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Identification of growth genes diversity of swamp buffalo using RFLP in Kabaena Island, Bombana District, Southeast Sulawesi, Indonesia

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Abstract. *Nafiu LO, Muzuni, Pagala MA, Kurniawan W, Rahadi S. 2020. Identification of growth genes diversity of swamp buffalo using RFLP in Kabaena Island, Bombana District, Southeast Sulawesi, Indonesia. Biodiversitas 21: 1901-1907.* Swamp buffalo (*Bubalus bubalis*) in Bombana District has been familiar in the socio-cultural life and used as a source of livelihood. Buffalo can adapt to the hard environment by utilizing a low-quality feed. However, it needs more attention from the public and the government to increase buffalo production, both from the genetic and environmental aspects. The objective of this study was to determine the diversity of swamp buffalo growth genes (GH and GHRH) in Kabaena Island, Bombana District, Southeast Sulawesi-Indonesia. The blood sample was taken from 58 heads of swamp buffaloes and analyzed using PCR technique to multiply the sequence of GH and GHRH genes with the target sizes of 327 bp and 451 bp. The Genes diversity determined using analysis of genotype frequencies and allele frequencies of each locus, Inbreeding Coefficient estimated using analysis of observed heterozygosity (Ho) and expected heterozygosity (He), while Population balance (Hardy-Weinberg equilibrium) specifically related to the presence/absence of selection determined using a chi-square analysis. The results of the study showed that the GH/*Msp*I and GHRH/*Hae*III locus were polymorphic with sizes of 327 bp and 451 bp, respectively, contain three genotypes; AA, AB, and BB. The frequency of GH/*Msp*I locus A and B locus were 0.562 and 0.438, respectively. Meanwhile, the frequency of A and B alleles at the GHRH/*Hae*III locus were0.700 and 0.300, respectively. Allele and genotype GH/*Msp*I - GHRH/*Hae*III locus frequency of swamp buffalo in Kabaena Island, Bombana District were in Hardy-Weinberg equilibrium, and it means that mating tends to occur randomly.

Keywords: GH/*Msp*I, GHRH/*Hae*III, growth genes

INTRODUCTION

Swamp buffalo (*Bubalus bubalis*) in Bombana District has been familiar in the socio-cultural life and used as a source of livelihood. Buffalo can adapt to the hard environment by utilizing a low-quality feed. However, it needs more attention from the public and the government to increase buffalo production, both from the genetic and environmental aspects (Nafiu et al. 2018). One of the buffalo's development areas in Bombana District is Kabaena Island, which is an isolated area.

Efforts to improve the genetic quality of livestock can be made through the selection of characters which have high economic value, such as the growth ability. The molecular markers utilization to assist selection (marker-assisted selection) is needed to get a faster selection response. The application of *Polymerase Chain Reaction* (PCR) techniques and *Restriction Fragment Length Polymorphism* (RFLP) allows selection to be carried out by using molecular markers that have been proven to control commercial properties. The use of marker selection can be done through two stages; identification of gene polymorphisms that control economic properties and search for gene types (alleles) associated with a superior character. The candidate genes that can be used are the *Growth Hormone* (GH) and *Growth Hormone Releasing Hormone* (GHRH) genes. These genes are controllers of the growth in which their existence and polymorphism are important to support the selection of the growth characters. Growth genes (GHRH) and their analogs have a direct effect on extrapituitic cells/tissue and play a role in the body's normal functions (Cui and Schally 2018). However, studies related to GH and GHRH in buffalo are still limited and have never been done in swamp buffalo in Bombana District.

The objective of this study was to identify growth genes (GH and GHRH) diversity of swamp buffalo in Kabaena Island, Bombana District, Southeast Sulawesi, Indonesia.

MATERIALS AND METHODS

The research was carried out in Kabaena island, Bombana District, Southeast Sulawesi Province, Indonesia for 10 months, started from June 6 of 2016 until April 4 of 2017. The research began with a field survey and buffalo blood sampling collection and continued with laboratory observations. The blood sample was taken from 58 heads of swamp buffaloes Forensic Laboratory and Genetic Laboratory, Faculty of Mathematics and Science, Universitas Halu Oleo, Kendari, Indonesia.

