

## PCR Based Genotyping of Lulu Cattle of Nepal for A1, A2 Type Beta-caseins

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### ABSTRACT

Lulu is an indigenous breed of cattle (*Bos taurus*) found in high altitude regions of western Nepal. Population of Lulu cattle has been declining due to introgression with other exotic breeds to increase milk productivity. Here we aimed at finding potential approach for conserving Lulu cattle and its assets by studying the milk contents and investigating which variant of beta-casein protein is present in this breed. Beta caseins are an abundant protein in cow milk with A1 and A2 being the most common genetic variants of this protein. Consumption of A1 type of milk has numerous health-related complications whereas A2 type of milk has numerous human health promoting factors. We used restriction fragment length polymorphism (RFLP) for determining the A1 and A2 variant of beta casein in Lulu cattle. For performing DNA extraction, we collected (n = 18) blood samples of Lulu from Mustang and (n=17) Nepal Agriculture research council farm. The amplified fragments in 3% agarose at 251bp and 213bp respectively confirmed the presence of both A1 and A2 gene in Lulu; however, A2 was of greater abundance. Our study indicated that Lulu has A2 variant of beta-casein predominantly. The gene frequency of A1A1 is 0, A1A2 is 0.06 and A2A2 is 0.94. We further found that the allele frequency of A1 and A2 is 0.03 and 0.97 respectively. We designed special primer for sequencing CSN2 genes since A2 type beta casein gene was predominantly seen on Lulu. The sequencing result further supports our RFLP result as most of our samples have “C” nucleotide SNP in amplified CSN2 gene sequence. The Chi-square value of the current study is 0.04 which supports Hardy-Weinberg equilibrium inferring that Lulu cattle are still in the pure state, where there is no genetic introgression with the exotic breed for the sake of improvement of productivity.

**Keywords:** Lulu, CSN2 gene, A2 variant, RFLP, sequencing

### सारांश

पश्चिम नेपालको उच्च पहाडी क्षेत्रमा पाईने लुलु एक जुरो नहुने स्थानिय गाईको नश्ल हो । मुस्ताङ्ग क्षेत्रका कृषकहरुले दुधको उत्पादन बढाउन अन्य विदेशी जातहरुको नश्लहरु भित्र्याउदा लुलु गाईको संख्या दिन प्रति दिन घट्दो क्रममा छ । सो अवस्थाको गाम्भिरेतालाई मध्यनजर गर्दै नेपाल कृषि अनुसन्धान परिषद अन्तर्गत पशु प्रजनन माहाशाखा(प.प्र.म)ले लुलु गाईको दूधमा कुन प्रकारको बीटा केसीन प्रोटीन छ त्यसको आधारमा लुलु को संरक्षण का लागि संभावित रणनीति खोज्ने प्रयास भईरहेको छ । गाईको दुधमा बिटा ए१ र बिटा ए२ केसीन प्रोटीन हुन्छ र यस प्रकारका प्रोटीन हुनका लागि गाईको आनुवंशिक गुणको प्रभाव रहन्छ । बिटा ए१ प्रकार को दूध सेवन गर्दा धेरै प्रकारका स्वास्थ्य सम्बन्धि जटिलता आउने विभिन्न अध्ययनबाट देखिइको छ । जबकि ए२ प्रकारको दूधमा मानव स्वास्थ्यको लागि धेरै लाभदायक गुणहरु हुन्छन् । लुलु गाईमा बीटा केसीन ए१ र ए२ को पहिचान गर्नका लागि Restriction Fragment Length Polymorphism (RFLP) को प्रयोग गरिएको थियो । त्यसका लागि मुस्ताङ्गबाट लुलु गाईको १८ र प.प्र.ममा रहेका बाट १७ वटा गरी जम्मा ३५ वटा रगत नमुना संकलन गरि डि.एन.ए छुट्याईएको थियो । ३ प्रतिशत

एगारोजमा २११ र २१३ बेसपियर (bp) मा विस्तारित टुक्राहरू (amplified fragments) ले लुलुमा ए१ र ए२ दुवैको उपस्थिति पुष्टि भएता पनि ए२ को बाहुल्यता अधिक थियो। यस अध्ययनले यो संकेत दियो कि लुलु मा मुख्य रूपमा ए२ बीटा केसिन छ। ए१ए१ जीन फ्रिक्वेन्सी ०.००, ए१ए२ को ०.०६ र ए२ए२ को ०.९४ पाईयो। ए१ र ए२ को एलिल फ्रिक्वेन्सी क्रमशः ०.०३ र ०.९७ पाईयो। CSN2 जीन अनुक्रमणका लागि विशेष प्राइमर डिजाइन गरीयो किनकी अध्ययनको नतिजाले मुख्यतया ए२ प्रकारको बीटा केसिन जीन लुलुमा देखिएको थियो। अनुक्रमणको परिणामले हाम्रो आर.एफ.एल.पीवाट आएको नतिजालाई पुष्टि गर्दछ किनकि प्रायः नमूनाहरू विस्तारित (amplified) CSN2 जीन अनुक्रममा “C” न्यूक्लियोटाइड एस.एन.पी.मा छन्। यस अध्ययनको काई सक्वायर मान ०.०४ छ जसले हार्डी वीनबर्ग सन्तुलनलाई समर्थन गर्दछ साथै लुलु गाईहरू अझै शुद्ध अवस्थामा छन् र विदेशी जातसँग कुनै आनुवंशिक संबन्ध नभएको पुष्टि गर्दछ।

