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Genetic Polymorphism of Three Genes (*PRL*, *K-CN* and *PIT-1*) Associated with Milk Traits in Egyptian Buffalo

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ABSTRACT

In dairy farm animals, the principal goal of selection is the improvement of milk yield and composition. The genes encoding milk protein and hormones are excellent candidate genes for linkage analysis with quantitative trait loci (QTLs) because of their biological significance on the quantitative traits of interest. Prolactin (*PRL*), a polypeptide hormone with multiple functions, secreted mainly by the anterior pituitary gland, shows many biological activities and plays essential roles in reproduction, lactation and immune functions. Casein proteins and their genetic variants have been reported as important factors associated with lactation performance, milk composition and the efficiency of cheese yield. Genetic variants of the bovine kappa-casein (*K-CN*) gene are associated with milk protein content and have a significant influence on rennet clotting time, firmness and cheese yield of milk. The pituitary-specific transcription factor (*PIT-1*) gene is responsible for pituitary development and hormone-secreting gene expression in mammals. *PIT-1* was studied as a candidate genetic marker for growth, carcass and also for milk yields traits. Genomic DNA extracted from 100 healthy buffaloes was amplified using primers that were designed for the cattle *PRL*, *K-CN* and *PIT-1* gene sequences. The amplified fragments of *PRL* (294-bp), *K-CN* (530-bp) and *PIT-1* (451-bp) were digested with *Rsa*I, *Hind*III and *Hin*fI restriction enzymes, respectively. All tested buffaloes were genotyped as GG for *PRL*, BB for *K-CN* and for *PIT-1*.

Keywords: *K-CN*, *PRL*, *PIT-1*, PCR, RFLP, *Bubalus bubalis*
Abbreviations: QTL, quantitative trait locus; ***K-CN***: kappa-casein; **PCR**: Polymerase chain reaction; ***PIT-1***, pituitary-specific transcription factor-1 gene; ***PRL***, Prolactin gene; **RFLP**, restricted fragment length polymorphism; **SNP**, single nucleotide polymorphism

INTRODUCTION

In marker-assisted selection of dairy livestock, some genes are proposed as potential candidates associated with dairy performance traits. Among different candidates, the prolactin gene (*PRL*) seems to be promising, because it plays a crucial role in mammary gland development and in the initiation and maintenance of lactation and expression of milk protein genes. Allelic variation in the structural or regulatory sequences of *PRL* would be of interest because of the possible direct and indirect effect on milk production. Single nucleotide polymorphisms (SNPs) occurring within *PRL* may influence the chemical composition of milk or at least be an effective DNA marker of a sub-region of dairy cattle genome (Brym *et al*. 2005).

Casein proteins and their genetic variants have been extensively studied, and reported as important factors associated with lactation performance, milk composition and cheese yield efficiency (Biase *et al.* 2005; Comin *et al.* 2008). The casein genes are tightly linked and inherited as a cluster so they have a potential value and can play an important role in marker-assisted selection for milk traits (Hamza *et al.* 2010). The kappa-casein (*K-CN*) gene has been broadly studied due to its influence on the manufacturing properties of milk. Nine variants have been described in bovine, the most frequent being the A and B alleles (Azevedo *et al.* 2008). The B allele was found to be associated with thermal resistance, shorter coagulation time, better curdles and micelles of different sizes, which are preferable in cheese making (Rachagani and Gupta 2008). The pituitary-specific transcription factor (*PIT-1*) gene was studied as a candidate genetic marker for growth, carcass and also for milk yields traits. *PIT-1* is responsible for pituitary development and hormone expression in mammals (Oprzadek *et al*. 2003). It was shown to control transcription of growth hormone (*GH*), *PRL* (Dybus *et al* 2004), thyroid-stimulation hormone β-subunit (*TSH-β*) (Viorica *et al*. 2007), growth hormone-releasing hormone receptor (*GHRHR*) genes (Edriss *et al*. 2008) and the *PIT-1* gene itself (Zhao *et al*. 2004). *PIT-1* polymorphism was found to be associated with milk yield and conformation traits in cattle (Hori-Oshima and Barreras-Serrano 2003).

In the present study, PCR-RFLP was used to detect genetic polymorphism within three genes (*PRL*, *K-CN* and *PIT-1*) associated with milk traits in Egyptian buffalo.

