

Effects of genetic polymorphism in Pit1, GH, GHR and KCN3 on milk yield and body weight of Khuzestan (Iran) water buffaloes

Efectos del polimorfismo genético en Pit1, GH, GHR y KCN3 sobre la producción de leche y el peso corporal del búfalo de agua de Juzestán, Irán

Efeitos do polimorfismo genético em Pit1, GH, GHR e KCN3 sobre produção de leite e peso corporal do búfalo de água do Cuzistão, Irã

Meysam Ahmadzadeh^{1, 2#}, MSc; Farzad Rashidi^{1, 2#}, MSc; Hamed Amirpour Najafabadi^{3#}, MSc.; Amir Jaferian⁴, PhD; Shahin Eghbalsaied^{1, 2, 5*}, PhD.

¹Transgenesis Center of Excellence, Isfahan (Khorasgan) branch, Islamic Azad University, Isfahan, Iran.

²Department of Animal Science, Faculty of Agriculture, Isfahan (Khorasgan) branch, Islamic Azad University, Isfahan, Iran.

³Faculty of Agriculture and Life Sciences, Department of Agricultural Sciences, Lincoln University, Christchurch, New Zealand.

⁴Department of Animal Science, Ramin University of Agriculture and Natural Resources, Mollasani, Ahvaz, Khuzestan, Iran.

⁵Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Mariensee, Neustadt am Rübenberge, Germany.

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Abstract

Background: Genetic information is necessary to devise strategic plans aimed to improve the genetic merit of buffaloes. **Objective:** To assess the effect of genetic polymorphisms in GH, Pit-1, GHR, GHRHR, and KCN3 genes on milk production and body weight of Khuzestan water buffaloes. **Methods:** Blood samples were collected from 60 buffaloes from the Khuzestan province, Iran. Using the PCR-RFLP technique, the amplified and digested fragments of GH/AluI, GHR/AluI, GHRHR/ HaeIII, Pit1/HinfI, and KCN3/HindIII were genotyped. **Results:** All animals were monomorphic for GHRHR. The frequency of mutant alleles for GH, GHR, KCN3, and Pit1 was 47.5, 74.2, 49.2, and 51.7%, respectively. There were significant differences ($p < 0.0001$) in the genotypic frequencies of GH, GHR, and Pit1 between high and low milk-yielding buffaloes.

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* Corresponding author: Shahin Eghbalsaied. Transgenesis Center of Excellence, Isfahan (Khorasgan) branch, Islamic Azad University, P.O. box 81595-158, Isfahan, Iran. E-mail address: shahin.eghbal@khuisf.ac.ir

These authors contributed equally to this work.

The GH ($p=0.0002$), GHR ($p<0.0001$) and Pit1 ($p<0.0001$) polymorphisms also had significant effects on body weight. Sequencing results revealed the presence of C496A, G495A, G498A and C1501T SNPs in the GH, and G1702T in the GHR gene of Khuzestan buffaloes. **Conclusion:** This study highlights the importance of GH, GHR, and Pit1 on milk production and body weight of Khuzestan buffaloes. The results suggest that devising an integrated breeding plan in Khuzestan water buffaloes can considerably benefit from the very high diversity in candidate genes.

Keywords: animal breeding, casein, genetic variation, growth hormone, milk production, SNP.

Resumen

Antecedentes: La información genética es necesaria para diseñar planes estratégicos con el objeto de mejorar el mérito genético de los búfalos. **Objetivo:** Evaluar el efecto de los polimorfismos genéticos en los genes GH, Pit-1, GHR, GHRHR y KCN3 sobre la producción láctea y peso corporal de búfalos de agua de la provincia de Juzestán, Iran. **Métodos:** Se recolectaron 60 muestras de sangre de búfalos de la provincia de Juzestán, en Irán. Los fragmentos amplificados y digeridos de GH/AluI, GHR/AluI, GHRHR/HaeIII, Pit1/HinI y KCN3/HindIII fueron clasificados genotípicamente, utilizando la técnica PCR-RFLP. **Resultados:** Todos los animales fueron monomórficos para el gen GHRHR. La frecuencia alélica de alelos mutantes para los genes GH, GHR, KCN3 y Pit1 fue 47,5, 74,2, 49,2 y 51,7%, respectivamente. Se encontraron diferencias significativas ($p<0,0001$) en las frecuencias genotípicas de GH, GHR y Pit1 entre búfalos de alta y baja producción. El efecto del polimorfismo GH ($p=0,0002$), GHR ($p<0,0001$) y Pit1 ($p<0,0001$) también fue significativo para peso corporal. Los resultados de la secuenciación revelaron la presencia de SNPs C496A, G495A, G498A y C1501T en GH, y G1702T en el gen GHR. **Conclusiones:** Este estudio resalta la importancia de los genes GH, GHR y Pit1 sobre la producción de leche y el peso corporal de búfalos de Juzestán. Los resultados sugieren que la elaboración de un plan de cruzamiento integrado en búfalos de agua de Juzestán puede beneficiarse considerablemente de la gran diversidad de genes candidatos.

