RESEARCH NOTE

GENETIC POLYMORPHISM OF β-CASEIN EXON 7 IN BUFFALOES (*Bubalus bubalis*)

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ABSTRACT

A1 and A2 are the most commonly observed milk variants of β-casein. Between these two types of milk, there is a difference of one amino acid located at exon 7 that influences the release of beta-casomorphin-7 (BCM-7), a peptide that can affect the digestibility of susceptible consumers. Thus, this study aimed to screen the β-casein exon 7 polymorphism in local water buffalo population. A total of 195 buffaloes and 49 cattle were genotyped for A1 and A2 allele variants using Restriction Fragment Length Polymorphism (RFLP) method with DdeI restriction enzyme. Analysis of the reported SNP C/A at position 8101 (Genbank: X14711) revealed a 100% A2 allelic frequency in buffaloes and 0.55 A2 and 0.45 A1 in cattle. A 509 bp fragment of exon 7 was also sequenced to determine the SNP 8101 C/A. This study demonstrates that the buffaloes still retain wildtype A2 milk.

Key words: A2 allele, beta-casein, buffalo, genotyping, milk variant degradability

INTRODUCTION

β-casein is the second most abundant casein in milk and has balanced amino acids (Sodhi *et al.*, 2012). Bovine β-casein (*CSN2*) is 8.5 kb long with nine exons and eight introns (Ramesha *et al.*, 2016). Mutations in exon 7 of the bovine β-casein (*CSN2*) led to different genetic variants. Among these variants, A1 and A2 are the most commonly expressed, resulting in two kinds of milk (Ramesha *et al.*, 2016). A2 milk is the natural β-casein variant for bovine but due to mutation and selective breeding of cows to produce higher milk volume, A1 milk has become widespread in most European cattle breeds (Farmaggioni *et al.*, 1999; Woodford, 2007).

The difference of A1 and A2 milk and its synthesis on the body system is due to one amino acid change, histidine (CAT) is found in A1 milk while proline (CCT) is in A2 milk. This one nucleotide change in the A to C base leads to a change in the expressed β-casein secondary structure. When A1 variant of β-casein was digested through the gastrointestinal proteolytic enzyme, a bioactive peptide known as beta-casomorphin-7 (BCM-7) is generated (Sodhi *et al.*, 2012). The release of BCM-7 through this enzymatic digestion is dictated

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differently on the amino acid sequence of the protein. The hypothesis on the effect of A1 variant to human health may have been suggested due to the effect of BCM-7, however strong evidence still needs to be established (Truswell, 2005).

Investigation of *CSN2* genetic variants could give information if native buffaloes and other breeds in the Philippines have the mutation at exon 7. The research was conducted to characterize the *CSN2* exon 7 in local water buffalo population, define the genotypes present and determine the allelic/genotypic frequencies in the different breeds of buffaloes for β-casein A1 and A2 allele in the Philippines.

MATERIALS AND METHODS

A total of 195 buffaloes were randomly selected, specifically, Bulgarian Murrah Buffalo (BMB; n=76), Brazilian Murrah Buffalo (BR, n=23), Italian Murrah Buffalo (IT, n=28) and Philippine Native Swamp (PC, n= 68) and 49 cattle of different breeds (Jersey-Holstein cross n=6, Jersey n=6, Holstein n=2, Holstein-Sahiwal cross n=3, Native Cattle n=5 and Girolando n=27) were used in the study. Blood samples were collected in vacutainers. The samples were kept cool in storage. Genomic DNA from the collected whole blood samples was isolated using Promega Wizard® Kit following the protocol provided by the manufacturer.