MATERIALS AND METHODS

Animals

A total of 100 blood samples of healthy unrelated female of Egyptian buffalo were collected from different farms in the Northern part of Egypt, Menoufia (lies in the South of the middle Delta region) and Kafr el Sheikh (lies along the Western branch of the Nile in the Nile Delta).

Genomic DNA extraction

Genomic DNA was extracted from the whole blood by phenol-chloroform method described by John *et al.* (1991) with minor modifications. Ten ml of blood taken on EDTA was mixed with 25 ml cold sucrose-triton (Merck, Germany) and the volume was completed to 50 ml by autoclaved double distilled water. The solution was mixed well and the nuclear pellet was obtained by spinning and discarding the supernatant. The nuclear pellet was suspended in lysis buffer (10 mM Tris base (Sigma-Aldrich,

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Table 1 DNA primers and restriction enzymes used in the present study.

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Restriction enzyme used	References
PRL	CCA AAT CCA CTG AAT TAT GCT T ACA GAA ATC ACC TCT CTC ATT CA	58	*Rsa*I	Brym *et al*. 2005
K-CN	ATA GCC AAA TAT ATC CCA ATT CAG T TTT ATT AAT AAG TCC ATG AAT CTT G	57	*Hin*dIII	Denicourt *et al*. 1990
PIT-1	AAA CCA TCA TCT CCC TTC TT AAT GTA CAA TGT GCC TTC TGA G	56	*Hin*fI	Renaville *et al*. 1997

Germany), 400 mM NaCl (Ran Baxy, Newdelhi, India) and 2 mM sodium EDTA (Sigma-Aldrich) pH 8.2, with 20% sodium dodecyl sulfate (SDS) (Merck) and proteinase K (10 mg/ml, Bioron, Germany), and incubated overnight in a shaking water-bath at 37°C.

Nucleic acids were extracted once with phenol (Merck), saturated with Tris-EDTA (TE) buffer (10 mM Tris, 10 mM NaCl and 1 mM EDTA), followed by extraction with phenol-chloroform-isoamyl alcohol (25: 24: 1, Loba Chemie, India) until there was no protein at the interface. This was followed by extraction with chloroform-isoamyl alcohol (24: 1).

To each extraction, equal volume of the solvent was added, followed by thorough mixing and centrifugation for 10 min. at 2000 rpm. The top layer was carefully transferred to another Falcon tube for the next extraction. To the final aqueous phase, 0.1 vol of 2.5 M Na acetate (Sigma-Aldrich) and 2.5 vol of cold 95% ethanol (Loba Chemie) were added. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The DNA was picked up with a heat-sealed Pasteur pipette and washed briefly in 70% ethanol. The DNA was finally dissolved in an appropriate volume of 1X TE buffer. DNA concentrations were determined and diluted to the working concentration of 50 ng/μl, which is suitable for PCR.

Polymerase chain reaction (PCR)

The primers used in this study (Sigma-Aldrich) were designed according to cattle gene sequences because of the high degree of nucleotide sequence conservation between cattle and river buffalo (**Table 1**). The PCR cocktail consisted of 1.0 μM forward and reverse primers and 0.2 mM dNTPs (Biotechnology, Cairo, Egypt), 10 mM Tris (pH 9), 50 mM KCl (Ran Baxy, New Delhi, India), 1.5 mM $MgCl_2$ (Sigma-Aldrich), 0.01% gelatin (Merck), 0.1% Triton X-100 (Merck) and 1.25 U of *Taq* polymerase (Bioron). The cocktail was aliquot into tubes with 100 ng DNA of buffalo. The reaction was run in a Programmable Thermal Controller (PTC-100), MJ Research Inc. The reaction was cycled for 1 min at 94°C, 2 min at optimized annealing temperature for each primer (**Table 1**) and 2 min at 72°C for 30 cycles. The PCR reaction products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (10 mg/ml) to test the amplification success.

RFLP and agarose gel electrophoresis

RFLP analysis for each gene was done using all 100-tested animals and each sample was ran duplicated. 20 μl of PCR products were digested with 10 U of the restriction enzyme (Fermentas, Germany) specific for each gene (**Table 1**) in a final reaction volume of 25 μl. The reaction mixture was incubated at 37°C in a water bath for 5 h. After restriction digestion, the restricted fragments were analyzed by electrophoresis on a 2.5% agarose/1X TBE gel stained with ethidium bromide. A 100-bp ladder (Biotechnology) was used as molecular size marker. The bands were visualized under UV light and the gels were photographed using an Mp4 plus Polaroid Camera.