Palabras clave: caseína, hormona del crecimiento, producción de leche, reproducción animal, SNP, variación genética.

Resumo

Antecedentes: Determinação informações genéticas é o passo crítico para elaborar planos estratégicos com o objetivo de melhorar o mérito genético dos búfalos. **Objetivo:** Avaliar o efeito de polimorfismos genéticos nos genes GH, Pit-1, GHR, GHRHR e KCN3 na produção de leite e no peso corporal dos búfalos de água do Cuzistão, Irã. **Métodos:** Amostras de sangue foram coletadas de 60 búfalos da província de Cuzistão, no Irã. Utilizando a técnica PCR-RFLP, os fragmentos amplificados e digeridos de GH/AluI, GHR/AluI, GHRHR/HaeIII, Pit1/HinI e KCN3/HindIII foram genotipados. **Resultados:** Todos os animais eram monomórficos para o gene GHRHR. A frequência alélica de alelos mutantes para os genes GH, GHR, KCN3 e Pit1 foi 47,5, 74,2, 49,2 e 51,7%, respectivamente. Uma diferença significativa ($p<0,0001$) foi encontrada nas frequências genotípicas de os genes GH, GHR e Pit1 entre búfalos de alta e baixa produção. O efeito do polimorfismo GH ($p=0,0002$), GHR ($p<0,0001$) e Pit1 ($p<0,0001$) também foi significativo para o peso corporal. Os resultados da sequenciação revelaram a presença de SNPs C496A, G495A, G498A e C1501T no GH, e G1702T no gene GHR dos búfalos do Cuzistão. **Conclusões:** Este estudo destacou a importância da GH, GHR e Pit1 na produção de leite e no peso corporal de búfalos do Cuzistão. Os resultados sugerem que a elaboração de um plano de melhoramento genético integrado em búfalos de água do Cuzistão pode beneficiar consideravelmente da grande diversidade de genes candidatos.

Palavras-chave: caseína, hormônio do crescimento, produção de leite, reprodução animal, SNP, variação genética.

Introduction

Based on archeological evidence, water buffaloes were domesticated in Iran from 2500 BC (Naserian and Saremi, 2007). Buffaloes adaptability to low quality grasses, high temperature and humidity, irregular rainfall, disease resistance along with having

a long productive life made them the first option as domestic animals for providing milk and meat in Iran (Dezfuli *et al.*, 2011). In comparison to cow milk, Khuzestan buffalo milk has more fat (6.6%) and solid content (Borghese and Mazzi, 2005). This prominent benefit of Khuzestan buffalo can compensate for the low fat percentage of Iranian Holstein dairy cows

(Eghbalsaied *et al.*, 2016), and is the subject of growing attention in the Iranian dairy market.

Several studies have been conducted to identify the major genes responsible for milk production and composition in dairy cows (Komisarek and Dorynek, 2009; Pasandideh *et al.*, 2015). The polymorphism in growth hormone (GH) was associated with selection for milk fat production in Red Danish and Norwegian Red cattle (Høj *et al.*, 1993). It controls energy metabolism in high-yield cows (Hart *et al.*, 1978), affects the size of mammary tissue (Capuco and Akers, 2009), reduces the apoptosis rate of the mammary cells (Accorsi *et al.*, 2002), and consequently increases milk production (Yao *et al.*, 1996). The growth hormone receptor (GHR) can also affect milk and fat yield of dairy cows (Jiang and Lucy, 2001; Viitala *et al.*, 2006). Growth hormone-releasing hormone receptor (GHRHR) is secreted by the hypothalamus and stimulates the release of pituitary growth hormones (Godfrey *et al.*, 1993). Besides these genes, a number of miscellaneous genes are involved in milk yield and milk contents. Among them, pituitary-specific transcription factor-1 (Pit1), also known as POU domain-class 1 transcription factor 1 (POU1F1) and Kappa-Casein (KCN3) are important genes involved in milk quality (Erwin *et al.*, 1983; Fries *et al.*, 1993; Lien and Rogne, 1993).