## INTRODUCTION

As per the report published by Department of Livestock Services (DLS) 2016/17, the total population of cattle in Nepal is 7,347,487. Some of the names of documented indigenous cattle are Lulu, Achhami, Siri, Khaila, Yak, Terai and Pahadi (Neopane et al 2002). A majority of the indigenous cattle are of zebu type while Lulu cattle are the only indigenous cattle of taurine breed (Fujise et al 2003). The total population of Lulu cattle as reported by District Livestock Services of Jomsom, Mustang in year 2016/17 is 7843. The cattle comprise 7% of the total indigenous population, and this population is in declining number because of their lower milk productivity level in comparison to other breeds in farmers' management system (DLS 2017).

In recent years, Beta-casein has been a popular topic of research in the animal breeding and dairy industry as it is a source for high-quality protein, and that bioactive peptide has numerous health implications (Kumar et al 2019). The polymorphic nature of beta-casein about milk production quality and composition has led in assessing the allelic distribution of beta-casein locus as a potential dairy trait marker (Dai et al 2016). The prime difference between A1 and A2 variant of beta-casein is in its 67<sup>th</sup> amino acid position in exon 7 of chromosome 6. Many research theories suggest that there has been the substitution of Proline with Histidine has, in turn, resulted in the change from A2 to A1. The substitution is mainly due to “C” nucleotide with “A” nucleotide in that corresponding nucleotide position (Farrell et al 2011). Moreover, many types of research have given positive correlation between A1 variant of beta casein and health-related problems (Sheng et al 2019).

To determine the presence of either A1 or A2 variant of beta casein, we used RFLP method for determining the single nucleotide polymorphisms (SNP). Also for further validation, we sequenced amplified CSN2 genes. Our research result implies to the government that the proper policy must be procured regarding genotyping indigenous cattle as A1 and A2 types since A1 milk has many health-related complications. The government of Nepal has been importing many different exotic breeds such as Holstein Friesian, Jersey for fulfilling the demand of milk in the country as they produce a larger volume of milk. Therefore, there requires a strong policy regarding genotyping them as A1 and A2 types by which we can control future health risks of populations and also prevent them from cross-breeding with our pure A2 indigenous breeds.

## MATERIALS AND METHODS

### Sample collection

Convenience sampling was employed for the study:thirty-five blood sample of Lulu cattle from Mustang (n=18) and Animal Breeding Division, NARC (n=17) were collected in sterilised EDTA tubes and transferred to genebank in Icebox. The blood collection was performed by professional veterinarians in accordance with the animal ethics guidelines.

### Specific Primers Sequence Used for PCR Amplification

Many research papers related to A1 and A2 variants of beta-casein have used the published primers (Lien et.1992) to amplify the fragment of exon 7 of CSN2 gene where SNP is present. Therefore, we also used the primer set of CASB67R and CASB122L for amplifying CSN2 gene of Lulu cattle (Lien et al 1992). For sequencing, we designed a set of primer by using Primer3plus and also, the primer set was further analyzed by using oligo6 software for its accuracy and reliability.

**Table 1. Primers used for amplifying and sequencing CSN2 gene**

SN	Name of primer	Nucleotide sequences
1	CASB67R	CCT GCA GAA TTC TAG TCT ATC CCT TCC CTG GGC CCA TCG
2	CASB122L	GAG TCG ACT GCA GAT TTT CAA CAT CAG TGA GAG TCA GGC CCT G
3	CSN2_F	TAC CAC TCT GCA GGC AAC TC
4	CSN2_R	GAA CAG GCA GGA CTT TGG AC

### DNA extraction

PROMEGA DNA extraction kit was used for DNA extraction. In order to get a good quality of DNA, DNA washing step by 70% of ethanol was performed twice, and the samples were left to be air dried for 1 hour. Afterwards, a good quality of DNA was obtained as the ratio of 260/280 was 1.8 and also good genomic band was seen in 1% agarose gel. The most samples had the DNA concentration about 50 ng/μl.

