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INVESTIGATION OF GROWTH HORMONE RELEASING HORMONE, GROWTH HORMONE AND PROLACTIN HORMONE GENE POLYMORPHISM IN ANATOLIAN WATER BUFFALO

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Abstract

The purpose of this work was to identify GHRH, GH and PRL gene polymorphisms in Anatolian water buffalo by means of the PCR-RFLP method. A total of 126 buffalo were included in this study. PCR amplification gave a 451 bp band for the GHRH gene, a 221 bp band for the GH gene and a 156 bp band for the PRL gene. The PCR products were digested by *Hae*III for the GHRH gene, *Alu*I for the GH gene and *Rsa*I for the PRL gene. The GH/*Alu*I and PRL/*Rsa*I polymorphisms were found to be polymorphic, while the GHRH/*Hae*III polymorphism was not found in Anatolian water buffalo. The frequencies of GH-L (0.87) and PRL-A (0.55) alleles were found to be high in the examined Anatolian water buffalo. The chi-square test showed that the Anatolian water buffalo were in Hardy-Weinberg (HW) equilibrium for the GH gene while significant deviation was observed from HW equilibrium for the PRL gene. The present study is the first to examine GHRH/*Hae*III, GH/*Alu*I and PRL/*Rsa*I polymorphisms in Anatolian water buffalo.

Key words: Anatolian water buffalo, growth hormone, growth hormone releasing hormone, prolactin hormone, RFLP

Domestic water buffalo are classified into two different classes as swamp buffalo (*Bubalus carabanesis*) which have 48 chromosomes, and river buffalo (*Bubalus bubalis*) which have 50 chromosomes (Mishra et al., 2015). While swamp buffalo are mostly reared for meat yield, freight transport and field cultivation, river buffalo are also reared for milk and meat yield (Atasever and Erdem, 2008). Water buffalo can adapt to poor environmental conditions such as low quality pasture and housing and also they are resistant to parasites and other infectious agents (Soysal, 2013). Anatolian water buffalo originate from the Mediterranean subgroup of river buffalo which generally have a dark colored coat, and it is the only water buffalo breed reared in Turkey (Soysal, 2013).

Water buffalo milk has a 7.85% fat content; this fat content is considerably higher than that of other livestock species (Atasever and Erdem, 2008). Compared to cattle of the same age, buffalo meat has 1% less muscle fat, 92% less saturated fatty acids, 25% less calories and 67% less cholesterol, while it has 11% more protein and 10% more minerals (Wanapat and Chanthakhoun, 2015). However, intensive farming of water buffalo has some disadvantages in terms of low milk yield and body weight gain, and long gestation period (average 320 days) which prevent its farming and breeding approaches (Soysal, 2013). For these reasons, over the last two decades, significant reductions have occurred in the buffalo population in Turkey and its number has markedly decreased from 366,150 in 1991 to 133,766 in 2015 (TSI, 2016). An improvement in water buffalo production traits is required in order to prevent further numerical decrease in Turkey.

Growth hormone releasing hormone (GHRH) is a hypothalamic hormone that regulates the secretion of growth hormone by the pituitary gland and plays an important role in the regulation of the metabolism and growth physiology in mammals (Kmieć et al., 2007). Therefore, it is assumed that the GHRH encoding gene might be used in livestock for increasing growth traits and milk yield traits (Dybus et al., 2003; Szatkowska et al., 2009). The GHRH/*Hae*III polymorphism was first reported by Moody et al. (1995) in cattle and is located on intron 1 of the GHRH gene (Kmieć et al., 2007). However there is not enough information about the molecular background of this polymorphism (Curi et al., 2005). In various cattle breeds, GHRH/*Hae*III polymorphism was found to be associated with milk production (Kmieć et al., 2007) and growth traits (Dybus et al., 2003). However, there are only a few studies on GHRH/*Hae*III polymorphism in various water buffalo breeds.

Growth hormone (GH) has a role in many physiological processes such as the regulation of growth, mammary gland development, initiation of lactation and muscle development (Kovács et al., 2006). GH is a peptide hormone secreted by the pituitary gland which is encoded by the GH gene (Ardiyanti et al., 2009). There are several polymorphisms in the bovine GH gene and the best known of these is GH/*Alu*I polymorphism, in which there is a substitution of a cytosine (C) to a guanine (G) at position 2141 on exon 5 of the GH gene in cattle (Balogh et al., 2009). Previous studies performed in cattle showed that GH/*Alu*I polymorphism could be used as a marker for selecting animals in terms of higher milk yield and milk quality (Khatami et al., 2005), live weight gain and meat amount in the carcass (Oprządek et al., 2005). However, as with GHRH/*Hae*III polymorphism, the number of studies related to GH/*Alu*I polymorphism in different water buffalo breeds is limited compared to those in cattle.

