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Genotyping of prolactin, kappa casein, and pituitary transcription factor 1 genes of the Anatolian water buffalo population in the Kızılırmak Delta

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Abstract: Thirty-five Anatolian water buffalo were selected from among a total of almost 7000 animals from 11 herds in the Kızılırmak Delta, which is known to be one of the most important areas for water buffalo farming in Turkey. The cleaved amplified polymorphic sequences (CAPS) method was used to identify single-nucleotide polymorphisms at 3 loci that are associated with milk production, namely *PRL*, *CSN3*, and *PIT-1*, which are associated with milk processing and milk yields, respectively. Sequence-tagged sites of *PRL*, *CSN3*, and *PIT-1* were digested by restriction endonucleases *Rsa*I, *Hind*III, and *Hinf*I, respectively. Capillary electrophoretic analysis identified all samples as belonging to the GG and BB genotypes, which are undesirable genotypes for *PRL* and *PIT-1* gene loci, but ideal for the *CSN3* locus. Then the samples were sequenced by an ABI 3730 DNA Analyzer (Applied Biosystems). DNA sequencing of the amplified *CSN3* fragment revealed one polymorphism at codon 135 (ThrACC > IleATC) in some samples. A homozygous structure was found for all three loci and the huge population loss between 1991 and 2008 suggests that a genetic bottleneck may have occurred in the population of Anatolian water buffalo in the Kızılırmak Delta.

Key words: Water buffalo, genetics, milk, DNA sequence

1. Introduction

The most significant wetland along the Black Sea coast of Turkey, the Kızılırmak Delta encompasses 56,000 ha within the districts of Ondokuzmayıs, Bafra, and Alaçam in the Central Black Sea province of Samsun [1]. The delta, which has broadly preserved its natural structure, contains endangered habitats such as trees and shrubs, fresh water and salt water lakes, fresh water swamp forests, reeds, pastures, sand dunes, sea, and farmlands. The water buffalo is one of the most influential members of the wetland ecosystem. The Anatolian water buffalo is a member of the river buffalo species (*Bubalus bubalis*), one of two species of domesticated buffalo, and has been bred in Turkey for over 1000 years [2,3]. Anatolian water buffalo are well adapted to the hot and humid climate of the Kızılırmak Delta and their presence is vital for rangeland vegetation, bird and fish species, and lake sedimentation. They are also an essential element of the regional economy, with many delta residents earning their livelihood by nature-dependent production of Anatolian water buffalo [4] bred principally for milk, kaymak (a type of cream traditionally made from buffalo milk), yogurt, and cheese.

The population decreased from 42,467 head in 1991 to only 8515 in 2008, with the majority of the population located in the Kızılırmak Delta (Figure 1). By 2017, the population in Samsun had increased to 17,944, the largest water buffalo population of any province in Turkey, which had a total of 142,073 head [5].

Anatolian water buffalo have been living in ecology-dependent conditions and under the stress of natural selection for centuries. Thus, these unique populations may be useful in the future for traits difficult to define today, e.g., disease and stress resistance, quality and composition of products, or adaptation to different environments [6]. Buffalo milk is rich in both fat and protein, making it economically more valuable than the milk of other dairy species.

Previous studies showed slight negative genetic correlations between milk yield, protein, and fat percentages, indicating that yields could be increased through selection without significant decreases in quality [7]. As with all dairy species, buffalo milk yield and characteristics are affected by numerous genes, the analysis of which can support breeding programs designed to improve milk production. Recent projects in the Kızılırmak Delta relied

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Figure 1. Water buffalo livestock in Samsun between 1991 and 2016 [5].

on pedigree records for the selection of water buffalo breeding stock [8]. However, without molecular data on specific yield-related genes, selection and breeding have failed to improve milk production.

Prolactin is a pleiotropic polypeptide hormone that is synthesized and secreted from lactotrophic cells of the anterior pituitary gland of bovines and other vertebrates that are active in lactation and reproduction [9]. The prolactin gene (*PRL*) plays a crucial role in the development of the mammary gland, in signal transduction during the process of milk production, and in preservation of the expression of milk protein genes [10,11]. Previous studies reported that *PRL* has a considerable impact on milk yield and quality in dairy breeds. The single-nucleotide polymorphisms (SNPs) that occur in this gene affect the chemical structure of the milk and can be used as a useful tool for the selection of animals with high milk yield characteristics in breeding.