RESULTS AND DISCUSSION

There is a considerable interest in the application of molecular genetic technologies in the form of specific DNA markers that are associated with various productivity traits to promote more efficient and relatively easy selection and breeding of farm animals with an advantage for inheritable traits of meat and milk productivity. Many candidate genes have been identified and selected for analysis based on a known relationship with productivity traits (Spelman and Bovenhuis 1998).

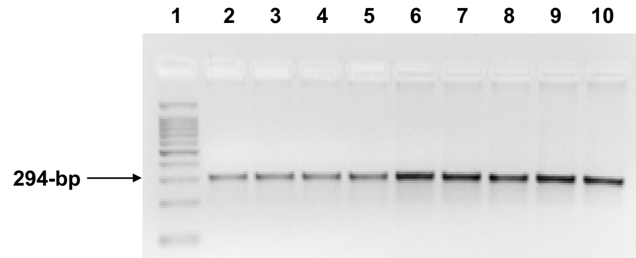

Fig. 1 Ethidium bromide-stained gel of amplified PCR products representing amplification of *PRL* gene in Egyptian buffalo. Lane 1: 100-bp ladder marker. Lanes 2-10: 294-bp PCR products amplified from Egyptian buffalo DNA.

Prolactin

The prolactin gene is 10-kb long and is composed of 5 exons and 4 introns (Cooke *et al*. 1981), and this gene was mapped to chromosome 23 in bovine (Hallerman *et al*. 1988). Prolactin is one of the most multifunctional hormones in the body and its activity consists of various roles in reproduction, lactation and a number of homeostatic biological functions, including immune functions (Brand *et al*. 2004).

Prolactin is a polypeptide hormone with multiple functions, secreted mainly by the anterior pituitary gland (Bole-Feysot *et al*. 1998). Gene disruption experiments have proved their mandatory role for mammary gland development, lactogenesis and expression of milk protein genes (Horseman *et al*. 1997). Therefore, the bovine *PRL* seems to be an excellent candidate for linkage analysis of quantitative trait loci (QTLs) affecting milk production trait.

In the present study, PCR-RFLP was used to detect the genetic polymorphism of *PRL* in Egyptian buffalo. Using the specific primers designed from the cattle *PRL* gene sequence, the PCR of all tested buffalo DNA (100 animals) gave specific amplified fragments at the expected size, 294-bp, involving the whole exon 4 and parts of introns 3 and 4 (**Fig. 1**).

The transition of G into A at position 8398 of the *PRL* gene creates a restriction site for *Rsa*I endonuclease. Digestion of the 294-bp PCR amplified fragments with this restriction enzyme results in two restriction fragments at 162- and 132-bp for AA genotype, one undigested fragment at 294-bp for GG genotype and three fragments at 294-, 162- and 132-bp for AG heterozygous genotype (Brym *et al*. 2005).

The present study declared that all tested animals are genotyped as GG homozygous genotype where all tested buffalo DNA amplified fragments were digested with *Rsa*I endonuclease and gave one undigested fragment at 294-bp (**Fig. 2**).

In mammals, especially dairy cattle, prolactin has important functions like the development of mammary gland affecting milk yield and composition (Kumari *et al*. 2008). Wojdak-Maksymiec *et al*. (2008) found a statistically significant association between somatic cell count (SCC) and *PRL* genotype (p=0.01). The highest SCC was recorded in the milk of BB cows while the lowest one in AA cows.

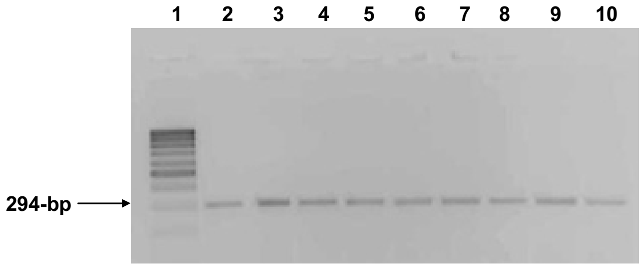

Fig. 2 The electrophoretic pattern obtained after digestion of PCR amplified buffalo *PRL* products with *Rsa*I. Lane 1: 100-bp ladder marker. Lanes 2-10: Homozygous GG genotypes showed one undigested fragment at 294-bp.