### Preparation of reaction mix for performing RFLP and sequencing

2X PCR master mix from Bioneer company of South Korea was used for amplifying CSN2 gene of Lulu cattle. Since most of our samples had DNA concentration about 50 ng/μl which was diluted it to 25ng/μl for performing PCR. The component of 2X master mix were bacterially derived Taq DNA polymerase, MgCl<sub>2</sub>, KCl, deoxynucleotides (dNTPs), Tris-HCL (pH-9), stabiliser and tracking dye. 25 μl of PCR amplification reaction mixture by keeping 13μl of PCR 2X Master Mix, 5 μl of template DNA, 5μl of molecular grade nuclease-free water, 1.5μl of forward primer (CASB67R), and 1.5 μl of reverse primer (CASB122L) were prepared. Subsequently, the PCR tubes consisting mixture to PCR thermocycler for amplifying CSN2 gene was transferred. PCR was performed on the following condition: denaturation at 95°C for 1 minute, annealing at 65.5°C for 1 minute and extension at 72°C for 2 minutes. For performing RFLP, the Taq I restriction enzyme of 20,000U/ml from New England Biolabs was used. 25μl of restriction digestion mixture was prepared. The digestion mixture includes 2.5μl of 10X RE assay buffer, 0.5ul of Taq I restriction enzyme (1U/μl), 10μl of PCR product and 12μl of molecular grade nuclease water. The reaction mixture was prepared for 5 hours at 65 degree Celsius. Agarose gel electrophoreses on 3% agarose gel with 2μl of ethidium bromide (10 mg/ml) for visualising the bands of digested DNA products was performed. The DNA bands of agarose gel under UV gel documentation by comparing with 200 bp DNA ladder was visualized.

For sequencing the CSN2 gene, a set of primer, CSN2\_F and CSN2\_R, was designed by using Primer3plus and validated its accuracy and reliability by using oligo6 software. The annealing temperature (tm) for the primer was 56.4 degree Celsius. Afterwards, the amplified PCR products were sent to Macrogen Inc., South Korea for sequencing.

### Statistical Analysis

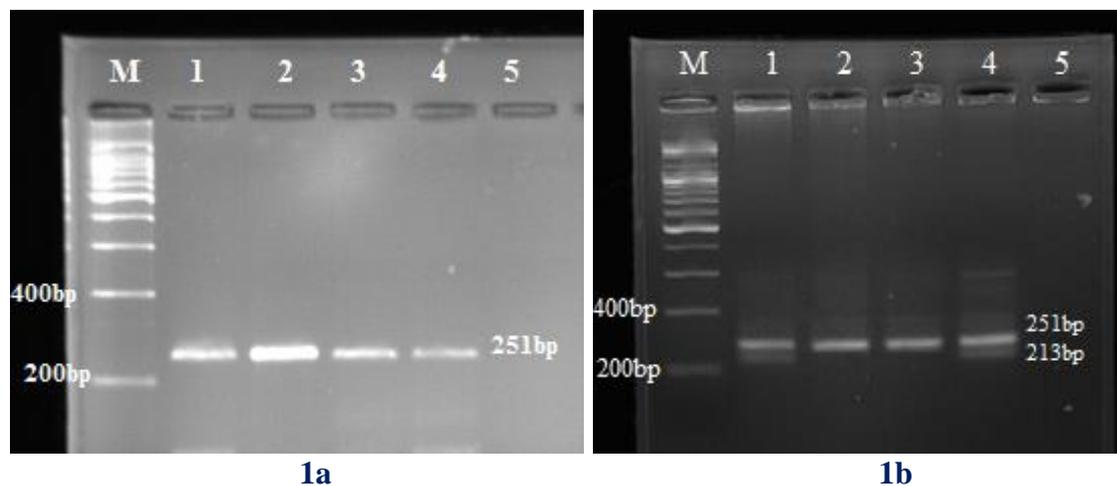
The data were collected by visualising bands on 3% agarose gel. Chi-square analysis was done by using IBM Statistical Package for the Social Sciences (version 23).