Prolactin (PRL) hormone is another peptide hormone secreted by the pituitary gland (Sodhi et al., 2011). It has been shown that the prolactin (PRL) hormone is responsible for more than 300 processes including the initiation and maintenance of lactation (Khatami et al., 2005) and other physiological processes such as the regulation of reproductive functions, cellular growth and differentiation (Sodhi et al., 2011). A few polymorphisms have been reported in the PRL gene in cattle (Hart et al., 1993; Mitra et al., 1995). Among these, PRL/*Rsa*I polymorphism, a substitution of an adenine (A) to a guanine (G) at position 103 on exon 3 of the PRL gene

(Sodhi et al., 2011), has been reported to be associated with milk yield traits in cattle (Alipanah et al., 2007).

The aim of this study was to determine the genetic variability of the GHRH, GH and PRL genes in a population of Anatolian water buffalo by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Material and methods

Animal materials and extraction of DNA samples

In the study, a total of 126 Anatolian water buffalo (male/female) from different provinces (Kayseri, Afyonkarahisar, Amasya and Çorum) of Turkey were genotyped for the GHRH, GH and PRL genes. Genomic DNA was isolated from whole blood with the phenol-chloroform-isoamyl alcohol (25:24:1) method.

Genotyping with PCR-RFLP

The PCRs for the GHRH, GH and PRL genes were fulfilled in 25 µL reaction mixtures, containing 1.5 mM $MgCl_2$, 200 µM dNTP mix, 5 pmol of each primer, 1 × PCR buffer, 1 U Taq DNA polymerase and 100 ng of genomic DNA.

A 451 bp fragment of the GHRH gene was amplified using a primer set (GenBank Access No. AF242855): forward 5'- GTA AGG ATG GCT CTG CCA GGT -3' and reverse 5'- TGC ATG ATG CTG TCC CTC TGG A -3' (Moody et al., 1995). The following cycles were applied: predenaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, at 60°C for 1 min, at 72°C for 1 min and the final extension at 72°C for 5 min. PCR products were digested using 5 U *Hae*III enzyme (MBI Fermentase) at 37°C for 4 h.

A 211 bp fragment of the GH gene was amplified using a primer set (GenBank Access No. EF592534.1): forward 5'- GCT GCT CCT GAG GGC CCT TC -3' and reverse 5'- CAT GAC CCT CAG GTA CGT CTC CG -3' (Othman et al., 2012). Thermal cycling condition was composed of a predenaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 1 min and the final extension at 72°C for 5 min. PCR products were digested using 5 U *Alu*I enzyme (MBI Fermentase) at 37°C for 3.5 h.

A 156 bp fragment of the PRL gene was amplified using a primer set (GenBank Access No. DQ287249.1) forward 5'- CGA GTC CTT ATG AGC TTG ATT CTT -3' and reverse 5'- GCC TTC CAG AAG TCG TTT GTT TTC -3' (Mitra et al., 1995). Thermal cycling conditions were composed of a predenaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 45 s, 60°C for 45 s, 72°C for 1 min and the final extension at 72°C for 5 min. PCR products were digested using 5 U *Rsa*I enzyme (MBI Fermentase) at 37°C for 3 h.

Statistical analysis

The allele and genotype frequencies of GHRH, GH and PRL genes were calculated by direct counting. The Chi-square (χ^2) test was used to analyze whether the

population was in Hardy-Weinberg equilibrium (HWE). For the statistical analyses, the PopGene32 software package was used (Yeh et al., 2010).

Results

A fragment of 451 bp was yielded for the GHRH gene and digested with *Hae*III enzyme. Anatolian water buffalo were found to be monomorphic for AA genotype for GHRH/*Hae*III polymorphism (Figure 1). Following digestion with *Hae*III enzyme for the GHRH gene, three fragments were expected to be seen for the homozygote AA genotype (312, 94 and 45 bp). However, due to the small size of the 45 bp band, this band could not be detected in agarose gel electrophoresis. Nevertheless, it was enough in the 312 and 94 bp bands genotyping of Anatolian water buffalo for GHRH/*Hae*III polymorphism.

