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Evaluation of DNA Polymorphism in Egyptian Buffalo Growth Hormone and Its Receptor Genes

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Abstract

Currently, the primary thrust of research in animal genetics is the identification of genes (so-called major genes), which affect the expression of quantitative traits markedly. Two of these major genes are growth hormone (*GH*) and its receptor (*GHR*) genes which are related to production and reproduction traits in livestock. There is extensive literature on the genetic polymorphism of *GH* and *GHR* in cattle, but perusal of literature has indicated paucity of information on these two genes in buffalo.

This study aimed to evaluate the genetic polymorphism within growth hormone and its receptor genes in Egyptian buffalo using PCR-RFLP technique. Genomic DNA extracted from 100 healthy buffaloes was amplified using primers that were designed from the cattle *GH* and *GHR* gene sequences.

All buffalo animals investigated in this study are genotyped as LL for *GH* gene where all tested buffalo DNA amplified fragments at 211-bp were digested with *Alu*I endonuclease and gave two digested fragments at 159- and 52-bp due to the presence of the *Alu*I restriction site at position 52^53 (AG^CT**)**.

The amplified fragments of *GHR* gene obtained from all tested buffalo DNA at 342-bp were digested with *Alu*I endonuclease. The result showed that all tested buffaloes are genotyped as GG for *GHR* gene, where amplified fragments were digested into two digested fragments at 241- and 101-bp due to the presence of *Alu*I restriction site at position 101^102 (AG^CT**).**

Key words: Buffalo, *GH*, *GHR*, PCR, RFLP

INTRODUCTION

Improvement of important livestock through selective breeding has received more attention so that annual optimum selective breeding programs may achieve improvement in most of the economic traits of different farm animals. Potentially, the genetic marker assisted selection can enhance progress in economic traits. Genetically superior animals are efficient in nutrient utilization and growth hormone exerts a key control in nutrient use, mammary development and growth (**Pawar et al., 2007**).

Growth hormone (*GH*) is necessary for tissue growth and fat metabolism, thus, it has an important role in reproduction, lactation and normal body growth (**Beauchemin et al., 2006**, **Curi et al., 2006** and **Yardibi et al., 2009**). Because of these important biological roles, *GH* is considered a promising candidate gene for improving milk and meat production in cattle and buffalo through marker-assisted selection programs.

The bovine growth hormone is a 22 KDa single-chain polypeptide hormone produced in the anterior pituitary gland. The encoding gene is approximately 1800 base pairs and consists of five exons and four introns (**Zhou et al., 2005** and **Sadeghi et al., 2008**) and assigned to chromosome region 19q26 in bovine genome (**Hediger et al. 1990**). The polymorphism within bovine growth hormone gene was reported by **Kovacs et al. (2006)** and **Curi et al. (2006**).

Growth hormone exerts its influence on growth and metabolism by interaction with growth hormone receptor (*GHR*) on the surface of the target cells (**Hradecka et al., 2008**). The changes in the functional regions of *GHR* can affect its binding capacity and signaling pathway, and therefore alter the activity of *GH* in the target tissues (**Oleński et al., 2010)**.

In cattle, the *GHR* gene is encoded by a single gene located on chromosome 20 (**Menon et al., 2001**). The gene coding for bovine *GHR* consists of 9 exons (from 2 to 10) in the translated part and of a long 5'-noncoding region (**Maj et al., 2004**). Several polymorphisms of bovine *GHR* were described by **Dybus and Grzesiak (2006**) and **Kmiec et al. (2007**).

The objective of this study was to detect the genetic polymorphism within growth hormone and its receptor genes in Egyptian river buffalo using PCR-RFLP technique.

MATERIALS AND METHODS

Genomic DNA extraction

Genomic DNA was extracted from the whole blood of 100 unrelated Egyptian buffaloes according to established protocol (**Miller et al., 1988**) with minor modifications. Briefly, 10 ml of blood taken on EDTA were mixed with 25 ml of cold 2X Sucrose-Triton and 15 ml double distilled water. The tubes were placed on ice for 10 min and mixed by inversion several times. After centrifugation, at 5000 rpm for 15 min at 4ºC, the pellet was re-suspended by 3 ml of nucleic lysis buffer. The content was mixed with 108 µl of 20% SDS and 150 µl of Proteinase K. The tubes were placed in a water bath at 37ºC overnight.

