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Breeding, genetics, and reproduction

Investigation of PRL-RsaI and HaeIII gene polymorphisms in Anatolian water buffaloes bred by using PCR-RFLP method

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ABSTRACT - The objective of this study was to investigate polymorphisms both in exons 1 and 3 of prolactin (PRL) gene for milk productivity of Anatolian water buffalo breed in Sivas province in Turkey. Blood samples were collected from 129 male and female water buffaloes and DNA was isolated by using phenol/chloroform method. Samples of DNA were amplified and resulting PCR products were digested with RsaI (for exons 1 and 3) and HaeIII (for exon 1). Allelic polymorphisms were determined by separation of fragments obtained from digested PCR products in 3% agarose gel electrophoresis. AA genotype (HaeIII) and BB genotype (RsaI) of exon 1 and only AA genotype (RsaI) of exon 3 were obtained. No polymorphisms were determined in Anatolian water buffalo breed and all loci were found as monomorphic. It can be stated that Anatolian water buffalo has a higher milk and milk fat yields since BB genotype was obtained.

Key Words: HaeIII, PCR-RFLP, prolactin, RsaI, Sivas

Introduction

Water buffaloes are raised in various countries for their economic value and as sources of workforce, meat, milk, leather, and horn (Michelizzi et al., 2010). It has been reported that all water buffalo breeds in Europe, including Anatolian water buffalo, belong to river water buffalo subspecies. However, it has been stated that Anatolian water buffalo breed is more similar to water buffaloes raised in the Mesopotamia region (FAO, 2005; Michelizzi et al., 2010).

Water buffaloes have many drawbacks in breeding, such as lower reproductive performance and higher infertility rates compared with cattle breeds, limited productivity rates, and lower survivability of their calves (Michelizzi et al., 2010). Therefore, molecular studies for determining their productivity traits are needed.

For selection of farm animals, choosing polymorphic genes as molecular markers is a useful approach. Milk fat and milk composition are important genetic selection traits for dairy animals. One of the most important hormones for prediction of milk yield and quality is prolactin (PRL), which has direct effects on milk yield. Therefore, PRL gene is a significant genetic marker. Studies regarding the polymorphisms in PRL gene have been conducted either in PRL exon 1 or exon 3; however, most of the studies were directed towards PRL exon 3. RsaI restriction enzyme (RE) digestion in exon 3 of PRL resulted in two allelic polymorphisms (B and b) associated with milk productivity (Freeman et al., 2000; Alipanah et al., 2007). According to a study carried out with water buffaloes of both Murrah and Nili-Ravi breeds, results showed that A_ allele has the highest frequency (0.93 and 0.84, respectively) and no BB genotype was obtained (Mitra et al., 1995). In another study conducted with Murrah water buffalo breed, RsaI RE digestion of PRL exon 3 resulted in no fragments and only monomorphic AA genotype was obtained, which was considered as breed characteristic (Biradar et al., 2014). When different RE were used to digest PRL exon 3 from different dairy breeds in India, the highest genotype frequency was found in AA (0.55) and the lowest was in BB (0.06) (Kumari et al., 2008). In another study, even though both AB and BB genotypes of PRL exon 3 were obtained from four different Indian breeds, no AA genotype was observed. The highest frequency of genotypes and alleles was found as AB (0.87-0.97) and B allele (0.50-0.57), respectively (Ladani et al., 2003a). According to a study conducted with Pandharpuri water buffalo breed, PRL exon 1 was digested by HaeIII RE and no polymorphism was found. Therefore, it was determined that Pandharpuri water buffalo breed was monomorphic for AA genotype (Madnalwar et al., 2010).

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Traditionally, Anatolian water buffalo is raised in various provinces of Turkey (Governmental Newspaper, 2012). However, with the grants from national domestication campaign of "National Domestication by Breeders", water buffalo breeding has increased throughout Turkey. It is known that Anatolian water buffalo breed is raised for both their meat and milk. The Anatolian water buffalo was declared as one of the national genetic resource in 2004 (Governmental Newspaper, 2004). There have been very sparse studies regarding molecular characterization of Anatolian water buffalo breed (Kaplan and Boztepe, 2000; Konca and Akyüz, 2017). Therefore, there is a need for the molecular characterization of Anatolian water buffalo breed. Since Anatolian water buffalo breed is mainly raised for its milk, this study focused upon PRL gene and both exons 1 and 3 were investigated by using high amounts of samples. The present study is among the first in literature in which both PRL exons were investigated at the same time.

