Original Research Article

IDENTIFICATION OF BOVINE GROWTH HORMONE (BGH)GENE POLYMORPHISM USING PCR-RFLP METHOD INBUFFALO BULLS

ABSTRACT

Growth Hormone (GH) is a single polypeptide chain synthesised and secreted from anterior pituitary gland by somatroph cells. The product of GH gene hastens metabolism and promotes the growth of many organs and tissues especially bone, muscle and visceral organs. It also regulates growth, mammary gland development and lactation and polymorphism in this gene is associated with increase in growth and development many tissues in the body.

Aim: The objective of this study was to investigate polymorphism of the bovine growth hormone (bGH) gene in buffalo bulls (*Bubalus bubalis*) using the PCR-RFLP (polymerase chain reaction–restriction fragment length polymorphism) technique.

Design: Genomic DNA was extracted from a total of 10 bulls, consisting of Murrah – Swamp crossbred and pure Swamp buffalo bulls. A 446 segment of the bGH gene was amplified. The DNA amplicons were detected in 2% agarose gel following 45 minutes of electrophoresis. They were thereafter digesting with AluI endonuclease restriction enzyme, and the digested DNA were detected in 2% agarose gel following electrophoresis for about 45minutes in all the samples

Result similar bands of approximately 300 and 146-bp each, with no variation, were detected in 2% agarose gel following electrophoresis in all the animals tesred

Conclusion: Based on the Alu1 digestion result, all samples produced the same allele of the gene, with no polymorphism detected.

Keywords: Buffalo, Bovine Growth Hormone (bGH), PCR-RFLP,Polymorphism

1. INTRODUCTION

Generally, buffaloes provide mechanical draught power, meat and milk for human consumption. In Malaysia, 60% of the buffaloes are mostly concentrated in the rice growing states of Kelantan, Terengganu, Kedah and Pahang. This is mainly due to their capability to be used as draught power in agriculture.

Buffalo population has seen a steady increase, it stood at 177.247 million in 2012 (1). According to (2), buffaloes are spread through 42 countries, with Asian buffalo population representing 97% of the world total. The rest of world therefore accounts for only 3%. Currently, one of the focus of studies in the field of animal genetics is the identification of genes with important influence on the expression of quantitative traits. Polymorphisms in these genes, which are also referred as candidate genes, plays an important role in animal selection and breeding. One of these genes, which is potentially polymorphic with a lot of selection benefit is the growth hormone gene (3). Several studies have indicated the association between polymorphism of the bovine growth hormone (GH) gene and production traits although with divergent results in some cases (4–6).Growth hormone is a polypeptide hormone secreted by somatroph cells from the anterior pituitary glands (7). GH gene has been assigned to 19q26q-ter position in thebovine chromosome(8).Polymorphism in the GH gene has been detected in the cattle after digestion with Alu restriction endonuclease(9–11). Two alleles have been identified following the use of this enzyme' the L and the V alleles. Animals with genotype LL were reported to be superior, in terms of milk production and other production traits, to those carrying the LV genotype(9)The buffalo (B b) growth hormone gene is similar to that of cattle (B t/i). The size of GH gene is approximately 1798 bp (12), consisting of five exons and four introns, with the mRNA being about 654 bp in length. GH gene of cattle and buffalo having 98.6% sequence homology (12). However, despite its potentials in animal breeding and selection, the amount of studies regarding GH gene polymorphism are scanty (13).

