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# Kappa-Casein Gene (*CSN3)* Polymorphisms Detection in Three Indigenous Iraqi Goat Breeds, Using PCR-RFLP and SNP Markers

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MILK contains a protein called kappa-casein, which controls the function and size of milk micelles as well as their ability to form and stabilize. Kappa-Casein gene (CSN3) polymorphisms were investigated in 70 Domestic (Native and Meriz goat) and Wild goat using the PCR-RFLP method and direct sequencing. *CSN3-Hea* III/RFLP revealed two homozygous genotypes AA and BB. For the AA and BB genotypes, the computed genotype frequencies were (0.87) and (0.13), respectively. The allelic frequency was 0.87 for the A allele and 0.13 for the B allele. The sequence data of *CSN3* gene of Meriz and Wild goats revealed 2 SNPs in functional region, one SNP of Wild (ACC. No: OR050625.1), and one in Meriz goat (ACC. No: OR050626.1). In position 415 in Wild goat, the amino acid Methionine changed to Isoleucine by changing (ATG) to (ATA). On the other hand, the point mutation in Meriz goat at the positions 449 led to change amino acid Valine to Isoleucine by alternation (GTC) to (ATC). The PCR-RFLP and SNP analysis is a powerful tool for the genetic study of *CSN3* variability in domestic and wild goats, allowing both the simultaneous identification of different alleles, and the detection of new variants. Establishing relationships between genotypes and both quantitative and qualitative milk qualities will require additional investigation.

**Keywords**: *Capra hircus*, *Hea* III, *CSN3* gene, *Capra aegagrus,* SNP

## Introduction

Milk is a readily available liquid food that contains a wealth of nutrients, including proteins, carbs, minerals, and vitamins [1]. About 80–83% of the total protein in milk is casein, which is made up of alpha, beta, and kappa casein [2]. On the chromosome six, the casein protein coding area has been identified in goats by the coding genes CSN1S1, CSN1S2, CSN2 and CSN3 [3, 4]. One of the most important characteristics of kappa-casein is its influence on the reduced milk micelle size. This has an impact on the milk's coagulation properties and is reflected in a stronger curd and the retention of a significant number of ingredients, which increases cheese yield [5, 6]. The four casein genes have drawn a lot of attention for research purposes because of the potential impact on milk quality [7]. The CSN3 gene in goats has five exons, and a 579 bp open reading frame mRNA, that codes for 171 amino acids in the mature protein (exons 3 (9 amino acids) through exon 4 (162 amino acids) and 21 amino acids in the signal peptide [8-11].

According to Moioli and his colleagues [12], the CSN3 gene is monomorphic in the sheep, however numerous investigations on goat CSN3 revealed that High polymorphism exists in this gene (13-15, 7]. Goat milk output and composition have been found to be highly variable [16], and this variability may be influenced by genetics [17]. One of the most often used techniques is the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The direct sequence is a useful method for

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identifying nucleotide changes in amplified DNA fragments, [18, 19]. Additionally, Single Nucleotide Polymorphism (SNP) Screening has been the preferred technique for locating and connecting traits with regions of the genome in many plants and animals [20, 21].

Goat milk is mostly used in the Kurdistan region of Iraq, generally for making cheese and confections. This product's economic value is mostly determined by the milk's total protein and fat content [22]. Therefore, casein polymorphism and their genetic variations that can alter the qualities of this milk product dependent on the variable composition that governs cheese manufacturing [23,24,6]. This article looked at the genetic polymorphism of the CSN3 gene in domestic (Mariz and Native goat) and wild Iraqi local goats in order to be able to analyze genotype distribution and apply this knowledge in forthcoming conservation initiatives for this breed. This is because milk quality may be influenced by genetics.

