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*Acu*I identifies water buffalo *CSN3* genotypes by RFLP analysis

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Introduction

Water buffalo population amounts to 185 million heads (http://www.fao.org). They are mainly present in Southeast Asia. Casein genes among other genes control the milk traits in water buffalo. The role of casein genotypes on composition and coagulation of milk, quality and yield traits has been investigated (Lien *et al.* 1995; Boettcher *et al.* 2004; Comin *et al.* 2008; Bonfatti *et al.* 2010, 2012; Vallas *et al.* 2012). The κ-casein gene has been broadly studied due to its influence on the properties of milk (Bonfatti *et al.* 2012).

All previous reports on genotyping of water buffalo *CSN3* gene (exon 4), by restriction fragment length polymorphism (RFLP) analysis used restriction enzymes *Hin*dIII, *Hin*fI and *Taq*I, which successfully identify different cattle *CSN3* genotypes. However in buffalo, they only resulted in BB monomorphic buffalo. This was reported in Egyptian (Othman 2005; Dayem *et al.* 2009; Mahmoud *et al.* 2010) Pakistani (Riaz *et al.* 2008), Indian (Gangaraj *et al.* 2008; Shende *et al.* 2009), Brazilian (Otaviano *et al.* 2005) buffalo and in the water buffalo genomic library (Masina *et al.* 2007).

In a previous study on Egyptian buffalo *CSN3* gene, using sequence analysis, the presence of two single-nucleotide polymorphism (SNP) in exon 4 (El Nahas *et al.* 2013) was reported. In the two SNP positions, C is replaced by T at codon 135 (ACC/ATC) and codon 136 (ACC/ACT) of the mature peptide. These two SNPs were also reported in Bulgarian Murrah breed (Beneduci *et al.* 2010), Italian buffalo (Bonfatti *et al.* 2012) and in Indian buffalo (Das *et al.* 2000). Using sequence analysis, three buffalo-*CSN3* genotypes were identified (AA, AB and BB) and using *in silico* analysis, the restriction enzyme *Acu*I was suggested for buffalo-*CSN3* genotyping (El Nahas *et al.* 2013). In this study, we show experimentally that *Acu*I restriction enzyme is useful in genotyping buffalo-*CSN3* gene by RFLP analysis.

Materials and methods

Genomic DNA was extracted from whole blood of 36 buffalo by salting-out method according to Miller *et al.* (1988). Polymerase chain reaction (PCR) using primer pairs F: 5′-TGTGCTGAGTAGGTATCCTAGTTATGG-3′; R: 5′-GCG TTGTCTTCTTTGATGTCTCCT-3′ was conducted. Each amplification reaction (25 μL) contained 2 μL of buffalo DNA (100 ng), 0.2 mM dNTPs, 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 1.0 μM upper and lower primers, 1.25 units dream *Taq* polymerase (Thermo Scientific, Waltham, USA). The reaction mixture was run in Q-Cycler (Quanta Biotech, England), under the following cycling conditions: 3 min at 94°C, 35 cycles for 1 min at 94°C, 45 s at 60°C, 80 s at 72°C and a final extension for 10 min at 72°C. PCR products were purified using GeneJET™ PCR Purification Kit (Fermentas #K0701, Thermo Scientific Fermentas, USA). PCR products were sequenced by Bioneer, ABI 3730XL DNA analyser. The primer pair amplified a 453-bp fragment of exon 4 of *CSN3* gene. By analysing buffalo sequences, samples were classified into AA, AB and BB genotypes as reported by El Nahas *et al.* (2013). RFLP analysis was performed on PCR products from the three buffalo genotypes using *Acu*I restriction enzyme (Thermo Scientific #FD0344) according to manufacturer's suggestion, with some modification. For each reaction, the enzyme was increased to 1.50 μL and the digestion time was increasedto 30 min. PCR amplified products and digested products of the three buffalo genotypes were electrophoresed on 2.0% agarose gel in 1× Tris acetate buffer (TAE) containing 0.8 μL of 10 mg/mL ethidium bromide and photographed using Gel Documentation System (Syngene Bio Imaging,).

Results and discussion

The PCR amplified fragment (453 bp) covers most of the coding region of *CSN3* exon 4 (483 bp). *CSN3* exon 4 contributes most of the *CSN3* gene coding region and has been used in cattle and buffalo genotyping using RFLP analysis.

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CSN3 exon 4 includes two SNPs reported in buffalo (Das *et al.* 2000; Beneduci *et al.* 2010; Bonfatti *et al.* 2012; El Nahas *et al.* 2013).

In this study buffalo-*CSN3* PCR products (453 bp) were sequenced and their genotypes (AA, AB and BB) were determined. The difference in genotypes resulted from the presence of two SNPs (C/T) at nt 315 and nt 319 as reported previously (El Nahas *et al.* 2013). Allele A has $135^{\text{ThrAC}\mathit{C}}/136^{\text{ThrAC}\mathit{C}}$, whereas allele B has $135^{\text{IleA}\mathit{T}\text{C}}/136^{\text{ThrAC}\mathit{T}}$.

