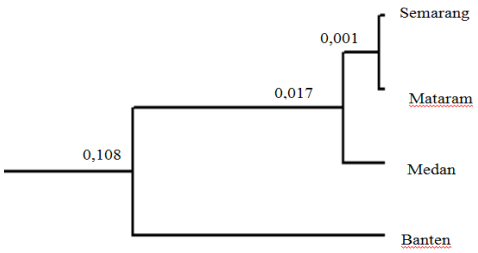

Figure 5. Genetic Tree Dendogram Based on GHRH Gene Local Buffaloes Population

CONCLUSIONS

GHRH gene in Indonesian local buffaloes were polymorphic, there were determined by two alleles; A and B. The frequency of B type was higher than A type in this study. Based on locations, buffalo's from Banten population has highest frequency of A allele (40%) and the lowest from Medan population (2%). Conversely, for frequency of B allele, the observed heterozygosis value was different among populations. The highest heterozygosis ($\hat{h}$) 0,485 for Banten population and the lowest was 0.037 for Medan population. Index fixation value of GHRH gene showed there was not fixed into one gene type ($F_{ski} \neq 0$). The highest genetic distance value of GHRH gene was found between Medan and Banten population (0.202) and the smallest between Semarang and Mataram population (0.001).

REFERENCES

Achtung, T.T., D.S. Buchanan, C.A. Lents, S.M. Barao dan G.E. Dahl. 2001. Growth hormone response to growth hormone-releasing hormone in beef cows divergently selected for milk production. J. Animal Sci., 79: 1295-1300.

Barendse, W., S.M. Armitage, L.M. Kosarek, A. Shalom, B.W. Kirkpatrick, A.M. Ryan, D.L. LI. Claytoni, H.L. Neibergs, N. Zhang, W.M. Grosse, J. Weiss, P. Creighton, F.Miccarthy, M. Ron, A.J. Teale, R. Fries, R.A. Mcgraw, S.S. Moore, M. Georges, M. Soller, J.E. Womack dan D.J.S. Hetzel. 1994. A genetic linkage map of the bovine genome. Nat. Genet. 6: 227-235.

Bonneau, M. dan B. Laarveld. 1999. Biotechnology in animal nutrition, physiology and health. Livestock Prod. Sci., 59: 223-241.Cheong, H.S., D.H. Yoon, L. H. Kim, B. L. Park, Y.H. Choi, E. R. Chung, Y.M. Cho, E.W. Park, I.C. Cheong, S.J. Oh, S.G. Yi, T. Park dan H.D. Shin. 2006. Growth hormone-releasing hormone (GHRH) polymorphisms associated with carcass traits of meat in Korean cattle. J. BioMed Central Ltd., 1471-2156.

Cheong, H.S., D.H. Yoon, L.H. Kim, B.L.Park, Y.H. Choi, E.R. Chung, Y.M. Cho, E.W. Park, I.C. Cheong, S.J. Oh, S.G. Yi, T.Park and H.D. Shin. 2006. Growth hormone-releasing hormone (GHRH) receptor. Agricultural Research Service. USDA. Beltsville. USA.

Connor E.E., M.S. Ashwell, dan G.E. Dahl. 2005. Characterization and expression of the bovine growth hormone-releasing hormone (GHRH) receptor.Agricultural Research Service.USDA. Beltsville USA.

Dybus, A. dan W. Grzesiak. 2006. GHRH/*Hae* III gene polymorphism and its associations with milk production traits in Polish Black-and-White cattle. Arch. Tierz., Dummerstorf 49 (5): 434-438.

Frohman, L.A., Bowns, T.R., Chomczynski, P. 1992. Regulation of growth hormone secretion. Front. Neuroendocrinol. **13** (1992), 344-405

Franco, M. M., R. C. Antunes, H. D. Silva, L. R. Goulart. 2005. Association of PIT1, GH and GHRH polymorphisms with performance and carcass traits in Landrace pigs. J Appl. Genet 46(2) pp. 195-200.

Hasinah, H. and Handiwirawan, E. 2006. Keragaman genetik ternak kerbau di Indonesia. Prosiding Lokakarya Nasional Usaha Ternak Kerbau Mendukung Program Kecukupan Daging Sapi. Pusat Penelitian dan Pengembangan Peternakan, Bogor.

Moody, D. E., D. Pomp, dan W. Barendse. 1995. Restriction fragment length polymorphism in amplification products of the bovine growth hormone releasing hormone gene. J. Animal Science. 73 : 3789.

Muladno. 2002. Seputar Teknologi Rekayasa Genetik. Pustaka Wira Usaha Muda. Bogor.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press. New York.

Ou, J.T., Zhong, J.C., Chen, Z.H., Wu, H. dan Rao, K.Q. 2002. T-A cloning and sequencing analysis on growth hormone releasing hormone gene of Yak. Journal College of Life Science and Technology, Southwest University for Nationalities, 282, Chengdu, Sichuan, China.

Tegelstrom, H. 1992. Mitochondrial DNA in natural population: an improved routine for screening of genetic variation based on sensitive silver staining. Electrophoresis. 7:226-229

Zhou P., G. W. Kazmer, dan X. Yang. 2000. *Bos taurus* growth hormone releasing hormone gene, complete cds. Gen Bank, F24285