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Three Previously Unseen Genotypes Detected in IGFBP-3 Gene in Buffalo Breed

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Abstract: Anatolian water buffalo breed is Turkey's sole water buffalo breed, and their numbers steadily increased with the national "Water Buffalo Breeding by Breeders Project". This study aimed to investigate of the gene region polymorphisms (Intron-2, Exon-2/Intron-3, Exon-3) of the meat-yield-related Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) gene in Anatolian water buffaloes by TaqI, HaeIII, and MspI restriction endonucleases. The phenol/chloroform method was used for DNA isolation from 151 blood samples, and extracted DNAs were amplified by touchdown PCR using specific primers. Amplified PCR products were digested with restriction endonucleases (REs) and separated in 3% agarose gel electrophoresis (AGE), then genotypes were determined. Results revealed two genotypes [AA (98.68%) and AC (1.32%)] and two alleles [A (0.99) and C (0.01)] for the Exon-2 to Intron-3 region from HaeIII digestion. TaqI digestion of the Intron-2 region revealed three genotypes [AA (7.94%), AB (3.97%), and BB (88.10%)] and two alleles [A (0.10) and B (0.90)]. MspI digestion of the Exon-3 region revealed only the AA genotype and A allele, thus revealing monomorphism. Overall, HaeIII digestion revealed insignificant polymorphism (P>0.05), and TaqI digestion revealed significant polymorphism (P<0.001) for their respective regions. Gene polymorphisms of these regions were investigated for the first time in Anatolian water buffaloes. Additionally, three novel genotypes for the IGFBP-3 gene (one from HaeIII and two from TaqI) were determined for the first time. The novel B allele from TaqI digestion was observed to have a substantial frequency.

Keywords: *Anatolian water buffalo, HaeIII, IGFBP-3, PCR – RFLP, Sivas, Taq I.*

Mandalarda IGFBP-3 Geninde Daha Önce Görülmemiş Üç Genotip Tespit Edildi

Özet: Anadolu su mandası Türkiye'de yetiştirilen tek manda ırkıdır ve sayıları ülke genelinde "Halk Elinde Manda Yetiştiriciliği Projesi" ile artmıştır. Bu çalışmanın amacı, Anadolu Mandalarında et verimi ile ilgili olan insülin benzeri büyüme faktörü bağlayıcı protein - 3 (IGFBP-3) geninin Taq I, Hae III ve Msp I restriksiyon enzimi (RE) ile gen polimorfizmlerinin (İntron 2, Exon 2 / İntron 3, Exon 3) araştırılmasıdır. Toplam 151 kan örneğinden Fenol/Kloroform yöntemi kullanılarak DNA izolasyonu yapılmıştır ve elde edilen DNA örnekleri spesifik primerler kullanılarak Touchdown PZR ile çoğaltılmıştır. Yükseltgenen PZR ürünleri restriksiyon enzimleri ile kesilmiş, %3 agaroz jel elektroforez yönteminde ayrıştırılmış ve genotipler belirlenmiştir. Sonuç olarak, Hae III RE'nde (Exon 2 – İntron 3) AA (%98.68) ve AC (%1.32) 2 genotip ve A (0.99) ve C (0.01) 2 allel tespit edilmiştir. Taq I RE'nde (İntron 2) AA (%7.94), AB (%3.97) ve BB (%88.10) 3 genotip ve A (0.10) ve B (0.90) 2 allel ve Msp I RE'nde (Exon 3) ise sadece AA genotipi ve A alleli tespit edilmiştir. IGFBP-3 geninde Msp I enzimi monomorfik (P>0.05), Hae III enzimi polimorfik fakat önemsiz (P>0.05) bulunurken Taq 1 enzimi yönünden hem polimorfik hem de önemlilik tespit edilmiştir (P<0.001). IGFBP-3 gen bölgelerinin polimorfizmleri Anadolu mandalarında ilk kez araştırılmıştır. Ayrıca IGFBP-3 geninde diğer manda ırklarında yapılan çalışmalardan farklı olarak ilk kez üç yeni genotip (Taq I enzim kesimi sonucunda iki, Hae III enzim kesimi sonucunda bir) tespit edilmiştir. Ayrıca Taq I enzim işlemi sonucu yeni tespit edilen B allelinin frekansı diğer alleline göre çok yüksek bulunmuştur.