Although the importance of the above-mentioned genes has been well documented in dairy cows, there are limited publications on their role in buffaloes. Therefore, providing the genetic polymorphism of these candidate genes is essential for devising an integrated breeding plan for water buffaloes. The goal of the present study was to assess the effect of genetic polymorphisms in GH, Pit-1, GHR, GHRHR, and KCN3 genes on milk production and body weight of water buffaloes in Khuzestan province, Iran.

Material and methods

Ethical considerations

The method for blood sampling was approved by the Animal Care and Ethics Committee of Isfahan Islamic Azad University, April 2014 (Nr. 15021393).

Animals and location

Blood samples were collected from 60 buffaloes from the Ahvaz (31°19'13"N 48°40'09"E) and Dezful (32°22'57"N 48°24'07"E) districts in the Khuzestan province, Iran. The buffaloes were 4 to 15 years old. Based on twice daily milking, animals were divided into low-yielding (<10 kg) and high-yielding (>13 kg) dairy buffaloes. In addition, based on their body weight, buffaloes were classified into three categories: light (<450 kg), moderate (450-500 kg), and heavy (>550 kg). Breeding and feeding conditions were similar in the rural riverine production for all animals.

DNA was extracted from blood samples using a SinaClon kit, and polymerase chain reaction (PCR) was carried out for amplification of GH, GHR, GHRHR, Pit1, and KCN3 genes using the specific primer sequences (Table 1) (Moody *et al.*, 1995; Woollard *et al.*, 1994); (Andreas *et al.*, 2010); (Balogh *et al.*, 2009); (Kalashnikova *et al.*, 2009). Amplification of segments of interest (Table 1) was performed in 20 µl reaction volume containing 100 ng of genomic DNA, 5 µM of each primer pairs, 0.2 mM dNTPs, 10X PCR buffer, 2 mM MgCl₂, and 1.5 units of Taq DNA polymerase (SinaClon, Iran). The PCR program for amplification of the desired targets was as follows: an initial denaturation at 94 °C for 4 minutes, 35 cycles comprising 94 °C for 50 seconds, 62, 53, and 53 °C (for GH, GHR, and GHRHR, respectively) for 50 seconds, and 72 °C for 50 seconds, followed by a final extension at 72 °C for 7 minutes. Then, the PCR product of GH and GHR genes underwent digestion with AluI, while GHRHR amplicon was digested with HaeIII restriction enzyme (Maga *et al.*, 2006). In addition, Pit1 and KCN3 amplicons were digested with HinfI and HindIII, respectively. Samples were genotyped using the restriction fragment length polymorphism (RFLP) assay (Table 1). Briefly, 10 µl of the PCR product was incubated with 2 IU of the corresponding restriction enzyme at 37°C for two hours. The digested products were electrophoresed on a 2% agarose gel. In addition, the PCR products of GH and GHR from a random sample of animals were sequenced.

Table 1. Primers used for PCR-RFLP (restriction fragment length polymorphism) on candidate genes for milk and meat production in Khuzestan water buffalo.

Gene	Gene ID	Sequence of Primer	Amplicon length (bp)	Restriction site	Reference
GH	NW_005781989	F:CGGACCGTGTCTATGAGAAGCTGAAG	432	AluI	(Balogh <i>et al.</i> , 2009)
		R:GTTCTTGAGCAGCGCGTCGTC			
GHR	NW_005785241	F:GCTTACTTCTGCGAGGTAGACGC	298	AluI	(Andreas <i>et al.</i> , 2010)
		R:GTCTGTGCTCACATAGCCAC			
GHRHR	NW_005782772	F:GTAAGGATGCCAGCTCTGGGT	451	HaeIII	(Moody <i>et al.</i> , 1995)
		R:TGCCTGCTCATGATGCTCTGGA			
KCN3	NW_005785429	F:ATAGCCAAATATATCCCAATTCAGT R:TTTATTAATAAGTCCATGAATCTTG	531	HindIII	(Kalashnikova <i>et al.</i> , 2009)
Pit1	NW_005785338	F:AAACCATCATCTCCCTTCTT R:AATGTACAATGTGCCTTCTGAG	435	HinfI	(Woollard <i>et al.</i> , 1994)

Statistical analysis

Genotypic and allelic frequencies were estimated using the PopGene software, Version 1.31 (Yeh *et al.*, 1999). Genotypic and allelic frequencies were compared between classes of milk yield and body weight using a Chi-square test. A p-value <0.05 was considered significant.

