Polymorphism Exploration of Growth Family (GH, GHRH and PIT-1) Genes Polymorphisms of Local Swamp Buffalo for Productivity Improvement in North Tapanuli Regency, North Sumatra

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Abstract. Genetic improvement of livestock productivity can be done through molecular selection on the genes controlling growth traits. Genetic polymorphism of the growth family (GH, GHRH, and PIT1) genes were studied in local swamp buffalo (106 hds.) from a government buffalo breeding station (46 heads) and smallholders (60 heads) in North Tapanuli District, North Sumatra Province. Genotype variants of the three genes were identified by PCR-RFLP method using restriction enzymes of MspI (GH gene), HaeIII (GHRH gene) and HinfI (PIT-1 gene). Genotyping on individual GH_g.1547T>C, GHRH_g.4666G>C, and PIT-1_g.1256G>A loci resulted only one type genotype, respectively TT, CC, and AA, with one type of allele, respectively T, C, and A. Heterozygosity observation (Ho) and expectation (He) values values and the PIC value for each locus was 0.00. It could be suggested to increase genotype frequenciest of the three growth genes that are positively associated with the growth traits and economic traits of the buffalo.

Keywords: Growth family genes, PCR-RFLP, swamp buffalo.

1 Introduction

Buffalo is part of the lifes of farmers and communities in various agro-ecosystems and contributes to the development of the livestock subsector in Indonesia. The most buffalo populations are traditionally maintained by farmers, instead buffalo has multiple functions as a source of red meat, labor, transportation, saving, organic fertilizer; likewise as religious, cultural and social events for the community [1]. In fact the population has not significantly increased instead of some decreases in some areas. National buffalo population for the last four years, 2016-2019, were successively 1,355, 1,322, 894, and 1,134 heads [2]. In 2018 the population declined, afterward it has increased possibly caused of the National Government Program of Productive Cattle and Buffalo Cows to be Pregnant. Buffalo population in North Sumatra Province (102,574 hds.) is the 4th highest after the Provices of East Nusa Tenggara (175,007 hds.), West Nusa Tenggara (121,575 hds.), and South Sulawesi (113,100 hds.). Buffalo population in North Sumatra exceeded those buffalo populations in central area in Java Island, such as from West Java (85,405 hds.), Central Java (59,478 hds.) and Banten (58,532 hds.).

Most of buffalo population in North Tapanuli Regency in North Sumatra are swamp buffalo instead of only a small number as river buffalo. These buffaloes have significance

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roles for farmers and local communities. In conditions of high demand for buffalo meat, semi-intensive management, and reducing habitat cause on decreased population and genetic degradation of swamp buffalo in this location similar another regions in Indonesia [3]. Mating programs using quality males and improving productivity through selection programs need to be done. The use of molecular genetic technology may be as an option to assist selection for genetic improvement. Molecular markers have a high level of polymorphism to provide an overview of genetic population, and to identify major genes controlling important traits such as growth traits [3, 4. 5, 6]. To make genetic improvement on growth traits of buffalo therefore can be assisted through exploration and use of molecular markers particularly in growth family genes such as Growth Hormone (GH) [3, 5, 6], Growth Hormone Releasing Hormone (GHRH) [3, 7], and Pituitary-Specific Transcription Factor 1 (PIT-1) [8,9].

Growth hormone (GH) is needed in tissue growth and metabolism, so it played important roles in the processes of reproduction, lactation and growth (Yardibi et al. 2009). In cattle, the GH gene was located on the 19th chromosome in the q26-qter band region with a size of about 1800 bp for having 5 exons and 4 introns [10]. Growth hormone releasing hormone (GHRH) was a hypothalamic hormone stimulating both the synthesis and secretion of growth hormone in the pituitary gland [7]. The GHRH gene and its analogues had a direct effect on extrapituitic cells / tissue and played a role on normal body function [11]. PIT-1 is a member of the POU domain taking an important role in cell differentiation and proliferation [12]. A number of previous studies have shown a fairly high genetic polymorphism of the GH, GHRH and PIT-1 genes in cattle and buffalo [9, 13, 14, 15]. Information on genetic polymorphisms of growth family genes in local swamp buffalo is still limited. Evaluation of polymorphisms is useful for determining the level of genetic diversity, controlling loss of genetic diversity, and determining selective breeding programs [16].