Table 1. PCR process condition

Process	Duration (minute/s)	Temperature (ºC)
Pre-PCR	5	95
Denaturation	1	94
Annealing	0.5	55
Extension	1.5	72
Post-PCR	5	72

Blood samples were taken from the jugular vein of about 2 ml using heparin-vacutainer tubes and soaked in 95% ethanol to avoid damage to blood cells. The PCR technique was used to multiply the sequence of GH and GHRH genes with the target sizes of 327 bp and 451 bp, respectively. Amplification of the GH gene used a pair of primers for GH1/*Msp*I and GH/M*sp*I (Table 1, Mitra et al. 1995), while the GHRH gene amplification used a pair of primers for GHRH1/H*ae*III and GHRH/H*ae*III locus (Table 1, Moody et al. 1995). The PCR technique used the Nested PCR method (thermocycler TC 3000) (Ono et al. 2007). The first PCR component uses GH1 and GHRH1 primers, in which the DNA template used is taken from the total genomic DNA by 10 times dilution. The second PCR component uses GH and GHRH primers, which the DNA template used is taken from the first PCR product. The PCR reaction used *Go Taq Green Master Mix 2x* PCR kit consist of DNA *template* 2 µl (100 ng), *Primer forward* 0,5µl (0,5 µM), *Primer reverse*: 0,5 µl (0.5 µM), *Taq Green Master Mix* 2x, 5 µl (1x), and dH_2O 2 µl.

The PCR reagent put together in a 0.5 ml sterile *Eppendorf* tube then vortexed and spin downed before placed in PCR *Thermocycler TC 3000* machine. PCR reaction was carried out for 30 cycles based on conditions in Table 1.

This amplification reaction took ± 2.03 hours. The results of the amplification were then electrophoresed in 1% at a constant voltage of 100 volts and 80A for 30 minutes then visualized over ultraviolet transilluminator and photographed.

Genotyping

PCR products were cut using restriction enzymes that were specific to the target genes. The restriction enzymes used for the GH and GHRH genes were *Msp*I and *Hae*III, respectively. The *Msp*I restriction enzyme recognizes the C*CGG restriction site while the *Hae*III restriction enzyme recognizes the GG*CC restriction site.

Visualization of RFLP

Visualization of the RFLP band pattern using 3% agarose gel electrophoresis. A total of 20 µl of RFLP product was mixed with 1 µl loading dye (bromthymol blue 0.01%, xylene cyanol 0.01 and 50% glycerol) and suspended before put it into the well of the gel. Electrophoresis is carried out at a constant voltage of 220 volts for 40 minutes. After electrophoresis, the gel was taken and immersed into fluorescent dye (EtBr) for + 10 minutes, then observed using photophoresis under ultraviolet light transilluminator. Each DNA band that appears is compared to a 100 bp DNA Ladder marker to determine its size.

Data analysis

The data obtained from this research *i.e.* genes diversity, inbreeding coefficient and population balance (Hardy-Weinberg equilibrium) were analyzed using the following methods. Genes diversity determined using analysis of genotype frequencies and allele frequencies of each locus according to Nei (1987).

Inbreeding Coefficient estimated using analysis of observed heterozygosity (Ho) and expected heterozygosity (He) according to HARTL (1988). Population balance (Hardy-Weinberg equilibrium) specifically related to the presence/absence of selection determined using a chi-square analysis according to HARTL (1988).

RESULTS AND DISCUSSION

The PCR reaction successfully amplified the 327 bp target gene in intron 3 and exon 4 (Figure 1). The GH gene amplification location covered the intron 3 and exon 4 areas along 327 bp (GenBank: KC852882). The length of this fragment was 270 bp, this was quite different from Othman et al. (2012) which is 329 bp. The position of the primary attachment and the mutation point in intron 3 in the sequence of the GH-*Msp*I gene were showed in Figure 2.

Table 2. Information on primer sequences used in the study

Locus	Primer sequences	Sources
GH1/*Msp*I	F : 5'-ATCCAAGTAGGGATGTGGTTAG-3' R : 5'-CCATTTTCCACCCTACCCTAC-3'	National Center for Biotechnology Information (NCBI)
GH/*Msp*I	F : 5'-CCCACGGGCAAGAATGAGGC-3' R : 5'-TGAGGAACTGCAGGGGCCCA-3'	Mitra et al. (1995)
GHRH1/*Hae*III	F : 5'-GGCAGAACTTGAACCCAGGTG-3' R : 5'-CCAGAAGTGACAGCTGCTGTG-3'	National Center for Biotechnology Information (NCBI) Singapore, GenBank
GHRH/*Hae*III	F : 5'-GTAAGGATGCCAGCTCTGGGT-3' R : 5'-TGCCTGCTCATGATGTCCTGGA-3'	Moody et al. (1995)