All animals were screened with *CSN2* Exon 7 for A1 and A2 alleles that influence the variation of milk due to SNP C/A at position 8101 (Genbank: X14711). DNA primers were used as described by McLachlan 2003 (a) and Vinesh, 2009 (b):

(a) *CSN2* 8101 C/A (121 bp digested by *DdeI*)
F: 5' – CCT TCT TTC CAG GAT GAA CTC CAG G – 3'
R: 5' – GAG TAA GAG GAG GGA TGT TTT GTG GGA GGC TCT – 3'

(b) *CSN2* Exon 7 (506 bp)
F: 5' – CCT TCT TTC CAG GAT GAA CTC CAG G – 3'
R: 5' – AAT AAT AGG GAA GGG TCC CCG G – 3'

The reaction mixture used was a total of 10 µL containing 4.3 µL of sddH2O, 2 µL PCR Buffer, 0.8 µL MgCl2, 0.8 µL dNTPs, 0.5 µL Reverse Primer, 0.5 µL Forward Primer, 0.1 µL *Taq* DNA polymerase and 1 µL of DNA. The following amplification parameters were applied: 95°C for 5 minutes followed by 30 cycles: 95°C for 40 seconds, 56°C for 60 seconds, 72°C for 90 seconds. The reaction was completed by the final synthesis of 72°C for 10 minutes. PCR products were then digested with *DdeI* (10 units) incubated at 37°C for 4 hours. The digested products were then electrophoresed in 3% agarose gel at 110V for 40 minutes. The gels were examined for different band patterns. Determination on the frequency of the genotypes A1A1, A1A2 and A2A2 were done to evaluate the status of buffalo's milk variant in the Philippines. The same procedure was applied to dairy cattle. Genotypic and allelic frequencies were calculated by direct counting divided by the population.

AB 3500 Genetic Analyser sequencer was used for sequencing. The nucleotide sequences were aligned and edited using Geneious Prime 2019.2.1 to confirm mutations and obtain images. Phylogenetic analysis was done using MEGA X 10.0.5.

RESULTS AND DISCUSSION

In this study, 506 bp fragment of exon 7 in Chromosome 6 for cattle and Chromosome 7 for buffaloes were sequenced to determine the SNPs present. Four breeds of buffaloes (BMB, BR, IT and PC) and two breeds of cattle (Girolando and Holstein) were sequenced. Different breeds of buffaloes have 99.40% homology percentage. Four polymorphic sites were observed in PC at position 8105 bp G/C, 8354 bp G/T, and 8480 bp C/T.

Phylogenetic analysis using the Maximum Likelihood method and Hasegawa-Kishino-Yano model showed grouping of all buffalo breeds except for PC diverging at different sub-clade whereas, all the cow breeds grouped together (Figure 1). Also, Clustal W sequence alignment of Buffaloes, Cattle, Goat, Sheep and Pig yielding 509 nucleotide sequences were shown in Figure 2. It was revealed that the A2 allele (Genbank: X14711, SNP 8101bp C/A) is highly conserved across breeds of buffaloes while cattle show both the A1 and A2 allele. This SNP from C to A transversion resulted in a missense mutation that replaces proline to histidine. The predicted amino acid sequences demonstrate the substitution of proline to histidine in A2 and A1 milk as presented in Figure 3. This amino acid change in the sequence is what generates the production of the BCM-7 caused by a weaker bond between Isoleucine and Histidine thus forming cleavage (Parashar and Saini, 2015).

A 121bp fragment of *CSN2* Exon 7 was used to amplify a segment of the beta-casein gene for the analysis of A1 and A2 allele genotypes through PCR-RFLP using *DdeI*. Allele A1 showed bands at 121 bp while Allele A2 produced bands at 86 bp and 35 bp. Thus, the heterozygous A1A2 exhibited bands at 121 bp, 86 bp and 35 bp (Figure 4).