## Material and Methods

### DNA extraction

Blood samples were obtained from 70 female goats of both domestic *(Capra hircus)* and wild (*Capra aegagrus*) Iraqi goats from various herds in Duhok province (28 Meriz, 15 Wild and 27 Native goats). Three ml of blood were collected into 2.7% EDTA tubes as an anticoagulant and kept at 4 °C until used. Blood genomic DNA was extracted using the phenol-chloroform method [25], the purity and concentration of genomic DNA were determined using a Nanodrop spectrophotometer.

### DNA amplification:

The specific primer (F:5′TCCCAATGTTGTACTTTCTTAACATC3′, R: 5′ GCGTTGTCCTCTTTGATGTCTCCTTAG 3′) provided by some studies[8] was used to amplify the exon four of the goat CSN3 gene. The master mix reaction included 10 µL of 2×PCR master mix (ADDBIO INC), 1 µL (10 pmol/µL) of each reverse and forward primer, 1 µL (100 ng) of genomic DNA and complete the volume to 20 µL by added 7 µL of deionized distilled water. The PCR programmer was composed of, an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, annealing at 63 °C for 45 s, and extension at 72 °C for 1 min, followed by one cycle of final extension at 72°C for 5 min and storage at 4 °C. The agarose gel electrophoresis (2%) was used to examine the PCR results. Red safe dye was used to stain the gels, before being illuminated by a UV trans-illuminator to confirm amplification.

### PCR-RFLP analysis:

The reaction combination, made up of 10 units of the *Hea* III restriction enzyme (Gena Bioscience) and 10 µL of PCR amplicons was employed to digest the PCR products. The reaction was done in a total volume of 25 µL of each sample and then incubated at 37 °C for 6 hours. Electrophoresis on a 2% agarose gel was used to separate the digested amplicon fragments, and 100 bp ladder DNA was run alongside the digested PCR products as a references to measure the bands size. Gels were stained with Red Safe Stain, and then photographed after being seen through a UV trans-illuminator. PopGene program version 1.31 was used to examine the data for this locus [26].

### CSN3 gene sequencing:

Macrogen (Seoul, Korea) sequenced the PCR products for different genotype of the CSN3 gene reveled in this investigation, there were a Wild goat with BB genotype and a Meriz goat with AA genotype. For sequence analysis and alignment, the NCBI/BLAST/blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch ) and Clastel W were used in order to detect each individual nucleotide substitution across different genotypes.

## Results

The PCR amplification products of specific primer of Kappa-casein (CSN3) gene, showed a band of 645 bp in each individual sample from Meriz, Wild, and Native goats (Figure 1).

Two distinct alleles (A and B) were found in the CSN3 gene after being digested with the restriction enzyme *Hae* III. The allele B was 645 bp fragments; while the allele A was split into two pieces, one measuring 416 bp and the other 229 bp. In this study, the examination of the digested 645 bp CSN3 fragment revealed polymorphisms with the two homozygote genotypes, AA (416 and 229 base pair) and BB (645). The outcomes also showed that heterozygotes AB genotype was absent (Figure 2).