This study therefore aimed at to identify genetic polymorphisms of GH, GHRH, and PIT-1 genes using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method in local swamp buffalo kept in a Government Breeding Stations and small farmers in North Tapanuli Regency in North Sumatra Province. Information of genetic polymorphisms of the three genes can be used in considering breeding program to improve productivity of swamp buffalo in North Tapanuli Regency in North Sumatra.

2 Materials and Methods

2.1. Location and Period of Research

Field research was conducted in 2013-2014 in North Tapanuli Regency in North Sumatra Province. North Tapanuli Regency is located at coordinates 1º20'00" - 2º41'00" North Latitude (NL) and 98º05 '- 99º16" East Longitude (EL). Area of this Tegency is about 3,800 km², with distribution of a land area by 3,793 km² and eater area of Toba Lake Toba by 6.60 km².

The field research consisted of collecting blood samples of local swamp buffalo from a Government Buffalo Breeding Station at BPTU HPT Siborong-borong and from small farmers in North Tapanuli Regency, North Sumatra Province.

Molecular research was carried out at the Laboratory of Animal Molecular Genetics, Animal Breeding and Genetic Division, Department of Animal Production Science and Technology, Faculty of Animal Husbandry, Bogor Agricultural University, West Java, Indonesia.

2.2. Materials

A total number of local swamp buffaloes by 106 hds were used as the research sample from BPTU HPT Siborong-borong (46 hds.) and from small farmers (60 hds.) in North Tapanuli Regency, North Sumatra Province, Indonesia.

2.3. Methods

Genetic polymorphisms of the GH, GHRH, and Pit1 genes were dobe by following a modified standard procedure. Working stages were done for DNA extraction, DNA isolation, gene amplification, electrophoresis, and restriction of base fragments from PCR products.

2.3.1. DNA extraction

DNA extraction was done by using blood samples, DW, 1xSTE, 10% SDS, Proteinase-K (5mg/ml), 5 M NaCl, phenol, CIAA, 70% EtOH, 80% TE buffer. Materials for PCR process consisted of forward and reverse primers, dNTPs, MgCl2, 10xBuffer, and Taq polymerase, destilated water. Primers used for amplification of GH gene in intron 3 at GH_g.1547T>C locus referred to [17], for GHRH gene in intron 2 at g.4666G>C locus referred to {18}, and for PIT-1 gene in exon 6 at g.1256G>A locus refered to [19]. Primer base pairs, annealing temperature, length of PCR products and references used for DNA extraction process are presented in Table 1.

Table 1. Primary sequence, annealing temperature, length of PCR product and reference in DNA extraction process of GH, GHRH and PIT-1 genes

Genes / Locus	Primary Sequence	Enzyme	Ref.
GH gene g.1547T>C locus	F: 5'-CCCACGGGCAAGAATGAGGC-3' R: 5'-TGAGGAACTGCAGGGGCCCA-3'	MspI	[17]
GHRH gene g.4666G>C locus	F: 5'-TGAAGGATGCTGCTCTGGGT-3' R:5'-TGCCTGTTCATGATATCCTGGA-3'	HaeIII	[18]
PIT-1 gene g.1256G>A locus	F: 5'-AAACCATCATCTCCCTTCTTCTT-3' R:5'-AATGTACAATGTGCCTTCTTCTG-3'	HinfI	[19]

Electrophoresis process used 1.5% agarose gel, 0.5 x TBE, Ethidium Bromide, loading dye, and 100 bp marker. Materials for RFLP included MspI, HaeIII, and HinfI restriction enzymes, buffers, and destilated water.

2.3.2. DNA isolation

DNA isolation used the phenol chloroform method according to [20]. For 200 µl of blood sample and 1,000 µl of 0.2% NaCl were centrifuged at 8000 rpm for five minutes. To lyse cells, degraded organic matter was added by 350 µl 1xSTE, 40 µl 10% SDS, and Prot-K

(10 mg / ml) and shaken in an incubator at 55 °C for two hours. Cell purification was carried out by adding 40 µl 5M NaCl, 400 µl phenol, and 400 µl CIAA (Chloroform: Isoamyl alcohol = 24: 1). DNA was separated from the phenol phase by centrifugation, the supernatant was added 800 µl of 70% EtOH and recentrifuged. DNA was dissolved with 100 µl TE 80% and stored in the freezer ready for use.