Restriction enzymes *Hin*dIII, *Hin*fI and *Taq*I have been used successfully in identifying the different *CSN3* genotypes in cattle. However, all investigations on buffalo that used these enzymes reported buffalo as BB monomorphic (Otaviano *et al.* 2005; Othman 2005; Masina *et al.* 2007; Riaz *et al.* 2008; Gangaraj *et al.* 2008; Dayem *et al.* 2009; Shende *et al.* 2009; Mahmoud *et al.* 2010).

PCR products of the three buffalo genotypes (AA, AB and BB) were digested with *Acu*I restriction enzyme. Alignment of alleles A and B sequence show that *Acu*I restriction site (CTGAAGN16^) is only present in buffalo-*CSN3* allele B (figure 1). Digestion of *CSN3* exon 4 amplified segment (453 bp) of the three buffalo genotypes using *Acu*I resulted in one band at 453 bp (undigested) in genotype AA, two bands (339 and 114 bp) in buffalo BB genotype and three bands at 453, 339 and 114 bp buffalo genotype AB (figure 2).

For the first time, the results showed that Buffalo-*CSN3* genotyping by RFLP analysis can be performed successfully using restriction enzyme *Acu*I. Restriction enzymes *Hin*dIII, *Hin*fI and *Taq*I have been used successfully in cattle-*CSN3* genotyping. However these enzymes are not suitable for Buffalo-*CSN3* genotyping. They all showed buffalo populations as BB monomorphic, this is because their restriction sites were found to be either present or absent in both buffalo alleles A and B (El Nahas *et al.* 2013). PCR-RFLP analysis has been used for decades in cattle (Denicourt *et al.* 1990), in screening large number of samples and cost low compared to

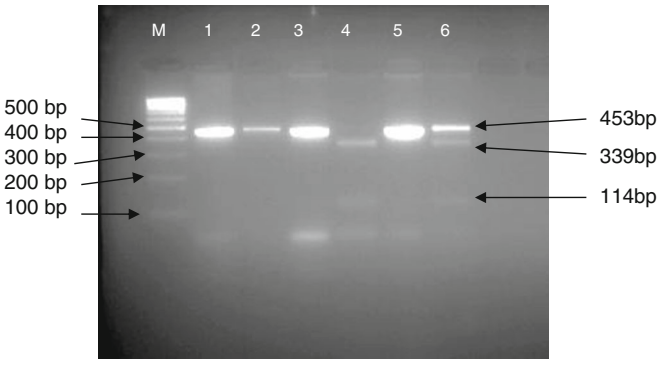

Figure 2. Genotyping buffalo-casein by RFLP analysis using *Acu*I. Lanes 1 and 3, and 5 are PCR products of buffalo genotypes AA, BB and AB (untreated with *Acu*I), respectively. Lane 2 (AA genotype, 453 uncut product), lane 4 (BB genotype, two fragments: 339 and 114) and lane 6 (AB genotype, three fragments: 453, 339 and 114). M, ladder 100.

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alleleA TGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTACCAACAGAAACCAGTTGCACT 60
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB TGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTACCAACAGAAACCAGTTGCACT 60

AlleleA AATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCTGCAGTTAGGTCACC 120
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB AATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCTGCAGTTAGGTCACC 120

AlleleA TGCCCAAATTCTTCAATGGCAAGTTTTGCCAAATACTGTGCCTGCCAAGTCCTGCCAAGC 180
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB TGCCCAAATTCTTCAATGGCAAGTTTTGCCAAATACTGTGCCTGCCAAGTCCTGCCAAGC 180

AlleleA CCAGCCAACTACCATGACACGTCACCCACACCCACATTTATCATTTATGGCCATTCCACC 240
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB CCAGCCAACTACCATGACACGTCACCCACACCCACATTTATCATTTATGGCCATTCCACC 240

AlleleA AAAGAAAAATCAGGATAAAACAGAAATCCCTACCATCAATACCATTGTTAGTGTTGAGCC 300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB AAAGAAAAATCAGGATAAAACAGAAATCCCTACCATCAATACCATTGTTAGTGTTGAGCC 300

AlleleA TACAAGTACACCTACCACCGAAGCAATAGAGAACACTGTAGCTACTCTAGAAGCTTCCTC 360
        ||||||||||||||| ||| ||||||||||||||||||||||||||||||||||||||||
AlleleB TACAAGTACACCTATCACTGAAGCAATAGAGAACACTGTAGCTACTCTAGAAGCTTCCTC 360

AlleleA AGAAGTTATTGAGAGTGTACCTGAGACCAACACAGCCCAAGTTACTTCAACCGTCGTCTA 420
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AlleleB AGAAGTTATTGAGAGTGTACCTGAGACCAACACAGCCCAAGTTACTTCAACCGTCGTCTA 420

AlleleA AAAACTCTAAGGAGACATCAAAGAAGACAACGC 453
        |||||||||||||||||||||||||||||||||
AlleleB AAAACTCTAAGGAGACATCAAAGAAGACAACGC 453
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Figure 1. Alignment of buffalo-CSN3 (453 bp) nucleotide sequences of alleles A and B showing two SNPs at nt 315 and nt 319 (in bold) and the presence of *Acu*I restriction site CTGAAGN16^ (underlined) in allele B.

sequencing. Buffalo has great potential. Identifying buffalo gene variants can be of value for selection of the best alleles within a breed for breeding programmes.

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