

Original Research Article

IDENTIFICATION OF BOVINE GROWTH HORMONE (BGH) GENE POLYMORPHISM USING PCR-RFLP METHOD IN BUFFALO 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 the growth, mammary gland development and lactation.

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 using GH2 primers (5'-CGG ACC GTG TCT ATG AGA AGC TGA AG-3') and reverse (5'-GTT CTT GAG CAG CGC GTC GTC A-3') designed and published previously for cattle.

Result: The DNA amplicons were detected in 2% agarose gel following 45 minutes of electrophoresis. After digesting the DNA amplicons with AluI endonuclease restriction enzyme, similar bands of approximately 300 and 146-bp each were detected in 2% agarose gel following electrophoresis for about 45minutes in all the samples.

Conclusion: Based on the Alu1 digestion result, all samples produced alleles 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 account 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 gene and production traits although with divergent results in some cases (4–6). Growth hormone (GH) is a polypeptide hormone secreted by somatroph cells from anterior pituitary glands (7). Growth hormone gene has been assigned to 19q26q-ter position of bovine chromosome(8). 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 (9), consisting five exons and four introns, with the mRNA being about 654 bp in length. Growth hormone gene of cattle and buffalo are having 98.6% sequence homology (9). However, despite its potentials the amount of studies regarding GH gene polymorphism is scanty (10).

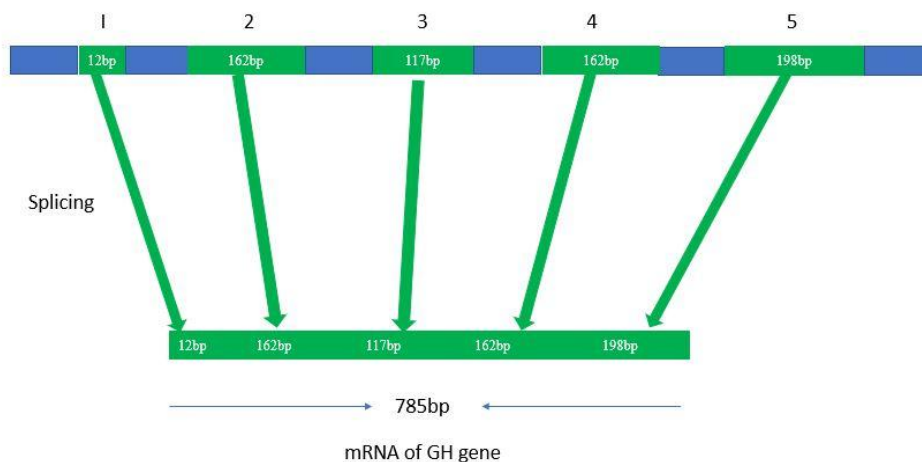


Figure 1. Length of bovine GH gene 1793bp with five exons and four introns.

2. MATERIAL AND METHODS

2.1 Samples 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. Ten millilitres of venous blood was collected from the jugular vein of each animal in a sterile 50 ml polypropylene vial containing 0.5 M EDTA as anticoagulant. The samples were subjected to analysis for bGH genomic DNA using PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism)

method. The EDTA tubes were centrifuged at 3,000 rpm for 5 minutes and the buffy coats were obtained from the blood. The buffy coats were transferred to 1.5 ml microcentrifuge tube and stored at -20 °C until further processing. Genomic DNA was extracted from the samples with GeneJET™ Genomic DNA 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 adding 400 µl Lysis Buffer Solution and 20 µl Proteinase K (10 mg/ml), which was then incubated at 56 °C for 10 minutes. After incubation, 200 µl of 96% absolute ethanol was added and the resulting mixture was applied into the GeneJET™ Purification Column. The column was centrifuged at 6000x g for 1 minute. DNA purification was conducted using spin column by adding 500 µl Wash Buffer I solution followed 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) were discarded, the DNA was eluted with 200 µl Elution Buffer and centrifuged at 8000x g 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 µl of each sample was placed on the lower optical pedestal, the lower lever 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 done using 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 436 bp fragments of the growth hormone gene. The PCR protocol was performed using Taq PCR Master Mix Kit (QIAGEN Germany) in a 25 µl reaction mixture containing 12.5 µl PCR master mix (250 units HotStarTaq DNA Polymerase, PCR Buffer with 3 mM MgCl₂, and 400 µM of each dNTP), 5 µl template genomic DNA, 0.5 µl of each GH2 forward and reverse primers, and 6.5 µl nuclease-free water. The PCR reactions were run at initial denaturation temperature at 95°C for 3 minutes, 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, which ended with 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 was visualised 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 restriction endonuclease enzyme, AluI. The restriction mixture for each sample was prepared into new microcentrifuge tubes by adding 17 µl of nuclease-free water followed by 2 µl of 10X FastDigest Green Buffer in each sample, 10 µl of the DNA (PCR product) and lastly 1 µl 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 a water bath for 15 minutes. Then, the enzyme was inactivated by further heating at 65°C for 5 minutes. The digested PCR products were electrophoresed on 2% agarose gel at (90V) for 45 minutes. Gel was stained by ethidium bromide and visualised on UV transilluminator 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 with 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). PCR was 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, were 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

