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Alpha S1-Casein Gene Polymorphism in Yankasa Sheep Breed of Mubi, Adamawa State, Nigeria

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ABSTRACT

The present study was conducted on five yankasa breeds of sheep to determine *alpha S1-CSNISI casein* gene polymorphism. Five blood samples were collected using sterile needle and syringe into sample tubes containing (EDTA, placed on ice in the cold-chain container and immediately transported to the laboratory for analysis. DNA extraction was carried out using Quick-DNA Miniprep ™ kit. Primers were used to amplify the DNA fragment of interest. Nucleotide sequences were subjected to the BLAST search against the NCBI database and a similar sequence NC-0040257.1 was retrieved for comparison. Single nucleotide polymorphism was analysed for variations. Multiple sequence alignment was performed using Clustal W and reconstructed using Multalin. A MEGAx Program incorporated in GenAleX 6.503 was used to construct a phylogenetic tree and multiple sequence alignment regenerated in the Muscle (3.8) to construct phylogenetic tree within as well as with the reference (NC-040257.1). Results from the analysis revealed that the extracted DNA was found on chromosome 6, intron 16 and exon 17. The breeds showed a total number of polymorphic and monomorphic site of 68 and 600 respectively, and percentage polymorphism of 10.18%. High frequency of 0.733 was observed at position 201. Amino acid substitution in yankasa sheep breed was 4 at positions 161, 163, 164 and 201, showing the existence of variation in yankasa species possibly indicating long term chances of survival. CSN1S1 isolated in yankasa sheep showed polymorphism, genetic variation within and between breeds. Present study have revealed polymorphisms in milk proteins with high frequency (0.733) in variant at position 201Thr > Ile. In further studies, it is therefore of interest to characterize, genotype and find the allele frequencies of casein gene of yankasa sheep breed, provide a complete picture about milk protein gene and milk protein variation to improve consumer preference.

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Introduction

With the increasing population in the country, the demand for milk proteins through sustainable animal agriculture is increasing. There is vigorous research for an efficient

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production system that will supplement nutrition. Valuable milk by-products have been documented to be suitable nutritional base for other foods due to their functional properties and health benefits [1]. Therefore, maintaining genetic variation is very important to avoid the loss of breeds by farmers and consumers. Already, numerous breeds are recorded extinct and others also are being classified at high risk of loss [2]. There is a need for characterization and description of casein gene polymorphism of ovine milk of native sheep breeds of Nigeria. It is important that casein DNA variation has been implicated in quantitative and qualitative parameters in milk, especially milk protein composition affecting properties of milk in cattle and goats [3, 4 5] and hence could be included in breeding strategies. Polymorphism in the casein gene might affect protein structure, which is a strong and biological fitness that suggests its important role of selection in the molecular evolution [4, 5, 6].

Improvement of livestock in the last century was restricted to the phenotypic selection, quantitative theory designs and accurate selection techniques. These provide economically important functions in bovine, ovine, sudae and avians. Recent advances in recombinant DNA techniques have contributed immensely towards genetic variation studies at the DNA level. The genetic variability in animals [7] can be assessed within and among the population for selection and preservation of the genetic structure. Preservation of genetic variation is crucial for breeding programs to match animal husbandry and consumer requirements. It has been shown that ovine genetic polymorphisms affect the physicochemical properties of milk hence, there is need for in-depth knowledge of the genetic polymorphism of indigenous ovine milk proteins for the improvement of the quality of ovine milk for its contribution to the Nigerian dairy industry. The genetic basis for diversity in sheep and its consequences on selection on gene polymorphism within the sheep breeds have not been assayed on a genomic scale worldwide [8]. Similarly, to our knowledge, there are no studies on variation in the alpha-casein gene of yankasa breed of sheep. There were genetic polymorphism studies in Ouda sheep breeds [9] and Balami sheep breed casein gene, which were both isolated within 154 bp of chromosome 6, intron 16 and exon 17 [10] and variations occurred within and between breeds. These reports prompted present similar study in yankasa sheep breeds.

Materials and Methods

Experimental location and metrological data

The study was conducted in Mubi South Local Government Area of Adamawa State, Nigeria. The area is located between Latitude 10° 05' N/ 10° 30' N and Longitude 13° 10' E/ 13° 30'E. The area has tropical weather. The minimum and maximum temperatures of the area are 23.4 ^{o}C and 29.3 ^{o}C. The highest and lowest rainfalls are 280 mm and 258 mm [11].