Allele A2 was 100% homozygous for all 195 samples of buffaloes from different breeds. This coincides with the results from the study of Mishra *et al.*, 2009 and Ramehsa *et al.*, 2015. All three genotypes were observed in cattle samples, A1A1 (n=5), A1A2 (n=23), and A2A2 (n=21). The frequency of A1 and A2 alleles vary depending on the breed. However, genotypic and allelic frequencies for A2 allele have a higher frequency than the A1 allele in most of the evaluated breeds of cattle. These results for cattle agree with the reports from EFSA, 2009 as the majority of black and white cattle (Holstein) has the A1 allele while

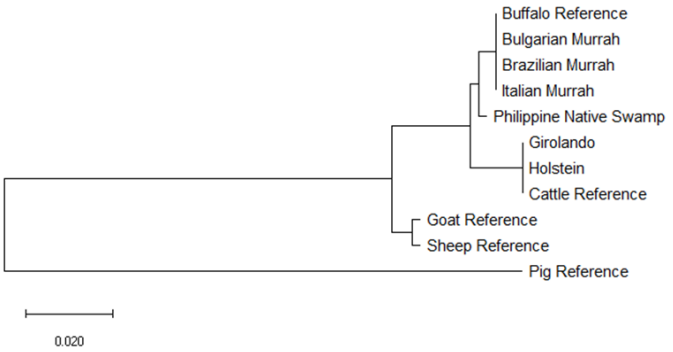

Figure 1. Phylogenetic relationship of *CSN2* Exon 7 between breeds of buffaloes and cattle with reference nucleotide sequences of buffalo, cow, goat, sheep and pig from NCBI.