2.3.3. Gene amplification

Amplification was carried out at 25 µl volume containing 0.3 µl 25 pmol primer, 0.3 µl dNTPs 10 mM, 2 µl MgCl2 25 mM, 3 µl 5x buffer, 0.1 µl taq polymerase enzyme 5 units/µl and removed water. The process was carried out on a thermal cycler machine with initial denaturation conditions (95 ºC; 5 minutes), 35 cycles consisting of denaturation (95 ºC for 10 sec.), annealing (60 ºC for 20 sec.), extension (72 ºC for 30 sec.), and final extension (72 ºC for 5 min.).

2.3.4. Electrophoresis

Electrophoresis was preceded by making 1.5% agarose gel. Agarose gel was prepared by dissolving agarose (0.45 g for large molds or 0.3 g for small prints) with 0.5xTBE (30 ml and 20 ml for each mold). The agarose solution was heated in a microwave (medium high temperature for ±5 minutes). The agarose solution was stirred with a magnetic stirrer and added with Ethidium Bromide (2.5 µl and 2 µl for each mold), then the solution was allowed to stand until becoming a gel.

Electrophoresis used 5 µl of the PCR sample mixed with 1 µl of loading dye to obtain a blue mixture and the sample was inserted into the well. 2 µl of 100 pb marker was inserted into the well, as a tool in determining the length of DNA fragments. Electrophoresis was carried out on a 100 volt power supply for ±35 minutes and the results were visualized with a UV transilluminator.

2.3.5. Restriction fragment leng polymorphism

A number of 5 µl PCR sample was put into a 0.5 ml tube using a micropipette and 2 µl of RE mix was added (0.7 µl buffer; 0.3 µl restriction enzyme; 1 µl destilated water). Samples were centrifuged and incubated in an incubator at 37 °C for 16 hours. PCR-RFLP samples were electrophoresed on 2% agarose gel at a voltage of 100 volts. The results of electrophoresis were visualized with UV transilluminator. Genotyping of genotype variant of each gene was carried out by pulling the sample DNA band fragments straight towards the marker, then compared to the marker to determine the length of the fragment.

2.4. Data analysis

Genotyping results of individual GH, GHRH, and Pit1 genes were calculated for genotype frequency, allele frequency, Hardy-Weinberg equilibrium value, heterozygosity observation (Ho) value and expectation (He) values by using the POPGENE32 software version 1.32. Genotype frequency was a ratio of the number of a certain genotype to the number of the observed population. Hardy-Weinberg equilibrium was tested using the Chi-square ($\chi 2$) calculation.

3. Results and Discussion

3.1. Allele and genotype frequenciest

The amplification results of fragments of the GH, GHRH, and PIT-1 genes resulted in PCR product lengthed by 327 bp, 451 bp, and 611 bp respectively. The PCR product of the GH gene stretched from 33 bp in exon 3 and 294 bp and in intron 3 (GenBank M57764.1) [17]. DNA amplicon of the GHRH gene included 86 bp in exon 2, 266 bp in intron 2, and 99 bp in exon 3 (GenBank AF242855) [18]. Meanwhile DNA amplicon of the PIT-1 gene stretched at 43 bp in intron 5 and 408 bp in exon 6 (GenBank Y15995) [19]. The PCR products of each GH, GHRH, and PIT-1 genes were successfully cut by the respective restriction enzymes of MspI, HaeIII, and HinfI [17, 18, 19]. MspI restriction enzymes recognized C*CGG restriction site, HaeIII enzymes recognized GG*CC restriction site and HinfI enzymes recognized G*ANTC restriction site. The results of allele frequency and genotype frequency of the GH gene at g.1547T>C locus, GHRH gene at g.4666G>C locus, and PIT-1 gene at g.1256G>A locus in the observed swamp buffalo are shown in Table 2.

3.1.1. GH_g.1547T>C locus

Genotyping of the GH gene at g.1547T>C locus produced only TT (BB/-) genotype of swamp buffalo populations both from the government breeding station at BPTU HPT Siborong-borong and smallholder breeders in North Tapanuli Regency. The TT genotype (BB/-) displayed only one fragment with the same length as the PCR product, namely 327 bp. If CC (AA /++) genotype was found, it would present 2 fragments, namely 223 pb and 104 bp, as a result of the cutting sites, by the MspI restriction enzyme, at C*CGG bases in intron 3 of the GH gene [17]. The CC genotype was not found in the swamp buffalo observed in this study. The polymorphism at the 1547th nucleotide of the GH gene was due to a base change of Thymine (T) to Cytosine (C), known as a transition mutation [21]. All of the observed swamp buffalo did not have base mutation site at the GH_g.1547T>C locus, that showed all buffalo having the TT genotype.