Anahtar Kelimeler: *Anadolu mandası, HaeIII, IGFBP-3, PZR-RFLP, Sivas, Taq I.*

Introduction

Water buffaloes naturally live in tropical and subtropical forests, water and rainfall-abundant wetlands, and marshlands. While water buffaloes are continental animals, they spend their time in mucks and river lines due to their quick dehydrating and water-dependent profiles (Hays, 2014; Michelizzi et al., 2010). The only water buffalo breed known to be bred in Turkey is the Anatolian water buffalo, a combined meat and milk-yielding breed (GDAR, 2011) classified under the river water buffaloes. The appearance of Anatolian water buffaloes was stated to be like the water buffaloes raised in the Mesopotamia region. Anatolian water buffaloes were subjected to artificial insemination in 2002 with semen imported from Italy (FAO, 2005). Migration routes of water buffaloes were stated to be from central Europe to Italy in the 6th century AD and from the Moroccan strait to Arabian-controlled Northern Africa region in the 7th century AD (Michelizzi et al., 2010). River water buffaloes, to which Anatolian water buffaloes belong, were stated to be originating from the Indian subcontinent and were domesticated 4 500 years ago (FAO, 2015).

Water buffalo meat is the capital product in Asian countries (FAO, 2005). For Turkey, water buffalo gross meat yield was 0.6% of total gross meat yield in 2021, which was calculated to be 8 424 tonnes in 2020, and 10 831 tonnes in 2021. With the effects of the "National Breeding Project of Water Buffaloes by the Breeders" in Turkey, the water buffalo counts have increased since 2010. The counts decreased only in 2021, but this decrease was coupled with increased meat production from water buffaloes (TurkStat, 2022a; TurkStat, 2022b).

It is reported that the cattle Insulin-like Growth Factor Binding Protein 3 (IGFBP-3) gene is located on bovine chromosome 4 (BTA 4) (Maciulla et al., 1997). However, no study currently indicates which chromosome contains the IGFBP-3 gene in water buffaloes. Other research on rat and human tissues identified six different IGFBP genes (IGFBP-1 to IGFBP-6). Of these six IGFBPs, the IGFBP-3 is the most abundant in human and animal serums. It is reported that the IGFBP-3 gene contains five exons and four introns (Shimasaki and Ling, 1991). It was demonstrated that there is a 93% amino acid similarity in the IGFBP-3 gene from water buffalo, cattle, and sheep species (Kumar et al., 2006).

Ramesha et al. (2015) studied the Intron-1 to Exon-2 regions of the IGFBP-3 gene in cattle and water buffaloes using the PCR-SSCP method. They found 2 SNPs in one cattle group and 3 SNPs in another, but they were seen as monomorphic in water buffaloes. The research were studied the IGFBP-3 gene in different cattle breeds by Choudhary et al. (2007), and HaeIII restriction endonuclease digestion was conducted in partial Intron-3, entire Intron-2 - Exon-3, and partial Exon-2 regions, which resulted in three genotypes (AA, AB, and BB) in two cattle breeds, and only one genotype (AA) in a cattle breed. These results stated that a significant relationship exists between these genotypes and birth and body weights; and the authors suggested that the AB genotype has more pronounced birth and body weights.

Padma et al. (2004) conducted PCR-RFLP research in 157 Indian water buffalo samples from breeds of Murrah, Surti, Jaffarabadi, and Nagpuri, where they digested entire Exon-3 and Intron-2, and partial Exon-2 and Intron-3 regions of the IGFBP-3 gene using three different restriction endonucleases (MspI, TaqI, and HaeIII). The authors stated that all samples provided a single genotype (AA) from HaeIII (Exon-2 - Intron-3), TaqI (Intron-2), and MspI (Exon-3) digestions.

Othman et al. (2014) investigated polymorphisms in the Exon-2 - Intron-3 region of the IGFBP-3 gene in 46 samples of Egyptian cattle by using three different restriction endonucleases (MspI, TaqI, and HaeIII). Their results provided three genotypes (AA, AC, and CC) from HaeIII digestion and a single genotype from MspI and TaqI digestions. While the HaeIII digestion results of their study were the same as other results obtained in cattle, the authors preferred using C in genotype naming. Othman et al. (2018) investigated gene polymorphism of the IGFBP-3 gene in 100 samples of Egyptian water buffalo using three different restriction endonucleases (MspI, TaqI, and HaeIII) but did not obtain polymorphism from any of the three digestions.