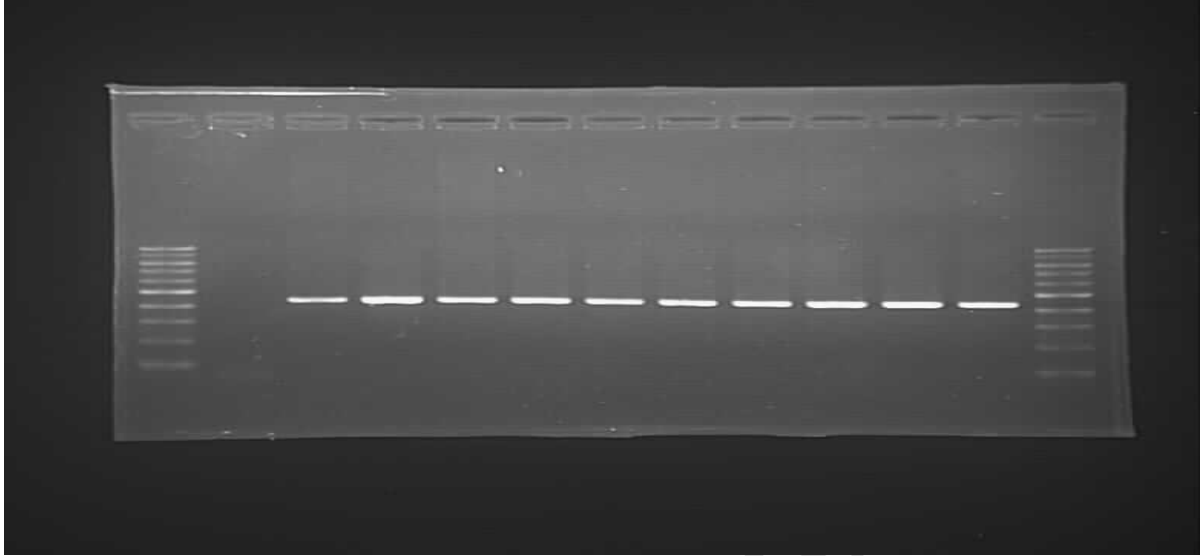


FIGURE 2. The PCR products of bGH gene with size of 446 bp, amplified using GH2 primer. Lane M and 12 DNA ladder (100-1000). Lane 1 is negative control. Lane 2-11 display the PCR product for each DNA sample

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

The digestion of PCR products of bGH gene with the 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 90 V for 45 minutes. Lane M displayed DNA ladder (100-1000). PCR was visualised under U.V light using Gel Doc™ system (Figure 2).

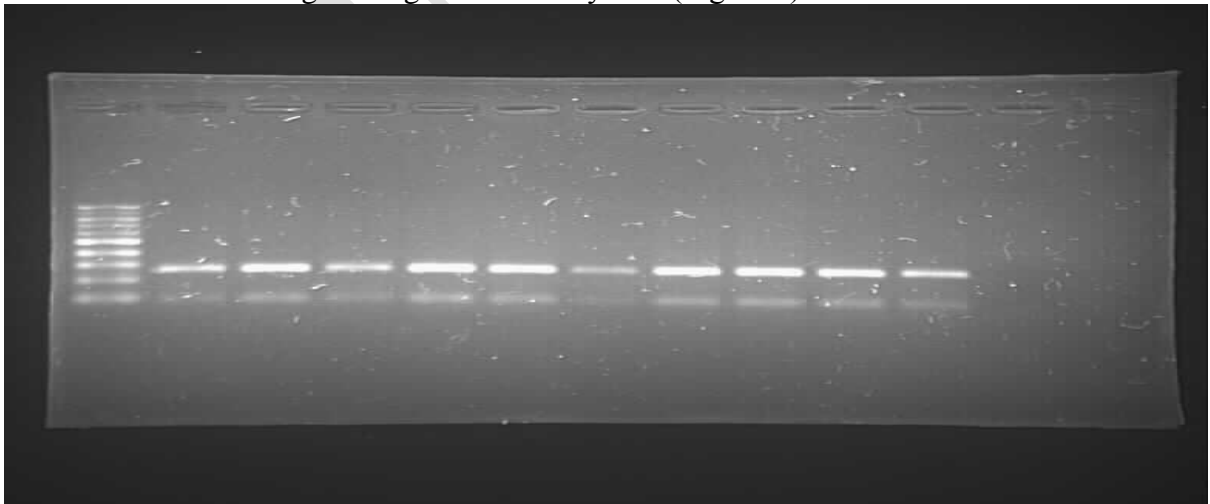


FIGURE 3. The digested PCR products with AluI restriction enzyme of bGH with size of 295- and 100-bp. Lane M shows DNA ladder (100-1000). Lane 1-11: PCR products with AluI restriction enzyme. Lane 12: Negative control.