Materials

Blood samples, needle and syringe, cooler, extraction kits, vortex, and centrifuge, and electrophoresis apparatus, gel documentation system, and thermocycler were used. Also used were software like: Genalex 6.503 [12], NCBI, Clustal W, BioEdit [13], Multalin [14]. Muscle 3.8 [15]. The laboratory analysis was conducted at African bioscience laboratory, Ibadan.

Experimental animals and collection of blood samples

Blood samples were randomly collected from five adult Yankasa female sheeps in Mubi, Adamawa State. Blood samples (5 mL) for DNA extraction were collected through the jugular vein, using a needle and syringe and preserved in EDTA an in EDTA tube. All the samples were transported to the laboratory inside cooler containing ice park.

Gene isolation

DNA isolation were performed according to Quick DNA™ microPrep Kit from Zymo Research following manufacturer's instruction. Four hundred (400) µl of Genomic Lysis Buffer was added to 100 µl of blood to make 4:1 volume and mixed by vortexing for about 4-6 seconds and left to stand for about 5-10 minutes at room temperature. The mixture was transferred to a zymo- spin™ ll column in a collection tube and was centrifuged at 10,000 × g for one minute, the collection tube was discarded with the flow-through.

The zymo-spin™ llC column was transferred into another sterile tube and added to 200 µl of DNA pre-wash buffer then centrifuged for 100,000 × g for 1 min. Again, 500 µl of gDNA

wash buffer was added to the spin column and eluted at 10, 00 × g for 1 min.

In the elution stage, the spin column content was poured into another tube and 50 µl DNA elution` buffer was added to the spin column and incubated at room temperature for about 2-5 minute and thereafter, it was centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was immediately used for molecular characterization.

All genomic DNA was checked on 1% agarose gel electrophoresis and all amplicons on 1.5% agaros gel electrophoresis. It was stained with ethidium bromide and visualized under blue light transilluminator.

For a 10 cm × 10 cm minigel cast of 1% agarose gel , 0.5 g agarose was dissolved in 50 cm^3 of 1 × TAE (Tris Acetate EDTA) buffer, while 1.5% agarose gel for the amplicons by dissolving 0.75 g of agarose in 5 ml of 1×TAE. The mixture swilled until colloids formed. This was then heated for 1-3 min until a clear solution formed.

The gel was cooled to 50°C under running water. Ethidium bromide was added to 0.5 µg/ml of 2.5 µl stock in 50 ml and vortexed till no trace of the stain was detected [16].

The gel was allowed to set for 20 min. The casted gel was removed from the tray and submerged in the running buffer (1× TAE). The comb then gently removed and DNA samples loaded in the gel wells.

Each PCR amplicon, prepared from ready–to-load master mix, was loaded, 5 µl per well, without the addition of loading dye. DNA samples were prepared for loading into the well by mixing 4µl of the extracted DNA sample with 1 µl of the 5 × loading dye. This proportion of loading dye to DNA was used when loading cleaned amplicons.

The electrophoresis was run at 100 volts for 40 min, after which the gel was viewed and photographed on biologix blue light transilluminator. Solisbiodyne 100 bp DNA ladder was run alongside DNA samples according to manufacturer instructions.

DNA amplification

DNA amplification was conducted using standard methods. Primers for Casein Alpha S1 gene was designed from reference genomic sequence NC_019463.2 as described by Zhao et al [17] with little modification. The Primers (CSN1S1F 5’-ACCCCTCAGGTACCCTAAGAAA-3’ and CSN1S1R 5’-GTTTATCCCCCACACTGCATTC-3’) spans Intron 16 – Exon 17 – Intron 17 of Casein

Alpha S1 gene on Oar_v17 genomic sequence assembly. Amplification was performed on Labnet Multigene Mini thermal cycler. A 20 µl reaction consisting of 4 µl of 5X master mix, 0.6 µl each of the primers, 12.8 µl of nuclease-free water and 2 µl of DNA template was prepared. PCR cycling condition was as follows: Initial denaturation at 95°c for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 40 seconds and a final extension at 72°C for 5 minutes. PCR product was run on 1.5% agarose, viewed and photographed on Biologix bluelight transilluminator.

Amplicons were cleaned up for sequencing using DNA Clean & Concentrator^{Tm}-5 from Zymo Research, according to the manufacturer's protocol. Cleaned amplicons were eluted with 30 µl nuclease-free water. The cleaned products were checked on 1.5% agarose.

