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YUSUF ÖZŞENSOY

HAKİ KARA

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Investigation of *GH* and *GHR Alu* I gene polymorphisms on meat yields in Anatolian water buffalo breed using PCR-RFLP method

Yusuf ÖZŞENSOY[1,*], Haki KARA[2,3]

[1]Department of Veterinary Biometrics and Genetics, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Sivas, Turkey
[2]Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Sivas, Turkey
[3]Anatolian Water Buffalo Breeding by Breeder Project, Sivas, Turkey

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Abstract: The Anatolian water buffalo is a native water buffalo breed only being reared in Turkey. The objective of this study was to investigate gene polymorphisms on exons 4 and 5 of the growth hormone (GH) and on exon 10 of the growth hormone receptor (GHR), which are thought to be related to meat yields of Anatolian water buffalo. Blood samples of 192 water buffaloes from Sivas Province were used in DNA extractions by phenol-chloroform method. DNA samples were amplified by using specific primers in PCR. PCR products were digested by *Alu* I (*GH*, n: 167) and *Alu* I (*GHR*, n: 192) restriction enzymes in order to determine polymorphisms. Digested PCR products were separated in 2.5%–3% agarose gel electrophoresis to determine allelic polymorphisms. As a result, the LL (78.44%) and LV (21.56%) genotypes and L (0.89) and V (0.11) alleles for the *GH* gene and the AA (7.81%) and AG (92.19%) genotypes and A (0.54) and G (0.46) alleles for the *GHR* gene were obtained. Gene polymorphisms were not detected ($P > 0.05$) on the *GH* gene, but a significant difference was found for the *GHR* gene ($P < 0.001$). Therefore, it can be said that Anatolian water buffaloes have increased meat yield due to the presence of genotypes in gene regions. This was the first investigation of enzyme polymorphisms of the *GHR* gene in Anatolian water buffaloes.

Key words: Growth hormone, growth hormone receptor, PCR-RFLP, Sivas, water buffalo

1. Introduction

Water buffalo is a species reared in various countries of the world for supporting agricultural economy and for its meat, milk, leather yield, and horns (Michelizzi et al., 2010). Water buffaloes are known to benefit from nutritionally deficient feeds, and they are tolerant to sudden changes in their feeds (GDAR, 2011). It has been reported that water buffaloes as a species migrated to Italy from Central Europe around the 6th century, and in approximately the 7th century they migrated towards the Arabian Peninsula through the Moroccan Gulf (Michelizzi et al., 2010). However, it has been reported that the swamp breed of water buffalo originated from Southeast China and was domesticated 5000 years ago. Similarly, it has been reported that the river breed of water buffalo originated from India and was domesticated 4500 years ago (FAO, 2015).

According to 2014 data (FAO, 2015), it is reported that 123 water buffalo breeds are present in the world. The highest number of breeds with a total of 90 breeds are found on the Asian continent, whereas only one breed is known in North America. It was also reported that a water buffalo breed formerly present in both Europe and the Caucasus has gone extinct (FAO, 2015). It is reported that all the water buffaloes reared in both Europe and the Near East belong to the river breed. However, the Anatolian water buffalo breed is being reared only in Turkey, and it is known to be valued for its combined meat and milk yield (FAO, 2005; GDAR, 2011).

There was a 23% increase in water buffalo meat yields worldwide during the years between 2004 and 2012, from 2,924,490 tons to 3,597,340 tons (FAO, 2015). Particularly in Asian countries, water buffalo meat is a primary product from its rearing (FAO, 2005). Water buffalo meat yields in Turkey were recorded as 1339 and 402 tons in 2017 and 2018, respectively. Water buffalo meat yields have thus shown a dramatic decline despite the increase in water buffalo head count (TÜİK, 2019a, 2019b).

There are some important hormones for meat yields. Growth hormone (GH), which is a part of the *GH* gene family that also includes prolactin and placental lactogens, is an anabolic hormone known to be secreted

* Correspondence: yusufozsensoy@yahoo.com

by somatotropic cells located in the frontal lobe of the hypophysis and regulated by the hypothalamus. In order to exhibit its endocrine functions in growth and metabolism, GH needs to bind to the surface of GH receptors located on the target cells. A single GH molecule binds to two GH receptors (Di Stasio et al., 2005; Ayuk and Sheppard, 2006). In addition to its primary function of growth that affects many organs and systems, GH is known to be responsible for changes in protein, lipid, and carbohydrate metabolisms (Ayuk and Sheppard, 2006). GH has a role in both growth and metabolism with protein storage and synthesis in tissues and organs (Pal and Chatterjee, 2010). It was reported that there is 97.1% nucleotide similarity in the *GH* gene between cattle and water buffalo species. It was also reported that when this gene region was amplified using the same primer pair the resulting fragment was of the same size. Genetic polymorphisms in the *GH* gene are the results of several point mutations located in the gene (Pal and Chatterjee, 2010). A single amino acid alteration in the 127th polypeptide position located in exon 5 of the *GH* gene is the cause of the polymorphism. The alteration occurs in this position by the substitution of valine instead of leucine. The *Alu* I (AG↓CT) restriction enzyme is used to determine this alteration in the said region (Mitra et al., 1995; Biswas et al., 2003).