After the incubation, the tube contents were transferred to a 15-ml polypropylene tube and 2 ml of saturated NaCl was added and shaken vigorously for 15 sec. After centrifuging at 3500 rpm for 15 min at 4ºC, the supernatant was transferred to a clean 15-ml polypropylene tube and mixed with absolute ethanol. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The precipitated DNA was picked up using the heat sealed pasture pipette, then washed twice in 70% ethanol and exposed to air to dry completely.

The DNA was dissolved in 200 µl TE buffer in 1.5-ml Microfuge tube and kept overnight in an incubator at 37ºC. DNA concentration was determined and diluted to the working concentration of 50 ng/µl, which is suitable for polymerase chain reaction using NanoDrop1000 Thermo Scientific spectrophotometer.

Polymerase chain reaction (PCR)

A PCR cocktail consisted of 1.0 mM upper and lower primers (specific for each tested gene (**Table 1**), 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin (w/v), 0.1 % Triton X-100 and 1.25 units of Taq polymerase. The cocktail was aliquot into PCR tubes with 100 ng of buffalo DNA. The reaction was cycled for 1 min. at 94°C, 2 min at an optimized annealing temperature that was determined for each primer (**Table 1**) and 2 min. at 72°C for 30 cycles. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide to test the amplification success.

Restriction fragment length polymorphism (RFLP)

The PCR products for the two tested gene were digested with the restriction enzyme *Alu*I. The restriction mixture for each sample was prepared by adding 2.5 µl of 10×restriction buffer to 10 units of the appropriate restriction enzyme and the volume was completed to 5 µl by sterile water. This restriction mixture was mixed with PCR product (~25 µl) and incubated overnight at the optimum temperature of the maximum activity for each restriction enzyme. The digested PCR products were electrophoresed on a 3% agarose gel staining with ethidium bromide to detect the different genotypes of the two tested genes.

Sequence Analysis

The PCR products of four animals for each tested gene were purified and sequenced by Macrogen Incorporation (Seoul, Korea). Sequence analysis and alignment were carried out using NCBI/BLAST/blastn suite. Results of endouclease restriction were carried out using FastPCR. The nucleotide sequences of tested genes in Egyptian buffalo were submitted to GenBank (NCBI, BankIt).

RESULTS AND DISCUSSION

The bovine growth hormone (*GH*) is a 22 KDa single-chain polypeptide hormone produced in the anterior pituitary gland. The encoding gene is approximately 1800 base pairs and consists of five exons and four introns (**Zhou et al., 2005** and **Sadeghi et al., 2008**). It is well known that it plays an important role in biological processes such as mammary development, lactation, growth and metabolism regulation (**Beauchemin et al., 2006**, **Curi et al., 2006** and **Yardibi et al., 2009**). Because of these important biological roles, *GH* is considered a promising candidate gene for improving milk and meat production in cattle and buffalo through marker-assisted selection programs.

The primers used in this study flanked a 211-bp fragment consisting of 49 base pairs from intron 4 and 162 base pairs from exon 5 of Egyptian buffalo *GH* gene. The amplified fragments obtained from all tested buffalo DNA (100 animals) were at 211-bp (**Fig. 1**).

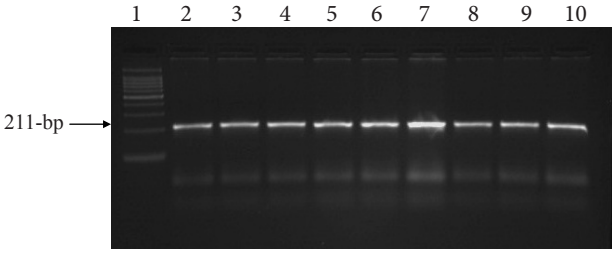

Fig. 1. Ethidium bromide-stained gel of PCR products representing amplification of *GH* gene in Egyptian buffalo.