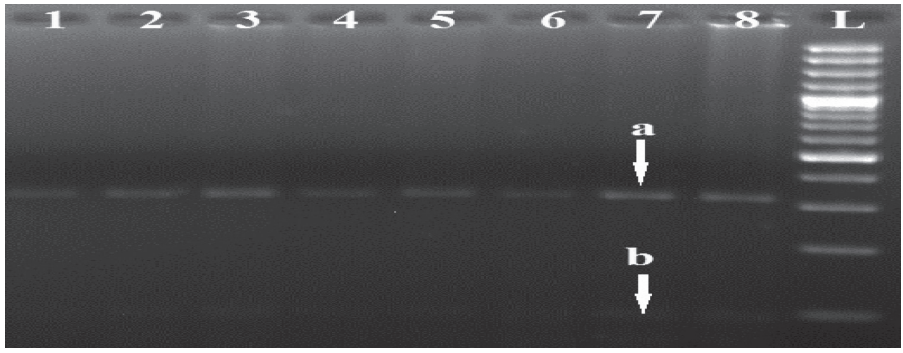

Figure 1. GHRH genotyping with PCR-RFLP method. L: DNA marker (100 bp); 1-6 AA genotyped samples (a: band of 312 bp; b: band of 94 bp)

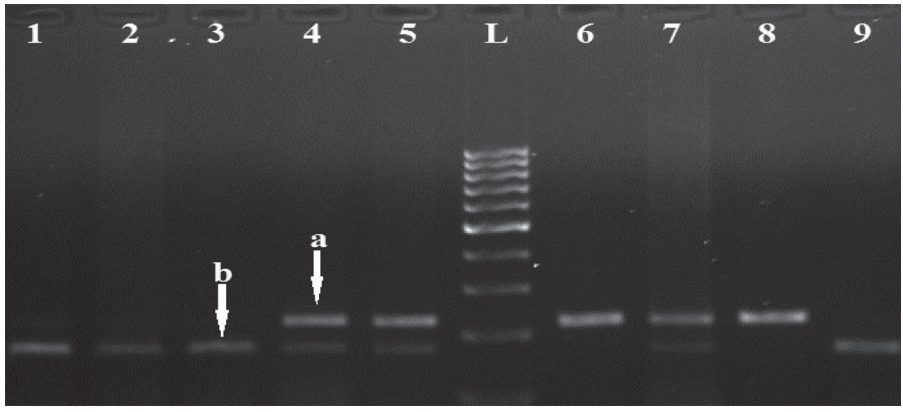

Figure 2. Patterns of restriction fragments of GH gene after digestion with *Alu*I enzyme. Lane L: markers (100 bp); lanes 1, 2, 3 and 9; LL genotype: 6 and 7; VV genotype: 4, 5 and 7; LV genotype (a: band of 211; b: band of 159 bp)

The PCR amplification yielded a 211 bp fragment for GH gene. Restriction digestion of PCR products with the *Alu*I restriction enzyme revealed three genotypes of VV (211 bp), LV (211, 159 and 52 bp) and LL (159 and 52 bp). However, because of 211 and 159 bp bands seen together and separately, genotyping was successfully fulfilled without observing 52 bp band in agarose gel electrophoresis (Figure 2).

GH/*Alu*I polymorphism for the Anatolian water buffalo breed is given in Table 1. The results showed that LL genotype frequency (0.755) was found to be higher, and VV genotype frequency (0.017) was found to be the lowest in the Anatolian water buffalo breed. L allele frequency (0.87) was found to be higher than V allele frequency (0.13) in Anatolian water buffaloes. The Anatolian water buffalo were in Hardy-Weinberg equilibrium (HWE) for the GH gene (Table 1).

Table 1. The allele and genotype frequencies of GH gene in Anatolian water buffalo

Breed	n	Genotype						Allele frequency		χ^2 (df=1)	P-value
		LL		LV		VV		L	V		
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F				
Anatolian water buffalo	126	95 (95.16)	0.755	29 (28.68)	0.228	2 (2.16)	0.017	0.87	0.13	0.02	0.8999NS

Obs: Observed genotype; Exp: Expected genotype; F: Frequency; df: degree of freedom.
NS: Non significant.

The 156 bp PCR products of the PRL gene were digested by the *Rsa*I enzyme. The following DNA restriction fragments were obtained for the PRL/*Rsa*I polymorphism: 156 bp (no digestion) for the AA genotype, 156, 82 and 74 bp for the AB genotype and 82 and 74 bp for BB genotype in Anatolian water buffalo (Figure 3).