PRL was mapped and found on bovine chromosome 23 (NCBI AC_000180.1). The entire gene was 9388 bp in length, with five exons and four introns, and it encodes 199 amino acids. In some studies, the genetic polymorphism of bovine *PRL* was screened and more than 20 SNPs were reported [9,10]. Although most of the identified SNPs are silent mutations and/or introns, the most important SNP affects the 4th exon and converts the guanine base to adenine at nucleotide position 8398. This SNP can be detected by the restriction endonuclease *Rsa*I [9–11].

Casein is a mammary gland secretion that affects milk, cheese, and butter quality in a number of ways, mainly through the formation of casein micelles, which control the stabilization of secondary milk products [12]. It accounts of 80% of milk protein and it has important roles in milk quality, cheese, and butter formation and the coagulation, stabilization, and collection of casein micelles [9, 13, 14]. Casein consists of four main proteins: CSN1S1 (α-s1), CSN1S2 (α-s2), CSN2 (β-), and CSN3 (κ-casein) [15,16]. The kappa casein gene (*CSN3*) has a crucial role in the quality and coagulation of milk and also in the formation, stabilization, and aggregation of casein micelles. Therefore, genetic variants of *CSN3* are associated with the protein content of milk, cheese yield, and frequency of yield and have a significant impact on clotting time. The great effect of *CSN3* on milk production led to numerous studies on this gene region, such as in cattle, goats, sheep, and buffalo [11,17,18]. The bovine *CSN3* is located on chromosome 6 (6q31) and its total length is 13 kb. It contains 5 exons and 4 introns and most of the mature protein-coding sequences are in the 4th exon [9]. Fourteen variants (A, B, B2, C, D, E, F1, F2, G1, G2, H, I, J, and A1) of *CSN3* have been identified in studies conducted to date in cattle. The most commonly described variants are *CSN3A* and *CSN3B*. They can be distinguished by differences in the structure of the primary protein [9,15,19,20].

These alleles are separated by differences in the two amino acid substitutions of the mature protein. At the *CSN3* locus (codon 136), the CSN3A allele has an ACC codon responsible for the synthesis of threonine amino acid (Thr), whereas the CSN3B allele has a C-to-T mutation in the same position (ACC→ATC) that produces an isoleucine amino acid (Ile) [16,21].

At the *CSN3* locus (codon 148), CSN3A has a GAT codon responsible for the synthesis of aspartic acid (Asp), whereas CSN3B has an A-to-C mutation in the same position (GAT→GCT) that produces an alanine amino acid (Ala). This mutation can be detected by the restriction endonuclease *Hind*III [16,21,22].

The *CSN3* in water buffalo was mapped on chromosome 7 and contained five exons and 4 introns [12]. The length of *CSN3* is 14.7 kb with 1074 bp upstream and 476 bp downstream (GenBank: AM900443.1). In studies related to water buffalo it was reported that *CSN3* and 4 SNPs are responsible for amino acid differences and that there are 2 SNPs at exon 2: codon 4 has two nucleotide variants (ArgAGG > SerAGT) and codon 8 has two nucleotide variants (ValGTT > GlyGGT) [20]. There are also 2 SNPs at exon 4: codon 135 has two nucleotide variants (ThrACC > IleATC) and codon 136 has two nucleotide variants (ThrACC > ThrACT). However, a mutation (third base position-silent mutation) at codon 136 is neutral [12]. CSN3A has threonine at both codons 135 and 136, whereas CSN3B has isoleucine and threonine at codons 135 and 136, respectively [12,16,19].

Pituitary-specific transcription factor 1 (*PIT-1*) is responsible for pituitary development and hormone production in mammals [11,23]. It is a gene that regulates growth hormone and *PRL* expression and plays a role in the expression of hormone-specific activators in somatotropic and lactotrophic cells [24]. It regulates the expression of the prolactin gene, growth hormone gene, thyroid stimulation hormone b-subunit, growth hormone-releasing hormone receptor genes, and the *PIT-1* gene itself. *PIT-1* is a candidate genetic marker for growth, carcass, and milk yield characteristics [9,11].

Bovine PIT-1, a 291 amino acid protein with a DNA-binding POU domain, was mapped onto chromosome 1 [11,23]. Several similar polymorphisms in *PRL* and *CSN3* were detected in the bovine *PIT-1* locus. *PIT-1* polymorphism was found to be related to milk yield and milk conformation characteristics in cattle. A SNP on *PIT-1* exon 6 has an A-to-G mutation that can be characterized by the restriction endonuclease *Hinf*I. This polymorphism is a silent mutation and was used to detect alleles PIT-1A and PIT-1B [11].