Results

The effect of polymorphisms using PCR-RFLP on milk yield and body weight

Using the RFLP technique, genetic polymorphism was assessed in five candidate genes in relation to milk yield of Iranian water buffalo. Using the HaeIII restriction enzyme, the previously detected mutation in GHRHR (Maga *et al.*, 2006) was not detected in the Iranian water buffaloes. Implementation of the restriction enzymes for GH, GHR, Pit1, and KCN3 resulted in detection of polymorphisms, corresponding to other studies on water buffalo around the world. Allelic frequencies of GH, GHR, Pit1, and KCN3 genes are presented in Table 2. The allelic frequencies of mutant alleles for GH/AluI, GHR/AluI, KCN3/HindIII, and Pit1/HinfI were 47.5, 74.2, 49.2, and 51.7%, respectively.

Table 2. Allelic and genotypic frequencies of GH/AluI, GHR/AluI, KCN3/HindIII, and Pit1/HinfI in Khuzestan water buffaloes.

Gene/Restriction enzyme	Genotype frequency (%)			Allelic frequency (%)	
	+/+	+/M	M/M	+	M
GH/AluI	5.0	75.0	20.0	42.5	47.5
GHR/AluI	6.7	38.3	55.0	25.8	74.2
KCN3/HindIII	8.3	85.0	6.7	50.8	49.2
Pit1/HinfI	33.3	30.0	36.7	48.3	51.7

There was a significant difference in GH/AluI genotypic frequency between high and low milk-yielding buffaloes ($p < 0.0001$). Frequency of the wild type allele was higher in the low-yielding group, while the mutant allele was more abundant in the high-yielding group (Figure 1).

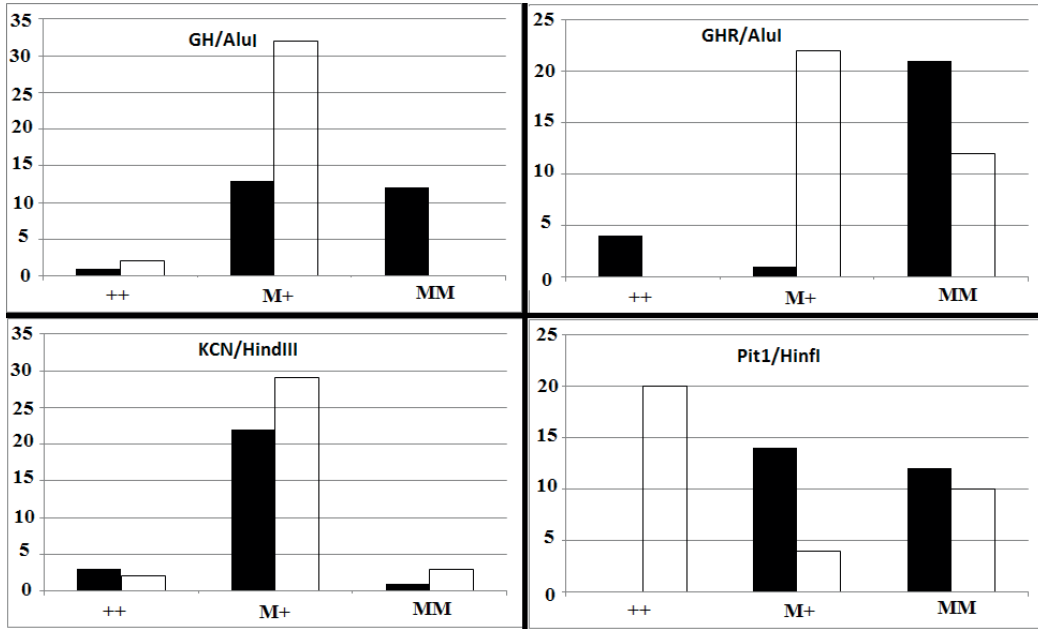


Figure 1. Genotypic distribution of GH/AluI ($p < 0.0001$), GHR/AluI, KCN3/HindIII ($p = 0.5729$), and Pit1/HinfI ($p < 0.0001$) in two classes of high- (black bar) and low-yielding (white bar) Khuzestan water buffalo. M and + indicate mutant and wild type alleles, respectively.

