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IDENTIFICATION OF ANATOLIAN BUFFALOES BREED IN THE PROVINCE OF TOKAT BASED ON COI (MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I) GENE REGION

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ABSTRACT

This study aimed to barcode the *COI* gene region for molecular identification and taxonomic classification of Anatolian buffaloes breed in the province of Tokat. In the study, blood samples were taken from 20 randomly selected Anatolian buffaloes breed in the Central, Turhal, and Pazar districts of Tokat. Sequence data of 709-base pair long *COI* gene region in Anatolian buffaloes were obtained. The sequences of *COI* gene region in buffalo species were analyzed in the MEGA program and a phylogenetic tree was created.

In light of the data, the intra-species genetic difference of the population in Tokat was calculated as 0.002 and the inter-species genetic distance was statistically insignificant. In addition, the inter-species distance was determined by a phylogenetic tree. As a result of the phylogenetic tree, it was confirmed that Anatolian buffaloes breed in Tokat belong to *Bubalus Bubalis* species. For five of the studied buffalo samples formed a subgroup. The other samples were divided into different branches.

In conclusion, it is thought that the study findings will significantly contribute to the literature, that DNA barcoding techniques can be used in determination the genetic characterization of Anatolian buffaloes, and that it will shed light on relevant studies to be carried out.

Keywords: *Bubalus bubalis*, Anatolian buffalo, DNA barcoding, phylogenetic tree, COI gene, molecular identification

INTRODUCTION

Buffaloes are generally raised in small-scale family-owned businesses under extensive conditions and are a species suitable for organic animal product production. Buffaloes are one of the sources of milk and red meat production in Turkey. They have many advantages such as the conversion of poor-quality raw feed into milk and meat, resistance to diseases compared to other livestock, high component content in milk, and low cholesterol and fat content in meat (Atasever and Erdem, 2008; Tamburrano *et al*., 2019) and are among the sources of living for families with low-income levels living in rural areas in Turkey. Anatolian buffaloes bred in Turkey originated

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from the Anatolian buffaloes, a subgroup of water buffaloes. Until today, since phenotypic and parental information has been used to estimate the individual breeding values of animals in breeding studies, the selection of breeding animals has been made according to the breeding values estimated based on phenotypic data. Pedigree information has been utilized to estimate the individual breeding values of farm animals; therefore, the individual performance records of the animals have constituted the source of the estimated breeding value in terms of the trait of interest in the herd or herds (Walsh, 2000). Significant improvements have been achieved as a result of many years of pedigree and performance-based selection studies that have been carried out to improve some yield traits until today.

Since many genes are effective in the formation of quantitative traits and the individual performances of animals, that is, their phenotypes do not fully reflect their genotypes, breeding value predictions based on phenotypic performance remain inadequate in some cases. Thus, it has become necessary to develop and utilize more accurate and reliable methods to estimate the breeding value for any quantitative trait (Ibtisham *et al*., 2017). The advances in molecular biology have led to new approaches in livestock breeding (Özcan *et al*., 2004). Integrating individual animal performance records and molecular genetic methods will increase success in the identification of animals to be selected for breeding. Anatolian buffaloes, which are one of the important gene sources, have been taken under protection by the public under the coordination of the Directorate General of Agricultural Research and Policies, and breeding studies have been initiated to increase their yield. With these studies, the number of Anatolian buffaloes, which are widely bred in the Black Sea, Aegean, Central Anatolia, Eastern Anatolia, and Southeastern Anatolia and Marmara regions of Turkey, reached 171,835 (Şahin *et al*., 2023). It is thought that integration of these studies with studies conducted based on molecular methods will be important in terms of the conservation and breeding strategies to be implemented and will increase the success of selection studies. The extinction of species that are indigenous gene sources may also lead to the extinction of species-specific traits. To avoid such a situation, species-specific genetic information should be identified, and the relevant species should be protected (Priyono *et al*., 2018). Despite many years of molecular studies, there are gaps in the identification of species.