Figure 2. DNA Estimated sequences of target GH genes (Source: sequences of buffalo GH gene, access number GenBank: KC852882) (Othman et al. 2012). Note: Blue Line: Primer (forward & reverse). Italic Letter: Intron Area, Yellow Line: Mutation Point/Cutting Site. Bold Letter: Exon Area

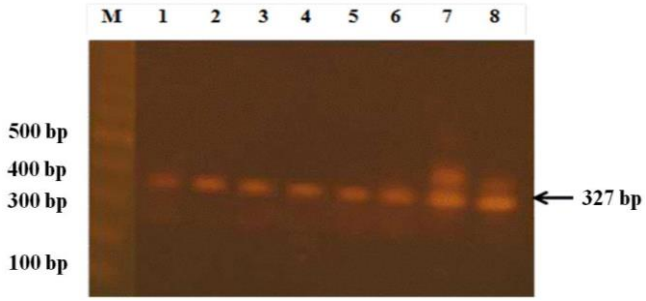

Figure 1. Results of GH gene amplification using PCR method on 1% agarose gel (M=100 bp ladder)

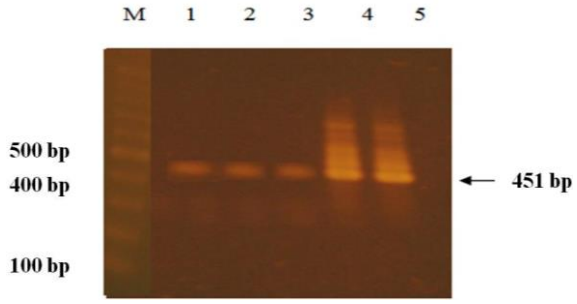

Figure 4. Results of the GnRH gene amplification using the PCR method on 1% agarose gel (M=100 bp ladder)

The RFLP technique was used to detect GH gene genotypes with *Msp*I restriction enzymes that recognize C * CGG restriction sites. Figure 3 showed the pattern of the cutting ribbon of the *Msp*I enzyme on the product of the 327 bp GH gene PCR resulting in two alleles: the A allele and the B allele. The mutation was observed in base 104, which was the C-T point mutation in introns 3 (Figure 3).

The occurrence of transition base mutation (single mutation) that changed the C-T base lead to the changing of the cutting site for the *Msp*I restriction enzyme changed. The transition mutations were due to substitution between base Adenine by Guanine (Purine) or cytosine bases and Thymine (Pyrimidine). It is well recognized that the mutation point of the GH- *Msp*I gene has appeared in the intron 3 area, which is a non-coding area. Since the non-coding area is spliced at the time of transcription, the mutations will not take effect on phenotypic changes. These kinds of mutations are also called a silent mutation. GH genes with AA genotypes were expressed clearly by two fragments, 104 and 223 bp. While the GH genes with AB genotype were indicated by three fragments, 104, 223, and 327 bp, and the BB genotype was suggested by the absence of a 327 bp of PCR product.

Amplification of the GHRH gene was carried out by the PCR technique using a pair of primers, according to Moody et al. (1995). The pair of primers used successfully amplified the GHRH gene with a length of about 451 bp (Figure 4). Figure 4 illustrates the amplified GHRH gene fragment was located in part of exon 2, intron 2, and part of exon 3. The length of the GHRH gene fragment from amplification in cattle and pigs is 455 bp and 455 bp, respectively (Franco et al. 2005). The GHRH gene in cattle is located on chromosome 13, and its presence is close to CSSM30 microsatellite and consists of 5 exons separated by 4 introns with a length of 9356 bp (Barendse et al. 1994).

The GHRH gene PCR product was cut using *the Hae*III restriction enzyme. The electrophoresis pattern of the GHRH/*Hae*III locus (Figure 5) showed the pattern of the *Hae*III enzyme cutting the ribbon on the 451 bp product of the GHRH gene PCR resulting in two alleles; A and B allele.

The mutations that occur in base 312, which was recognized by the *Hae*III restriction enzyme on the GG*CC restriction site (Figure 6). The GHRH gene with AA genotype was observed with perfect pieces of three fragments; 312, 94, and 45 bp. The GHRH gene with AB genotype was indicated by four fragments; 451, 312, 94, and 45 bp. While BB genotype was suggested by the uncut of 451 bp PCR product, the 45 bp DNA band could not be displayed because agarose with a concentration of 3% was incorrectly used to separate DNA with a length of less than 60 bp (Muladno 2002).