Also, there was a significant difference in genotypic frequency of GH between body weight classes ($p = 0.0002$) (Figure 2).

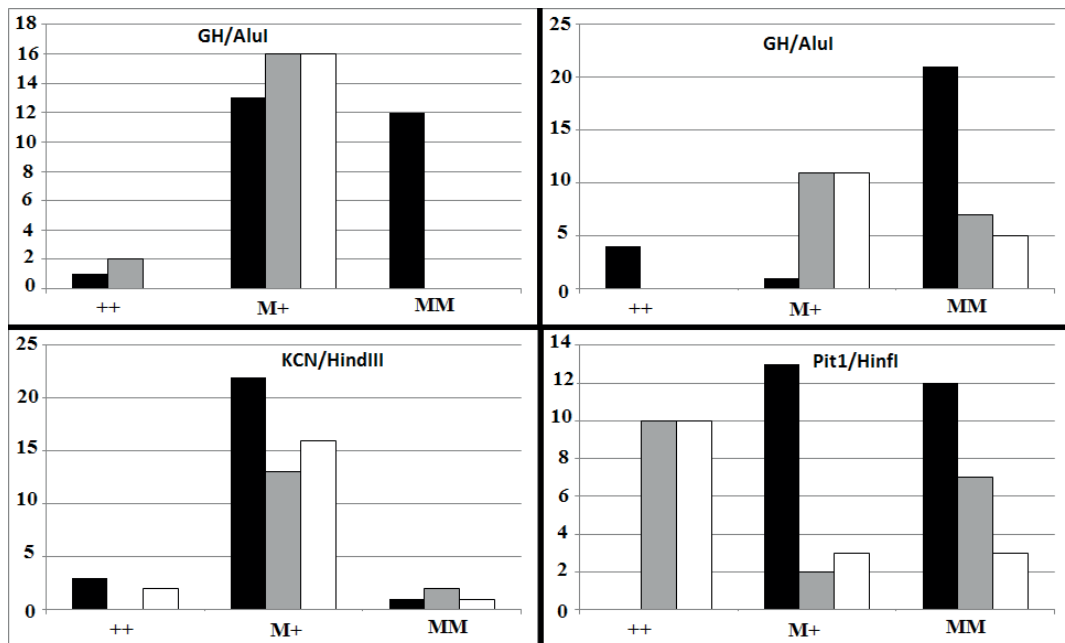


Figure 2. Genotypic distribution of GH/AluI ($p = 0.0002$), GHR/AluI ($p < 0.0001$), KCN3/HindIII ($p = 0.5489$), and Pit1/HinfI ($p < 0.0001$) in three classes of light- (black bar), moderate- (gray bar), and heavy-weight (white bar) Khuzestan water buffalo. M and + indicate mutant and wild-type alleles, respectively.

The effect of the GHR allele detected by the AluI restriction enzyme was also significantly different between high and low milk-yielding groups ($p < 0.0001$). Although the majority of high-yielding buffaloes were homozygote for MM, all of the low-yielding buffaloes carried at least one mutant allele for the GHR/AluI restriction site. There was a significant difference in distribution of genotypic frequencies of GHR/AluI between body weight classes ($p < 0.0001$). The majority (84.0%) of heavy-weight buffaloes were homozygote mutant for GHR/AluI.

No significant difference was observed for KCN3 allelic and genotypic frequencies among either milk production or body weight classes. The effect of detected Pit1 polymorphism on milk production was statistically significant ($p < 0.0001$). Fifty-eight percent of low-yielding buffaloes were PP homozygous, while no animals from the high-yielding group were homozygous PP. In addition, Pit1/HinfI genotypic frequencies

were significantly different among body weight groups ($p < 0.0001$).

Sequencing results of GH and GHR revealed multiple SNPs in Iranian water buffalo

Results of sequencing revealed four mutations in GH and one mutation in GHR genes of Iranian water buffaloes, compared to the NCBI reference sequences of NM_001290929.1 and NM_001290971.1, respectively (Figure 3). The mutations in DNA of GH correspond to G495A, C496A, and G498A conversions in the mRNA sequence. One of these mutations, C496A, was a missense mutation, which causes Q166K (Glutamine to Lysine) (Figure 3). Both the G495A and G498A were silent mutations for Lys (Lysine) and Thr (Threonine). In addition, one mutation was detected in intronic region 4, C1501T of GH. The only detected mutation in the GHR gene corresponded to the G1702T mutation in mRNA sequence, which was a silent mutation for Ile (Isoleucine) at position 561.