Since it has become more and more difficult to determine intra- and inter-species relations based on morphological traits, phylogenetic trees formed by molecular traits play an important role in filiation. Today, the use of genetic barcoding has become necessary for the identification of animal species and the origin of animal-derived foods such as milk and meat (Haider *et al*., 2012; Spychaj *et al*., 2016; Hassan *et al*., 2018). The term DNA barcoding has been widely used in the literature and the method is based on the use of a standardized region of mitochondrial 600 to 700 base pair-long DNA for rapid, accurate, and automated species identification (Hebert and Gregory, 2005). The DNA barcoding method reveals species-specific DNA profiles and has played a significant role in the definition of species through DNA sequences, which act as “barcodes” and play a key role in eliminating the deficiency in this field. In the last century, advances in DNA sequencing technology have enabled variations in short sequences of DNA to be used as markers for species in the process of DNA barcoding. DNA barcoding enables efficient

identification of a known species. In many studies, it has been reported that an appropriate barcode is usually 600-800 base pairs long in animals (Hebert *et al*., 2003a; Kress *et al*., 2005). For this purpose, the Consortium for the Barcode of Life (CBOL), which goals to provide such a DNA barcode for each species, was established (Hebert *et al*., 2003b; Waugh, 2007). Chloroplast DNA is usually used for this in plants, whereas mitochondrial DNA is utilized in animals. In animals, mitochondrial DNA (mtDNA), known as a single double-stranded circular molecule, contains 13 protein-coding genes, 2 ribosomal genes, a non-protein-coding control region, and several tRNAs (Waugh, 2007). One of the most widely used mitochondrial DNA markers for the identification of taxonomic parts is the cytochrome C oxidase subunit I (COI) gene marker (Priyono *et al.,* 2018). In many studies, it has been reported that the utilization of the COI gene as a DNA barcode marker is the correct method for species identification and phylogenetic interpretations (Hebert *et al*., 2003a). The COI gene is usually present in all eukaryotes and has an important function in metabolism. COI is one of the most conserved mitochondrial protein-encoding genes in animals and is preferred in molecular barcoding studies as it provides a clear distinction between species even though it does not show variation within species (Priyono *et al*., 2018; Saputra *et al*., 2014). In this study, blood samples were collected from 20 Anatolian buffaloes breed in the Central, Turhal, and Pazar districts of Tokat, which is one of the provinces where Anatolian buffaloes are widely bred in Turkey. The sequence of the COI gene region, which is a fragment of mtDNA, was identified and barcoded, and the phylogenetic relationship of the region was evaluated. Thus, genetic similarities and differences within the species in the population in this region were revealed, and the results of the research will significantly contribute to the databases and literature.

MATERIALS AND METHODS

Sample collection

In this study, blood samples collected from 20 male Anatolian buffaloes breed in the province of Tokat and its districts (Central, Turhal, and Pazar) were used as materials to examine the barcoding of the mitochondrial protein-encoding gene region of the Cytochrome Oxidase Subunit I (COI) gene. The buffaloes from which the blood samples were collected were randomly selected.

DNA Extraction

GeneJET Genomic DNA Purification kit was used for DNA isolation. The DNA samples were stored at -20°C until amplification with Polymerase Chain Reaction (PCR). The quality of the DNA samples to be used was tried by running on a 1% agarose gel.

PCR procedure

The gene region used for DNA barcoding, Cytochrome Oxidase Subunit I (*COI*), was selected as it is one of the mitochondrial protein-coding genes. The sequences of primers for this gene sequence were taken from the study conducted by Hassan *et al.* (2018). Detailed information about the primers is given in Table 1.

For PCR amplification of the 709 bp *COI* gene region, a total volume of 40 µl of PCR mixture was prepared with 2 µl genomic DNA (100 ng), 5X HOT FIREPol Blend Master Mix (15 mM $MgCI_2$), 10 µM of each primer, and water.

The PCR mix prepared to amplify the COI

gene region was used in a thermal cycler (TC96-CM-10, MultiGene Gradient Thermal Cycler). The temperature conditions required for each PCR step are shown in Table 2.

Imaging of PCR Products

For imaging of PCR products, the samples were run on agarose gel electrophoresis. For this purpose, 5 µl of PCR product was loaded sequentially into the wells in 2% TAE gel. GeneRuler 1 kb DNA ladder was used to determine whether the products were obtained at the desired length. The ladder (5 µl) was loaded into the middle well between the samples The samples on the gel were run for 1 hour by applying a voltage of 95 volts and were imaged using the MiniLumi (Bio-Imaging Systems) device in Kırşehir Ahi Evran University Faculty of Agriculture, the laboratory of the Department of Agricultural Biotechnology DNA Sequencing and Alignment.