Growth hormone receptor (GHR) is a part of the class I cytokine receptor superfamily. This receptor is a transmembrane protein with high affinity and specificity towards binding GH (Flores-Morales et al., 2006; Andreas et al., 2010). This receptor needs to be expressed for GH to have cellular activity (Andreas et al., 2010). It was reported that GHR has 70% similarity in amino acid sequences between different species (Kelly et al., 1991). GHR was reported to have 84% similarity in amino acid sequences between rabbits and humans (Leung et al., 1987). The exon 10 region of the *GHR* gene was previously determined as a candidate locus for investigations of meat yield genes in cattle (Di Stasio et al., 2005).

Aside from the many beneficial traits of water buffaloes, such as their natural resistance to various diseases, they have some disadvantages (Michelizzi et al., 2010). Further studies are required to better understand the DNA sequence variations in water buffalo species that are related to meat yields to better utilize these animals in molecular domestication procedures. Therefore, molecular studies on yields are particularly important. The objective of this study was to investigate meat yield-related *GH* gene and *GHR* gene polymorphisms by using the PCR-RFLP method in water buffaloes reared in Sivas Province, Turkey.

2. Materials and methods

A total of 192 water buffalo (140 female and 52 male) that were bred in Sivas Province and born between 2014 and 2015 were used. Genetically unrelated animals were selected. The standard phenol/chloroform method was used for DNA isolation from blood samples (Sambrook et al., 1989). Isolated genomic DNAs were amplified in polymerase chain reaction (PCR) by using synthesized primers (Table 1) for two gene regions (*GH* and *GHR*). PCR mixtures were prepared in volumes of 15 µL. Each reaction was prepared as described by Doğan et al. (2019).

A touchdown PCR protocol was used for amplification reactions of the two gene regions (Don et al., 1991). The touchdown PCR protocol was performed as previously described by Özşensoy (2018); the only modification of that method was that 45 s and 1 min were applied for the annealing and elongation steps, respectively. Amplified fragments were electrophoresed in 1.5% agarose gel electrophoresis at 100 V for 60 min and visualized at 365 nm under a UV transilluminator.

Amplified PCR products were digested using the *Alu* I restriction enzyme. Enzyme digestions were prepared by using 10 µL of PCR product, 10 U of FastDigest restriction enzyme (Thermo), 2 µL of enzyme buffer (Thermo), and ddH_2O at a total volume of 31 µL. Digestion mixes were incubated at 37 °C for 20–25 min. Digested fragments were electrophoresed in 2.5%–3.0% agarose gel electrophoresis stained using EtBr at 100 V for 60 to 85 min and visualized at 365 nm under a UV transilluminator.

Band sizes for determination of the obtained genotypes as a result of enzyme digestions are shown in Table 2. Allele

Table 1. Primer pairs and restriction enzyme used in the study.

Locus	Primer sequence (5′ –› 3′)	PCR (bp)	Restriction enzymes	Reference
GH	F: 5′ - CCGTGTCTATGAGAAGC - 3′ R: 5′ - GTTCTTGAGCAGCGCGT - 3′	428	*Alu* I	Pal and Chatterjee, 2010
GHR	F: 5′ - GCTTACTTCTGCGAGGTAGACGC - 3′ R: 5′ - GTCTGTGCTCACATAGCCAC - 3′	298	*Alu* I	Andreas et al., 2010

F: Forward, R: reverse, bp: base pairs.

Table 2. Length of PCR products and each genotype obtained for *GH* and *GHR* genes in Anatolian water buffalo.

GH gene, exon 4 and 5	PCR (bp)	LL (bp)	LV (bp)	VV (bp)
	428	16, 51, 96, 265	16, 51, 96, 147, 265	16, 147, 265
GHR gene, exon 10	PCR (bp)	AA (bp)	AG (bp)	GG (bp)
	298	81, 217	81, 217, 298	298

bp: Base pairs.

frequencies and genotypic structures were determined by using gene counting and differences were calculated by conducting chi-square analysis using Microsoft Office Excel 2013 (Microsoft Inc., USA).

Ethics approval of the study was granted by the Local Ethics Committee for Animal Experiments of Cumhuriyet University on 23.02.2016, Number 65202830-050.04.04-24.