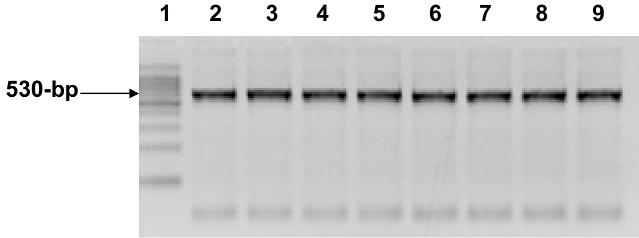

Fig. 3 Ethidium bromide-stained gel of amplified PCR products representing amplification of *K-CN* gene in Egyptian buffalo. Lane 1: 100-bp ladder marker. Lanes 2-9: 530-bp PCR products amplified from Egyptian buffalo DNA.

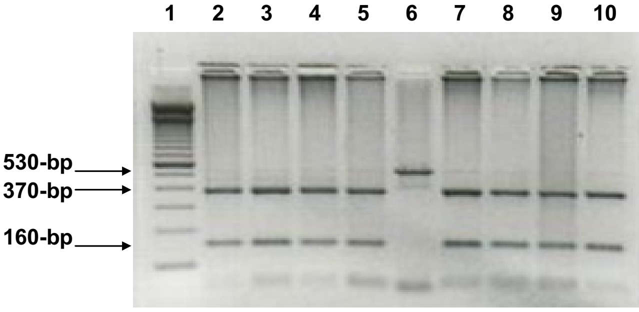

Fig. 4 The electrophoretic pattern obtained after digestion of PCR amplified buffalo *K-CN* products with *Hind*III. Lane 1: 100-bp ladder marker. Lane 6: Undigested fragment at 530-bp. Lanes 2-5, 7-10: Homozygous BB genotypes showed two restricted fragments at 370- and 160-bp.

Cows with the BB genotype, which is least desirable due to the high SCC, were also characterized by the lowest daily milk yield and lactose content and the highest fat, protein and dry matter content compared to other cows.

In contrast to the results of Alipanah *et al.* (2007), which showed that the highest milk, milk fat yield and milk protein yield were obtained by cows with the genotype *PRL-Rsa*I BB, different results for milk and milk fat were reported by Chung and Kim (1997), Dybus (2001) and Khatami *et al.* (2005), who found that cows with the *PRL* genotypes AA and AB yielded more milk fat than BB animals.

The associations were analyzed between polymorphisms of the prolactin gene (*PRL-Rsa*I) and milk production traits of Montebeliard cows (Ghasemi *et al.* 2009). PCR-RFLP was used for genotype identification. Frequencies of genotypes were 0.81, 0.15 and 0.04 for A/A, A/B and B/B, respectively. The frequency of the *PRL* A allele is 0.89. The results show that AA cows yielded high milk compared to other groups.

Brym *et al.* (2005), using the same primer and restriction enzyme used in the present study, assessed allele frequencies in Black-and-White cows (0.113 and 0.887 for A and G, respectively) and in Jersey cows (0.706 and 0.294 for A and G, respectively). Black-and-White cows with genotype AG showed the highest milk yield, while cows with genotype GG showed the highest fat content. The high frequencies of allele G in different cattle breeds were reported previously and ranged from 0.61 in Brown Swiss breed (Mitra *et al.* 1995) to 0.95 in Holstein breed (Chrenek *et al.* 1998).

The effect of A and G alleles on milk performance was analyzed also in Iranian Holstein bulls by Mehmannavaz *et al.* (2009). The frequencies of A and G alleles were 0.069 and 0.931, respectively. The allelic substitution effect was significant for milk and protein yield ($p<0.05$). The G allele was unfavorable for milk and protein yield. Genetic trends for all analyzed traits were significant ($p<0.01$) and that was progressive for milk, fat and protein yield, but diminishing for fat and protein percent. The effects of prolactin SNP on genetic trends and the difference between genetic trends produced by A and G alleles were not significant for all studied traits.

The present results showed that all 100 tested buffalo animals are GG genotyped and according to the results of Brym *et al.* (2005), the Egyptian buffalo population which possesses the fixed G allele yields milk with the favorable highest fat content other than milk yield or milk protein content.

Kappa-casein

The *K-CN* gene is located on bovine chromosome 6q31 and the overall length of the *K-CN* gene is close to 13-kb. Out of known kappa-casein genetic variants, the A and B are the most common in the majority of cattle breeds (Erhardt 1989). Genetic variants of bovine *K-CN* gene are associated with protein content of milk and have a significant influence on rennet clotting time, firmness and cheese yield of milk with a superiority of milk from cows with *K-CN* BB compared to *K-CN* AA milk (Rachagani and Gupta 2008).