Gene sequence analysis

The sequencing was carried out according to Sanger [17] at Xcelri Genomics, India. The sequences were searched for identity and similarity using BLAST against NCBI database. The yankasa sheep breed casein gene and reference sequences (NC_040257.1) [19] were used for multiple sequence alignment using Multalin [14]. Phylogenetic and molecular evolutionary analyses were conducted using MEGAx [20].

Result and Discussion

Alpha S1-casein **gene sequencing**

Amplicons were cleaned up for sequencing using Commercial DNA clean kit, with 30 µl nuclease-free water, the cleaned products were checked on 1.5 % agarose. The amplified DNA extracted were sequenced and sequences of all yankasa sheep breed are presented in figures 1 - 5. Yankasa sheep showed variation in the nucleotide sequence within breeds, the longest DNA fragment with the highest number of nucleotide sequence was found in sheep breed C as shown in figure 3 (718), sheep breed E showed the shortest DNA fragment with the lowest number of the nucleotide sequence (526), sheep breed B and D have 707 and 701 respectively as shown in figures 3, 4 and 5 respectively.

Variation of nucleotide sequence within yankasa sheep was also observed, at positions 706, 702, 700, and 564. The nucleotides were similar to those reported by Ramunno *et al.* [21], that casein gene CSN1S2 encoding αs_2 had a length of 18438 nucleotide and divided into 19

exons ranging from 24 to 266 nucleotide, the observed differences may be due to differences of classes of casein, exon and breed. Gencheva and Georgieva [22] also reported two genetic variants of CSNISI gene and two genotypes in Bulgarian autochthonous sheep breeds based on nucleotide variation in alpha SI-casein gene.

The present study showed that, there was a consensus at a various position as shown, at position 120-123(ATT), 160-165 (T TT), 184-186(CTT), and 476- 478 (ATT) as depicted in figure 6. The consensus was observed in yankasa sheep breed with the reference sequence in different positions, at position 14637-14640 (AGA), and 14647- 14647 (TTC) among the many consensus found as depicted in figure 7. A phylogenetic tree within yankasa sheep breed showed that 2B and 2A, 2C and 2D are genetically closer, while and yankasa sheep breed 2E is genetically far away from sheep breeds 2B and 2A, 2C and 2D as shown in figure 8. Corpet [18] used the same method which resulted in similarity in the result obtained in this study. Phylogenetic tree of yankasa sheep breed with reference sequence to 2E is genetically closer to the reference sequence than 2A, 2B, 2D and 2C as showed in figure 9 and confirmed using Muscle phylogenetic tree 3.0 [15]. Yankasa sheep breed showed the same frequency of amino acid substitution of 0.067 at two different positions, the highest being 0.733 at position 201 and, at position 164 was 0.133 (Table 1).

> CSN1S1-Yankasa-1

CAAGGGGGGGGGTGATACACAATACCTAGGGAGAGTTTGGTTTCTCTGTTTTC
CTCACAGAGTAAACATCTCTTGTGATGCGAATAGCCATGTCTGAAATGAATGC
AATGATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCA
ATGGTCTTTCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGACGCCTATCCATC
TGGTGCCTGGTATTACCTTCCACTAGGCACACAATACACTGATGCCCCCTCATT
CTCTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGACTACTATGCC
ACTGTGGTGGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAA
ATAGAAGAAAACATAATATAAAAATAAATTTAGAATAAGCATGACACTTAAAT
GCTTAGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCT
GATATATGGCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCT
ATTTGATAAGTGATAATCATTCTGATGAAAATAGGAGGAAAATTTTCTCTCCAA

AGTAAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTC
TTTGGATTATACCCATGATATACATTAGAATGCAGTGAGGGGGAAAAATAACA
CAAA

Fig 1 Nucleotide sequence of yankasa sheep breed A

>CSN1S1-yankasa-2

CAGGGTGGGGATGAGCATCATCAGTAAGGAAGAGTTTGGTTTCTCTGTTTTCCT
CACAGAGTAAACATCTCTTGTGATGCGAATAGCCATGTCTGAAATGAATGCAA
TGATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCAAT
GGTCTTTCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGACGCCTATCCATCTG
GTGCCTGGTATTACCTTCCACTAGGCACACAATACACTGATGCCCCCTCATTCT
CTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGATTACTATGCCAC
TGTGGTGGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAAAT
AGAAGAAAACATAATATAAAAATAAATTTAGAATAAGCATGACACTTAAATGC
TTAGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCTGA
TATATGGCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCTAT
TTGATAAGTGATAATCATTCTGATGAAAATAGGAGGAAAATTTTCTCTCCAAAG
TAAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTCTT
TGGATTATACCCATGATATACATTAGAATGCAGTGTGGGGGAAATAAAAACAA