3. Results

A total of 167 samples were used to amplify exons 4 and 5 of *GH* (Figure 1), and polymorphisms were determined according to restriction enzyme digestions (Figure 2). Observed and expected allele genotypes and their frequencies are shown in Table 3.

Two genotypes (LL and LV) and two alleles (L and V) were determined in the *GH* gene (Figure 2; Table 3). Among the samples, the LL genotype (78.44%) and L allele (89%) were determined as the highest. According to these results, no statistical significance was determined in Anatolian water buffaloes for *GH* gene polymorphisms ($P > 0.05$).

A total of 192 samples were used for the amplification of the exon 10 region of the *GHR* gene (Figure 3). Polymorphisms were determined by digesting this region (Figure 4). Observed and expected allele genotypes and their frequencies are shown in Table 4.

Two different genotypes (AA and AG) were determined from digestions (Figure 4). Among the samples, the AG genotype was the highest at 92.19%, and the A allele was the highest at 54% (Table 4). According to these results, statistical significance for the exon 10 region of the *GHR* gene was determined in Anatolian water buffaloes ($P < 0.001$).

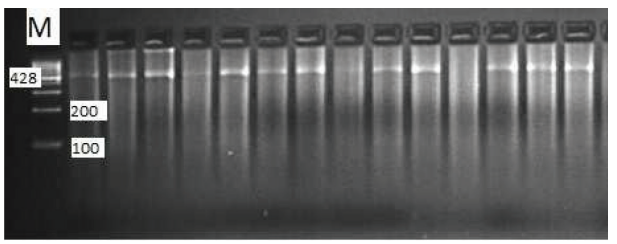

Figure 1. PCR products of exons 4 and 5 of the *GH* gene (428-bp band, M: 100-bp DNA ladder).

4. Discussion

Utilization of polymorphic genes as molecular markers has a significant role in farm animals for their selections for economical traits. *GH* and *GHR* were previously reported as particular gene regions for meat yield in farm animals, and they were selected as candidate genes for molecular selection (Biswas et al., 2003; Andreas et al., 2010). Specific primer pairs that were previously described for cattle (Maciulla et al., 1997) were used for these two gene regions in water buffaloes, and PCR products were obtained with similar fragment lengths from water buffalo samples (Andreas et al., 2010; Ramesha et al., 2015).

The same exon region of the *GH* gene in this study was investigated in 5 water buffalo breeds of Indonesia (Andreas et al., 2010), in Egyptian breeds (Othman et al.,

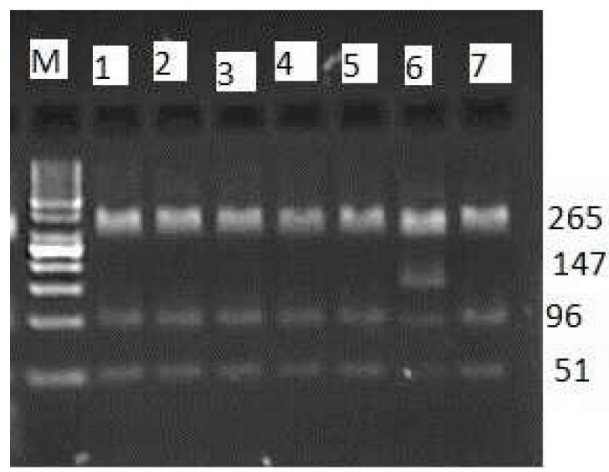

Figure 2. Enzyme digestion results of exons 4 and 5 of the *GH* gene (M: 50-bp DNA ladder; 1–5 and 7: LL genotype, 6: LV genotype).

Table 3. Observed and expected genotypes and allele frequencies of *GH* gene.

Gene	n	Genotype frequency			Allele frequency		χ^2	P-value (df = 1)
		LL	LV	VV	L	V		
		O (E)	O (E)	O (E)				
Exons 4 and 5 (*Alu* I)	167	131 (132.94) 78.44%	36 (32.12) 21.56%	0 (1.97) -	0.89	0.11	2.437	0.1185 ns

O: Observed genotype; E: expected genotype; n: sample count; df: degrees of freedom; χ^2: chi-square value, ns: P > 0.05.

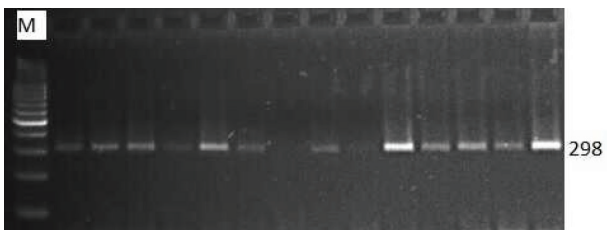

Figure 3. PCR results of the exon 10 region of the *GHR* gene (298-bp band, M: 100-bp DNA ladder).