Purification and bidirectional sequencing of 35 µl of product amplified with the PCR method were made by BM Labosis service provider. Sequencing results were delivered in ab.1-format files. Sequencing results were checked in the Finch TV program. After the control, all sequences were imported into the MEGA 7 program and their forward and reverse sequences were aligned. Segments of the sequences that were defective during the alignment process were removed and corrected in the MEGA 7 program, and after the controls, data analysis was initiated. The sequences of the reference gene region with accession number KU932121.1 in the NCBI gene bank and the sequences of the relevant gene region of buffalo and buffalo-related species in NCBI (Table 3) were imported into the MEGA 7 program in fasta format and aligned. The aligned and corrected files were saved in the appropriate formats.

Phylogenetic Analyses

Statistical parameters (GC% (Guanine-Cytosine) content, number of conserved base pairs, number of diverse base pairs, number of base pairs of sequences with parsimony information, number of sequences with a single base change, and average genetic distance between species) of the aligned sequences were calculated in the MEGA 7 program. To show the inter-species distance, the phylogenetic tree was created using the Jukes-Cantor model in the MEGA 7 package program. In addition, the phylogenetic tree was drawn using the bootstrap method in 1000 repetitions, the ML (Maximum Likelihood) method, and Neighbor-Join and BioNJ algorithms to show inter-species distance.

RESULTS AND DISCUSSIONS

Amplification of PCR products

As a result of the molecular study, PCR products were successfully obtained from the DNA samples of 20 Anatolian buffaloes. The 709 bp bands belonging to the COI gene region were obtained from all 20 samples (Figure 1).

Sequencing and statistical analysis

The sequencing data of PCR products were obtained for each sample because of bidirectional reading. Necessary corrections were made for the statistical parameters of 621 bp-long sequence of the COI gene region of 20 Anatolian buffaloes bred in the province of Tokat in the MEGA 7 and the parameter values are presented in Table 4. According to these statistical data, while 615 base pairs constituted the conserved sequences, 4 base pairs were found to contain diversity. Two of the diversities were due to single

nucleotide substitutions and 3 of them were due to base sequences with parsimony information. The G-C ratio of the COI gene region was 45.2%, indicating that the region was correctly amplified. The average genetic difference between the species studied in the research was 0.002, indicating that the genetic distance between the species was small.

Intra-species genetic distance was calculated in the MEGA 7 program and shown in Table 5. The intra-species genetic distance of Anatolian buffaloes bred in Tokat was close. When genetic distances were analyzed, it was found that the most distant species were between buffalo 14 and those numbered 2, 4, 6, 10, and 12 (0.007) and the closest distance was 0.00 between many breeds.

For a clearer representation of the distance between species in phylogenetic tree construction, the species in Table 3, which are close to the species, were also included in the study as outgroups. The phylogenetic tree was drawn using the Maximum Likelihood method and Jukes-Cantor parameters in the MEGA 7 program and is shown in Figure 2. The tree was organized as two main groups, *Bos taurus* (cattle) and *Bubalus* (buffalo) species. Within the group of *Bubalus* species, *Bubalus carabenensis* was separated as a subgroup, while all other *Bubalus bubalis* species were grouped into one. Among the *Bubalus bubalis* species studied, buffalo samples 2, 4, 6, 10, 12, and 15 formed a distinct subgroup from the other studied species (Bootstrap value: 90). Among the species in this subgroup, species 15 was separated from buffaloes 2, 4, 6, 10, and 12 with a bootstrap value of 56 (statistically, it can be said that this is due to the number of base pairs with 2 variations and the number of sequences containing single base substitutions). The closest buffaloes based on the COI gene region studied were buffaloes 2, 4, 6, 10, and 12.

In the study, it was observed that the barcode region of the COI gene in Anatolian buffaloes was 621 bp-long in total. As an end of the statistical analysis, it was detected that the region containing parsimony information was 3 bp long. As this information is a criterion for measuring genetic distances, it shows that the species are very close to each other according to the study on Anatolian buffaloes.

The closest intra-species genetic distance was 0.000 and the largest genetic distance was 0.007. It was determined that the genetic closeness between the species was very high. A similar result was obtained in a study (Priyono *et al*., 2018) in which the COI gene region of 10 Anoa buffaloes was barcoded. The same researchers found that the largest genetic distance was 0.039. It was determined that the finding of this study in terms of genetic distance and the genetic distance between species determined by Priyono *et al.* (2018) was similar.

A phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Maximum Composite Likelihood (MCL) methods for the barcoding of COI in river and swamp buffaloes (Hassan *et al*., 2018) in Egypt. According to the phylogenetic tree, there were two sub-groups: *Bubalus carabensis* (swamp buffalo) and *Bubalus bubalis* (river buffalo). In this study, the phylogenetic tree drawn using the ML method and JC parameters was consistent with the tree obtained by Hassan *et al.* (2018). Similarly, the tree of the COI gene region of swamp buffaloes, including an outgroup of river buffalo, drawn using the Neighbor-Joining Method and the Kimura-2 parameter model (Saputra and Sumantri, 2014), supports the finding of the present study.

In the tree drawn by Priyono *et al.* (2018)

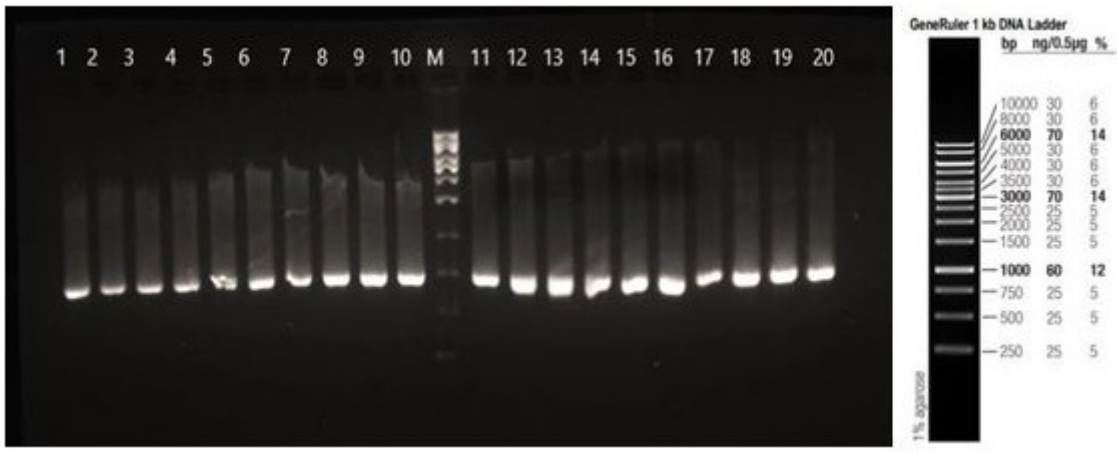

Figure 1. The image of the PCR product of the COI gene (709 bp) region of buffalo DNA on agarose gel.