Lane 1. 100-bp ladder marker.
Lanes 2-10. 211-bp PCR products amplified from Egyptian buffalo DNA

Table 1. The sequences and information of primers used in this study

Gene	Sequences 5′ -------- 3′	PCR conditions (35 cycles)	PCR product size	Restriction enzyme used	References
GH	GCT GCT CCT GAG GGC CCT TC CAT GAC CCT CAG GTA CGT CTC CG	95°C 1 min 62°C 2 min 72°C 2 min	211 bp	*Alu*I	Reis et al (2001)
GHR	GCT AAC TTC ATC GTG GAC AAC CTA TGG CAT GAT TTT GTT CAG	94°C 1 min 53°C 2 min 72°C 2 min	342 bp	*Alu*I	Di Stasio et al (2005)

GCTGCTCCTGAGGGCCCTTCGGCCTCTCTGTCTCTCCCTCCCTTGGCAGGAGCTGGAAG
ACGGCACCCCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACACAAAC
ATGCGCAGTGACGACGCGCTGCTCAAGAACTACGGTCTGCTCTCCTGCTTCCGGAAGGA
CCTGCACAAAA**CGGAGACGTACCTGAGGGTCATG**

Fig. 2. The sequence analysis of Egyptian buffalo *GH* amplified fragment.

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Query   1  GCTGCTCCTGAGGGCCCTTCGGCCTCTCTGTCTCTCCCTCCCTTGGCAGGAGCTGGAAGA 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  24  GCTGCTCCTGAGGGCCCTTCGGCCTCTCTGTCTCTCCCTCCCTTGGCAGGAGCTGGAAGA 83

Query  61  CGGCACCCCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACACAAACAT 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  84  CGGCACCCCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACACAAACAT 143

Query 121  GCGCAGTGACGACGCGCTGCTCAAGAACTACGGTCTGCTCTCCTGCTTCCGGAAGGACCT 180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 144  GCGCAGTGACGACGCGCTGCTCAAGAACTACGGTCTGCTCTCCTGCTTCCGGAAGGACCT 203