Fig 2 Nucleotide sequence of yankasa sheep breed B

> CSN1S1-yankasa-3

TAGGGCCATGTTAAGGAAGATTAGCAAAAGTGCAAGGAGGATAAAGTTGAATT
TTTACTTCTGTGAGAGAAAATTTTCCTCCTATTTTCATCAGAATGATTATCACTT
ATCAAATAGATAGCACTGCTCCACATGTTCCTGAGTAATGGATTAACATTAGCC
ATATATCAGAAAGTTATCATCAATTTTCCATTTCAGAAAATTCTAGCATAGGAC
ACTAAGCATTTAAGTGTCATGCTTATTCTAAATTTATTTTTATATTATGTTTTCTT
CTATTTCCCTTGATACGGCAGCAATATGCAGTCATTTAAATGAACTTACCACCA
CAGTGGCATAGTAATCTTTCCACTGTTCTCAGAGCCAATGGGATTAGGGATGTC
AGAGAATGAGGGGGCATCAGTGTATTGTGTGCCTAGTGGAAGGTAATACCAGG
CACCAGAAGGATAGGCGGCGAGCTGGTAGAATTGTCTGAAAAGCTA

Fig 3 Nucleotide sequence of yankasa sheep breed C

> CSN1S1-yankasa-4

GGTGGGTGCGCTCGCAAAATGGTGCTCATGTTAAGTGATTAGCAAAAGTGCAA
GGAGGATAAAGTTGAATTTTTACTTTGGAGAGAAAATTTTCCTCCTATTTTCAT
CAGAATGATTATCACTTATCAAATAGATAGCACTGCTCCACATGTTCCTGAGTA
ATGGATTAACATTAGCCATATATCAGAAAGTTATCATCAATTTTCCATTTCAGA
AAATTCTAGCATAGGACACTAAGCATTTAAGTGTCATGCTTATTCTAAATTTAT
TTTTATATTATGTTTTCTTCTATTTCCCTTGATACGGCAGCAATATGCAGTCATT
TAAATGAACTTACCACCACAGTGGCATAGTAATCTTTCCACTGTTCTCAGAGCC
AATGGGATTAGGGATGTCAGAGAATGAGGGGGCATCAGTGTATTGTGTGCCTA
GTGGAAGGTAATACCAGGCACCAGATGGATAGGCGTCCACCTGGGAGAATTGT
CTGAAAAGCTAGAGAGAAAGACCGTTGAAAAAGCAACAGTGTATGATAAATC
AGTTTTGAATCTCTGAAAATGAATCATT

Fig 4 Nucleotide sequence of yankasa sheep breed D

> CSN1S1-yankasa-5

TTACCTTACTGTGATTTACCATAGGGAAGAGTTTAGGTTTCTCTAGTTTTCCTCA
CAGAGTAAACATCTCTTGTGATGCGAATAGCCATGTCTGAAATGAATGCAATG
ATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCAATGG
TCTTTCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGACGCCTATCCATCTGGT
GCCTGGTATTACCTTCCACTAGGCACACAATACACTGATGCCCCCTCATTCTCT
GACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGATTACTATGCCACTG
TGGTGGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAAATAG
AAGAAAACATAATATAAAAATAAATTTAGAATAAGCATGACACTTAAATGCTT
AGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCTGATA
TATGGCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCTATTT
GATAAGTGATAATCATTCTGATGAAAATAGGAGGAAAATTTTCTCTCCAAAGT
AAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTCTTT
GGATTATACCCATGATATACATTAGAATGCATTGGGGGAAAAAAAAAAAA

Fig 5 Nucleotide sequence of yankasa sheep breed E

Multiple sequence alignment (Figure 6 and 7) presents the nucleotide sequence variation among yankasa sheep within 154 bp of intron 16 and exon 17 are in positions 706 (highest) and 479 (lowest), nucleotide positions when compared to reference gene NC_040257.1 [19]. The dot represents the variation and similarity between the species and the reference gene, while the variation is represented as G, A, C, and T by using Multalin multiple sequences with hierarchical clustering [14].

Evolutionary relatedness of the sequences studied is presented in figures 8 and 9. The genetic distance between the reference gene [19] and yankasa breeds was genetically far from each other by 0.022 observed in the phylogenetic tree constructed in MEGA X using Nei's genetic distance [23] generated in GenAlex 6.50. This was also in tandem with multiple sequenced alignments generated in Muscle (3.8) neighbour-joining tree (Figure 6).