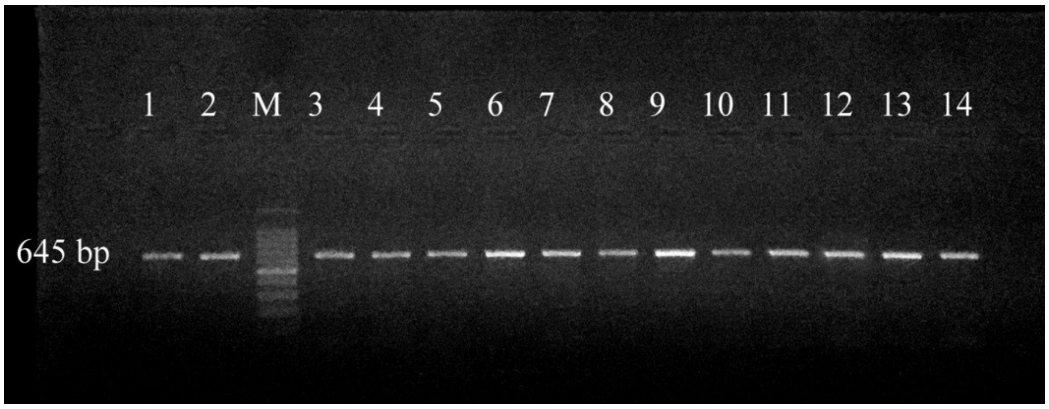


**Figure 1.Represent 645 bp PCR product of *CSN3* run on 2% agarose gel electrophoresis, M: represent 100 bp DNA marker, Lane 1 to 4 represent Wild, 5 to 9 represent Native and 10 to 14 represent Meriz goats**

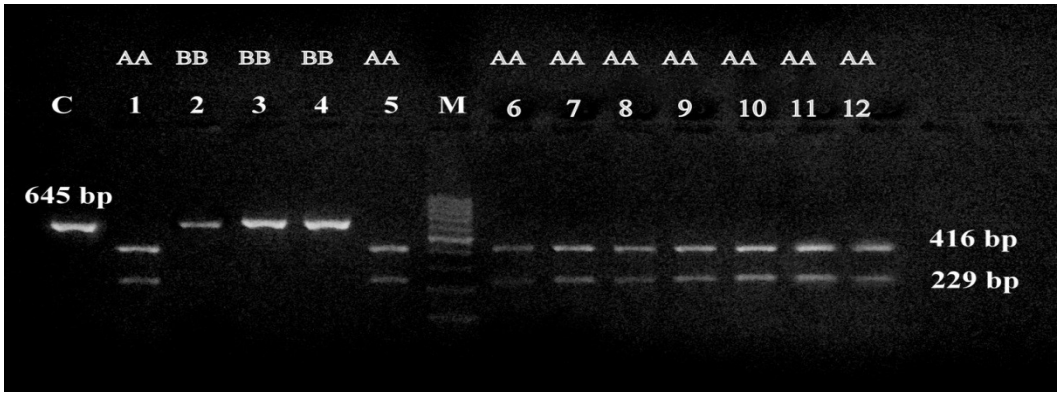


**Figure 2. Shows CSN3 PCR-RFLP patterns on 2% agarose gel electrophoresis using Hae III. M: stands for a 100 bp DNA marker, C: represents an undigested amplified PCR product as the control. Lane 1 to 4 represents Wild goats, Lane 5 to 8 represents Meriz goats and lane 9 to 12 represents Native goats**

The data analysis of genotype and allele frequencies in this investigation was described in Table (1). The table showed that the genotype frequencies of all Native and Meriz goats carried AA genotype but the Wild goats carried both AA and BB genotypes. The average of genotype frequencies in AA (0.87) was higher than BB (0.13). The total allele frequency of allele A was 0.87 and allele B was 0.13.

The sequence information gleaned from the PCR products of Meriz (NCBI accession number: OR050626) with AA genotype and wild goat (NCBI accession number: OR050625) with BB genotypes were aligned with reference sequences of *CSN3* gene mRNA of *Capra hircus* (JX889419.1) that shown in Figure (3)

The sequence data when aligned with JX889419.1sequence showed two new SNPs, one SNP in functional region of Wild goat (OR050625.1), and one in Meriz goat (OR050626.1). In position 415 in Wild goat, the amino acid Methionine changed to Isoleucine by changing (ATG) to (ATA). On the other hand, the point mutation in Meriz goat at the positions 449 led to change amino acid Valine to Isoleucine by alternation (GTC) to (ATC) Figure (4).