Query 181  GCACAAAACGGAGACGTACCTGAGGGTCATG  211
           |||||||||||||||||||| ||||||||
Sbjct 204  GCACAAAACGGAGACGTACCTGCGGGTCATG  234
```

Fig. 3. Sequence alignment of Egyptian buffalo *GH* and published sequence.

In order to verify the PCR product, two-way sequence analysis of the *GH* amplified PCR product of buffalo DNA (amplicon) was conducted, using both the forward and reverse primers. The buffalo amplicon obtained was found to be 211-bp (**Fig. 2**). The Egyptian buffalo *GH* nucleotide sequence was submitted to nucleotide sequences database NCBI/ Bankit/ GenBank and has the accession number **JN809916**.

The sequence alignment of Egyptian buffalo *GH* and published sequence (accession number. JF293086.1) was carried out using BLAST and showed that the Egyptian buffalo possess identities at 99% with only one SNP (**A/C**) at position 203 (**Fig. 3**).

These PCR amplified fragments (211-bp) were digested with *Alu*I endonuclease. Depending on the presence or absence of the restriction site at position 52^53 (AG^CT), we can easily differentiate between 3 different genotypes. VV with undigested one fragment at 211-bp, LL with two digested fragments at 159- and 52-bp and LV with three digested fragments at 211-, 159- and 52-bp.

All buffalo animals investigated in this study are genotyped as **LL** where all tested buffalo DNA amplified fragments were digested with *Alu*I endonuclease and gave two digested fragments at 159- and 52-bp (**Fig. 4**) due to the presence of the restriction site at position 52^53 (AG^CT) (**Fig. 5**).

Kovacs et al. (**2006**) studied the polymorphisms of bovine growth hormone gene. Two variants (L and V) of the bovine growth hormone gene digested with *Alu*I enzyme were identified in the experiment. Distribution of the three genotypes was 87.05% (LL), 12.40% (LV) and 0.55% (VV). On the basis

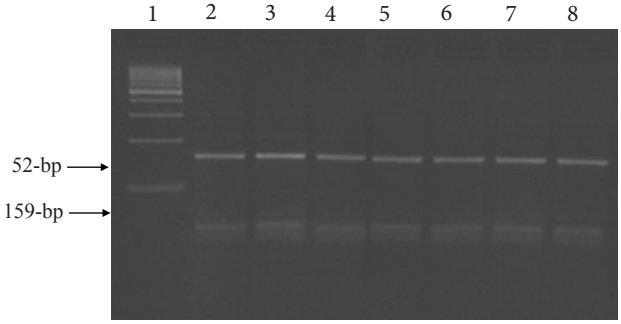

Fig. 4.. The electroploretic pattern obtained after digestion of PCR amplified buffalo *GH* with *Alu*I restriction enzyme.

Lane 1. 100-bp ladder marker.
Lanes 2-8. Homozygous LL genotypes showed two restricted fragments at 159- and 52-bp.

GCTGCTCCTGAGGGCCCTTCGGCCTCTCTGTCTCTCCCTCCCTTGGCAGG**AG^CT**GGAAG
ACGGCACCCCCCGGGCTGGGCAGATCCTCAAGCAGACCTATGACAAATTTGACACAAAC
ATGCGCAGTGACGACGCGCTGCTCAAGAACTACGGTCTGCTCTCCTGCTTCCGGAAGGA
CCTGCACAAAACGGAGACGTACCTGAGGGTCATG

Fig. 5. Endonuclease restriction of Egyptian buffalo *GH* using FastPCR

of statistical analyses it can be found that LL genotyped dams produced milk with significantly higher milk fat and protein percent. The same association between LL genotype of *GH* gene with higher milk fat and protein percent was reported by **Sadeghi et al. (2008)**. Also, **Reis et al. (2001)** reported the frequencies of L and V alleles in different cattle breeds as 0.759 and 0.241, respectively. These authors reported an association between LL and LV genotypes with the average live body weight in these cattle breeds.