Research is necessary for water buffaloes to reveal their yield potentials and utilization of molecular selection, which is due to the variation present in their DNA sequences. Therefore, the importance of molecular studies on yield potentials is increasing. Investigation of gene polymorphisms of the meat yield-related IGFBP-3 gene using the PCR-RFLP method in Anatolian water buffaloes that were part of the National Breeding Project of Water Buffaloes by the Breeders in Sivas province was aimed in this research.

Material and Methods

The study material was composed of 151 Anatolian water buffalo blood samples that were present in the laboratory (Animals were born between 2014 and 2015, were unrelated to each other, and were clinically healthy. Of the 151 animals, 112 were female, and 39 were male). DNA isolation from blood samples was conducted using the standard phenol/chloroform method (Sambrook et al., 1989). DNA isolated from each sample was amplified by using touchdown (TD) PCR (Don et al., 1991) protocol with the IGFBP-3-specific primer pairs (Table 1). Each PCR mixture was prepared in 25 uL volumes and contained 12.5 uL 2x PCR MasterMix (Ampliqon), 10 pM from each primer, approximately 100 ng of DNA template, and ultrapure water. Each PCR mixture was subjected to the following TD-PCR profile: (1) first denaturation at 96 °C for 10 min, 16 cycles of (2) denaturation at 96 °C for 30 sec, (3) primer annealing starting at 60 °C and decreasing by 0.5 °C at each cycle to 52 °C for optimal annealing for 45 sec, (4) extension at 72 °C for 1 min, 25 cycles of (5) denaturation at 96 °C for 30 sec, (6) primer annealing at 52 °C for 45 sec, (7) extension at 72 °C for 1 min, and (8) final extension at 72 °C for 10 min for complete adenylation. Each amplified PCR product was subjected to 2% agarose gel electrophoresis (AGE) at 100 VA for 65 min and visualized in a UV transilluminator at 365 nm wavelength.

Obtained PCR products were digested with restriction

endonucleases detailed in Table 1. Digestion mixtures were prepared in 31 uL volumes and contained in each mixture 10 uL PCR product, 1 uL respective FastDigest RE (10 U per uL), 2 uL digestion buffer solution, and 18 uL ultrapure water. Prepared digestion mixtures for HaeIII and MspI were incubated at 37 °C for 20 to 25 min, and mixtures for TaqI were incubated at 65 °C for 20 to 25 min. Digested products were separated in 3% AGE at 100 VA for 60 to 70 min and were visualized in a UV transilluminator at 365 nm wavelength. The band sizes to determine genotypes resulting from digestions are shown in Table 2.

Genotype forms and allele frequencies of the samples were determined by gene counting. Polymorphic differences were assessed by conducting a Chi-square analysis. This study was approved by the Cumhuriyet University Animal Experiments Local Ethics Committee (02.23.2016, 65202830-050.04.04-24 Number Ethics Committee Decision).

Results

According to the PCR results of the IGFBP-3 gene region amplifications, all 151 samples were used for HaeIII and MspI digestions for their respective regions, and 126 samples were used for TaqI digestions (Figure 1). HaeIII digestion was used for polymorphism investigations in Exon-2 - Intron-3 region, TaqI digestion was used for the Intron-2 region, and MspI digestion was used for the Exon-3 region (Figure 2). Observed and expected allele genotypes and frequencies were determined from the obtained polymorphisms (Table 3).

Results provided genotypes of AA (98.68%) and AC (1.32%) in the Exon-2 to Intron-3 region of the IGFBP-3 digested with HaeIII (Figure 2). The novel AC genotype and C allele (0.01) *were determined for the first time in this study.* Since there is no prior information about these genotypes, the AC genotype, which is a 655 bp band presence together with the AA genotype, *was named for the first time in this study.*

The TaqI digestion of the Intron-2 region of the IGFBP-3 gene provided the previously documented AA genotype (7.94%) but provided two novel genotypes of AB (3.97%) and BB (88.10%), which were not reported previously. Since there is no prior information about these genotypes, the genotype with 655 bp band together with 415 bp and 240 bp bands was named AB, and the genotype with only 655 bp band *was named BB for the first time in this study* (Figure 2). The novel B allele was determined to have a substantial frequency of 0.90 (Table 3).