The objective of this study was to investigate polymorphisms in both PRL exons 1 and 3 related to milk productivity by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in Anatolian water buffalo breed in Sivas province in Turkey.

Material and Methods

Blood sampling was carried out from Anatolian water buffalo breed raised by various breeders throughout Sivas province and care was taken not to choose closely related animals. A total of 129 animals, of which 89 were females and 40 were males, were used. Blood samples were collected into K_3-EDTA tubes and then kept in −20 °C until analyses. Extraction of DNA from blood samples was done by using standard phenol/chloroform method (Sambrook et al., 1989).

Samples of DNA were amplified by using gene specific primers (Table 1). Polymerase chain reaction was performed in 15 µL volume containing 1 × Mg^{++} free PCR buffer (Thermo Scientific, USA), 200 mM dNTPs (Thermo Scientific, USA), 1.5 mM $MgCl^{++}$ (Thermo Scientific, USA), 0.375 U *Taq* polymerase (Thermo Scientific, USA), 5 pM of each primer, and 50-100 ng DNA.

Polymerase chain reactions were amplified by using touchdown PCR profile (Don et al., 1991). In this profile, denaturation was achieved at 95 °C for 4 min. In the first phase, steps included 30 sec denaturation at 94 °C, 30 sec annealing which started at 60 °C and lowered by 0.5 °C in each cycle for ideal primer hybridization, and 30 sec elongation at 72 °C. Steps cycled 16 times. In the second phase, steps included 30 sec at 94 °C, 30 sec at 52 °C, and 30 sec at 72 °C. Steps cycled 25 times. The final extension of 72 °C for 10 min was applied in all reactions. For the amplification of 857 base pairs (bp) long region of PRL gene, durations of annealing and elongation increased by 1 min. Amplified PCR products were then separated in 2% agarose gel electrophoresis.

Digestions of RE (Table 1) were carried out in 31 µL reaction volumes. Reaction volumes contained 10 µL PCR product, 1 µL RE (10 U μL^{-1}) (Thermo Scientific, USA), 2 µL enzyme buffer, and 18 µL ddH_2O and reactions were incubated overnight at 37 °C. Fragments obtained from digestions were run in 3% agarose gel electrophoresis at 100 V for 1 h. Fragment localizations were then visualized under 365 nm UV.

Genotypic and allelic frequencies were determined by gene counting (Table 2). Differences in polymorphisms were then determined by chi-square analysis.

Research on animals was carried out with the permission granted by the local Ethics Commission for Animal Experimentations (case no. 65202830-122).

Table 1 - Primers and restriction enzymes used in the study

Locus	Primer sequence (5' –> 3')	PCR (bp)	Restriction enzyme	Reference
PRL Exon 1	F: ATTATCTCTCTCATTTCCTTTCA R: ACTCTGCTGTCACTGTCTGTATT	857	RsaI HaeIII	25
PRL Exon 3	F: CGAGTCCTTATGAGCTTGATTCTT R: GCCTTCCAGAAGTCGTTTGTTTTC	156	RsaI	11

PCR - polymerase chain reaction; PRL - prolactin; bp - base pair.
F - forward primer; R - reverse primer.

Table 2 - Obtained PRL genotypes

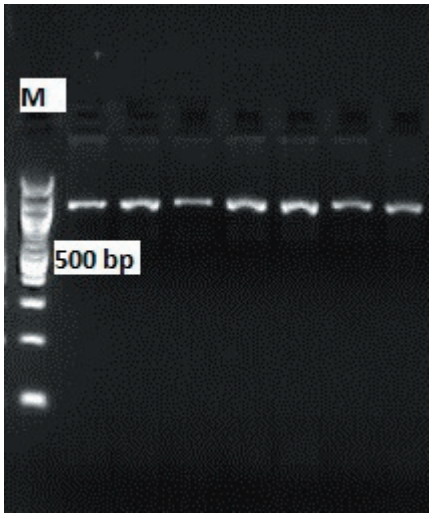
Gene	PCR (bp)	Restriction enzyme	AA (bp)	AB (bp)	BB (bp)
Exon 3	156	RsaI	156	156, 82, 74	82, 74
Exon 1	857	RsaI HaeIII	857 857	857, 323, 243, 125, 89, 77 857, 663, 194	323, 243, 125, 89, 77 663, 194

PCR - polymerase chain reaction; PRL - prolactin; bp - base pair.