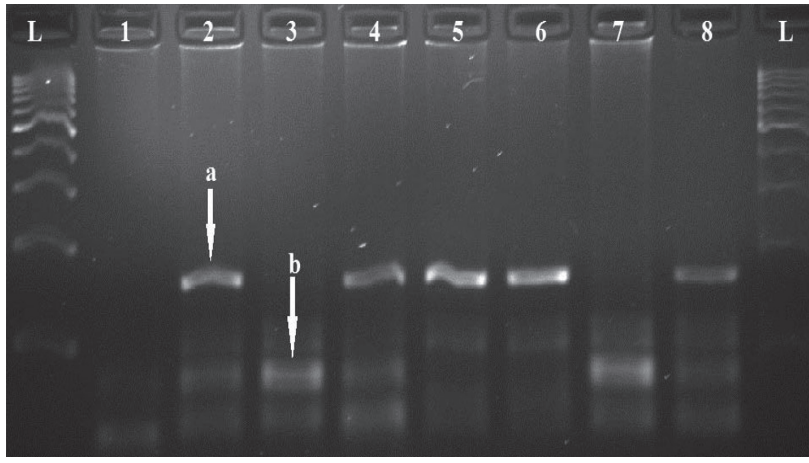

Figure 3. Gel image for the PRL genotypes by PCR-RFLP analysis. Lane L: marker (100 bp); lanes 5 and 6; AA genotype: 1, 3 and 7; BB genotype: 2, 4 and 8; AB genotype (a: band of 156 bp; b: band of 82 and 74 bp)

The AB genotype had the highest frequency (0.81) and BB had lowest genotype frequency (0.047). The A allele frequency (0.55) was found to be higher than that of B allele frequency (0.45) (Table 2). For the PRL gene, the AB genotype was identified as the most common genotype in Anatolian water buffalo. The PRL gene chi-square test results showed the deviation from HWE in the examined Anatolian water buffalo samples (Table 2).

Table 2. The genotype and allele frequencies of PRL gene in Anatolian buffalo

Breed	n	Genotype						Allele frequency		χ^2 (df=1)	P-value
		AA		AB		BB		A	B		
		Obs (Exp)	F	Obs (Exp)	F	Obs (Exp)	F				
Anatolian water buffalo	126	18 (37.79)	0.143	102 (62.43)	0.81	6 (25.79)	0.047	0.55	0.45	χ^2 =50.63	0.00***

Obs: Observed genotype; Exp: Expected genotype; F: Frequency; df: degree of freedom.
*** significant (P<0.001).

Discussion

The aims of selection programs are to select animals with high breeding value and to achieve a rapid genetic gain in livestock farming. In this study, the GHRH/*Hae*III, GH/*Alu*I and PRL/*Rsa*I polymorphisms were investigated in Anatolian water buffalo. In the literature search, we found only one study on polymorphisms in the PRL gene in Anatolian water buffalo (Kaplan and Boztepe, 2010), but none on polymorphisms in the GHRH and GH genes. Therefore, the present study is the first report on GHRH and GH gene polymorphisms in Anatolian water buffalo.

Growth Hormone Releasing Hormone (GHRH)

It has been reported that GHRH, also known as growth hormone releasing factor, can increase cattle milk production and the growth rate of beef cattle (Dybus et al., 2003; Kmieć et al., 2007). The GHRH/*Hae*III polymorphism was found to be polymorphic; three genotypes (AA, AB and BB) and two alleles (A and B) were reported in cattle (Kmieć et al., 2007; Szatkowska et al., 2009; Rini et al., 2013). However, AA genotype frequency was found to be lowest in cattle breeds such as the Holstein (Kmieć et al., 2007; Szatkowska et al., 2009; Rini et al., 2013), Jersey (Szatkowska et al., 2009), Simmental (Rini et al., 2013) and Limousin (Rini et al., 2013). In contrast to those breeds, AA genotype frequency was found to be highest in Angus cattle (Rini et al., 2013), whereas the BB genotype was found only in the Brahman breed which originates from *Bos indicus* (Rini et al., 2013). On the other hand, BB genotype frequency was found to be highest in dairy cattle breeds such as the Holstein (Kmieć et al., 2007; Szatkowska et al., 2009; Rini et al., 2013) and Jersey (Szatkowska et al., 2009) breeds.

Only a few studies have been conducted to determine GHRH/*Hae*III polymorphism and its effect on production parameters in different buffalo breeds. In Indonesia, in two experiments, GHRH/*Hae*III polymorphism was investigated in swamp buffalo. The frequency of the B allele was higher than that of the A allele (Sumantri et al., 2010, 2013). In another study, to determine GHRH/*Hae*III polymorphism, BB genotype frequency was found to be highest and the AA genotype was not detected in Indonesian swamp buffalo (Primasari et al., 2009). However, in our study, Anatolian water buffalo were found to have the AA genotype for GHRH/*Hae*III polymorphism. Similar to our results, the only AA genotype was found in Egyptian water buffalo (Othman et al., 2015), which originate from the Mediterranean river buffalo subgroup. It is thought that these genetic differences in the GHRH/*Hae*III polymorphism may be specific to river buffalo or to the Mediterranean river buffalo subgroup. However, to obtain more conclusive results, further research with a larger sample number is needed.