Polymorphism and diversity were indicated by the presence of two or more alleles in a population. Genes categorized as polymorphic (diverse) if one of the alleles has a frequency of less than 99% (Nei and Kumar 2000). The frequency of the genotypes of GH/*Msp*I and GHRH/*Hae*III gene fragments of swamp buffalo in Bombana can be seen in Table 3.

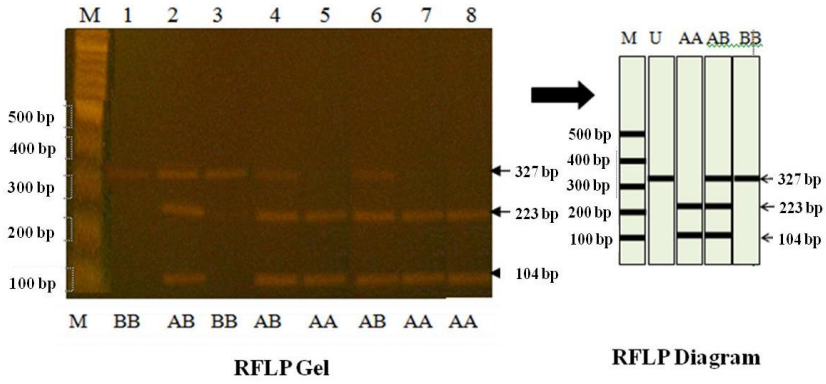

Figure 3. Example of the pattern of cutting ribbon GH along 327 bb in 3% agarose gel. *U = PCR product*

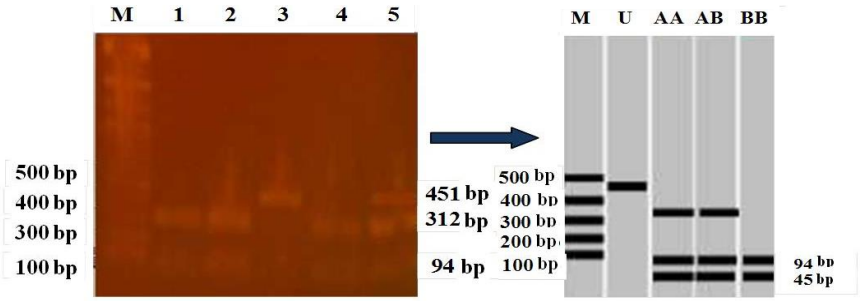

Figure 5. The pattern of the cutting band of the GnRH gene along 451 bp in 3% agarose gel. U = PCR product

Primer F

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  1 tgaaggatgc tgctctgggt gttcttcctc gtgaccctca ccctcagcag cggctcccac
 61 ggttccctgc cttcccagcc tctcaggtaa gcagttctga gaagagaagc aagagagg*cc
121 ctttgaggat gcgactcgag ctggtcccca gctgggtcct caggcagcct cccttgctca
181 tctctgggag ggtggcagac tgagccccag agaggtcacc acccagccct ggttccagcc
241 ctctctgggg acgagcaggg caagaggcga cagaaagacc tcacagagac caagtgagca
301 cagtcccctg gg*cctcccac cccacccttt gacctctgac tccttctact aggattccac
361 ggtacgcaga tgccatcttc actaacagct accggaaggt tctgg*ccag ctgtctgccc
421 gcaagctact ccaggatatc atgaacaggc a
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Primer R

Figure 6. Estimated sequences of GNRH target genes (Source: GHRH cow gene sequence, GenBank access number: AF242855) (Zhou et al. 2000). Note: Blue Line: Primer (forward & reverse). Italic Letter: Intron Area, Yellow Line: Mutation Point/Cutting Site. Bold Letter: Exon Area

Table 3. Frequency of genotypes and alleles GH/*Hae*III and GHRH/*Hae*III genes locus of swamp buffalo in Kabaena Island, Bombana District, Southeast Sulawesi, Indonesia

Location	GH/*Msp*I				GHRH/*Hae*III			
	Genotype	Allele	Allele Freq.	Genotype Freq.	Genotype	Allele	Allele Freq.	Genotype Freq.
Kabaena Island	AA	A	0.562	0.375	AA	A	0.700	0.600
	AB	B	0.438	0.375	AB	B	0.300	0.200
	BB			0.250	BB			0.200