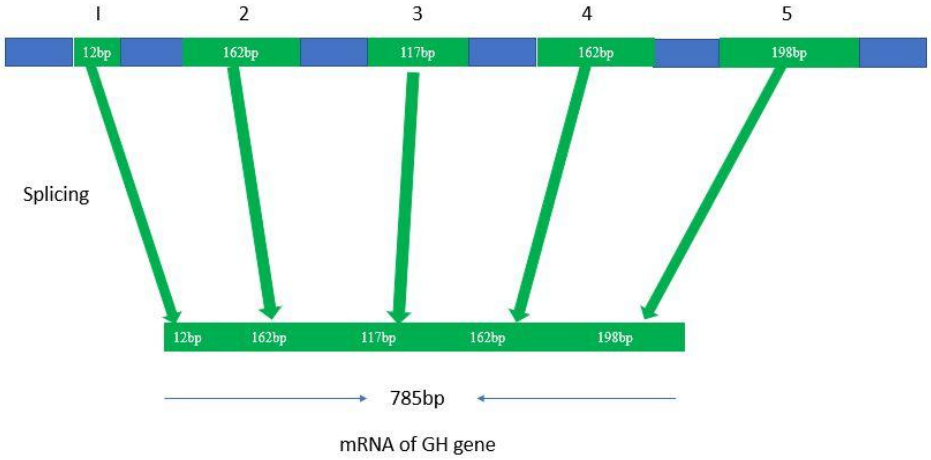

Figure 1. Length of bovine GH gene 1793bp with five exons and four introns.

2. MATERIAL AND METHODS

2.1Samples collection and storage

Blood samples were taken from 10 buffalo bulls maintained at the Buffalo Breeding and Research centre, Department of Veterinary Services and Animal Industry, Telupid, Sabah. Tenmillilitresof venous blood were collectedfrom the jugular vein of

each animal in a sterile 50 ml polypropylene vial containing0.5 M EDTA as anticoagulant. The samples were subjected to analysis for bGHgenomic DNA using PCR-RFLP (polymerase chain reaction – restriction fragmentlength polymorphism) method. The tubes containing EDTA were centrifuged at 3,000 rpm for 5minutes and the buffy coats were obtained from the blood. The buffy coats were transferred to 1.5 ml microcentrifuge tube and stored at -20 ºC until further processing. Genomic DNA was extracted from the samples with GeneJET™ GenomicDNA Purification Kit (Thermo Scientific, USA) according to manufacturer's protocol.

2.2 Genomic DNA Extraction

A total of 200 µl blood sample (buffy coat) was lysed by adding400 µl Lysis Buffer Solution and 20 µl Proteinase K (10 mg/ml), which wasthen incubated at 56 °C for 10 minutes. After incubation,200 µl of 96% absolute ethanol was addedand the resulting mixture was applied into the GeneJET™Purification Column. The column was centrifuged at 6000x g for 1 minute. DNApurification was conducted using spin column by adding 500 µl Wash Buffer I solutionfollowed by centrifugation at 8000x g for 1 minute. After the supernatants (flow-through) were discarded, the DNA was washed again with 500 µl of Wash Buffer II and centrifuged at 13000x g for 3 mins. After the supernatants (flow-through) werediscarded, the DNA was eluted with 200 µl Elution Buffer and centrifuged at 8000xg for 1 min.The flow-through elution was collected and stored at -20°C. DNA quality, quantity and purity, were assessed using a Nanodrop spectrometer (DNA TECH, UCDAVIS GENOME CENTER); 1 ul of each sample was placed on the lower optical pedestal, the lower leveer arm was closed and the sample read via a computer application. The concentration (ng/ul) and the purity at 260/280 and 260/230 were recorded.

2.3 Polymerase chain reaction (PCR)

Amplification of bovine growth hormone (bGH) gene fragments was doneusing polymerase chain reaction (PCR) method(Bio-Rad Thermal Cyclers). GH2 forward (5'-CGG ACC GTG TCT ATG AGA AGC TGA AG-3') and reverse (5'-GTT CTT GAG CAG CGC GTC GTC A-3') primer sequences published by Reis *et al*. (2001) and Balogh *et al*. (2009) were used to amplify the 436bp fragments of the GH gene. The PCR protocol was performedusing Taq PCR Master Mix Kit (QIAGEN Germany) in a 25µl reaction mixturecontaining 12.5µl PCR master mix (250 units HotStarTaq DNA Polymerase,PCR Buffer with 3 mM MgCl2, and 400 µM of each dNTP), 5µl template genomicDNA, 0.5µl of each GH2 forward and reverse primers, and 6.5µl nuclease-free water.The PCR reactions were run at initial denaturation temperature at 95°C for 3minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, followed by extension at 72°C for 1 minute and one cycle of final extension at 72°C for 12 minutes, whichendedwith infinity duration at 4°C.