By using PCR, the buffalo DNA was amplified using oligonucleotide primers that were designed from the cattle *K-CN* gene sequence. The amplified fragments obtained from all tested buffalo DNA were of 530-bp (**Fig. 3**).

The PCR-amplified fragments resulted from buffalo DNA appeared at 530-bp were digested by *Hind*III to detect the genetic polymorphism located within exon IV and intron IV of the buffalo *K-CN* gene. We can easily differentiate between 3 different genotypes: AA with undigested one fragment at 530-bp, BB with two digested fragments at 370- and 160-bp and AB with three fragments at 530-, 370- and 160-bp. All buffalo animals investigated in the present study were genotyped as BB when all tested buffalo DNA amplified fragments were digested with *Hind*III, giving two digested fragments at 370- and 160-bp (**Fig. 4**).

Otaviano *et al.* (2005) examined the existence of polymorphism in the *K-CN* gene in 115 Brazilian female buffaloes. PCR-RFLP and SSCP demonstrated that the studied animals were monomorphic for this gene. Only allele B was observed in these animals, which was homozygous. The same BB genotyping pattern was also reported in all Indian buffalo breeds (Mitra *et al.* 1998; Pipalia *et al.* 2001). Riaz *et al.* (2008) studied polymorphism at the *K-CN* locus in Nili-Ravi buffalo in Pakistan using three restriction enzymes (*Hin*fI, *Hae*III and *Mae*II). Analysis of 163 animals revealed that all animals were monomorphic, showing only the BB genotype. The monomorphism for the BB *K-CN* gene observed in different buffalo populations in Brazil, India and Pakistan confirmed the results obtained in the present study for Egyptian buffalo populations.

Rachagani and Gupta (2008) analyzed the allelic variants of the *K-CN* gene in Sahiwal and Tharparkar cattle breeds. Genotype BB of the *K-CN* gene had more influence on the milk yield, solids-not-fat yield and protein yield in Sahiwal cattle. According to Marziali and Ng-Kwai-Hang (1986), cheese production can be increased by 10% if milk is from cows of the BB genotype of *K-CN* when compared with milk from AA animals. Therefore, to increase the frequency of *K-CN* B within breeding program, preferring sires with the favorable *K-CN* genotypes is proposed.

The relation between *K-CN* polymorphism and milk performance traits in Holstein-Friesian heifer cows was reported in Poland by Beata *et al.* (2008). In contrast to the previously mentioned results, the authors reported that the AA genotype of the *K-CN* gene was characterized by the highest milk, fat and protein yield, while the lowest fat and protein contents were observed in milk of cows with the BB genotype. This association between AA genotype with higher milk production agreed with the results of Curi *et al.* (2005) in Brazilians cows.

Pituitary-specific transcription factor

Bovine *PIT-1*, a 291 amino acid protein with DNA-binding POU domain (de Mattos *et al.* 2004), is a pituitary-specific transcription factor that is responsible for pituitary development and hormone-secreting gene expression in mammals (Cohen *et al.* 1997). *PIT-1* gene was sequenced by Bodner *et al.* (1988) and it was mapped to the centromeric region of bovine chromosome 1 and located between TGLA57 and RM95 (Woollrad *et al.* 2000).

In the bovine *PIT-1* gene, the RFLP – using *Hin*fI – was identified by Moody *et al.* (1995). The molecular basis of this polymorphism was the silent mutation (G→A) located within exon 6 of the *PIT-1* gene (Dierkes *et al.* 1998). There are many reports on allelic and genotypic frequencies of the *PIT-1* gene in some bovine breeds and the relationships between these frequencies and production traits (Renaville *et al.* 1997; Zwierzchowski *et al.* 2002; Dybus *et al.* 2004; Zhao *et al.* 2004; Beauchemin *et al.* 2006).

In the present study, PCR-RFLP was used to detect genetic polymorphism of the *PIT-1* gene in Egyptian river buffalo. The *PIT-1*/*Hin*fI genotypes were analyzed using primers designed from cattle *PIT-1* gene sequence; the primers were designed from intron 5 and exon 6. All tested Egyptian buffaloes DNA used in the present study were amplified using these primers and gave PCR products at the expected size, 451-bp (**Fig. 5**).