Table 2. Genotype frequency and allele frequency of GH, GHRH, and PIT-1 genes of local swamp buffalo in North Tapanuli Regency

Genes / Locus	Number of Genotype			Genotype Frequency			Allele Frequency	
GH gene	TT	TC	CC	TT	TC	CC	T	C
g.1547T>C locus	106	0	0	1.00	0.00	0.00	1.00	0
GHRH gene	GG	GC	CC	GG	GC	CC	G	C
g.4666G>C locus	0	0	106	0.00	0.00	1.00	0.00	1.00
PIT-1 gene	GG	GA	AA	GG	GA	AA	G	A
g.1256G>A locus	0	0	106	0.00	0.00	1.00	0.00	1.00

For all of the observed buffalo of possessing only one genotype, the TT genotype (BB/-) resulted in TT genotype frequency of 1.00 (100%), so the T allele frequency was also equal to 1.00 (100%). An allele was said to be monomorphic for its frequency exceeded 0.99 [22]. The observed swamp buffalo was therefore monomorphic.

Study on the bGH gene in intron 3 on 17 breeds of Indian zebu cattle (750 hds.) in two geographics [23] found the same length of PCR products (329 bp) and identified two types of alleles, namely T (-) and C (+) with the frequenciest about 0.06 - 0.33; and had two

genotypes TT (-/-) and TC (- /+) with the frequenciest about 0.67 to 0.94. Neither of these breeds were with CC (+/+) genotype. The highest frequency of these two genotypes was expressed as a unique of the *Bos taurus* breesd over those from northern Europe, Mediterranean, and American countries. When compared with the TT genotype of the observed swamp buffalo, it can be stated that local swamp buffalo in North Tapanuli District owned high TT genotype frequency that corresponded to *Bos indicus* cattle and buffalo breeds from the previous study in India [23].

3.1.2. GHRH_g.4666G>C locus

Genotyping results of the GHRH gene in intron 2 at locus g.4666G>C also showed that all the observed swamp buffaloes had CC (BB) genotype. The CC (BB) genotype was identified by finding three cutting sites at nucleotides of 4472*4473 (intron 2), 4666*4667 in intron 2, and 4760*4761 in exon 3, resulting in four fragments, i.e. 194 pb, 118 bp, 94 bp, and 45 pb. The fragment length45 bp was not seen due to running off during electrophoresis. Contrastly, for the GG (AA) genotype was identified if two cutting sites were recognized by the HaeIII enzyme (GG*CC), at nucleotides of 4666*4667 in intron 2 and 4760*4761 in exon 3, generating three fragments, i.e. 312 bp. 94 pb, and 45 pb (Wollard et al, 1994). From this study observed that all of the observed swamp buffalo possessed a base mutation at the GHRH_g.4666G>C locus, namely from guanine to cytosine, resulting in all buffalo had the only CC genotype (100%) and owned the only C allele (100%).

Study by [24] in Polish HF cattle from two farms found that GHRH gene at g.4666G>C locus had high CC (BB) genotype (0.631 and 0.704), followed by heterozygous GC (AB) genotype (0.327 and 0.242) , and the lowest for GG (AA) genotype (0.042 and 0.054). Thus, buffaloes with the C (B) allele (0.673 and 0.794) were higher than the G (A) allele (0.206 and 0.175). Further an association study of the GHRH_ g.4666G>C SNP on milk quality revealed that cows with genotype GG (AA), compared to genotype CC (BB), produced higher levels of milk fat (4.26%) and milk protein (3. 43%). Results from this study seemed similar with the results of previous studies [7, 25] which obtained that GG (AA) genotype cows were benefit on milk production and milk quality. By consdering the results of the association of the GHRH_ g.4666G>C SNP with lactation trait from previous studies, it can be stated that the observed swamp buffalo had the CC (BB) genotype which was not related to the benefits for milk traits. This probably corresponds to the type of swamp buffalo as as a meat type instead of milk type (such as river buffalo).