**TABLE 1. Allele and Genotype frequency of *CSN3* in three goat breads**

| Population | Individual number | Observed AA allele | Observed BB allele | Genotype frequency | | Allele frequency | |
|---|---|---|---|---|---|---|---|
| | | | | AA | BB | A | B |
| **Wild goat** | 15 | 6 | 9 | 0.4 | 0.6 | 0.4 | 0.6 |
| **Meriz** | 28 | 28 | 0 | 1 | 0 | 1 | 0 |
| **Native goat** | 27 | 27 | 0 | 1 | 0 | 1 | 0 |
| **Average** | | 61 | 9 | 0.87 | 0.13 | 0.87 | 0.13 |

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JX889419.1                                  TGCTGTGAGAAAGATGAAAGATTCTTCGATGACAA      178
OR050626.1                                  TGCTGTGAGAAAGATGAAAGATTCTTCGATGACAA      156
OR050625.1                                  TGCTGTGAGAAAGATGAAAGATTCTTCGATGACAA      156

JX889419.1  AATAGCCAAATATATCCCAATTCAGTATGTGCTGAGTAGGTATCCTAGTTATGGACTCAA  238
OR050626.1  AATAGCCAAATATATCCCAATTCAGTATGTGCTGAGTAGGTATCCTAGTTATGGACTCAA  216
OR050625.1  AATAGCCAAATATATCCCAATTCAGTATGTGCTGAGTAGGTATCCTAGTTATGGACTCAA  216

JX889419.1      TTACTATCAACAGAGACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATATTA
298
OR050626.1      TTACTATCAACAGAGACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATATTA
276
OR050625.1      TTACTATCAACAGAGACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATATTA
276

JX889419.1      TGCAAAGCCAGTTGCAGTTAGGTCACCTGCCCAAACTCTTCAATGGCAAGTTTTGCCAAA
358
OR050626.1      TGCAAAGCCAGTTGCAGTTAGGTCACCTGCCCAAACTCTTCAATGGCAAGTTTTGCCAAA
336
OR050625.1      TGCAAAGCCAGTTGCAGTTAGGTCACCTGCCCAAACTCTTCAATGGCAAGTTTTGCCAAA
336

JX889419.1      TACTGTGCCTGCCAAGTCCTGCCAAGACCAGCCAACTACCCTGGCACGTCACCCACACCC
418
OR050626.1      TACTGTGCCTGCCAAGTCCTGCCAAGACCAGCCAACTACCCTGGCACGTCACCCACACCC
396
OR050625.1      TACTGTGCCTGCCAAGTCCTGCCAAGACCAGCCAACTACCCTGGCACGTCACCCACACCC
396

JX889419.1      ACATTTATCATTTATGGCCATTCCACCAAAGAAAGATCAGGATAAAACAGAAGTCCCTGC
478
OR050626.1      ACATTTATCATTTATGGCCATTCCACCAAAGAAAGATCAGGATAAAACAGAAATCCCTGC
456
OR050625.1      ACATTTATCATTTATAGCCATTCCACCAAAGAAAGATCAGGATAAAACAGAAGTCCCTGC
456

JX889419.1      CATCAATACCATTGCTAGTGCTGAGCCTACAGTACACAGTACACCTACCACCGAAGCAAT
538
OR050626.1      CATCAATACCATTGCTAGTGCTGAGCCTACAGTACACAGTACACCTACCACCGAAGCAAT
516
OR050625.1      CATCAATACCATTGCTAGTGCTGAGCCTACAGTACACAGTACACCTACCACCGAAGCAAT
516

JX889419.1      AGTGAACACTGTAGATAATCCAGAAGCTTCCTCAGAATCGATTGCGAGTGCATCTGAGAC
598
OR050626.1      AGTGAACACTGTAGATAATCCAGAAGCTTCCTCAGAATCGATTGCGAGTGCATCTGAGAC
576
OR050625.1      AGTGAACACTGTAGATAATCCAGAAGCTTCCTCAGAATCGATTGCGAGTGCATCTGAGAC
576