2.4 Gel electrophoresis and documentation

The PCR products were electrophoresed on 2% agarose gel stained with Ethidium Bromide at a constant voltage (90V) for 45 minutes. The result wasvisualised under U.V light using Gel Doc™ system.

2.5 Restriction fragment length polymorphism (PCR-RFLP*)*

The PCR products for the tested fragments were digested with the restrictionendonuclease enzyme, AluI. The restriction mixture for each sample was prepared intonew microcentrifuge tubes by adding 17µl of nuclease-free water followed by 2µl of 10X FastDigest Green Buffer in each sample, 10µl of the DNA (PCR product) and lastly 1µl of FastDigest enzyme containing AluI. The restriction mixture was then gently mixed and spun down using vortex for a few seconds and incubated at 37°C in awater bath for 15 minutes. Then, the enzyme was inactivated by further heating at65°C for 5 minutes. The digested PCR products were electrophoresed on 2% agarosegel at 90V for 45 minutes. Gel was stained by ethidium bromide and visualised onUV transiluminator and finally documented in Gel Doc system.

3. RESULTS AND DISCUSSION

3.1 Amplification of bGH gene using GH2 primers.

The PCR product of bGH gene witha fragment length size of 436 bp was amplified with primer GH2, which was electrophoresed on 2% agarose gel stained with ethidium bromide at a constant 90V for 45 minutes. Lane M showed DNA ladder (100-1000). The ampliconswere visualised under U.V transilluminator light using Gel Doc™ system.

The PCR products were sequenced using Sanger's method. The complete sequence of all 10 bGH genes was analysed using MEGA version 10 and BioEdit software. The sequences were later submitted to the GenBank database and Accession Numbers were obtained.

Table 1. Growth hormone DNA sequences and their corresponding GenBank Accession numbers

s/no	Sequence ID	Accession number
1	BankIt2060151 UPM3926	MG450554
2	BankIt2060151 UPMTBN1	MG450555
3	BankIt2060151 UPMTBN2	MG450556
4	BankIt2060151 UPM1SAWAH	MG450557
5	BankIt2060151 UPM2SAWAH	MG450558
6	BankIt2060151 UPM3SAWAH	MG450559
7	BankIt2060151 UPM3898	MG450560
8	BankIt2060151 UPM839	MG450561
9	BankIt2060151 UPM830	MG450562
10	BankIt2060151 UPMTBN3	MG450563