Results

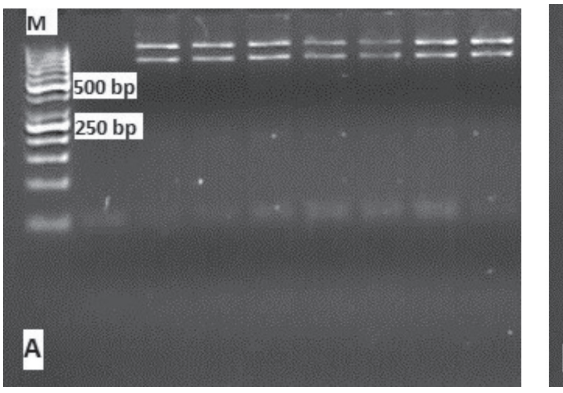
According to RE digestions of PCR products, amplifications from PRL exon 1 (Figure 1) and exon 3 (Figure 3) were observed. Polymorphisms of these exons were determined by RE digestion (Figures 2 and 4).

As seen from the PCR result of exon 1 (Figure 2), an additional band was located close to the expected fragment. The band mentioned above was observed as two bands in the same region when digested with HaeIII (Figure 2a). HaeIII digestion of the same exon resulted in a single allele (A) and a single genotype (AA) for all samples. Similarly, RsaI digestion of the same exon led to a single genotype (BB) and a single allele (B) for all samples (Figure 2b). RsaI digestion of PRL exon 3 was presented in a single genotype (AA) and a single allele (A) for all samples (Table 3). Hence, no PRL gene polymorphisms in Anatolian water buffalo breed were observed (P>0.05).



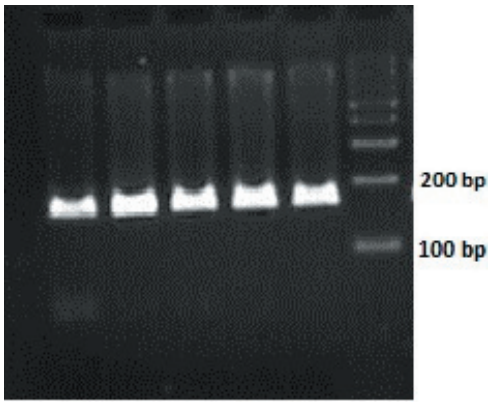
PCR - polymerase chain reaction; PRL - prolactin; bp - base pair.
857 bp band; M for 100 bp Ladder.

Figure 1 - Results of PCR of exon 1 of PRL gene.



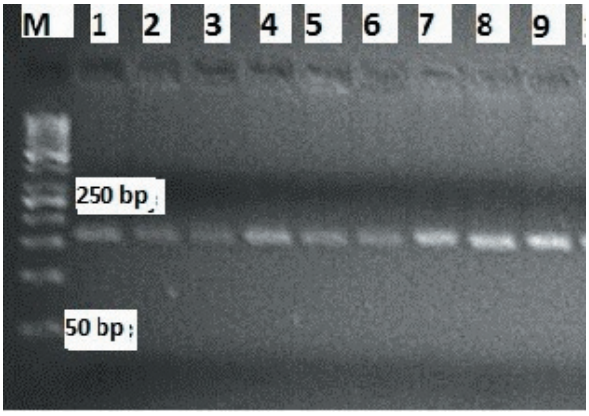
PRL - prolactin; bp - base pair.
A: HaeIII digestion; samples with AA genotype; M for 100 bp Ladder.
B: RsaI digestion; samples with BB genotype; M for 100 bp Ladder.

Figure 2 - Digestion results of exon 1 of PRL gene.



PCR - polymerase chain reaction; PRL - prolactin; bp - base pair.
156 bp band; M for 100 bp Ladder.