Growth Hormone (GH)

For the detection of GH gene polymorphism, mostly *Alu*I and *Msp*I restriction enzymes have been used in different cattle breeds. Therefore, the *Alu*I enzyme was used to determine GH polymorphisms in Anatolian water buffalo in this study.

In high milk yield cattle such as the Holstein, Brown Swiss and Simmental breeds, it was reported that the frequency of the L allele was higher than that of the V allele in terms of GH/*Alu*I polymorphism (Kovács et al., 2006). In contrast, in beef cattle breeds such as the Limousin, Charolais, Piedmontese, Angus and Hereford, it was reported that the frequency of the V allele was higher than that of the L allele (Khatami et al., 2005). Also, it was shown that in cattle originating from the *Bos indicus* cattle breeds, the frequency of the L allele was much higher than that of the V allele in terms of GH/*Alu*I polymorphism (Zhou et al., 2005). Nevertheless, there are limited studies in buffalo on GH/*Alu*I polymorphism compared to those in cattle. In Indonesia, one experiment showed that only the VV genotype has been reported in swamp buffalo (Sumantri et al., 2010). However, in two other studies it was reported that all the animals with the LL genotype in the GH/*Alu*I polymorphism were swamp buffalo (Andreas et al., 2010; Sumantri et al., 2010). On the other hand, three genotypes (LL, LV and VV) were observed and the LL genotype was the most common with a 0.871 frequency, and the least common was the VV genotype with a 0.005 frequency in the Egyptian water buffalo breed (Othman et al., 2012). Similarly, in our study, the LL genotype was the most common genotype (0.755) and the VV genotype was the least common (0.017) in the Anatolian water buffalo. Similar genotypic frequencies to those observed in water buffalo of the Mediterranean basin were found, which might be due to their shared genetic properties.

Prolactin (PRL)

The PRL-*Rsa*I locus was found to be polymorphic and three genotypes were observed for this locus in cattle (Kaplan and Boztepe, 2010). However, in the literature, the frequency of the A allele was found to be higher than that of the B allele in different dairy cattle breeds such as the Holstein (Khatami et al., 2005; Kumari et al.,

2008) and Jersey (Kumari et al., 2008). Similarly, two alleles (A and B) have been found in different buffalo breeds; however, three genotypes have not been found in water buffalo (Ishaq et al., 2013; Kaplan and Boztepe, 2010). In a previous report, PRL/*Rsa*I polymorphism studies showed that all buffalo had the BB genotype in Nili-Ravi water buffalo, which are reared in Pakistan (Ishaq et al., 2013). Controversially, Kaplan and Boztepe (2010) reported that the AA genotype had the highest frequency (0.63) and the BB genotype was not detected in a PRL/*Rsa*I polymorphism study carried out in 30 Anatolian water buffalo. In the present study, a total of 126 Anatolian water buffalo (PRL/*Rsa*I) were examined. PRL/*Rsa*I polymorphism was investigated and all three genotypes were observed. Contrary to the results of Kaplan and Boztepe (2010), although the frequency of the BB genotype (0.047) was very low, six buffalo were identified to have this genotype and, AB genotype frequency (0.81) was found to be highest in this study.

In this study, we observed a deviation from H-W equilibrium in the Anatolian water buffalo population in terms of the PRL gene. This may be due to the decreasing size of the population, inbreeding or unplanned selection according to milk yield among animals. In order to reach a more definite conclusion, it is necessary to conduct genotyping studies on more animals throughout Turkey.

In conclusion, to the best of our knowledge, this is the first study on GHRH/*Hae*III and GH/*Alu*I polymorphisms in Anatolian water buffalo. In the literature search, we were unable to find studies related to GHRH, GH and PRL gene polymorphisms in different buffalo breeds. Only the AA genotype was found for the GHRH/*Hae*III polymorphism in Anatolian water buffalo. Therefore, it is thought that this polymorphism is less likely to be used to improve the yield traits of Anatolian water buffalo. On the other hand, the GH and PRL genes were, for the first time, found to have three genotypes in Anatolian water buffalo. Therefore, new experiments should be planned to investigate the association between GH/*Alu*I and PRL/*Rsa*I polymorphisms with milk and meat yield traits in Anatolian water buffalo.

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