Curi et al. (2006) estimated the allele and genotype frequencies of the *GH*/*Alu*I and their association with growth and carcass traits in Zebu and crossbred beef cattle. The results showed significant associations between the LL genotype of the *GH*/*Alu*I polymorphism and higher weight gain and body weight at slaughter ($p< 0.05$). On the other hand, **Yardibi et al. (2009)** reported that *Alu*I polymorphism with VV genotype cows had higher milk fat percentage compared to other genotypes.

Growth hormone actions on target cells depend on the *GH* receptor (*GHR*) (**Burton et al., 1994**). The *GH* binding to *GHR* causes its dimerisation, activation of the *GHR*-associated JAK2 tyrosine kinase, and tyrosyl phosphorylation of both JAK2 and *GHR* (**Zhu et al., 2001**). These events activate a variety of signaling molecules, including MAP kinases, protein kinase C, and STAT transcription factors (**Maj et al., 2004**).

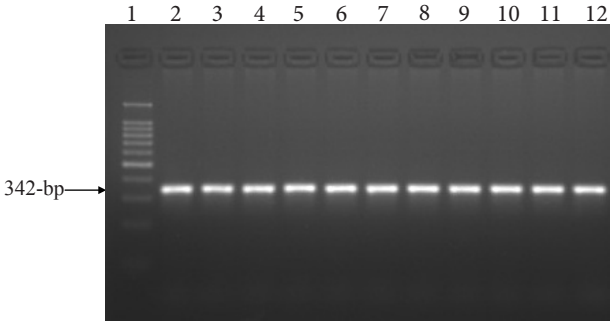

Fig. 6. Ethidium bromide-stained gel of PCR products representing amplification of *GHR* gene in Egyptian buffalo.

Lane 1. 100-bp ladder marker.
Lanes 2-12. 342-bp PCR products amplified from Egyptian buffalo DNA

GCTAACTTCATCGTGGACAACGCTTACTTCTGCGAGGTAGACGCCAAAAAGTACATTG
CCCTGGCCCCTCACGTCGAGGCTGAATCACACGTAGAGCCAAGCTTTAACCAGGAAGAC
ATTTACATCACCACAGAAAGCCTTACCACTACAGCCGGGAGGTCGGGGACAGCAGAACA
TGTTCCAAGTTCTGAGATACCTGTCCCAGATTATACCTCCATTCACATAGTACAGTCTCC
TCAGGGCCTCGTACTCAATGCGACTGCCCTGCCCTTGCCTGACAAAGAGTTTCTCTCATC
ATGTGGCTATGTGAGCACAGACCAA**CTGAACAAAATCATGCCATAG**

Fig. 7. The sequence analysis of Egyptian buffalo *GHR* amplified fragment.

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Query 1   GCTAACTTCATCGTGGACAACGCTTACTTCTGCGAGGTAGACGCCAAAAAGTACATTGCC 60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 157 GCTAACTTCATCGTGGACAACGCTTACTTCTGCGAGGTAGACGCCAAAAAGTACATTGCC 216

Query 61  CTGGCCCCTCACGTCGAGGCTGAATCACACGTAGAGCCAAGCTTTAACCAGGAAGACATT 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 217 CTGGCCCCTCACGTCGAGGCTGAATCACACGTAGAGCCAAGCTTTAACCAGGAAGACATT 276

Query 121 TACATCACCACAGAAAGCCTTACCACTACAGCCGGGAGGTCGGGGACAGCAGAACATGTT 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 277 TACATCACCACAGAAAGCCTTACCACTACAGCCGGGAGGTCGGGGACAGCAGAACATGTT 336

Query 181 CCAAGTTCTGAGATACCTGTCCCAGATTATACCTCCATTCACATAGTACAGTCTCCTCAG 240
          |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct 337 CCAAGTTCTGAGATACCTGTCCCAGATTATACCTCCATTCACATAGTACAGTCTCCACAG 396

Query 241 GGCCTCGTACTCAATGCGACTGCCCTGCCCTTGCCTGACAAAGAGTTTCTCTCATCATGT 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 397 GGCCTCGTACTCAATGCGACTGCCCTGCCCTTGCCTGACAAAGAGTTTCTCTCATCATGT 456

Query 301 GGCTATGTGAGCACAGACCAACTGAACAAAATCATGCCATAG 342
          |||||||||||||||||||||||||||||||||||||||||
Sbjct 457 GGCTATGTGAGCACAGACCAACTGAACAAAATCATGCCATAG 498
```