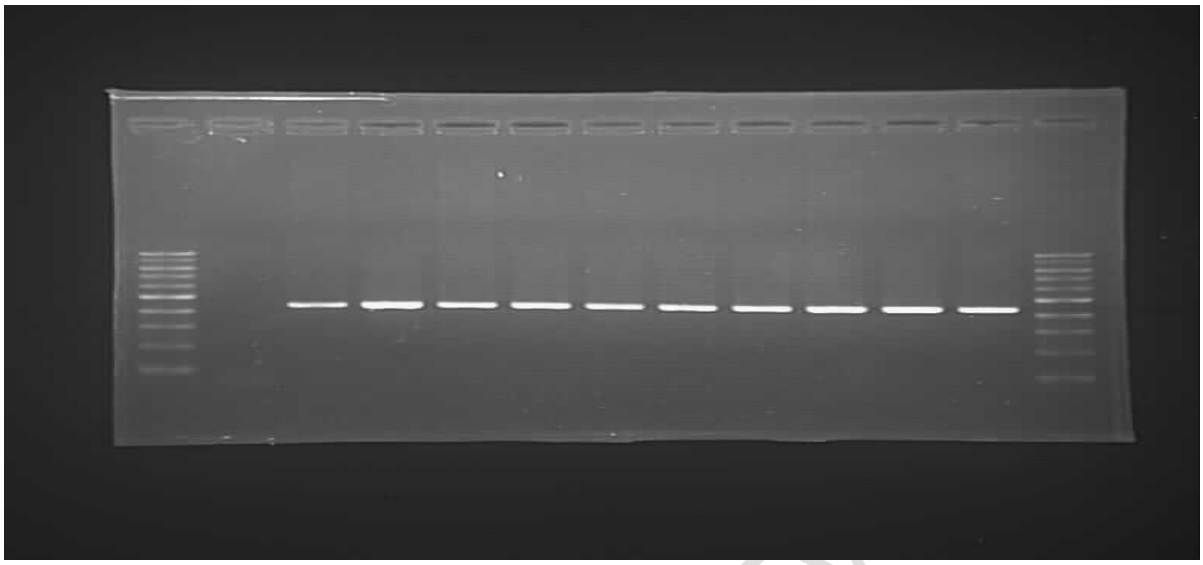

FIGURE 2. The PCR products of bGH gene with size of 446 bp, amplified using GH2 primer. Lane M and 12 DNA ladder (100-1000). Lane 1 is negative control. Lane 2-11 display the PCR product for each DNA sample

3.2 Digestion of PCR product of bGH gene with AluI enzyme.

The digestion of PCR products of bGH gene withthe size of 295- and 100- bp was conducted using AluI endonuclease restriction enzyme. The product was electrophoresed on 2%agarose gel stained with 10X FastDigest Green Buffer at constant 90V for 45 minutes.Lane M displayed DNA ladder (100-1000). PCR was visualised under U.V light usingGel Doc™ system (Figure 2).

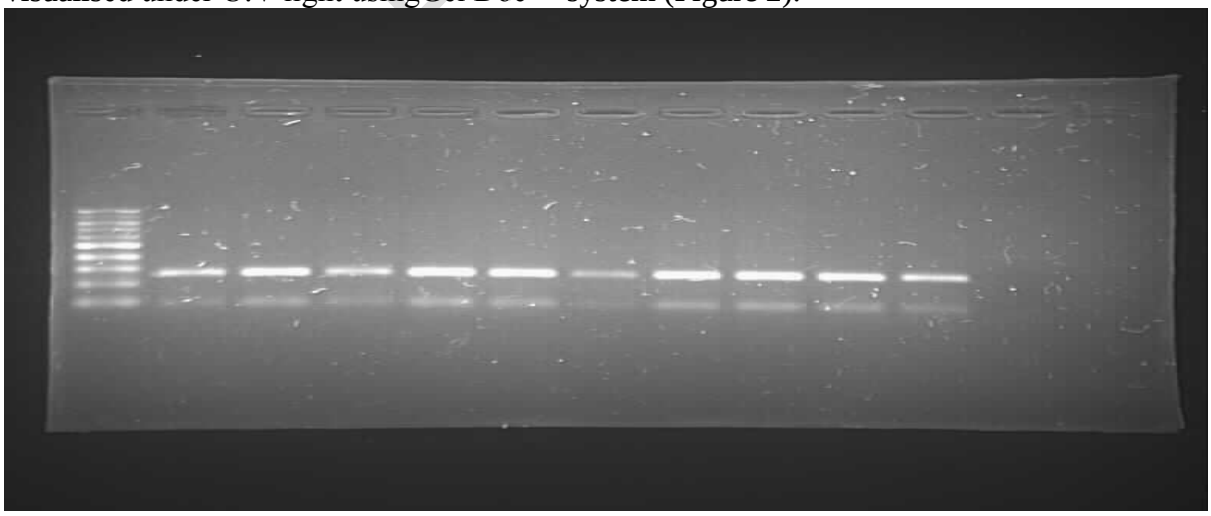

FIGURE 3.The digested PCR products with AluI restriction enzyme of bGH with size of 295- and 100-bp. Lane M shows DNA ladder (100-1000). Lane 1-11: PCR products with AluI restriction enzyme. Lane 12: Negative control.