The amplified DNA fragments were digested with *Hin*fI and separated electrophoretically to detect the genetic polymorphisms of the Egyptian buffalo *PIT-1* gene. The point mutation (A→G) in exon VI, affecting a *Hin*fI restriction site, was used to differentiate between two alleles, A and B (Woollard *et al.* 1994). The restriction fragments obtained for the *PIT-1* gene polymorphism were: 244- and 207-bp for BB genotype; 451-, 244-, 207-bp for AB genotype and 451-bp (undigested fragment) for AA genotype.

All Egyptian buffaloes investigated in the present study are genotyped as BB homozygous genotype where the amplified fragments of all tested DNA samples were at 451-bp and digested with *Hin*fI giving two digested fragments at 244- and 207-bp (**Fig. 6**).

The highly frequency of B allele compared to the A allele in cattle *PIT-1*/*Hin*fI polymorphism was reported in many studies. The frequency of the B allele was 0.812 in Italian Holstein Friesian bulls (Renaville *et al.* 1997), 0.79 in Canadian Holstein bulls (Sabour *et al.* 1996), 0.74, 0.75 and 0.757 in Polish Black-and-White cattle (Klauzińska *et al.* 2000; Oprzadek *et al.* 2003; Dybus *et al.* 2004, respectively), 0.845 in California Holstein cattle (Hori-Oshima and Barreras-Serrano 2003), 0.744 in Iranian Holstein cows (Edriss *et al.* 2008) and 0.78 in Romanian Simmental cattle (Viorica *et al.* 2007).

The *PIT-1* locus has potential as a marker for genetic variation in milk production traits. Polymorphism within the bovine *PIT-1* gene detected with *Hin*fI was described by Woollard *et al.* (1994) and Renaville *et al.* (1997). They found that allele A seemed to be linked to higher milk yield, more protein yield and less fat percentage (Zwierzchowski *et al.* 2002).

Hori-Oshima and Barreras-Serrano (2003) studied *PIT-1* gene polymorphism in Baja California Holstein cattle. The authors revealed that the AA genotype for *PIT-1* had significant effect ($p<0.05$) on milk yield as was reported by Renaville *et al.* (1997). Viorica *et al.* (2007) revealed that the digestion of PCR products of the *PIT-1* gene with *Hin*fI in Romanian Simmental cattle resulted in two alleles, A and B; the A allele was superior for milk and protein yield and inferior for fat percentage (Zwierzckowski *et al.* 2002; Vlaic *et al.* 2003; Zhao *et al.* 2004). This result can be interpreted as a single positive action of the A allele on protein yield and, to a lesser extent, on milk yield and fat content.

It is concluded that the milk of Egyptian buffalo is characterized by super protein content- where it possesses the BB genotype for the *K-CN* gene and is also characterized by highest fat content where it possesses the GG genotype for the *PRL* gene and the BB genotype for the *PIT-1* gene.

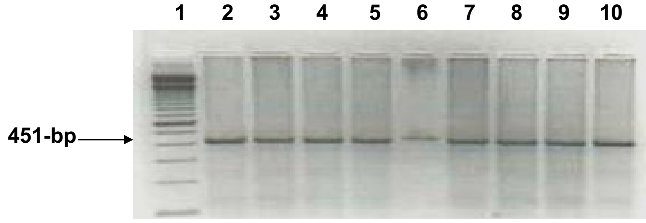

Fig. 5 Ethidium bromide-stained gel of amplified PCR products representing amplification of *PIT-1* gene in Egyptian buffalo. Lane 1: 100-bp ladder marker. Lanes 2-10: 451-bp PCR products amplified from Egyptian buffalo DNA.

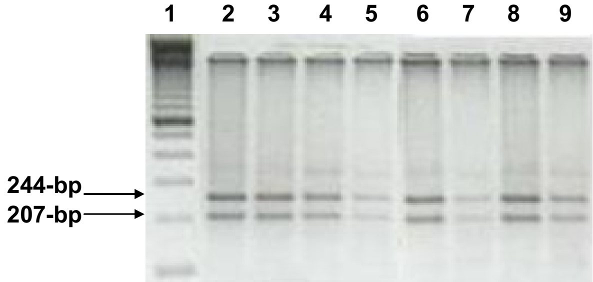

Fig. 6 The electrophoretic pattern obtained after digestion of PCR amplified buffalo *PIT-1* products with *Hin*fI. Lane 1: 100-bp ladder marker. Lanes 2-9: Homozygous BB genotypes showed two restricted fragments at 244- and 207-bp.

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