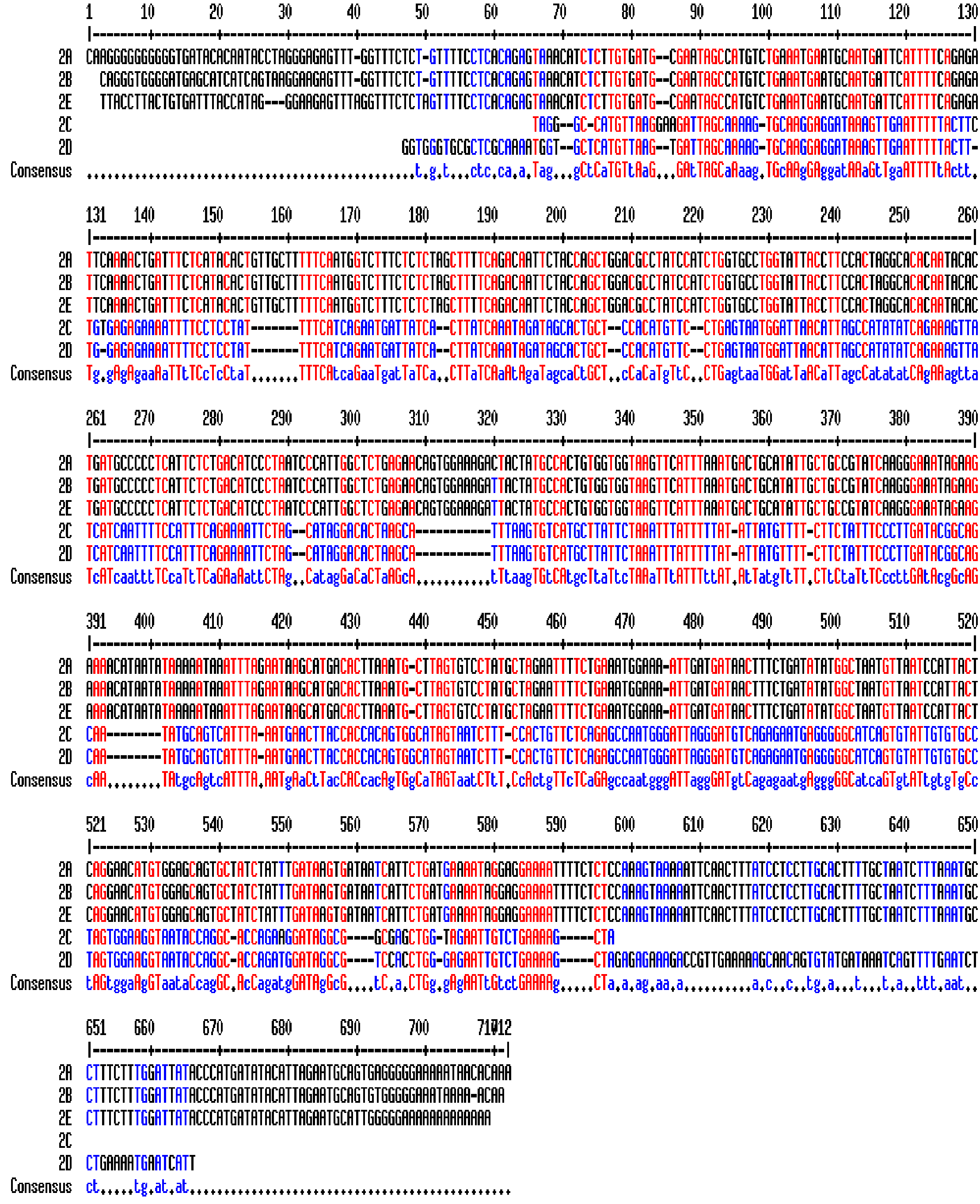

Fig 6 Multiple sequence alignment of nucleotide within yankasa sheep breed

Note: 2A= Nucleotide sequence for yankasa sheep 1
2B= Nucleotide sequence for yankasa sheep 2
2E= Nucleotide sequence for yankas sheep 5
2C= Nucleotide sequence for yankasa sheep 3
2D= Nucleotide sequence for yankasa sheep 5