The MspI digestion of the Exon-3 region of the IGFBP-3 gene provided only the AA genotype and was thus determined as monomorphic. The polymorphism observed in the Exon-2 - Intron-3 region (for HaeIII) was determined as statistically insignificant ($P>0.05$), but the polymorphism observed in the Intron-2 region (for TaqI) was determined as statistically significant ($P<0.001$) (Table 3).

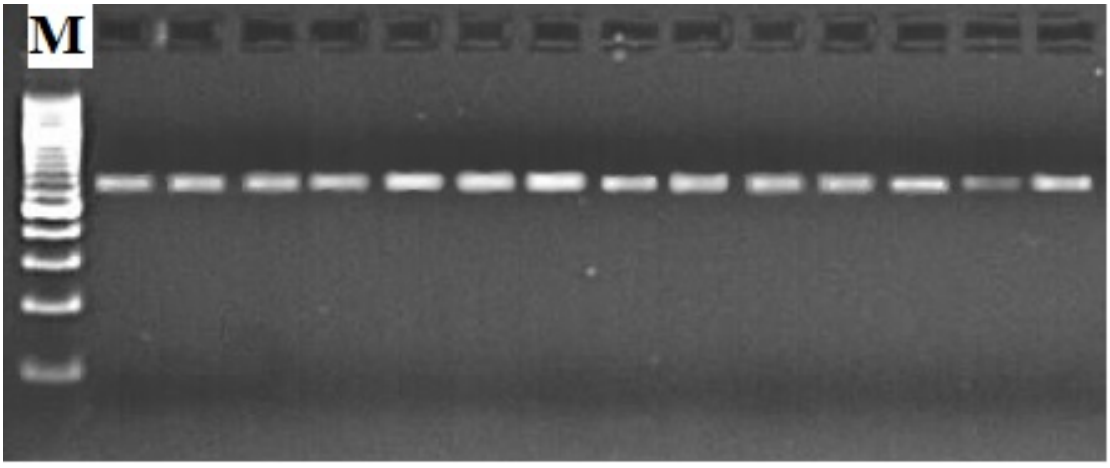

Figure 1. PCR results of the IGFBP-3 gene region (M: 100 bp DNA Ladder; Product sizes: 655 bp).

Table 1. List of the primer sequences and restriction endonucleases.

Locus	Primer sequence **(5' –> 3')**	PCR(bp)	RE	Reference
IGFBP - 3	F: 5′- CCAAGCGTGAGACAGAATAC - 3′ R: 5′- AGGAGGGATAGGAGCAAGTT - 3′	655	*HaeIII* *TaqI* *MspI*	Maciulla et al., 1997 Padma et al., 2004

F: Forward, R: Reverse, RE: restriction endonuclease, bp: base pair.