2012), and in Indian breeds (Mitra et al., 1995; Biswas et al., 2003; Pal and Chatterjee, 2010; Jammeda and Vataliya, 2014). These studies reported only the LL genotype as monomorphic. In this study, more animals were used compared to other studies and two genotypes (LL and LV) and two alleles (L and V) were determined. However, no statistical significance (P > 0.05) was determined in the Anatolian water buffalo breed. In contrast to this study, various studies conducted on 3 different *GH* gene regions (exon 5 – *Alu* I; intron 3 – *Msp* I; 3'UTR – *Hae* III) in water buffalo breeds reported them as monomorphic (Mitra et al., 1995; Jammeda and Vataliya, 2014, 2017; Eriani et al., 2019).

In another study conducted on 126 Anatolian water buffaloes in Turkey, *GH* gene polymorphisms were determined as LL (75.5%), VV (1.7%), and LV (22.8%) genotypes, and the L allele frequency was reported as 87% (Konca and Akyüz, 2017). In this study, more animals were used, but the VV genotype was not determined. However, the LL genotype (78.44%) and L allele frequency (89%) were determined to be higher. Similar to this study, in a study conducted on cattle, LL genotype and L allele frequencies were reported as higher (Biswas et al., 2003).

The same exon region of the *GHR* gene was investigated in Indonesian water buffalo breeds and only the AA genotype was reported (Andreas et al., 2010). Again, the same region was investigated in Egyptian breeds and only the GG genotype was reported (Othman et al., 2012). In contrast, Mae II enzyme digestion of the same region in the Indian Surti breed was studied, but all samples were reported as monomorphic (Jammeda and Vataliya, 2014). In this study, very high sampling size was used and two different genotypes were determined in the Anatolian water buffalo breed, which are AA (7.81%) and AG (92.19%), together with two different alleles of A (0.54) and G (0.46). All of these findings were statistically significant (P < 0.001), with the AG genotype being highest. In a study conducted on 60 Iranian water buffalo breeds, 3 genotypes were determined in both the *GH* and *GHR* genes, and a statistically significant effect on body weight was determined (Ahmadzadeh et al., 2019).

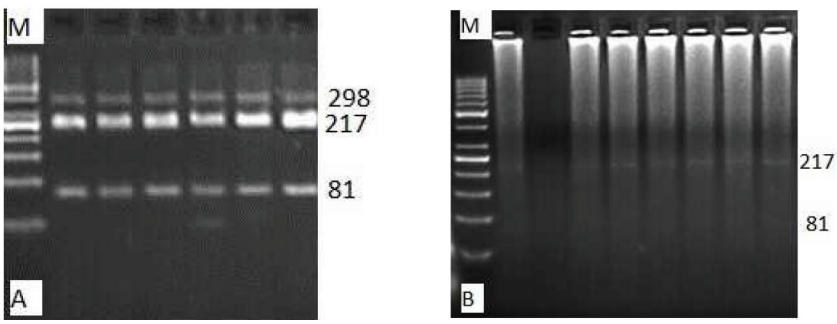

Figure 4. Enzyme digestion results of the exon 10 region of the *GHR* gene (M: 50-bp DNA ladder, A: AG genotype, B: AA genotype).

Table 4. Observed and expected genotypes and allele frequencies of *GHR* gene.

Gene	n	Genotype frequency			Allele frequency		χ^2	P-value (df = 1)
		AA	AG	GG	A	G		
		O (E)	O (E)	O (E)				
Exon 10 (*Alu* I)	192	15 (55.79) 7.81%	177 (95.41) 92.19%	0 (49.79) -	0.54	0.46	140.381	0.0000 ***

O: Observed genotype; E: expected genotype; n: sample count; df: degrees of freedom; χ^2: chi-square value, ***: $P < 0.001$.

The LL genotype of the *GH* gene was reported as a possibly species-related trait for water buffaloes (Mitra et al., 1995; Pal and Chatterjee, 2010; Jammeda and Vataliya, 2014). It was also reported that the animals with this genotype had higher birth weights (Biswas et al., 2003). In this study, LL genotype frequency was determined as the highest one, and the LV genotype was determined as well.

Two different genotypes from the exon 10 region of the *GHR* gene were stated to be related to different meat yields (Di Stasio et al., 2005), and that was reported as a candidate gene for investigations of meat and milk yields in Bovidae (Andreas et al., 2010; Othman et al., 2012). In this study, two different genotypes were determined and the *GHR* gene was investigated in the Anatolian water buffalo breed for the first time.

In conclusion, it can be stated that the Anatolian water buffalo breed has a meat yield trait since birth weight-related genotypes were determined in this breed.

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