The present study used the cleaved amplified polymorphic sequences (CAPS) method to identify SNPs at 3 sequence-tagged site (STS) loci of the *PRL*, *CSN3* and *PIT-1* genes in an Anatolian water buffalo population in the Kızılırmak Delta. The aim of the study was to provide a molecular characterization of the population in order to support the development of breeding programs geared to improve milk yield and processing.

2. Materials and methods

Sterile tubes containing anticoagulant K_3EDTA were used to collect blood specimens from 35 Anatolian water buffalo of the same age (3–4 months) selected from among a total of almost 7000 animals from 11 herds in the Kızılırmak Delta (Figure 2).

Genomic DNA was isolated using a peqGOLD Blood DNA Extraction kit (Peqlab, Germany) in accordance with the instructions provided. DNA quality and quantity were checked using a NanoDrop spectrophotometer (Thermo, USA). Samples with OD ratios between 1.7 and 1.9 were used for polymerase chain reactions. Due to difficulties in DNA extraction and gene amplification, genotyping could be performed only on samples from 18 male and 17 female water buffalo. Polymerase chain reaction (PCR) was performed according to Othman et al. [11] using the following primers of the genes: A and G alleles for *PRL*: 5'–CCAAATCCACTGAATTATGCTT–3' (PRL-F), 5'–ACAGAAATCACCTCTCTCATTCA–3' (PRL-R); A and B alleles for *CSN3*: 5'–ATAGCCAAATATATCCCAATTCAGT–3' (*CSN3*-F), 5'–TTTATTAATAAGTCCATGAATCTTG–3' (*CSN3*-R); A and B alleles for *PIT-1*: 5'–AACCATCATCTCCCTTCTT–3' (*PIT-1*-F), 5'–AATGTACAATGTGCCTTCTGAG–3' (*PIT-1*-R). PCR amplification was performed in a thermocycler (Bio-Rad, USA) with a total volume of 20 µL comprising 40 ng DNA, 1.25 µM primer, 4 µL master mix (Promega, USA), and ultrapure water, as follows: initial denaturation for 4 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 56–58 °C, and 2 min at 72 °C, with a final extension of 10 min at 72 °C. Amplification products were loaded on 2% agarose for visualization and verification. PCR products were digested with FastDigest restriction endonucleases (Thermo, USA) *Rsa*I, *Hind*III, and *Hinf*I for *PRL*, *CSN3*, and *PIT-1*, respectively (total volume of 20 µL each). Reactions were incubated at 37 °C for 5–10 min and stopped by heat inactivation at 65 °C for 10 min. Genotyping of alleles was performed according to the fragment size, which was analyzed using an automatic microcapillary electrophoresis system (Qsep100 DNA Analyzer, Bioptic Inc., Taiwan; 300 s running time, 6 kW voltage, 0.5 mm capillary diameter). Sequencing analysis was performed by selecting two samples for each gene based on fragment results. PCR products of the samples were sequenced by the ABI 3730 DNA Analyzer (Applied Biosystem).

3. Results and discussion

A 320-bp STS fragment of *PRL* was amplified by PCR. Amplicons were used to digest *Rsa*I but no digestion occurred at the STS of the *PRL* locus. This indicated that all samples belonged to the GG genotype at the *PRL* STS locus (Figure 3a).

Similarly, for genotyping of STS of *CSN3*, a 556-bp fragment was amplified by PCR. Amplicons were digested using *Hind*III and two fragments were detected, namely a 406-bp and a 150-bp allele, indicating that all samples belonged to the BB genotype at the STS of the *CSN3* locus (Figure 3b).