3.3 Determination and amplification of bGH gene fragments

In well 2 to 10, same bands were produced on 2% agarose gel under the view of UV - transilluminator light. The fragments' size/length produced was ranged between 400- and 500 bp. In order to confirm the DNA of bovine growth hormone (bGH) gene fragments' 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 length amplification product was done by matching the primers set alignment with bGH gene sequence. Previous studies on bGH gene polymorphisms in buffaloes (*Bubalus bubalis*) by Balogh *et al.* (2009) and Andreas *et al.* (2010) showed the fragments length of bGH gene of 428 bp and 432 bp, respectively. This result is different with that produced from other studies(10). However, no studies demonstrated the GH gene base pair value of 436 bp as in this study. Even though the base pair (bp) produced was different, it matched with GH2 primer (F = 5'- CGGACCGTGTCTATGAGAAGCTGAAG - 3' and R = 5'- GTTCTTGAGCAGCGTCGTCA - 3' (11) used in this study producing 436 bp on 2% agarose gel electrophoresis. Primer sequences used by (11) 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 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 this study was done using PCR-RFLP method. This method used endonuclease restriction enzyme, AluI, which has a cutting site at 5' AG|CT 3' and 3' TC|GA 5'. Based on DNA sequences of bGH genes amplified segment, there were two AluI cutting sites that produced fragments of length 295 and 100-bp in each well in the presence of the restriction site at position 100[^]101 (AG[^]CT). This result is supported by that of (12) in their studies on Indian and Egyptian buffaloes as well as cattle.

The AluI restriction endonuclease enzyme has a specific target on PCR fragments of the bGH gene containing exon 5 and the alleles found were designated with L and V. The L allele is responsible for the formation of bGH with an amino acid residue of Leucine at position 127th, whereas the V allele is responsible for an alternative form with a Valine residue at the same position (13). (14) reported a polymorphic site for AluI restriction endonuclease localised in the exon 5 in bovine GH gene and characterised by the substitution of cytosine [C] for guanine [G] at position 2,141 causing an amino acid change from leucine to valine at residue 127. The presence of homozygous LL genotype suggests that the AluI restriction endonuclease enzyme recognised the sequence on both helixes of DNA and cut at the two restriction sites, AG|CT. Besides, presence of recessive V allele on DNA fragment indicated no

restriction site recognised by the *AluI* restriction endonuclease. However, in contrary, this result is in partially disagreement with that by (15) with the presence of VV genotype through their study on GH/ *AluI* gene polymorphisms in Slovak cattle, which might be due to product size that was considered large and has more than one restriction site for the same enzyme. Thus, to obtain more accuracy, another primer was used to shorten the PCR product 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 LL where 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 of restriction site at position 100[^]101 (AG[^]CT). According to (12), the L allele indicates the presence of restriction site while its absence 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 total length of amino acid in growth hormone (191) occurs in 127th position of polypeptide (16) According to (14), the distribution of bGH genotypes and alleles contains the already known phenomenon where the L allele is predominant 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 bands produced with the same fragment length size, it can be deduced that these DNA carried the same genotypes, thus supporting the statement above. There are no other studies done on the polymorphisms of the same fragment length; however, a study done by (11) demonstrated GH gene with 211 bp that produced two separate bands with same values of fragment length at 159- and 52-bp in each well, respectively. Therefore, buffalo bulls used in this study were having monomorphic gene loci and as a result, no polymorphism was identified in the 10 samples of buffalo bulls.

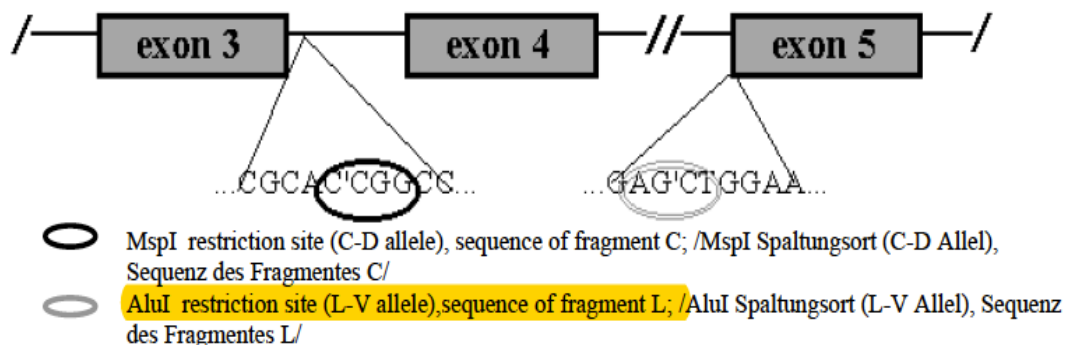


FIGURE 4. The *AluI* RE enzyme specifically target exon 5 containing cutting site of AG|CT

4. CONCLUSION

CONCLUSION

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

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. 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>
 10. 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.
 11. 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>

12. 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>
13. 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>
14. 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.
15. 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.
16. 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.

UNDER PEER REVIEW