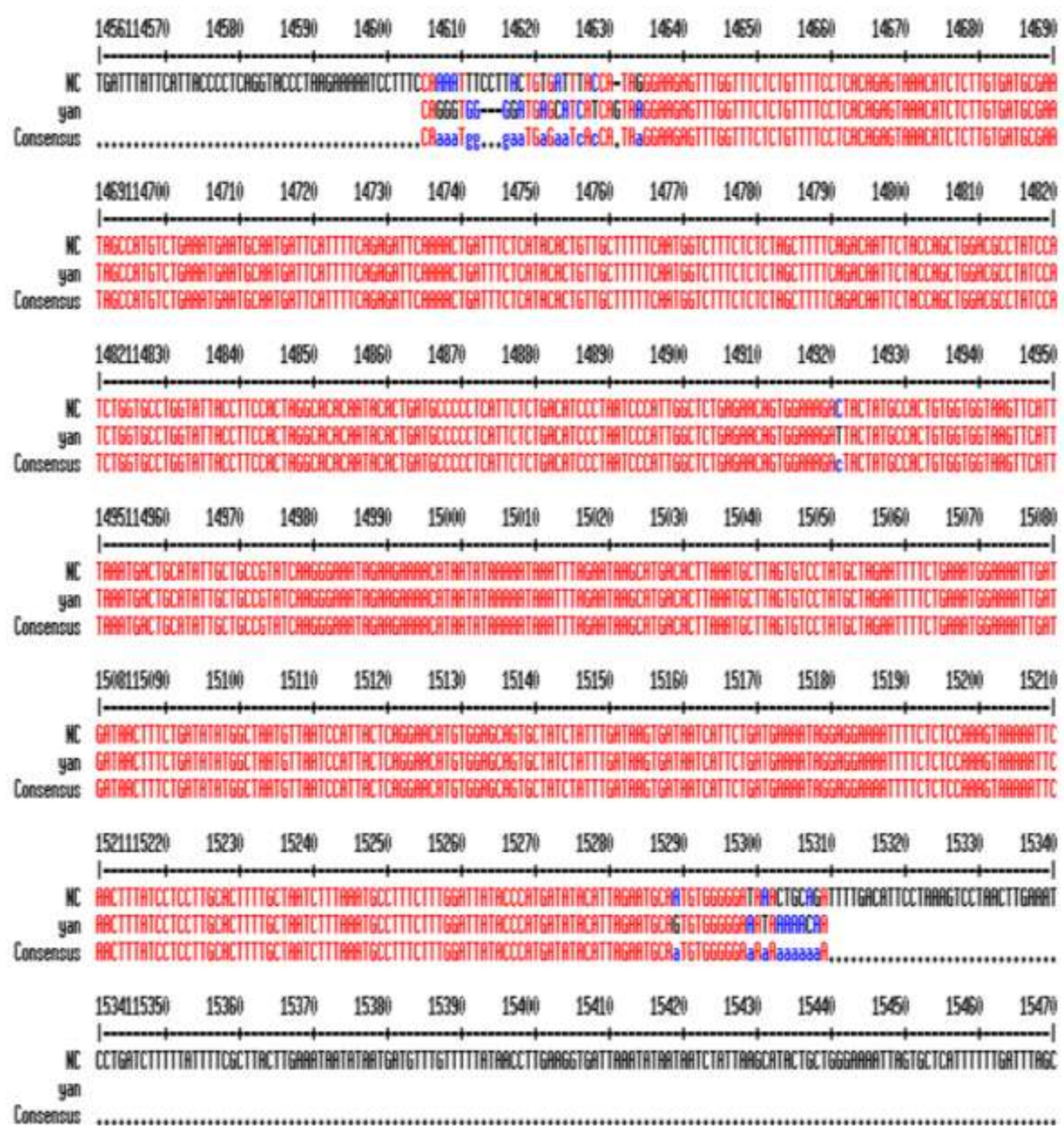

Fig 7 Pairs wise sequence alignment of nucleotide of yanksa sheep breed with reference sequence
Note: NC= Nucleotide sequence for reference NC- 040257.1
Yan= Nucleotide sequence for yankasa sheep