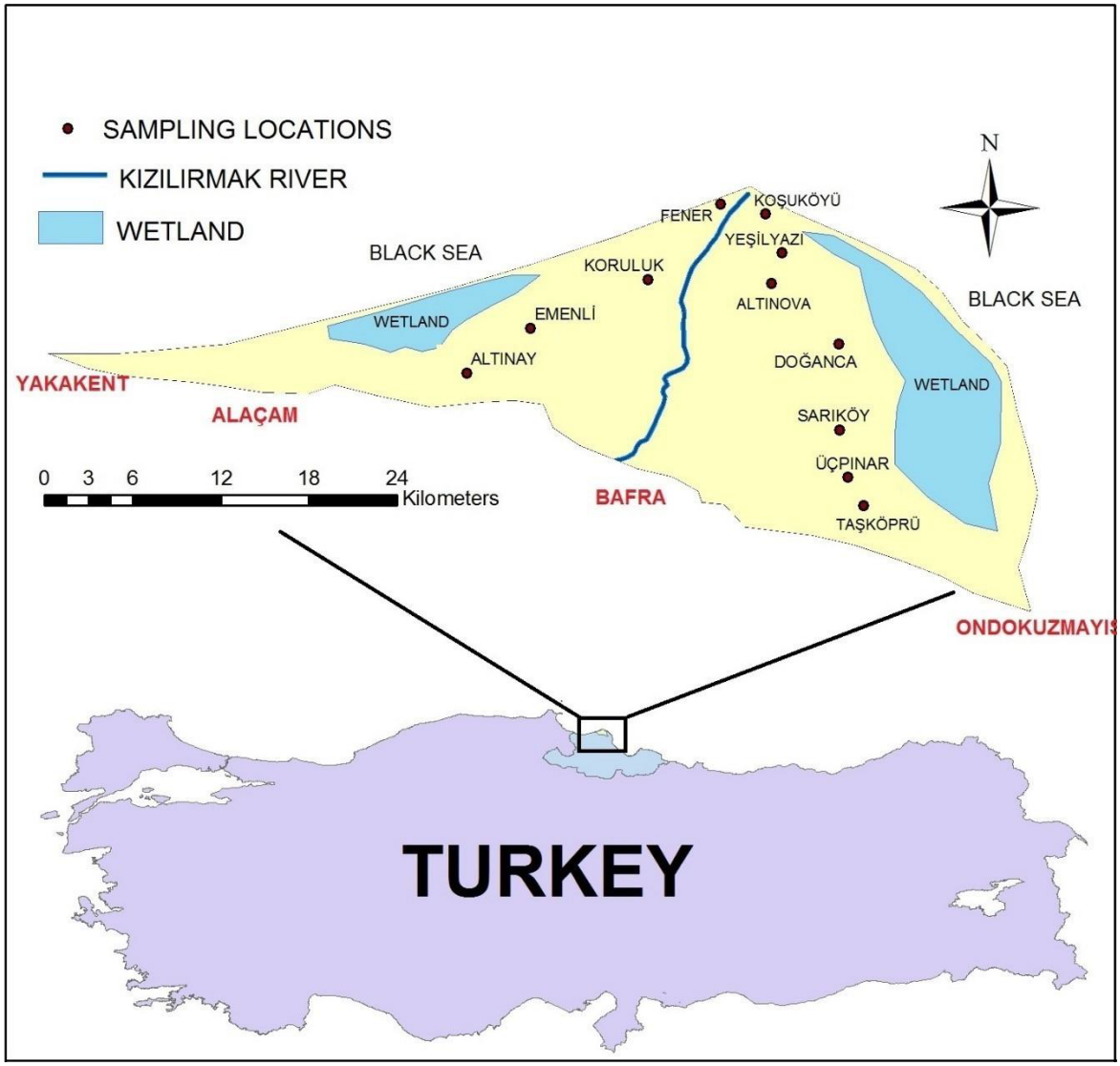

Figure 2. Sampling locations of Anatolian water buffalo in the Kızılırmak Delta.

A 447-bp STS fragment of *PIT-1* was amplified by PCR. Then PCR amplicons were used to digest *Hinf*I and two fragments were detected, namely a 244-bp and a 203-bp allele, indicating that all samples belonged to the BB genotype at the STS of *PIT-1* locus (Figure 3c).

On the other hand, sequencing outputs of each sample were compatible with the result of CAPS and microcapillary electrophoresis. Previous studies [9,11] reported *PRL*, a 294-bp undigested fragment in the GG homozygous genotype, as two digested fragments of 162 bp and 132 bp in the AA homozygous genotype and as three fragments (one undigested 294-bp fragment and two digested 162-bp and 132-bp fragments) in the AG heterozygous genotype. The same authors reported that the AA homozygous genotype had the highest milk yield, whereas yields of the GG homozygous genotype were considered insufficient for milk and milk protein production. This study found that *Rsa*I detected no SNPs for *PRL* in any of the examined samples, indicating that all samples belonged to the GG homozygous genotype.