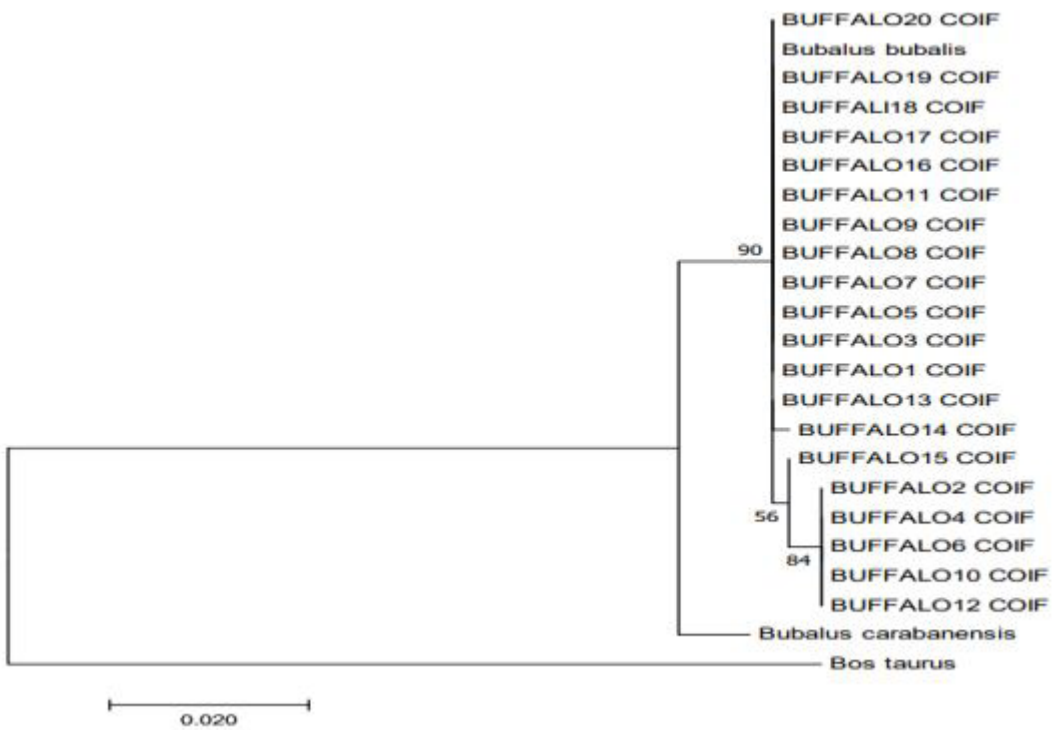

Figure 2. According to the Juke Conter model, a phylogenetic tree was drawn using the Maximum Likelihood method and bootstrap method. The analysis involved 23 species (20 studied and 3 outgroups). All analyses were performed in the MEGA 7 program.

Table 1. Primers to be utilized for the COI gene region and their lengths (Hassan *et al*., 2018).

Primer name	Sequence	Length of regions
COI (Forward)	5′-TCTCAACCAACCATAAAGATATCGG-3′	709 bp
COI (Revers)	5′-TATACTTCAGGGTGTCCGAAGAATCA-3′	

Table 2. Temperature cycles of COI gene in PCR reaction.

PCR Stages	Temperature	Duration	Cycle
Pre-denaturation	:95 ℃	4 minnutes	1
Denaturation	:95 ℃	1 minute	35
Annealing	:54 ℃	30 seconds	
Extension	:72 ℃	1 minute	
Final Extension	:72 ℃	10 minnutes	1

Table 3. Accession information on buffalo and buffalo-related species in NCBI.

Species	Acession numbers	References
Bos taurus	HM102289.1	Cooper *et al*., 2007
Bubalus bubalis	KU932121.1	Hassan *et al*., 2018
Bubalus carabanensis	KF714291.1	Saputra *et al*., 2014

Table 4. Parameter values for Anatolian buffaloes.

Parameters	Values
Number of base pairs conserved (C)	615/621
Number of base pairs showing diversity	4/621
Number of bases with parsimony information (Pi)	3/621
Number of bases with a single base substitution (S)	1/621
Average G+C content (%)	45.2
Average intra-species genetic distance	0.002

Table 5. Genetic distance values between Anatolian buffalo species.

Genetic distances between Anatolian buffalo breeds	BUFFALO1_COIF	BUFFALO2_COIF	BUFFALO3_COIF	BUFFALO4_COIF	BUFFALO5_COIF	BUFFALO6_COIF
BUFFALO1_COIF						
BUFFALO2_COIF	0.005					
BUFFALO3_COIF	0.000	0.005				
BUFFALO4_COIF	0.005	0.000	0.005			
BUFFALO5_COIF	0.000	0.005	0.000	0.005		
BUFFALO6_COIF	0.005	0.000	0.005	0.000	0.005	
BUFFALO7_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO8_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO9_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO10_COIF	0.005	0.000	0.005	0.000	0.005	0.000
BUFFALO11_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO12_COIF	0.005	0.000	0.005	0.000	0.005	0.000
BUFFALO13_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO14_COIF	0.002	0.007	0.002	0.007	0.002	0.007
BUFFALO15_COIF	0.002	0.003	0.002	0.003	0.002	0.003
BUFFALO16_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO17_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALI18_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO19_COIF	0.000	0.005	0.000	0.005	0.000	0.005
BUFFALO20_COIF	0.000	0.005	0.000	0.005	0.000	0.005

Table 5. Genetic distance values between Anatolian buffalo species. (Continue).