Table 4. GH and GHRH gene index point (heterozygosity and chi-square point) of Swamp Buffalo in Kabaena Island, Bombana District, Southeast Sulawesi, Indonesia

Locus	Heterozygosity value		Hardy-Weinberg equilibrium/χ^2(p)
	H_0	H_e	
GH/*Msp*I	0.375	0.492	(0.89 > 0.05) ^{ns}
GHRH/*Hae*III	0.200	0.420	(0.70 > 0.05) ^{ns}

Based on the GH/*Msp*I gene locus, swamp buffalo in Kabaena Island had three genotypes with frequencies of 37.5% for AA genotype, 25% of BB genotype, and 37.5% of AB genotype. The frequency of A allele was slightly higher (56.2%) compared to the B allele (43.8%). The high frequency of A allele was also found in several cattle populations (Sumantri et al. 2010). The GH/AluI and *Msp*I genes are also found in local Indonesian swamp buffalo (Sumantri et al. 2013), Anatolian water buffalo (Konca and Akyuz, 2107) and Simeulue buffalo of Aceh, Indonesia (Eriani et al. 2019).

The results of allele frequency and GHRH/*Hae*III gene genotype frequency in Kabaena Island swamp buffalo were presented in Table 3. GHRH/*Hae*III locus is polymorphic. The frequency of AA, AB and BB genotypes were 0.600 (60.0%), genotype AB 0.200 (20.0%) and genotype BB 0.200 (20.0%), respectively. The frequency of A allele for the GHRH/*Hae*III gene in Kabaena Island swamp buffalo was greater than 0.700 (70.0%) than B, allele (30.0%). AA genotypes for the GHRH/*Hae*III gene were rarely found or have low frequencies in the bovine population (Moody et al. 1995). The GHRH/*Hae*III gene is found in the Anatolian river buffalo with a frequency of A 0.55 and B 0.45 genes (Konca and Akyuz, 2107). The presence of the GHRH gene is also found in Egyptian buffalo (Othman et al. 2015).

The frequency of A allele for the GHRH/*Hae*III gene in Kabaena Island swamp buffalo was 0.700 (70.0%). There is the diversity of the GHRH/*Hae*III locus in Red and White dairy cows with the frequency of A and B alleles are 0.28 and 0.72, respectively, as well as the genotype frequencies of AA (0.09), AB (0.38) and BB (0.53) (Kmiec et al. 2007). The study showed the presence of sequences diversity of GHRH/*Hae*III genes and expressed higher values for the milk production characteristic which included milk production (kg), milk fat production (kg), milk protein (kg), total fat and protein (kg) and fat and protein content (%) in AA genotype cattle. The growth hormone can enhance the semen quality in Nili Ravi buffalo bulls (Masood et al. 2016).

Kabaena Island is one of the islands in Bombana District, Southeast Sulawesi, with an area of 873 km^2. It takes 4-6 hours by ferry or motorboats to Kabaena island from the mainland. The island is relatively isolated, especially towards the mobility of buffaloes. Several locations on this island are swamps that are suitable for buffalo habitat. The local people have been familiar with swamp buffalo, but it is not too clear when they commence raising buffaloes. Local people explained that their ancestors had kept buffaloes for decades from one generation to next. They raise buffaloes for some purposes, such as saving or for social status. The live buffaloes are also exported to another place like Tanah Toraja in South Sulawesi, while the buffalo meat is made as salty jerky and is traded locally. There is no clear information where the origin of buffalo raising in Kabaena island, so it was predicted that swamp buffaloes in Kabaena island have specific characters based on the growth genes diversity, such as GH and GHRH.

Based on the diversity of growth genes (GH and GHRH), swamp buffalo in Kabaena Island have a distinctive phenomenon. GH/*Msp*I locus in the study were polymorphic (the frequency of $GH^{+\ (A)}$ alleles was 0.562, and $GH^{-\ (B)}$ was 0.438), whereas the swamp buffalo in Pandeglang District, Banten Province, Indonesia (Sumantri et al. 2013) and in Simeulue Province Aceh, Indonesia (Eriani et al. 2019) were monomorphic, with the frequency of $HG^{+\ (A)}$ reaching 1.0 or 100%). The Growth hormone is encoded by the GH gene (Ardiyanti et al. 2009), which has an essential role in the regulation of growth, development of mammary glands, initiation of lactation, and muscle development (Kovacs et al. 2006). The frequency of A allele at the GH/*Msp*I locus in Beijing Holstein cattle reaches 0.875, with AA genotypes producing high milk production and AB genotypes expressing high-fat milk content (Zhou et al. 2005). GH/*Alu*I polymorphism can be used as a selection indicator to increase milk production and quality (Khatami et al. 2005), weight gain, and the amount of meat in the carcass (Oprzadek et al. 2005).