JX889419.1  CAACACAGCCCAAGTTACTTCAACCGAGGTCTAA----------                    632
OR050626.1        CAACACAGCCCAAGTTACTTCAACCGAGGTCTAA----------
610
OR050625.1        CAACACAGCCCAAGTTACTTCAACCGAGGTCTAA----------
610
```

**Figure 3. Represents DNA sequences of functional region of CSN3 (exon 4) gene of OR050626.1(Meriz goat) and OR050625.1 (Wild goat) that aligned with JX889419 sequence showing detected SNPs**

**JX889419.1**

ccekderffd dkiakyipiq yvlsrypsyg lnyyqqrpva linnqflpyp
yyakpvavrs paqtlqwqvl pntvpakscq dqpttlarhp hphlsfmaip
pkkdqdktev paintiasae ptvhstptte aivntvdnpe assesiasas
etntaqvtst ev

**OR050626.1**

ccekderffd dkiakyipiq yvlsrypsyg lnyyqqrpva linnqflpyp
yyakpvavrs paqtlqwqvl pntvpakscq dqpttlarhp hphlsfmaip
pkkdqdktei paintiasae ptvhstptte aivntvdnpe assesiasas
etntaqvtst ev

**OR050625.1**

ccekderffd dkiakyipiq yvlsrypsyg lnyyqqrpva linnqflpyp
yyakpvavrs paqtlqwqvl pntvpakscq dqpttlarhp hphlsfiaip
pkkdqdktev paintiasae ptvhstptte aivntvdnpe assesiasas
etntaqvtst ev

**Figure 4. Represents the JX889419, OR050626.1 (Meriz goat) and OR050625.1 (Wild goat) amino acid sequences of CSN3 gene (exon 4).**

## Discussion

The 645 bp amplified bands of CSN3 were found to be consistent with studies published by [8] in 210 animals belonging to different Spanish, Italian and French goat breed. In this study, the examination of the digested 645 bp CSN3 fragment with the restriction enzyme *Hae* III revealed polymorphisms with the two homozygote genotypes were AA (416 and 229 base pair) and BB (645) genotypes. The allele B (645 bp) left uncut, due to the lack of *Hae* III enzyme restriction sites on this allele (Figures 2). These findings differed from that of Patel and his colleagues [27] who revealed monomorphism with AA genotypes in on Zawaladi goat.

In this investigation, the allelic frequency of the allele A (0.87) was higher than that of the allele B (0.13). In line with our findings, Di Gerlando and his colleagues [7] revealed that the allele (A) was most frequent allele in *Girgentana* goat breed also, Mahmoodi *et al.,* [28], found that allele A (0.7) was higher than allele (B) in Kermani sheep. Different results were reported by Ahmed and Othman, [29] who reported that B allele was the highest frequency in the Zaraibi breed among the several goat breeds raised in Egypt (90%), also Catota-Gómez and his colleagues [6] found that the allele B was most frequency allele in Saanen goats. The genetic flow given by the selection of carrier animals, whether directly or indirectly, can be used to explain why the (A) allele is more prevalent in the population. This segregation of the (A) allele to future generations could also be a contributing factor [6].

The sequence information gleaned from the PCR results of Meriz (NCBI accession number: OR050626) with AA genotype and wild goat (NCBI accession number: OR050625) with BB genotypes were aligned with reference sequences of *CSN3* gene mRNA of *Capra hircus* (JX889419.1) which revealed two new SNPs in functional region of the gene that led to change amino acid sequence. In position 415 in Wild goat, the amino acid Methionine changed to Isoleucine by changing (ATG) to (ATA). On the other hand, the point mutation in Meriz goat at the positions 449 led to change amino acid Valine to Isoleucine by alternation (GTC) to (ATC) Figure (4). In order to comprehend the impact of SNPs and amino acid alterations, it is necessary to examine the milk quality of these three native Iraqi goat breeds, as there is currently no sequence data available on them. This study was the first to look into this matter.