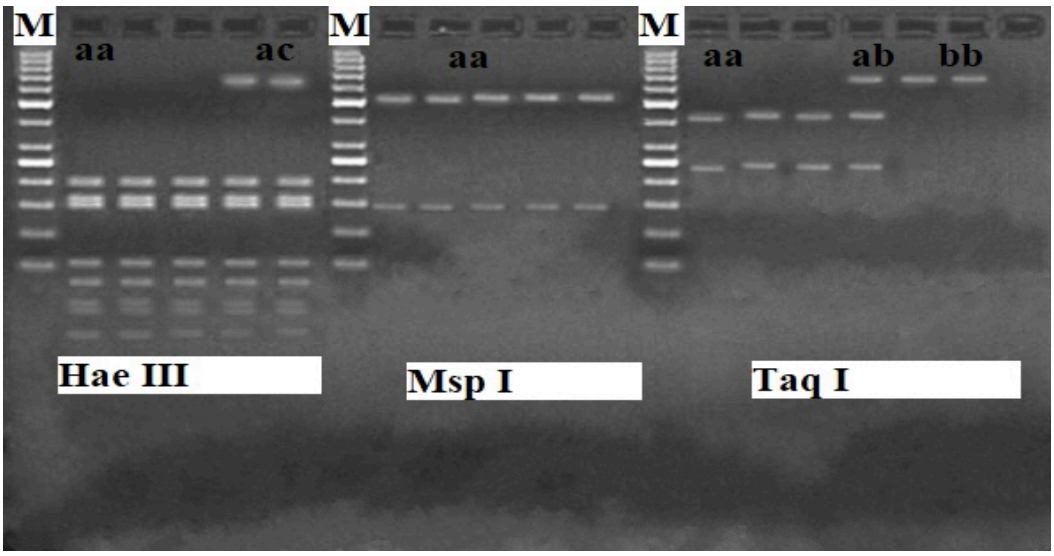

Figure 2. Restriction endonuclease digestion results of the IGFBP - 3 gene (M: 50 bp DNA Ladder).

Table 2. Obtaining of genotypes from the Insulin - like Growth Factor Binding Protein - 3 (IGFBP - 3) gene.

Gene	PCR (bp)	RE Digestion	AA (bp)	AB (bp)	BB (bp)	AC (bp)
Exon 2 - Intron 3	651 (Cattle)	HaeIII	199, 164, 154, 56, 36, 18, 16, 8	215, 199, 164, 154, 56, 36, 18, 16, 8	215, 164, 154, 56, 36, 18, 8,	
Exon 2 - Intron 3	655 (Buffalo)	*HaeIII*	**201, 165**, 154, 56, 36, **19**, 16, 8	215, **201**, **165**, 154, 56, 36, **19**, 16, 8	215, **165**, 154, 56, 36, **19**, 8,	**655, 201, 165**, 154, 56, 36, **19**, 16, 8 (in this study)
Intron 2	655	*TaqI*	415, 240	655, 415, 240 (in this study)	655 (in this study)	
Exon 3	655	*MspI*	510, 145	-	-	

RE: restriction endonuclease, bp: base pair.

Table 3. Chi - square analysis, frequencies, and significances of genotypes and alleles obtained from the Insulin - like Growth Factor Binding Protein - 3 (IGFBP - 3) gene.

		Genotype Frequency			**Allele Frequency**		**χ2**	**P – Values (df = 1)**
Gene	**n**	**AA** O (E)	**AC** O (E)	**CC** O (E)	A	C		
Exon 2 -Intron 3 (*HaeIII*)	151	149 (149.01) %98.68	2 (1.99) %1.32	-	0.99	0.01	0.007	0.9347 ns
Gene	n	**AA** O (E)	**AB** O (E)	**BB** O (E)	A	B	**χ2**	**P – Values (df = 1)**
Exon 3 (*MspI*)	151	151 (151)	-	-	1	0	0	1.00 ns
Gene	n	**AA** O (E)	**AB** O (E)	**BB** O (E)	A	B	**χ2**	**P – Values (df = 1)**
Intron 2 (*TaqI*)	126	10 (1.24) %7.94	5 (22.52) %3.97	111 (102.24) %88.10	0.10	0.90	76.26	0.0000***

O: Observed genotype; E: Expected genotype; n: Sample count; df: Degree of freedom; χ2: Chi - square value; ns: not significant (P > 0.05); ***: P < 0.001.

Discussion and Conclusion

The same primer sequences for the IGFBP-3 gene regions result in 651 bp PCR product in cattle (Maciulla et al., 1997), 655 bp product in water buffaloes (Padma et al., 2004), and 654 bp product in sheep (Kumar et al., 2006). The similarity of the IGFBP-3 gene between these three species was reported as 88.54% - 95.06% (Saleh et al., 2019).

Phylogenetic results conducted on four species (cattle, buffalo, goat, and sheep) on the IGFBP gene family summarily revealed a closer relationship between Bos taurus and Bubalus bubalis for genes IGFBP-1 to IGFBP-6 but not for IGFBP-7. Close relationship is also apparent between Capra hircus and Ovis aries, except for IGFBP-2 and IGFBP-7 genes (Rehman et al., 2022). Therefore, it is suggested that the IGFBP-3 gene region can be used as a marker for specie determination (Padma et al., 2004). Within the scope of the present study, polymorphism in three regions of the IGFBP-3 gene was investigated in Anatolian water buffaloes for the first time.