3.1.3. PIT-1_g.1256G>A locus

Genotyping of the PIT-1 gene in intron 6 at locus g.1256G>A also showed all of the observed swamp buffalo had AA genotype. The AA genotype was due to the recognation of HinfI enzyme restriction site on the fragment of G*ANTC at nucleotides of 1256*1257 in exon 6, so that two fragments were obtained, i.e. 207 bp and 244 bp. In contrast, for GG genoype displayed by only one fragment of 451 bp [19]. The AA genotype had a base mutation at 357th base or at locus g1256 in exon 6 of the PIT-1 gene known as a transitional mutation form Guanine (G) to Adenine (A) [9]. However, this mutation did not change amino acids or called as a silent mutation [8]. All of local buffalo in this study generated only the AA genotype without the GG genotype.

Previous studies also obtained genetic polymorphisms of the PIT-1 gene at the g1256 locus both in cattle and in buffaloes [9, 26]. However, for genotype naming of the PIT-1_g.1256G>A locus in this study was by considering the form of the base mutation (G>A) that was different to the genotype naming from previous studies. The non-mutated (wild

type) genotype in this study was expressed as GG genotype (AA genotype for previous studies), rather than that mutated genotype as AA (BB) genotype. The PIT-1 gene at the same locus in a number of local Iranian cattle breeds (17 breeds) were polymorphic [9]. For cattle, the frequenciest of the AA (BB) genotype ranging by 00.000-0.375, GG (AA) genotype by 0.451-0.842, and GA (AB) genotype by 0.000-0.341; so the frequenciest of A (B) allele by 0.173-0.789 and G (A) allele by 0.622-0.921. For buffalo the frequency of AA (BB) genotype by 0.033, GG (AA) genotype by 0.567, and GA (AB) genotype by 0.400; so frequenciest of A (B) allelle by 0.233 and e G (A) allele by 0.766. The G (A) allele related to a higher effect on milk production and milk components [26]. The observed swamp buffalo had AA (BB) genotype for no association to the benefit on milk production and milk quality. This probably corresponded to the observed swamp buffalo of as a beef type instead of a milking type.

3.2. Hardy-Weinberg equilibrium

The results of Hardy-Weinberg analyses for the three GH_g.1547T>C, GHRH_g.4666G>C, and PIT-1_g.1256G>A loci of the observed buffalo showed $\chi 2_{\text{calculation}}$ were unable to be analyzed as those three loci were monomorphic. Thus the number of genotype did not meet the assumption to analyze the Hardy-Weinberg equilibrium. The degree of freedom $\chi 2$ was the result of a reduction between the number of genotypes and alleles [27]. Meanwhile, the observed buffalo had only one genotype resulting in zero degrees of freedom. A population was said to be in equilibrium if genotype and allele frequencies were always constant from generation to generation and this equilibrium would be achieved in condition of no selection, mutation, migration, and genetic drift in the population [28].

3.3. Heterozygosity Value and Degree of Polymorphism (PIC)

The degree of polymorphism of a gene can be determined by calculating the values of heterozygosity observation (Ho), heterozygosity expectation (He), and PIC. Examination on individual GH_g.1547T>C, GHRH_g.4666G>C, and PIT-1_g.1256G>A loci resulted in Ho = 0.00 and PIC = 0. This indicated no genetic diversity of the three targetted loci in the observed swamp buffalo. This confirmed the previous results that all buffaloes were with the same genotypes for all the three loci of the growth family genes. Hildebrand et al. (1992) stated that if one gene only had two alleles, a maximum PIC value would be 0.375, PIC = 0 value occurred in case of only one type of allele identified. However study on 17 breeds of Iranian cattle and one local buffalo, for example, reported the PIT-1 g.1256G>A locus had heterozygosity observation ranging from Ho = 0.1454-0.4730 for cattle and Ho = 0.3578 for buffalo [9]. Heterozygosity less than 0.5 indicates low genetic variation of a gene in a population. It was suggested that the introduction of new genetic diversity and cross-breeding to improve gene diversity and conservation.

4 Conclusion

The GH gene at g.1547T>C locus, GHRH gene at g.4666G>C locus, and PIT-1 gene at g.1256G>A locus of the observed swamp buffalo had succesively only TT, CC, and AA genotypes, resulting only T, C, and A allelles. Monomorphic of the three loci caused of very low heterozygosity level (Ho = 0 and PIC = 0). However, the genotypes possessed by the swamp buffalo were positively related to growth traits.

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