3.3 Determination and amplification of bGH gene fragments

In wells 2 to 10, similar bands were observed on 2% agarose gel under the view of UV - transilluminator light. The fragments' size/length produced ranged between 400- and 500 bp. In order to confirm the DNA of bovine growth hormone (bGH) genefragments' size/length, semi-log graph was used and plotted the axis using *GraphPad PRISM®7*. The bGH gene fragments' size/length was determined by plotting the x-axis(distance migrated) and y-axis (inverted log base pair) in GraphPad PRISM®7 and 436-bp was observed when compared with DNA ladder on Lane M. Fragments lengthamplification product was done by matching the primers set alignment with bGH genesequence. Previous studies on bGH gene polymorphisms in buffaloes (Bubalus*bubalis*) by Balogh *et al*. (2009) andAndreas et al. (2010) showed the fragmentslength of bGH gene of 428bp and 432bp, respectively. This result is different from that of other studies(13).However,any of the studies demonstratedthe 436 bp GH fragment as in this study. Eventhough the base pair (bp) produced was different, it matched with GH2 primer (F= 5'-CGGACCGTGTCTATGAGAAGCTGAAG - 3' and R = 5'-GTTCTTGAGCAGCGCGTCGTCA - 3' (14) used in this study producing 436bp on 2% agarose gel electrophoresis. Primer sequences used by (14) have been frequently used in determining GH gene sequence of the DNA fragments length. The primers were rechecked through BLAST® NCBI *(https://blast.ncbi.nlm.nih.gov/Blast.cgi)* for confirmation. All the amplicons showed very high similarity with those on the GenBank.Therefore, it can be assured that the bands produced on 2% agarose gel electrophoresis in this work were those of GH gene with 436 bp fragment length.

3.4 PCR-RFLP analysis of bGH gene polymorphisms

Determination of bovine growth hormone (bGH) gene polymorphism in thisstudy was done using PCR-RFLP method. This method usedendonuclease restriction enzyme, AluI, which hasa cutting site at 5' AG|CT 3' and 3'TC|GA 5'. Based on DNA sequences of bGH genes amplified segment, there weretwo AluI cutting sitesthat produced fragments of 295 and 100-bp long in the presence of the restriction site at position 100^101 (AG^CT). This result is supported by that of (11)in their studies on Indian and Egyptian buffaloes as well as cattle.

The AluI restriction endonucleaseenzyme has a specific target on PCR fragments of the bGH gene containing exon 5 and thealleles found were designated with L and V. The L allele is responsible for the formationof bGH with an amino acid residue ofleucine at position 127, whereas the V allele isresponsible for an alternative form with a valine residue at the same position (15).(16) reported a polymorphic site for AluI restrictionendonuclease localised in the exon 5 in bovine GH gene and characterised by thesubstitution of cytosine [C] for guanine [G] at position 2,141 causing an amino acidchange from leucine to valine at residue 127. The presence of homozygous LL genotype suggests that the AluI restriction endonuclease enzyme recognised the sequence on bothhelixesof DNA and cut at the two restriction sites, AG|CT. Besides, presence of recessive Vallele on DNA fragment indicated no restriction site recognised bythe AluI restriction endonuclease. However, in contrast, this result is in partial disagreementwiththat by (17) with the presence of VV

genotypethrough their study on GH/ AluI gene polymorphisms in Slovak cattle, which might be due toproduct size that was considered large and has more than one restriction site for the sameenzyme. Thus,to obtain more accuracy, another primer was used to shorten the PCRproduct giving only one site for the AluI enzyme GH2. This showed that each well had a similar genotype. All buffaloes investigated in this study were genotyped as LLwhere all tested buffalo DNA amplified fragments were digested with*AluI*endonuclease and gave two digested fragments at 295- and 100- bp due to the presence ofa restriction site at position 100^101 (AG^CT). Accordingto (11), the L allele indicates the presence of restriction site while itsabsence is assigned as allele V. In L allele, the restriction site contains nucleotide C, while a transition with G at the same site indicates the absence of*AluI*restriction site. The presence of nucleotide C in the total length of amino acid in growthhormone (191) occurs in position 127of the polypeptide (18)According to (16), the distribution of bGHgenotypes and alleles contains the already known phenomenon where the L allele ispredominant in dairy cattle, whereas the V allele occurs more frequently in beef cattle.The presence of nucleotide (C) at triplet codon encodes the amino acid Leucine,while nucleotide G encodes the amino acid Valine.This Leucine/Valine substitution indicates polymorphism. In this study, no polymorphism was identified. The values of the fragments' length produced were calculated using the same method as bGH gene fragment length base pair values by plotting a semi-log graph in GraphPad PRISM®7. By observing two bandsproduced with the same fragment length size, it can be deduced that these DNAcarried the same genotypes,thus supporting the statement above. There are no otherstudies done on the polymorphisms of the same fragment length; however, a study done by (14)demonstrated GH gene with 211bp that produced twoseparate bands with same values of fragment length at 159- and 52-bp, respectively. Therefore, buffalo bulls used in this study were having monomorphicgene loci and as a result, no polymorphism was identified in the10 samples of buffalo bulls.