CSN3 was previously reported to present as a 530-bp undigested fragment in the AA homozygous genotype, as two digested fragments (370 bp and 160 bp) in the BB homozygous genotype, and as three fragments (undigested 530 bp; digested 370 bp and 160 bp) in the AB heterozygous genotype [9,11]. The same studies reported that cheese yields of the BB genotype were 10% higher than those of the AA genotype, suggesting that the BB allele is important for breeding. Othman et al. [11] stressed the importance of CSN3B for milk and cheese yields, and Cinar et al. [25] reported the CSN3B of the Anatolian water buffalo to be significantly related to the

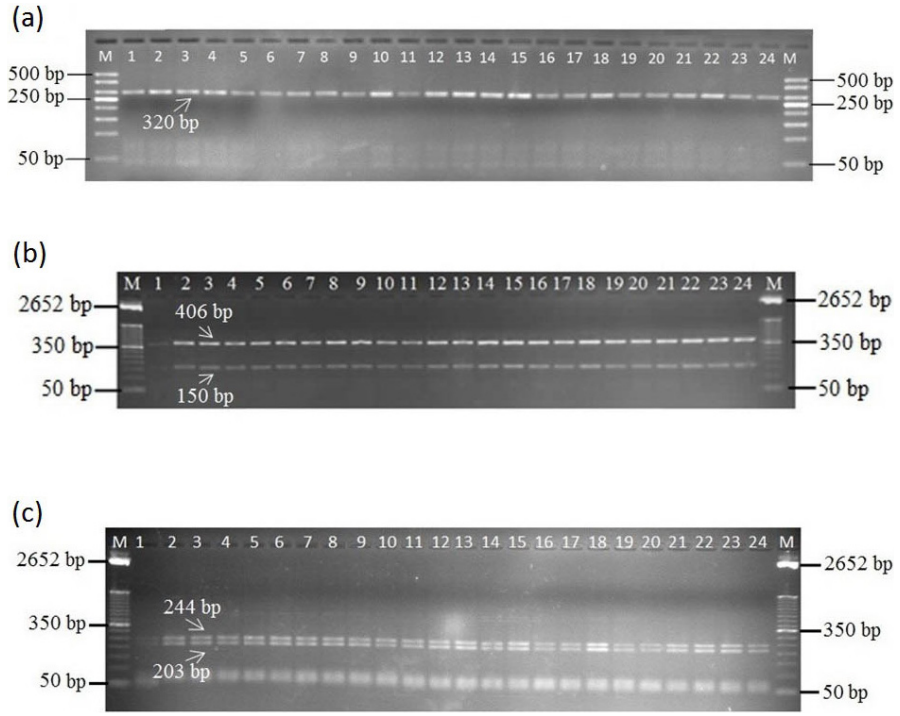

Figure 3. Ethidium bromide-stained 2% agarose gel showing DNA amplified at the *PRL* STS locus undigested with *Rsa*I (a), at the *CSN3* STS locus digested with *Hind*III (b), and at the *PIT-1* STS locus digested with *Hinf*I (c).

characteristics associated with efficient cheese production in cattle and goats. This study identified a single restriction point for *CSN3* in all samples, indicating a homozygous population of the BB genotype.

In studies performed with buffalo, CSN3A was identified with 135Thr/136Thr, while *CSNB* was identified with 135Ile/136Thr [20,24,26]. The 135Thr/Ile variation was reported in the case of Egyptian buffalo [16,26], Italian buffalo [12], Indian buffalo [19], and Bulgarian Murrah buffalo [27].

In this study, partial sequence results showed that the amplified *CSN3* fragment length containing partial exon IV was 480 bp. The fragment contained 405 nucleotides encoding 134 amino acids of the mature peptide (open reading frame 1) and 75 nucleotides of 3'UTR (Figure 4), but sequence results of *CSN3* showed that while Sample1 has the T nucleotide at codon 135 (Ile/ATC) and codon 136 (Thr/ACT), Sample2 has the C nucleotide at codon 135 (Thr/ACC) and codon 136 (Thr/ACC) on the *CSN3* STS locus (Figure 4). As a result, Sample1 was determined as CSN3B (135Ile/136Thr), whereas Sample2 was determined as the CSN3A allele (135Thr/136Thr). These transitions led to differentiation in primary amino acid sequences. Findings from this study were completely consistent with the studies in the literature and the GenBank database [12,16,28].