Fig. 8. Sequence alignment of Egyptian buffalo *GHR* and published sequence.

The *GHR* is a member of the cytokine/hematopoietin superfamily of receptors. It contains a single transmembrane domain comprising 24-amino acids, extracellular hormone binding domain, and a long cytoplasmic domain (**Kopchick and Andry, 2000**). In cattle, the *GHR* gene is encoded by a single gene located on chromosome 20 (**Menon et al., 2001**). The gene coding for bovine *GHR* consists of 9 exons (from 2 to 10) in the translated part and of a long 5'-noncoding region (**Maj et al., 2004**).

The primers used in this study (**Table 1**) flanked a 342-bp fragment from exon 10 of Egyptian buffalo *GHR* gene. The amplified fragments obtained from all tested buffalo DNA (100 animals) at 342-bp (**Fig. 6**)

In order to verify the PCR product, two-way sequence analysis of the *GHR* amplified PCR product of buffalo DNA (amplicon) was conducted, using both the forward and reverse primers. The buffalo amplicon obtained was found to be 342-bp (**Fig. 7**). The Egyptian buffalo *GHR* nucleotide sequence was submitted to nucleotide sequences database NCBI/ Bankit/ GenBank and has the accession number **JN809917**.

The sequence alignment of Egyptian buffalo *GHR* and published sequence (accession number. AY053568.1) was carried out using BLAST and showed that the Egyptian buffalo possess identities at 99% with only one SNP (**T/A**) at position 237 (**Fig. 8**).

These PCR amplified fragments (342-bp) were digested with *Alu*I endonuclease. Depending on the presence or absence of the restriction site at position 101^102 (AG^CT) in these amplified fragments, we can easily differentiate between 3 different genotypes. AA with undigested one fragment at 342-bp, GG with two digested fragments at 241-and 101-bp and AG with three digested fragments at 342-, 241- and 101-bp.

All buffalo animals investigated in this study are genotyped as **GG** where all tested buffalo DNA amplified fragments were digested with *Alu*I endonuclease and gave two digested fragments at 241- and 101-bp (**Fig. 9**) due to the presence of restriction site at position 101^102 (AG^CT) (**Fig. 10**).

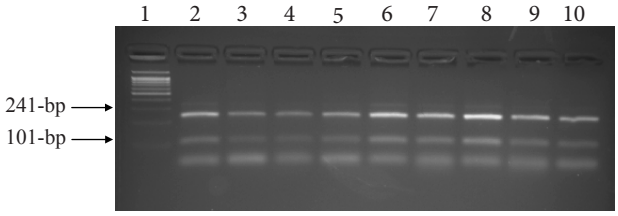

Fig. 9. The electroploretic pattern obtained after digestion of PCR amplified buffalo *GHR* with *Alu*I restriction enzyme.

Lane 1. 100-bp ladder marker.
Lanes 2-10. Homozygous GG genotypes showed two restricted fragments at 241- and 101-bp.

Maj et al. (**2004**) examined the effect of *GHR* gene on the traits related to feed intake and meat production in beef cattle. The results showed that genetic variants at the 5'-noncoding region of the bovine *GHR* gene had a marked effect on beef production traits. The (–) allele at the polymorphic *Alu*I site appeared favorable for weight of carcass and percentage of valuable cuts.

Di Stasio et al. (**2005**) analyzed the polymorphism at position 257 in exon 10 of the *GHR* and investigated its relationship with four meat characteristics in cattle. The statistical analysis did not show significant nucleotide substitution effect on growth, size and meat conformation traits. As for meat characteristics, a significant nucleotide substitution of GHR^{A} over GHR^{G} was observed for drip losses at day 3, with the allele GHR^{A} associated with higher value.

Oleński et al. (2010) evaluated the significance of associations between missense mutation S555G in bovine *GHR* gene and two sets of data. milk performance data of cows and breeding values of bulls. To generate genotypes, the polymorphic region of *GHR* exon 10 (S555G) was amplified and genotyped using PCR-RFLP method. With the use of the Linear Mixed Model analysis, it was shown that A allele has positive effect on milk performance traits in cows and breeding value of bulls. The A allele is significantly related to fat yield, protein yield and fat content.

Zulkharnaim et al. (2010) estimated genetic diversity of the *GHR*|*Alu*I gene in different cattle breeds. Single nucleotide polymorphisms (SNP) had been found in exon 10, coding for the cytoplasmic domain of *GHR*, which was located at position 81 bp (A/G) induced amino acid substitutions Ser/Gly.

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GCTAACTTCATCGTGGACAACGCTTACTTCTGCGAGGTAGACGCCAAAAAGTACATTGC
CCTGGCCCCTCACGTCGAGGCTGAATCACACGTAGAGCCA**AG^CT**TTAACCAGGAAGAC
ATTTACATCACCACAGAAAGCCTTACCACTACAGCCGGGAGGTCGGGGACAGCAGAACA
TGTTCCAAGTTCTGAGATACCTGTCCCAGATTATACCTCCATTCACATAGTACAGTCTCC
TCAGGGCCTCGTACTCAATGCGACTGCCCTGCCCTTGCCTGACAAAGAGTTTCTCTCATC
ATGTGGCTATGTGAGCACAGACCAACTGAACAAAATCATGCCATAG

Fig. 10. Endonuclease restriction of Egyptian buffalo *GHR* using FastPCR

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