Figure 3 - Results of PCR of exon 3 of PRL gene.



PRL - prolactin; bp - base pair.
1 to 16: samples with AA genotypes (156 bp); M for 100 bp Ladder.

Figure 4 - Digestion results of exon 3 of PRL gene.

Table 3 - Chi-square analyses, genotype and allelic frequencies, and significance of PRL genotypes

Gene	n	Genotype frequency			Allelic frequency		$\chi 2$	P-value (df = 1)
		AA	AB	BB	A	B		
		O (E)	O (E)	O (E)				
Exon 1 (HaeIII)	129	129 (129)	0	0	1	0	0.00	1.00 ns
Exon 1 (RsaI)	129	0	0	129 (129)	0	1	0.00	1.00 ns
Exon 3 (RsaI)	129	129 (129)	0	0	1	0	0.00	1.00 ns

PRL - prolactin; O - observed; E - expected; df - degree of freedom; χ^2 - Chi-square; ns - not significant at P>0.05.

Discussion

Polymorphism investigations in both cattle and water buffalo breeds were carried out for PRL exons. There are studies for exon 3 (Mitra et al., 1995; Kaplan and Boztepe, 2000; Alipanah et al., 2007; Kumari et al., 2008; Biradar et al., 2014; Konca and Akyüz, 2017) and for exon 1 (Ladani et al., 2003b; Madnalwar et al., 2010). However, PRL exon 3 has been the most extensively studied. Up to this point, only the HaeIII RE is used for digesting the PRL exon 1. There are only two previous studies conducted with Anatolian water buffalo breed for PRL gene polymorphisms and only exon 3 was investigated (Kaplan and Boztepe, 2000; Konca and Akyüz, 2017).

In this present study, for the first time, both PRL exons 1 and 3 were examined at the same time in Anatolian water buffalo breed. Additionally, polymorphisms in PRL exon 1 was investigated for the first time by using both HaeIII and RsaI RE. Therefore, the present study is a novel addition to literature for Anatolian water buffalo breed and also for other studies conducted in different countries. Besides, the sample amount used for the present study is the highest for studies conducted in Anatolian water buffalo breed and is even higher than most studies conducted for different breeds.

HaeIII digestion of PRL exon 1 resulted in only AA genotype for Anatolian water buffalo breed. Similarly, only AA genotype was obtained from Pandharpuri water buffalo breed using the same RE (Madnalwar et al., 2010). In addition, PRL exon 1 was digested with RsaI RE in this study and resulted only BB genotype.

PRL exon 3 digestion with RsaI resulted only in AA genotype and only the A allele for Anatolian water buffalo breed. Similarly, only AA genotype was obtained from the Iranian (Tabar et al., 2010), the Murrah (Biradar et al., 2014), and the Anatolian (Kaplan and Boztepe, 2000) breeds. However, it has been reported that AB and BB genotypes, in addition to AA genotype, were found in Anatolian water buffalo breed (Konca and Akyüz, 2017). In previous studies, in which PRL exon 3 polymorphisms in Anatolian water buffalo breed were investigated, 45 animals were used by Kaplan and Boztepe (2000) and 126 animals were used by Konca and Akyüz (2017). Significantly more animals were used in the present study. Polymorphism studies carried out in other countries by using RsaI RE in both cattle and water buffalo breeds reported that the highest genotype received was AA, with a ratio of 55-93%, and the highest allelic frequency was A with a ratio of 79-82% (Mitra et al., 1995; Kaplan and Boztepe, 2000; Alipanah et al., 2007; Kumari et al., 2008). Nonetheless, there is another study in which AA genotype was not reported (Ladani et al., 2003a).

It has been reported that Anatolian water buffalo breed is similar in appearance to Mesopotamian breeds (FAO, 2005). Accordingly, it has been previously reported that monomorphic AA genotype was found as the breed characteristic for the Murrah water buffalo breed (Biradar et al., 2014). The AA genotype was mostly obtained from water buffalo breeds in Mesopotamia and in the present study, monomorphic AA genotype was obtained from Anatolian water buffalo breed.

Conclusions

Anatolian water buffalo breed has a dairy performance with high milk and milk fat yields since BB genotype was obtained in the present study.

Acknowledgments

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