Previous studies reported *PIT-1* to be present as an undigested fragment of 451 bp in the AA homozygous genotype, as two digested fragments (244 bp and 207 bp) in the BB homozygous genotype, and as three fragments (undigested 451 bp; digested 244 bp and 207 bp) in the AB heterozygous genotype [9,11]. The AA allele was reported to be necessary for high milk yields and desirable for high percentages of milk proteins and low fat percentages, whereas the BB allele was associated with low yields, low protein contents, and high fat contents [11]. This study found that restriction endonuclease digestion of *PIT-1* revealed all samples to belong to the BB genotype.

Given that only undesirable alleles for *PRL* and *PIT-1* were identified in the buffalo in this study, it is very important to identify animals that carry the more desirable *PRLA* and *PIT-1A* to select for breeding in the region. In contrast to the situation with *PRL* and *PIT-1*, the study population was found to possess the ideal *CSN3* allele for ease of milk processing, CSN3B; therefore, the animals identified can be selected and bred for this characteristic.

The homozygosity of the *PRL*, *CSN3*, and *PIT-1* loci found in this study was unexpected, considering that buffalo were not intensively selected for these in the past [7]. It is likely that the high homozygosity in all the studied loci is a sign of serious genetic erosion that accompanied the loss of nearly 80% of the population between 1991 and

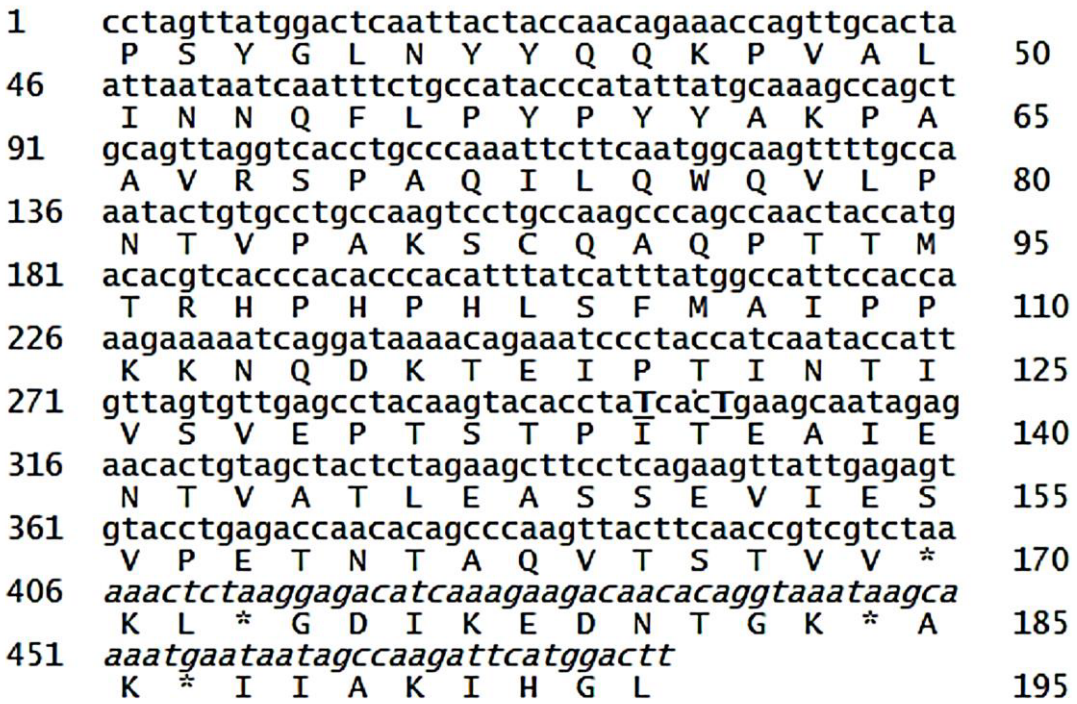

Figure 4. Capital and underlined letters in nucleotide sequence (open reading frame 1) belonging to Sample1 showed polymorphism at codons 135/Ile and 136/Thr. Sample2 has C nucleotide at both codons 135/Thr and 136/Thr. The 3'UTR region is indicated in italics.

2008. This large loss of population size is a clear warning for the future.

It is imperative that more animals be examined to identify the exact genetic diversity in the region. Moreover, breeding programs should be developed and planned immediately to prevent the loss of genetic diversity. For instance, other water buffalo from different parts of the country can be used as breeding animals. Small-scale family enterprises could be encouraged with regards to buffalo farming as a source of alternative income, such as ecotourism.

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