The detection of DNA polymorphism in CSN3 gene has been reported by several scientists in different goat breeds. According to Prinzenberg and his colleagues [13], the domestic goat has a total of 16 DNA variants, in which three are silent mutations and thirteen are protein variants involving a total of 15 polymorphic sites. In the other study, Kiplagat *et al* [15] revealed the nine point mutations corresponding to base transitions in nine indigenous goat groups from five different nations in Eastern Africa. Additionally, Gautam and his colleagues [16] found 12 additional polymorphisms at nucleotide locations in the Indian goat CSN3 exon 4 areas.

In this study, the most frequent allele was A (0.87) but, the most of researchers concluded that the kappa-casein B variant is linked to increased levels of fat, protein, and casein in milk and significantly affects milk's ability to make cheese [30]. As compared to the AA genotype, the genotypes BB and

AB were significantly related with higher amounts of total casein and protein content, highlighting the significance of the CSN3 genotype when selecting dairy goats for milk composition [7].

**Conclusions:**

The outcomes of this investigation show that genetic polymorphism for k-casein exists. There are no enough phenotypic data available at this time for this three (Meriz. Native and Wild goat) indigenous Iraqi goat breed; Therefore, additional research could determine whether polymorphisms affect milk's quantitative and qualitative properties and whether they have any associations with those properties, and use larger sample sizes and inclusion of other milk genes can now be considered.

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*Conflicts of interest*: There is no conflict of interest disclosed by the authors.

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## الكشف عن تعدد أشكال جين كابا-كازين (CSN3) في ثلاث سلالات ماعز عراقية محلية باستخدام مؤشرات PCR-RFLPوSNP

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[٢] مركز البحوث العلمية - كلية العلوم - جامعة دهوك - إقليم كردستان - العراق.

يحتوي الحليب على بروتين يسمى كابا-كازين، الذي يتحكم في وظيفة وحجم مذيلات الحليب وكذلك قدرتها على التكوين والاستقرار. لقد تمت دراسة تعدد أشكال جين كابا-كازين (CSN3) في ٧٠ من الماعز المحلي (ماعز محلي و ميريز) و الماعز البري باستخدام طريقة PCR-RFLP والتسلسل الجيني المباشر. CSN3-Hea III/RFLP كشفت عن نمطين وراثيين متماثلين AA وBB . بالنسبة للطرازين الوراثيين AA وBB، كانت ترددات النمط الجيني المحسوبة (٠,٨٧) و (٠,١٣) على التوالي. وكان التردد الأليلي ٠,٨٧ للأليل A و٠,١٣ للأليل B . وكما كشفت البيانات التسلسلية لجين CSN3من الماعز ميريز و البري عن وجود ٢ SNPsفي المنطقة الوظيفية، وواحد SNP من البري (ACC.NO:OR050625.1)، وواحد في ماعز ميريز (ACC.NO:OR050626.1).
في المواضع ٤١٥ في الماعز البري، لقد تغير الحمض الأميني ميثيونين إلى آيزوليوسين عن طريق تغيير(ATG) الى (ATA). ومن الناحية الأخرى، أدت الطفرة النقطية في الماعز ميريز في المواضع ٤٤٩ إلى تغير الحمض الأميني فالين إلى آيزوليوسين بالتناوب (GTC) إلى (ATC) . كما يعد التحليل PCR-RFLP و SNP كأدوات قوية للدراسة الجينية لتقلب CSN3 في الماعز المحلية والبرية، مما يسمح بالتعرف المتزامن على الأليلات المختلفة، ولاكتشاف المتغيرات الجديدة. و لإنشاء علاقات بين الأنماط الجينية ونوعية الحليب الكمية والنوعية سوف يتطلب تحقيقًا إضافيًا.

**الكلمات المفتاحية**: كابرا هيركوس- *Hae* III- جينCSN3 - كابرا إيغاجروس- SNP .