Present results provided a single genotype (AA) from MspI digestion of the Exon-3 region in all samples; however, HaeIII digestion of the Exon-2 to Intron-3 regions revealed two genotypes (*one of them is for the first time in this study*), and TaqI digestion of Intron-2 region revealed three genotypes (*two of them are for the first time in this study*) in this study (Figure 2; Table 3). Of the two polymorphic regions, only the region digested with TaqI was found to be statistically significant. The same Exon-2 - Intron-3 region digested with HaeIII in cattle was provided with three genotypes (AA, AB, BB) in two cattle breeds and a single genotype (AA) in one cattle breed (Choudhary et al., 2007). Gene region polymorphisms of the IGFBP-3 in water buffaloes (Ramesha et al., 2015; Saleh et al., 2019), Egyptian sheep (El-Hanafy and Salem, 2009; Saleh et al., 2019), and Indian sheep (Kumar et al., 2006) provided monomorphic results. A short segment of the IGFBP-3 investigated in Egyptian goats provided monomorphic results (Saleh et al., 2019), whereas Chinese goats provided polymorphic results for the same segment (Lan et al., 2007; Lan et al., 2009). Overall, the HaeIII digestion region is considered monomorphic for water buffaloes and sheep but is polymorphic for cattle with three different genotypes (Kumar et al., 2004; Saleh et al., 2019; Saleh et al., 2022). For the first time in this study, both HaeIII and TaqI digestion regions of the IGFBP-3 gene were found to be polymorphic in Anatolian water buffaloes.

The HaeIII digestion of the IGFBP-3 gene in the Anatolian Black cattle breed, which is one of the native Turkish cattle breeds, and in one culture cattle breed (Holstein-Friesian) provided monomorphic results in Anatolian Black samples, whereas Holstein-Friesian samples provided polymorphic results (Fadhıl et al., 2020). In the present study, the same gene region digested with HaeIII in Anatolian water buffaloes, a native breed for Turkey, provided polymorphic results in contrast to the Anatolian Black cattle breed.

The same regions of the IGFBP-3 gene studied in the present study were investigated previously and provided the AA genotype as monomorphic (Padma et al., 2004). The same regions of the IGFBP-3 gene were studied previously using the same REs in Egyptian cattle (Othman et al., 2014) and Egyptian water buffaloes (Othman et al., 2018), and a single genotype was obtained from both cattle and water buffaloes digested with TaqI and MspI REs. In contrast, water buffaloes were determined as monomorphic from HaeIII digestion. In the present study, more water buffalo samples were studied compared to the previous studies conducted on water buffalo breeds, and three novel genotypes, one from the HaeIII digestion region and two from the TaqI digestion region, were determined for the first time. These novel genotypes were not obtained in prior studies on different species in other countries (Ali et al., 2009; Fadhıl et al., 2020; Kumar et al., 2006; Lan et al., 2007; Ramesha et al., 2015). Since there is no prior information about these novel genotypes, naming these genotypes and alleles was done in this study for the first time. Again, in contrast to previous studies, the B allele obtained from TaqI digestion was found to have a higher frequency. It is considered that this novel allele and the genotypes might be inherent to the Anatolian water buffalo breed.

In conclusion, the gene regions of IGFBP-3 were reported typically as monomorphic in previous studies in water buffaloes. However, in the present study, three novel genotypes from HaeIII and TaqI digestion regions were obtained and subsequently named for the first time. Although the study population was limited only to Sivas province and no phenotypic analysis was conducted for the revealed polymorphisms, novel genotypes are noteworthy. To precisely determine these genotypes and upload their data to the GenBank, it is important to conduct sequence analysis on these samples. Especially for TaqI polymorphisms, further and concise research is necessary to reveal its phenotypic effects in Anatolian Water Buffalo breed.

Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Cumhuriyet University Animal Experiments Local Ethics Committee (02.23.2016, 65202830-050.04.04-24 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Similarity Rate

We declare that the similarity rate of the article is 6% (excluding abstract and references) as stated in the report uploaded to the system.

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Explanation

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Author Contributions

Motivation / Concept: YO
Design: YO
Control/Supervision: YO
Data Collection and / or Processing: YO, İB
Analysis and / or Interpretation: YO, İB
Literature Review: YO
Writing the Article: YO, İB
Critical Review: YO

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