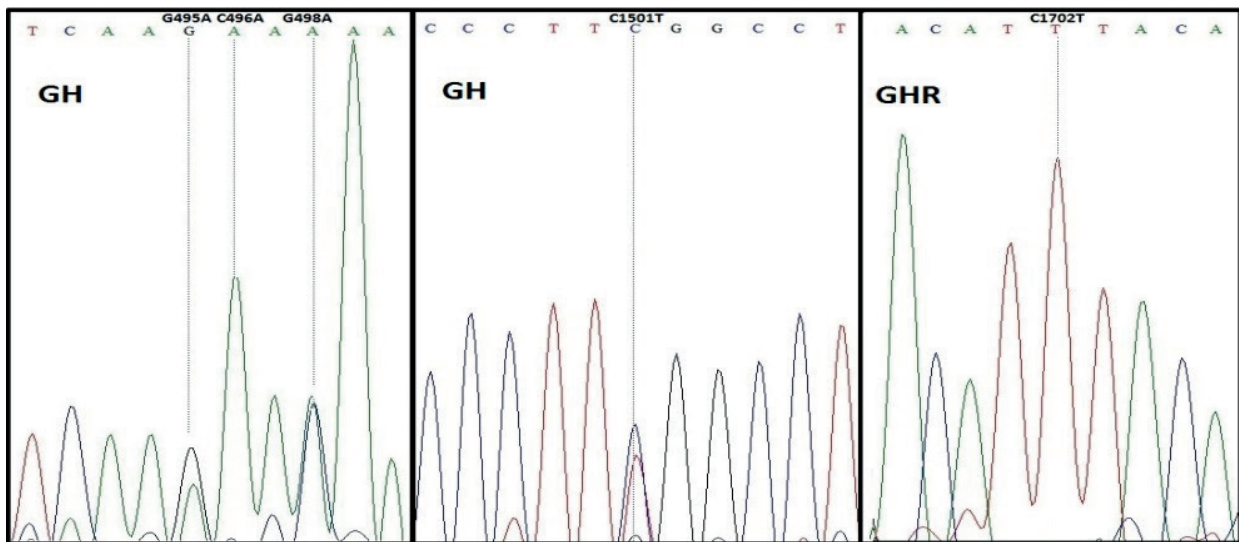


Figure 3. Presence of SNPs in GH and GHR of Iranian Khuzestan water buffalo.

Discussion

Khuzestan buffaloes are distributed in the tropical zone of Iran and Iraq with a population of more than 104,000 and 120,000 water buffaloes, respectively (Borghese, 2010). An average annual population growth of 2.6% has been reported in Iranian Khuzestan

buffaloes (Kamalzadeh *et al.*, 2008). Defining important genes responsible for growth rate and milk yield is critical to uncover the genetic mechanism controlling these attributes in water buffalo. However, documentation is limited on the importance of these genes in water buffalo while several studies have documented the importance of growth factors on the