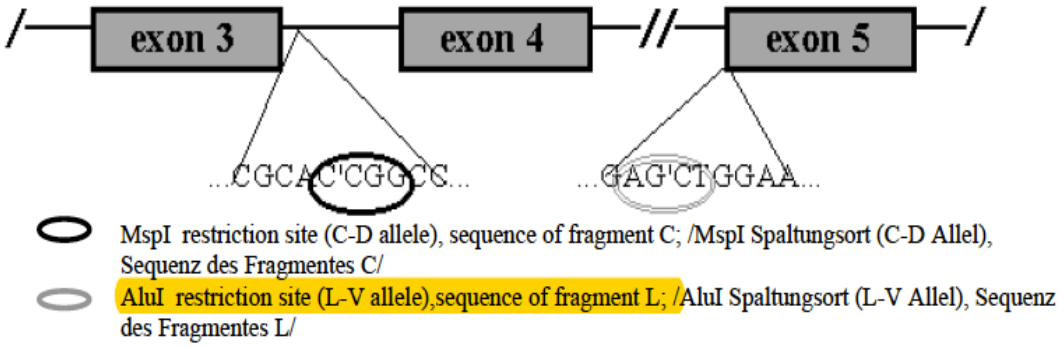

FIGURE 4. The AluI RE enzyme specifically targets exon 5 containing cutting site of AG|CT

4. CONCLUSION

CONCLUSION

In conclusion, all buffalo bulls investigated in this study were genotyped as LL and the diversity of GH| AluI was verylow, showing no polymorphisms in their bGH genes. This could have resulted from the low diversity of bGH genes among the buffalobulls due to limited number of males in the farm and high inbreedingfrequency. Therefore, for future study, it is recommended to use more buffalo bullsfrom different locations and farms to increase gene diversity in the samples to be examined for gene polymorphism. For the farms involved in this study, itis highly recommended to increase the number of breeding bullsby purchasing new bulls from other locations or breeding farms to increase genetic diversity and to avoid inbreeding practice. The use of different restriction endonuclease enzymesin PCR method could be considered since the genes may be having differentsites of polymorphism. Therefore, the potential of bGH gene as candidate gene forgenetic-marker to be used in breeding programme in buffaloes could only be consideredonce the presence of bovine growth hormone (bGH) gene polymorphism can bedetected.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee

REFERENCES

1. FAO. The state of food and agriculture,. 2012.

2. Habib MR, Haque MN, Rahman A, Aftabuzzaman M, Ali MM, Shahjahan M. Dairy buffalo production scenario in Bangladesh: a review. Asian J Med Biol Res. 2017;3(3):305–16.

3. Dolmatova II, Il'iasov IG. [Association of cattle growth hormone gene polymorphism with milk productivity]. Genetika [Internet]. 2011 Jun [cited 2019 Jul 16];47(6):814–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21866862

4. Arango J, Echeverri J, Lopez A. Association of the bovine growth hormone gene with Holstein cattle reproductive parameters Asociación del gen de la hormona de crecimiento bovino con. Rev MVZ Cordoba. 2014;19(3):4249–58.

5. Amiri S, Jemmali B, Amine Ferchichi M, Jeljeli H, Boulbaba R, Ben Gara A. Assessment of growth hormone gene polymorphism effects on reproductive traits in Holstein dairy cattle in Tunisia. Arch Anim Breed. 2018;61(4):481–9.