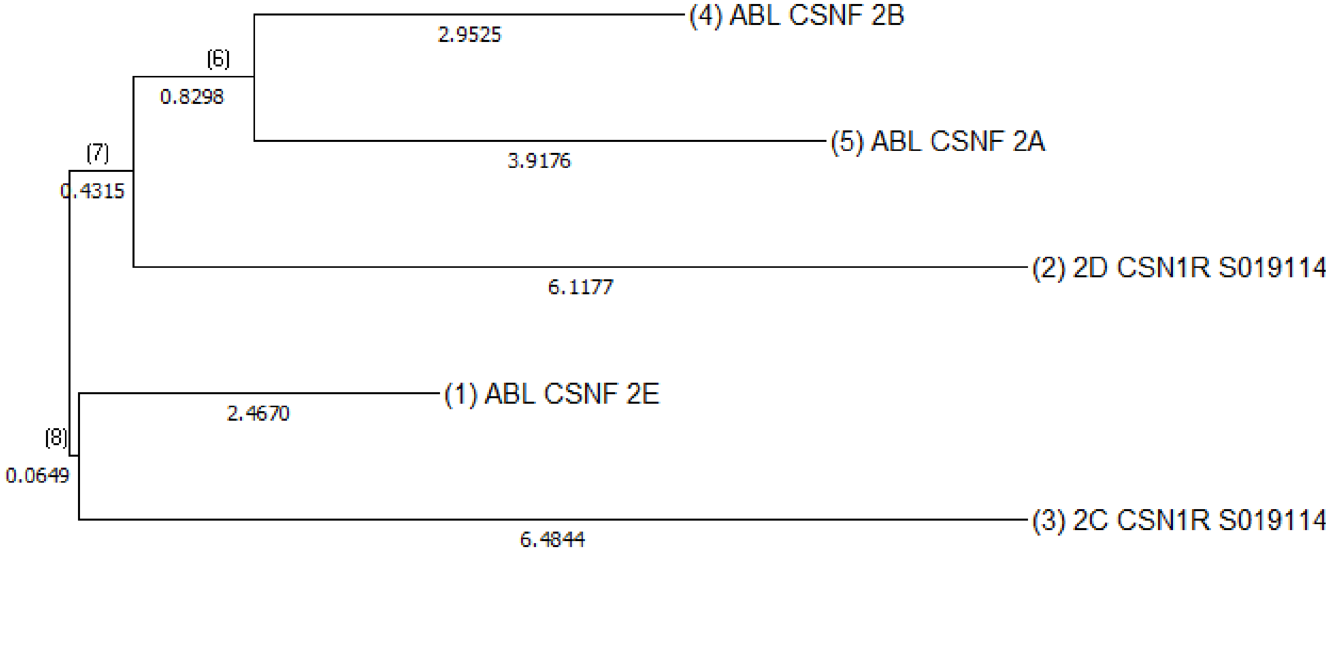

Fig 8 Phylogenetic tree within yankasa sheep breeds

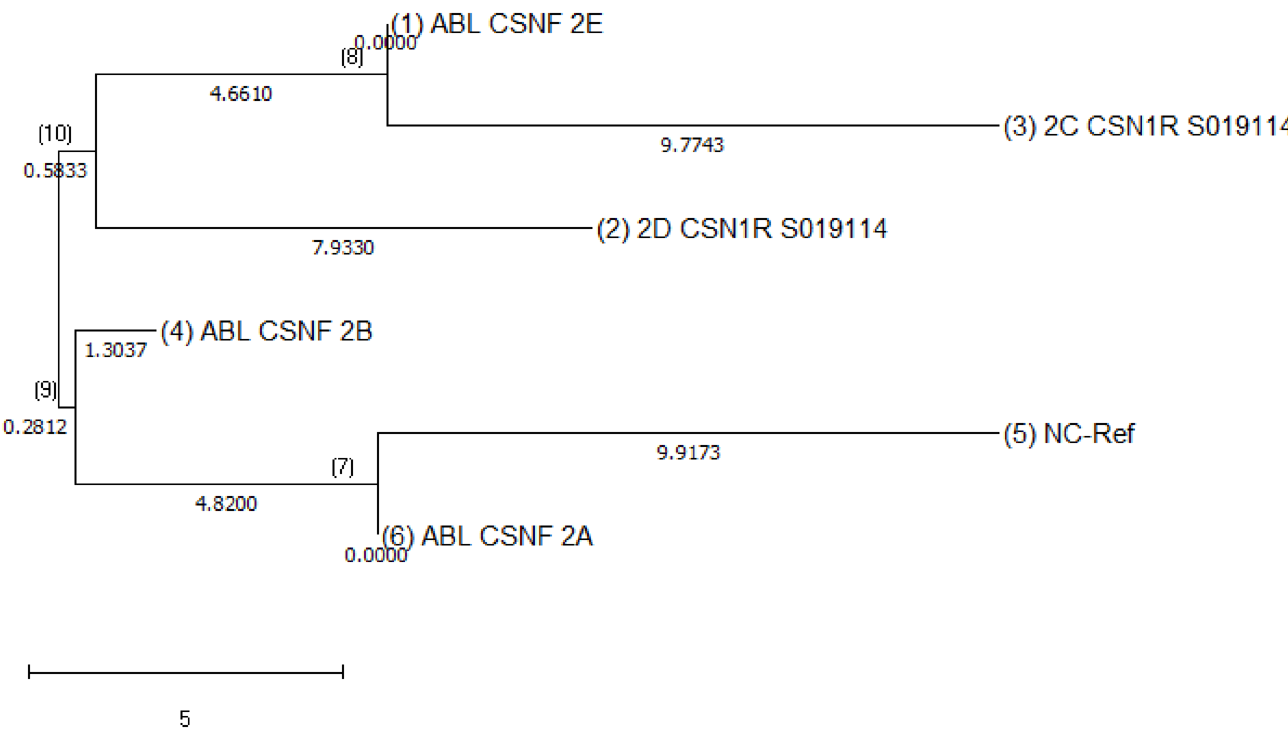

Fig 9 Phylogenetic tree of yankasa sheep breed with the reference gene

Genetic variation studies in sheep were mostly at phenotypic level. The polymorphism of *alpha S1-casein* was reported among some local Bulgarian sheep breeds at DNA level [22, 23]. Single nucleotide polymorphism (SNP) was detected in traditional sheep populations in Bulgaria at position 13 of the third exon in CSNIS1 gene with two genotypes (homozygous – CC and heterozygous – AC) [22]. The result of amino acid substitution in yankasa breeds of sheep in Mubi, Nigeria is presented in Table 1. Polymorphism in *alpha SI casein* gene in yankasa breed of sheep in Nigeria have not been reported previously by either phenotypic or molecular methods. In present study, the polymorphic sites and frequency of polymorphism confirmed the variation and similarity in the multiple sequence alignment, where amino acids substitution and polymorphism were identified within the open reading frame of the CSN1S1 gene [24] as compared with the reference sequence. This result is similar to the finding of Calvo *et al.* [25] who observed polymorphism in Assaf sheep breed on exon 17. However, yankasa sheep breed showed the percentage polymorphism of 1.65%, and 11 number of polymorphic sites and monomorphic site of 657 with reference [19]. Amino acid substitution

were found at position 161 Try> Ser, 163 Leu>Val, with the same frequency of 0.067 respectively, at position 164 Asp> Ala with a frequency of 0.133, and a synonymous exchanged at position 163 Leu>Leu (Table 1). Similar to the finding of Ceriotti, et al. [26] that detected a single nucleotide polymorphism in casein CSN1S1 on exon 17 and also observed amino acid exchange at position 186 Ile>Thr, 183 Met> Val and 104 Ser> Leu. The observed difference may be due to difference on the base pairs used as well as geographical location.