performance of dairy cows (Bauman *et al.*, 2015; Berry *et al.*, 2016; Bines and Hart, 1982; Hadi *et al.*, 2015; Machlin, 1973). Therefore, defining candidate genes that can play key roles in milk and meat production is an essential step for the genetic improvement of water buffaloes. Subsequently, identifying missense and nonsense mutations in the candidate genes can be helpful in animal genetic/genomic selection. In this study, polymorphisms in GH, GHR and GHRH as well as KCN3 and Pit1 genes in Khuzestan buffalo were investigated. The results indicated that except for GHRH, all the assessed genes using the RFLP technique were polymorphic. The mutant alleles were highly abundant in the studied population, ranging from 47.5 to 74.2% in GH and GHR, respectively. This result confirmed the findings of previous studies indicating the presence of high genetic diversity in Iranian buffalo and water buffalo (Heydarian *et al.*, 2014; Javanmard *et al.*, 2005; Ranjbar *et al.*, 2016; Shokrollahi *et al.*, 2009; Taghizadeh *et al.*, 2014). The results of PCR-RFLP for GH/AluI and GHR/AluI genes showed that the frequency of mutant allele was 3.5% in Iraqi water buffaloes, while all animals were monomorphic for the GHR/AluI allele. No polymorphism was detected using the RFLP method for GH and GHR genes in Egyptian water buffaloes (Hussain, 2015). A study of Bangladesh water buffaloes for GHRHR showed the presence of both mutant and wild type alleles (Andreas *et al.*, 2010). All animals in the present study were monomorphic wild type for GHRHR. Our results showed that both Pit1 and KCN3 are highly polymorphic in Khuzestan buffaloes. Although the Pit1/HinfI mutation was characterized in Indian and Indonesian buffaloes (Jakaria and Noor, 2015; Parikh and Rank, 2013). Assessment of this mutation in Egyptian buffaloes showed a lack of polymorphism (Nasr *et al.*, 2016). Moreover, KCN3/HindIII was wild type monomorphic in Pakistani buffalo (Riaz *et al.*, 2015). Our results indicate that local buffaloes can be substantially variable for the assessed genes. Iranian Khuzestan water buffaloes have some similarity with Iraqi Khuzestan buffaloes and may have originated from the same ancestor (Watanabe *et al.*, 1994). However, the results of the present study showed that, unlike Iraqi water buffaloes, Iranian water buffaloes are polymorphic for GHR/AluI. Recently released data indicated the presence of high genetic diversity of African buffalo in nine African countries (Smitz *et al.*, 2016) the African

buffalo (*Syncerus caffer*). Therefore, the present study highlighted the importance of sequencing candidate genes in buffaloes from each specific local area to devise a proper strategy for genetic selection.

A single mutation in the Pit1 gene causes a substantial deficiency in anterior pituitary Pit-1 hormone production and subsequently an aberrant activation of GH and prolactin genes (Cohen *et al.*, 1996). A polymorphism in Pit1 gene was associated with milk traits in Egyptian and Indian buffaloes (Othman *et al.*, 2011; Parikh and Rank, 2013). In addition, water buffaloes carry polymorphism in the KCN gene (Cinar *et al.*, 2015; Mitra *et al.*, 1998), which can affect their milk yield as well as feta cheese processing parameters (Shakerian *et al.*, 2016).

We then looked at whether the detected polymorphism was associated with milk production and body weight traits. The results indicated that the majority of buffaloes from the high-yielding group carried the mutant allele for GH, GHR, and Pit1. Moreover, the genotypic distribution of GHR and Pit1 was different between body weight classes, so that the mutant alleles were more abundant in buffaloes from the heavy weight class. The effect of GH as a metabolic modifier in livestock has been well documented (Dunshea *et al.*, 2016). An association of Pit1 with milk yield in buffalo was suggested by Parikh and Rank (2013). In line with our results, a recent study on Egyptian water buffalo verified the importance of Pit1 polymorphism on milk yield and body weight (Nasr *et al.*, 2016). Therefore, our results indicate that besides employing growth hormones on dairy farms, there is a high potential for genetically selecting and upgrading GH and Pit1 as growth factors as well as GHR in dairy buffaloes. However, KCN3 polymorphism was not associated with either milk yield or body weight traits. This indicates that the mutation has not been under selection pressure either naturally or artificially for milk and meat production in Iranian water buffalo.

Finally, the PCR amplicons from GH and GHR were sequenced to see if further mutations exist in these genes. Our results showed the presence of several mutations in GH and one mutation in GHR. Among them, the C496A causes Q166K (Glutamine to Lysine) in GH polypeptide. None of these

mutations have been reported in the literature. The detected mutation in GHR has not been reported in the literature and is different from the detected mutation by Shi *et al.* (2012). Therefore, to the best of our knowledge, no literature has been published on the association of detected mutations and milk/meat attributes of buffalo. Nonetheless, an association study with a larger sample size is yet to be explored if the detected SNPs in the present study are associated with production performance of water buffalo.

In conclusion, our results suggest that a high number of SNPs may segregate in GH and GHR genes of Iranian buffalo. It should be noted that the majority of polymorphism studies, including this study, have been based on the PCR-RFLP results from bovine genome. Results of this study provides information for one missense mutation in GH and highlighted that sequencing the candidate genes in buffaloes can be very practical to survey the prevalence and importance of possible SNPs in buffaloes. These data can provide information about genetic diversity as well as association analysis of the above-mentioned genes for further application in selection programs of Khuzestan buffaloes.

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Conflicts of Interest

Authors declare they have no conflicts of interest with regard to the work presented in this report.

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