Genetic distances between Anatolian buffalo breeds	BUFFALO7_COIF	BUFFALO8_COIF	BUFFALO9_COIF	BUFFALO10_COIF	BUFFALO11_COIF	BUFFALO12_COIF
BUFFALO1_COIF						
BUFFALO2_COIF						
BUFFALO3_COIF						
BUFFALO4_COIF						
BUFFALO5_COIF						
BUFFALO6_COIF						
BUFFALO7_COIF						
BUFFALO8_COIF	0.000					
BUFFALO9_COIF	0.000	0.000				
BUFFALO10_COIF	0.005	0.005	0.005			
BUFFALO11_COIF	0.000	0.000	0.000	0.005		
BUFFALO12_COIF	0.005	0.005	0.005	0.000	0.005	
BUFFALO13_COIF	0.000	0.000	0.000	0.005	0.000	0.005
BUFFALO14_COIF	0.002	0.002	0.002	0.007	0.002	0.007
BUFFALO15_COIF	0.002	0.002	0.002	0.003	0.002	0.003
BUFFALO16_COIF	0.000	0.000	0.000	0.005	0.000	0.005
BUFFALO17_COIF	0.000	0.000	0.000	0.005	0.000	0.005
BUFFALI18_COIF	0.000	0.000	0.000	0.005	0.000	0.005
BUFFALO19_COIF	0.000	0.000	0.000	0.005	0.000	0.005
BUFFALO20_COIF	0.000	0.000	0.000	0.005	0.000	0.005

Table 5. Genetic distance values between Anatolian buffalo species. (Continue).

Genetic distances between Anatolian buffalo breeds	BUFFALO13_COIF	BUFFALO14_COIF	BUFFALO15_COIF	BUFFALO16_COIF	BUFFALO17_COIF	BUFFALI18_COIF
BUFFALO1_COIF						
BUFFALO2_COIF						
BUFFALO3_COIF						
BUFFALO4_COIF						
BUFFALO5_COIF						
BUFFALO6_COIF						
BUFFALO7_COIF						
BUFFALO8_COIF						
BUFFALO9_COIF						
BUFFALO10_COIF						
BUFFALO11_COIF						
BUFFALO12_COIF						
BUFFALO13_COIF						
BUFFALO14_COIF	0.002					
BUFFALO15_COIF	0.002	0.003				
BUFFALO16_COIF	0.000	0.002	0.002			
BUFFALO17_COIF	0.000	0.002	0.002	0.000		
BUFFALI18_COIF	0.000	0.002	0.002	0.000	0.000	
BUFFALO19_COIF	0.000	0.002	0.002	0.000	0.000	0.000
BUFFALO20_COIF	0.000	0.002	0.002	0.000	0.000	0.000

Table 5. Genetic distance values between Anatolian buffalo species. (Continue).

Genetic distances between Anatolian buffalo breeds	BUFFALO19_COIF	BUFFALO20_COIF
BUFFALO1_COIF		
BUFFALO2_COIF		
BUFFALO3_COIF		
BUFFALO4_COIF		
BUFFALO5_COIF		
BUFFALO6_COIF		
BUFFALO7_COIF		
BUFFALO8_COIF		
BUFFALO9_COIF		
BUFFALO10_COIF		
BUFFALO11_COIF		
BUFFALO12_COIF		
BUFFALO13_COIF		
BUFFALO14_COIF		
BUFFALO15_COIF		
BUFFALO16_COIF		
BUFFALO17_COIF		
BUFFALI18_COIF		
BUFFALO19_COIF		
BUFFALO20_COIF	0.000	

based on the 681 bp-region according to the Bootstrap value with 1000 repetitions using the Neighbor-Joining method, among Anoa species (Mountain anoa and Lowland anoa), *Bubalis bubalus* (river buffalo) was grouped in the tree separately from the Anoa species. In the relevant study, it was concluded that the buffaloes were separated as mountain and lowland subgroups, that the genetic distance was close, and that the river buffalo was separated as a different group from the two Anoa species.

CONCLUSION

In the study, barcoding of the COI gene region was performed in Anatolian buffaloes raised in the Central, Turhal, and Pazar districts of the province of Tokat. As a result, intra-species and inter-species genetic distance was determined and it was concluded that barcoding is important for the identification of species. The distance of species to each other was determined by creating a phylogenetic tree.

In the study, it was thought that the number of samples taken was effective in the determined very close genetic distance between the species. Since the research involves the DNA barcoding method in Anatolian buffalo herds raised in the province of Tokat for the first time, it is thought that it will contribute to the future breeding studies to be planned and carried out on this species.

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