Table 1 Amino acid substitutions and frequencies in yankasa sheep breed

Positions	Amino Acid Substitution	Frequency
161	161Tyr > Ser (161Y > S)	0.067
163	163Leu > Val (163L > V)	0.067
164	164Asp >Ala (P>A)	0.133
201	201Thr > Ile (201T > 201I)	0.733

The breeds showed a total number of polymorphic and monomorphic site of 68 and 600 respectively, percentage of polymorphism of 10.18%, and amino acid substitution was four, it showed variation exists within breeds these are very important for species long term survival. High frequency of 0.733 was observed at position 201 (201Thr > Ile). However, Cerotti *et al*. [26] reported an amino acid exchange on exon 17 positions 183 Met>Val with a frequency of 0.12 to 0.26 this difference could be as a result of differences in targeted segments on the exon as well as the position of the exchanged protein.

Conclusion

In conclusion, casein CSN1S1 was isolated in yankasa sheep within 154 bp of chromosome 6, intron 16 and exon 17. Present study has shown variations of nucleotides in the casein gene coding for milk protein at position 201Thr > Ile of balami breed of sheep indigenous to Mubi, Nigeria. It was characterized and showed polymorphism and genetic variation within and between breeds. These sequence obtained from yankasa sheep breeds will be deposited on

NCBI database for further research. This will assist in conserving the genes of the native animals for breeding purposes. There is need for complete characterization, genotyping and finding the allele frequencies of casein gene of indigenous sheep breeds, this will offer the possibility to get a complete picture about milk protein gene and then consider milk protein variation in a specific breeding programme for improving consumer preference.

Conflict of interest

The authors hereby declare that there is no conflict of interest.

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