The locus of the GHRH/*Hae*III gene in swamp buffalo in Kabaena Island was also polymorphic, consisting of 3 genotypes, namely AA, AB, and BB, with A allele of 0.700 and B allele of 0.300. This result also showed a specific phenomenon, because it was very different from swamp buffaloes in Pandegang District which were generally monomorphic, with the frequency of A allele reaching 1.0 or 100%, except in Menes and Cisata, Pandeglang District, and Cibadak, Lebak District (Sumantri et al. 2013). The frequency of BB genotypes was the highest one, but AA genotypes are not detected in Indonesian swamp buffalo (Primasari et al. 2009). Anatolian swamp buffalo in Egypt has AA genotypes at the GHRH/*Hae*III locus with very low frequencies (Othman et al. 2015; Konca and Akyuz 2017). The GHRH gene plays a vital role in regulating the secretion of growth hormone by the pituitary gland and plays an important role in regulating metabolism and physiological growth in mammals (Kmiec et al. 2007), so it can be assumed that the GHRH coding gene can be used to improve the growth and traits of milk production trait (Dybus et al. 2003; Szatkowska et al. 2009). However, studies related to GH and GHRH on the buffalo production traits, both meat, and milk, are still limited, so the research refers to those issues that are necessary.

The value of the GH/*Msp*I and GHRH/*Hae*III locus index (heterozygosity and chi-square value) in swamp buffalo in the Bombana District was presented in Table 4.

The data in Table 4. revealed that the value of observational heterozygosity (H_o) and expected heterozygosity (H_e) at the GH/*Msp*I locus were 0.375 and 0.492, respectively, while at the GHRH/*Hae*III locus were 0.200 and 0.420. The value of H_o's heterozygosity observation was lower than the expected heterozygosity (H_e). The results of this research indicate that there are factors that affect population balance such as selection. The buffalo population in this area is thought to have undirected, thus effect on the heterogeneity value at the GH/*Msp*I and GHRH/*Hae*III locus. The difference between observed heterozygosity values (Ho) and expected

heterozygosity values (He) can be used as indicators of genotype imbalance in the population (Hedrick 2005; Tambasco et al. (2003).

Moreover, the price of proven buffalo bull was relatively high so that many were sold, causing the availability of proven buffalo bull in the field to be less. This condition increased inbreeding, which could reduce buffalo productivity. Also, the low value of heterozygosity at the GH/*Msp*I and GHRH/*Hae*III locus indicated a close genetic relationship between individuals in the population.

Based on the results of the chi-square test it was concluded that the heterozygosity values of the observations were not significantly different from the expected values. These results revealed that the genotype frequencies of the GH and GHRH genes in swamp buffalo populations in Kabaena Island, Bombana District, Indonesia were in Hardy-Weinberg equilibrium.

The results of the chi-square test for the GH/*Msp*I and GHRH/*Hae*III locus between the observed values (H_e) and the expectation value (H_o) showed no significant difference. This revealed that random breeding existed among buffalo populations in these regions (Vasconcellos et al. 2003), which made the allele and genotype frequencies ton relatively constant from one generation to the next. Moreover, it also showed that the buffalo population in Kabaena Island was at the equilibrium of Hardy-Weinberg. Random mating indicated no selection in the buffalo population, or the selection had not significantly changed the gene frequency. Selection is an effective way to increase productivity by improving genetic quality.

Lately, the traits of buffalo production have been linked to variations in growth genes and controlling genes for milk production such as PIT, GH and GHRH (Franco et al. 2005; Kmiec et al. 2007; Eriani et al. 2019), DGAT, PRL and PRLR (Shi et al. 2011), GHR, IGF, IGFBP (Ramesha et al. 2015; El-Magd et al. 2017; Othman et al. 2018) and TRL (Sonawane et al. 2018). In the future, the use of genes controlling growth, milk production, and disease resistance are expected to have implications for the acceleration of buffalo productivities, both meat, and milk.

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