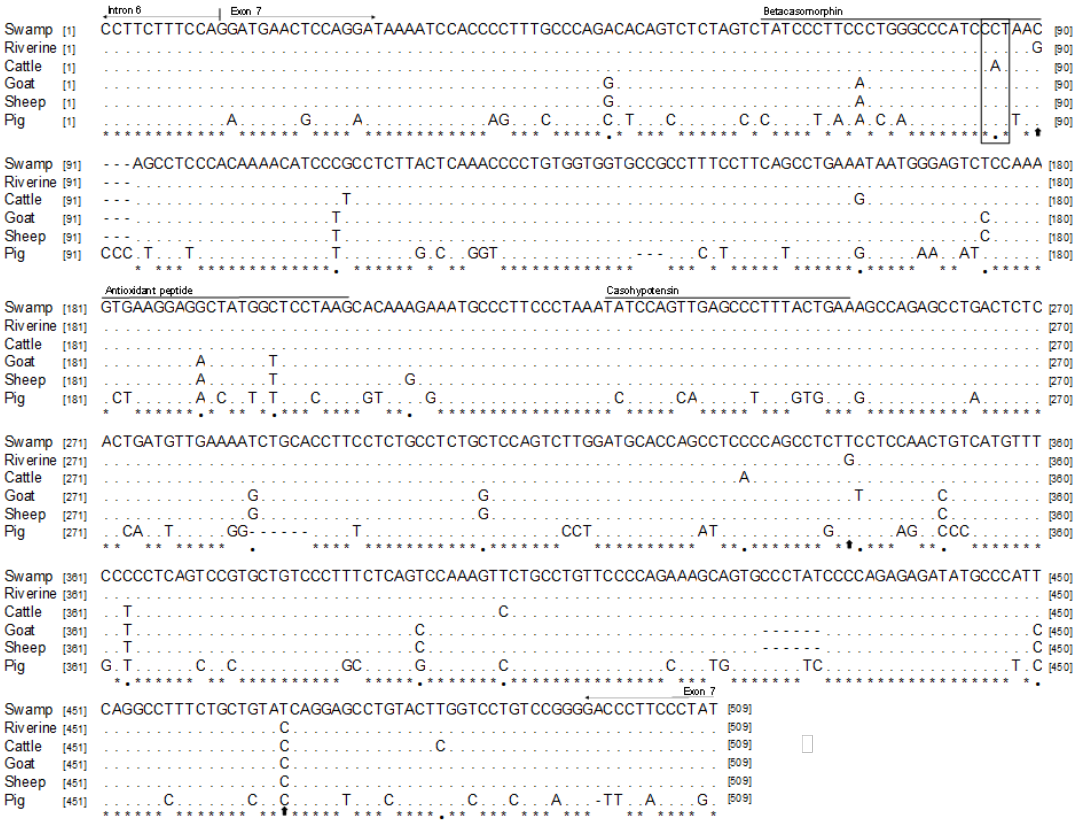

Figure 2. Nucleotide sequence alignment of *CSN2* Exon 7 of Philippine Native Swamp and Riverine Buffaloes with Cattle (Genbank MK426695), Goat (Genbank AJ011018), Sheep (Genbank X79703.1) and Pig (Genbank NC_010450). (-) indicates indel, (*) indicates conserved sites, (⇑) indicates nucleotide substitution between swamp and riverine, and (•) indicates nucleotide substitution between swamp and other species except pig. Codon that influences the two variances in milk is enclosed in box which shows the SNP 8101 C/A.

	20									30			
1. Buffalo	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Lys	Ser	Leu
2. Cattle	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	His	Asn	Ser	Leu
3. Bulgarian	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Lys	Ser	Leu
4. Brazilian	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Lys	Ser	Leu
5. Italian	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Lys	Ser	Leu
6. Swamp	Leu	Val	Tyr	Pro	Phe	Pro	Gly	Pro	Ile	Pro	Asn	Ser	Leu
7. Goat	Leu	Val	Tyr	Pro	Phe	Thr	Gly	Pro	Ile	Pro	Asn	Ser	Leu
8. Sheep	Leu	Val	Tyr	Pro	Phe	Thr	Gly	Pro	Ile	Pro	Asn	Ser	Leu

BCM-7

Figure 3. Amino acid sequence showing the change (boxed) from proline (A2 milk) to histidine (A1 milk).

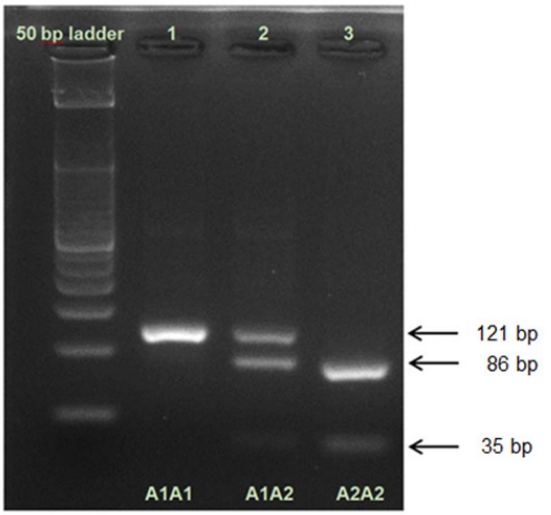

Figure 4. Genotype variants of β-casein exon 7 observed using RFLP with 50 bp ladder.

most of the Jersey breed has the A2 allele. Further, nearly quarter of the population has the A1 allele and almost half having the B allele (EFSA, 2009). The presence of the A1 allele suggests that the breeds of cattle included in the study produce milk that has the detrimental type of β-casein which generates BCM-7.

In the studies of Tailford *et al.* (2003) and Kaminski *et al.* (2007) it was shown that the presence of A2 allele indicates that the animal produces high-quality milk associated with a reduction in cholesterol and triglycerides. Due to the presence of β-casein A1 in milk, peptide bonds cleave as a result of the digestion process, releasing bioactive BCM-7; the presence of the A2 allele prevents the hydrolysis of the peptide bond between residues 66a and 67a, inhibiting the release of BCM-7. From the observed results, 100% homozygous A2 allele frequency shows that the breeds of buffaloes do not have the deleterious variant of β-casein causing low or no BCM-7 production (Rangel *et al.*, 2017). The presence of the A1 allele suggests that the breeds of cattle included in the study produces milk that generates BCM-7.

The screening of buffaloes for β-casein genotypes belonging to four breeds from different herd under the Philippine Carabao Center showed that the animals are 100% homozygous for the wild type A2 allele while all three genotypes are present in cattle with the majority of the animals still carrying the A2 allele. It is therefore concluded that buffaloes screened in the study carry only the favorable A2 allele.

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