## RESULTS

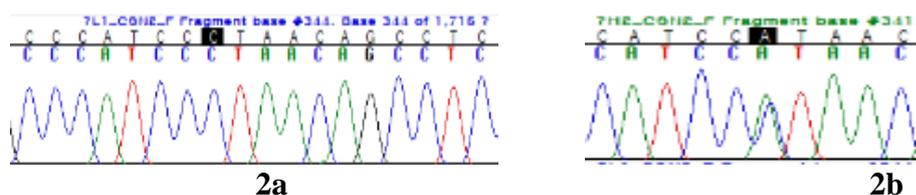
Among 35 samples, 33 predominantly gave one band of 251bp while two samples of Lulu from Mustang gave two bands 251bp and 213bp as presented in Table 2. 38bp in 3% agarose gel wasn't detected. Since 38bp is very short DNA sequence, it was very hard to trap in 3% agarose gel. The photo of gels is provided in [Figure 1](#). The presence of only 251 bp after restriction digestion illustrates the cattle samples have only A2 homozygous variant of beta casein whereas the presence of 251bp and 213bp indicate that those samples have A1A2 heterozygous variant of beta casein. The PCR products were sent for sequencing and the sequenced data were further observed. The chromatograms observed were shown in [Figure 2](#). The figure 2a has only one peak in SNP. The single peak displays the presence of A2 homozygous variant of beta casein. The figure 2b has overlapping peak in SNP. This overlapping peak illustrate the presence of A1A2 varaint of beta casein.

**Table 2. Result of Restriction fragment length polymorphisms**

SN	Gene type	Fragment size (bp)	Number of animals (n = 35)
1	A1A1	213 bp, 38 bp	0
2	A1A2	251bp,213bp,38bp	2
3	A2A2	251bp	33



**Figure 1.** Gel is showing PCR products of CSN2 gene of Lulu cattle after restriction digestion Lane M: 200-bp DNA marker. **Figure 1a** showing only band A2 variant of beta-casein (251bp), Lane 1,2,3,4: A2 homozygous CSN2 gene (251bp) and Lane 5: Negative control. **Figure 1b** is showing band A1 and A2 variant of beta casein. Lane 2,3: A2 homozygous CSN2 gene (251bp), Lane 1,4: A1A2 heterozygous CSN2 gene (251bp and 213bp) and Lane 5: Negative control.



**Figure 2.** Chromatogram of sequenced data. **Figure 2a** showing A2 homozygous variant of beta casein gene (Non overlapping peak in SNP). **Figure 2b** showing A1A2 heterozygous variant of beta casein gene (Overlapping peak in SNP).

## DISCUSSION

In this study, a potential approach for conserving Lulu cattle was established and its assets by studying the milk contents and investigating which variant of beta-casein protein was presented in this breed. The results indicated that the A2 variant of beta casein was abundant in Lulu cattle from sampled population. The sequencing results further helped in validating our findings from RFLP that Lulu cattle predominantly share A2 type beta casein. This result indicates that the A2 type of milk can be promoted to farmers so that the declining population of indigenous breeds can be conserved.

In recent times, many researchers have been using different variants of Milk protein as a valuable tool for characterising breeds and assessing diversity and phylogeny (Caroli et al 2009). Alpha s1-Casein B and beta-casein A phenotypes were reported to have a higher yield of milk, protein and fat than other variants in the two casein systems (Ng-Kwai-Hang et al 1984). However, Proteins in bovine milk are found to be a common source of bioactive peptides which has detrimental effects on human health (Cieślińska et al 2012). The bioactive peptides are released when human digest the casein and whey of milk component. The name of the bioactive peptide is beta-casomorphin 7 (BCM-7). The proteolytic digestion of bovine beta-casein variants A1 and B releases BCM-7.

Nevertheless, the BCM-7 is not formed in the case of bovine variant A2 milk, human milk and goat casein (Elliot et al 1990). Dairy cattle breeds have abundant variants A1 and A2 of beta-casein. Many research reports BCM-7 as a major cause of human diseases such as ischemic heart disease, neurological disorders and type I diabetes. Many research papers have proven that the populations consuming beta-casein A2 milk have a lower incidence of type 1 diabetes (Kamiński et al 2007).

As all the Indian indigenous dairy animals (cattle and buffaloes) endowed with A2 milk protecting the human population from ill effects of A1 milk (Mishra et al 2009), Nepalese indigenous dairy animals possibly to be having A2 variant (allele frequency, 0.96). The present study gave the evidence that even Nepalese taurine cattle, Lulu cattle, exhibited A2 variant of beta-casein while globally most of the taurine population such as Red (0.710) cattle, Ayrshire (0.432-0.720) and Holstein-Friesian (0.310-0.660) are predominantly with A1 variant. Nevertheless, Guernsey (0.880-0.970) and Jersey (0.490-0.721) cattle have a high frequency of A2 (Boro et al 2016). The milk yield corresponds with the A1 variant of beta casein. Nowadays many animal breeders have been deliberately selecting A1 variant type high yielding dairy breeds for the breeding purpose. Fortunately, the Chi-square value of the current study supports Hardy-Weinberg equilibrium inferring that Lulu cattle are still in the pure state, where there is no genetic introgression with the exotic breed for the sake of improvement of productivity. The population of Lulu, however, is declining drastically to which the government has to give priority to conserve, utilise and promote this valuable genetic unique animal of high land of Nepal.

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