6. Hernández N, Martínez-González JC, Parra-Bracamonte GM, Sifuentes-Rincón AM, López-Villalobos N, Morris ST, et al. Association of polymorphisms in growth hormone and leptin candidate genes with live weight traits of Brahman cattle. Genet Mol Res. 2016;15(3).

7. Ayuk J, Sheppard MC. Growth hormone and its disorders. Postgrad Med J [Internet]. 2006 Jan [cited 2019 Jul 16];82(963):24–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16397076

8. Hediger R, Johnson SE, Barendse W, Drinkwater RD, Moore SS, Hetzel J. Assignment of the growth hormone gene locus to 19q26-qter in cattle and to 11q25-qter in sheep by in situ hybridization. Genomics [Internet]. 1990 Sep 1 [cited 2019 Jul 16];8(1):171–4. Available from: https://linkinghub.elsevier.com/retrieve/pii/088875439090241L

9. Pal A, Chakravarty AK, Bhattacharya T, Joshi BK, Sharma A. Detection of Polymorphism of Growth Hormone Gene for the Analysis of Relationship between Allele Type and Growth Traits in Karan Fries Cattle. Asian-Australasian J Anim Sci. 2004;17.

10. Akçay A, Akyüz B, Bayram D. Determination of the AluI polymorphism effect of bovine growth hormone gene on carcass traits in Zavot cattle with analysis of covariance. Turkish J Vet Anim Sci. 2015;39(1):16–22.

11. Biswas TK, Bhattacharya TK, Narayan AD, Badola S, Kumar P, Sharma A.

Growth Hormone Gene Polymorphism and Its Effect on Birth Weight in Cattle and Buffalo. Asian-Australasian J Anim Sci [Internet]. 2003 Jan 1 [cited 2019 Jul 16];16(4):494–7. Available from: http://ajas.info/journal/view.php?doi=10.5713/ajas.2003.494

12. Verma S, Ghorpade A, Tiwari G, Das P, Garg LC. cDNA cloning and sequence analysis of bubaline growth hormone. DNA Seq [Internet]. 1999 [cited 2019 Jul 16];10(2):101–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10376211

13. Hussain DA, Ghareeb AM, Salo WH. Evaluation of Dna Polymorphism in Bovine Growth Hormone Gene By Pcr-Rflp Method. Internarional J Sci Nat. 2014;5(3):407–11.

14. Balogh O, Kovács K, Kulcsár M, Gáspárdy A, Zsolnai A, Kátai L, et al. AluI polymorphism of the bovine growth hormone (GH) gene, resumption of ovarian cyclicity, milk production and loss of body condition at the onset of lactation in dairy cows. Theriogenology [Internet]. 2009 Mar 1 [cited 2019 Jul 16];71(4):553–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19111333

15. Fedota OM, Lysenko NG, Ruban SY, Kolisnyk OI, Goraychuk I V. The effects of polymorphisms in growth hormone and growth hormone receptor genes on production and reproduction traits in Aberdeen-Angus cattle (Bos taurus L., 1758). Cytol Genet [Internet]. 2017 Sep 1 [cited 2019 Jul 16];51(5):352–60. Available from: http://link.springer.com/10.3103/S0095452717050024

16. Lucy MC, Hauser SD, Lucy MC, Hauser SD, Eppard PJ, Krivi GG CR. Genetic polymorphism within the bovine somatotropin (bST) gene detected by polymerase chain reaction and endonuclease digestion. J Dairy Sci. 1991;74((Suppl 1)):284.

17. Moravcikova N, Trakovicka A, Hazuchova E. The Association of Bovine Growth Hormone Gene Polymorphism with Milk Performance Traits in Slovak Spotted Cows. Anim Sci Biotechnol. 2012;45(1):206–10.

18. Tety Hartatik, Slamet Diah Volkandari MPR and S. Polymorphism leu/val of growth hormone gene identified from limousin cross local cattle in Indonesia. In: The 3rd International Conference on Sustainable Future for Human Security SUSTAIN. Elsevier B.V.; 2012. p. 105–8.